



Edwardsiella ictaluri in *Pangasianodon* catfish: antimicrobial resistance and the early interactions with its host

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List of Abbreviations

AdiA	Arginine decarboxylase	
ATCC	American Type Culture Collection	
BHI	Brain heart infusion	
BHIB	Brain heart infusion broth	
BNP	Bacillary Necrosis in Pangasius	
bp	Base pair	
CCUG	Culture Collection of the University of Goteborg	
CFU	Colony Forming Units	
CHSE-214	Chinook salmon embryo cell line	
CLSI	Clinical and Laboratory Standards Institute	
dhfr1	Trimethoprim resistance gene	
DNA	Deoxyribonucleic acid	
EIA	Edwardsiella ictaluri agar	
ELISA	Enzyme-linked immunosorbent assays	
EMB	Eosin Methylene-Blue Lactose sucrose Agar	
EMEA	European Medicines Agency	
ENR	Enrofloxacin	
ESC	Enteric septiceamia of catfish	
FAO	Food and Agriculture Organization	
FATs	Fluorescent antibody techniques	
FCS	Fetal calf serum	
FDA	The Food and Drug Administration	
FHM	Fat head minnow cell line	
FM	Flumequin	
IHC	Immunohistochemistry	
Inc	Incompatibility	
IP	Intraperitoneal injection	
Kb	kilobase pairs	
kDa	kilodalton	
LMG	Laboratorium voor Microbiologie Gent	
LPS	Lipopolysaccharide	
LYM	Lyophilization medium	
Henle 407	Human embryonic intestinal epithelium cell line	

IEC-6	Rat small intestinal epithelium cell line	
MAbs	Monoclonal antibodies	
MEM	Minimum Essential Medium	
MIC	Minimal inhibitory concentration	
Mofi	Ministry of Fisheries in Vietnam	
MOI	Multiplicity of infection	
MRHA	Mannose-resistant hemagglutination	
MSHA	Mannose-sensitive hemagglutination	
NCCLS	The National committee for Clinical Laboratory Standards	
NEAA	Non essential amino acids	
OiE	International Office of Epizootics	
OriT	Origin of transfer	
OTC	Oxytetracycline	
OXO	Oxolinic acid	
PBS	Phosphate-buffered saline	
PCR	Polymerase Chain Reaction	
R1 214	Rainbow trout liver cell-line	
rDNA	Ribosomal Deoxyribonucleic acid	
SEM	Scanning electron microscopy	
SM	Streptomycin	
strA-strB	Streptomycin resistance gene	
sul 2	Sulfonamide resistance gene	
tDNA	Transport Deoxyribonucleic acid	
tDNA-PCR	tDNA intergenic lengh polymorphism PCR	
TEM	Transmission electron microscopy	
TET	Tetracycline	
<i>tet</i> (A)	Tetracycline resistance gene A	
TMP	Trimethoprim	
TSA	Tryptic soya agar	
TTSS	Type III secretion systems	
USA	United States of America	
USDA	United States Department of Agriculture	
WHO	World Health Organization	

1. REVIEW OF THE LITERATURE

Preface

The freshwater catfish *Pangasianodon hypophthalmus* has grown into a global giant faster than any other aquaculture species in history. *Pangasianodon* production was more than one million metric tons in 2007, a target originally set for 2010 (FAO Globefish 2007). It is widely recognized that culture intensification often leads to disease problems. Bacterial pathogens cause the most overall disease problems and infections with Gram-negative bacteria often result in high mortality in finfish (Austin and Austin 1999; USDA 2003). Of the infectious diseases diagnosed in catfish, Bacillary Necrosis of *Pangasianodon* (BNP) caused by *Edwardsiella ictaluri* is the most frequently occurring (USDA 2003; Crumlish et al. 2002). Besides the Vietnamese freshwater production, the American channel catfish (*Ictalurus punctatus*) industry also suffers massively from *E. ictaluri* infections which have been termed "Enteric Septicemia of Catfish" (ESC). Both diseases are responsible for important economic losses to the catfish industry across the world (Plumb 1999; Wagner 2002; Crumlish et al. 2002). Problems associated with epizootics include high mortality rates, increased susceptibility to disease and high treatment costs.

This indigenous fish species is high in demand from global consumers. Antimicrobial agents are widely used for treatment of bacterial infections, including ESC and BNP. The continued use of antimicrobial agents increases the risks for the presence of antibiotic residues in fish meat and fish products. It also results in the emergence of antimicrobial resistance in fish-associated bacteria and bacteria in the aquatic environment. These may transfer their antimicrobial resistance determinants to bacteria of terrestrial animals and human beings, including pathogens (Sørum 2006; Cabello 2006). Therefore, emphasis should be focused on preventive measures to control disease. A prerequisite to achieve this, is the understanding of the mechanisms through which the bacterium interacts with its host and causes disease. However, little information is available in the literature relating to the pathogenesis of *E. ictaluri* infections and most of these publications deal with the pathogenesis of ESC.

In this Chapter, the main characteristics of *E. ictaluri* are first reviewed. Thereafter, the epidemiology, clinical signs, pathogenesis, diagnostic procedures, treatment and control strategies of BNP and ESC are considered. Finally, antimicrobial resistance with emphasis on fish-associated and aquaculture environmental bacteria is discussed. Special attention is given to tetracycline resistance because resistance to this antimicrobial agent was specifically investigated in the experimental studies.

1.1. Edwardsiella ictaluri infections in catfish: a review

1.1.1. The agent

Taxonomy

The genus *Edwardsiella* was first described in 1965 by Ewing et al. (1965) and harboured a collection of 37 strains biochemically distinct from other taxa within the family *Enterobacteriaceae*. A Japanese group studying bacterial isolates from reptiles identified a distinct yet homogeneous group, referred to as the Asakusa group (Sakazaki et al. 1962), which appeared to be very similar to the newly described species *Edwardsiella tarda*. It was not until 1980 that a second species, *Edwardsiella hoshinae*, was added to this genus by Grimont et al. (1980) who isolated it from reptiles and birds. Finally, Hawke et al. (1981) added the last species to the genus, *Edwardsiella ictaluri*, which was isolated from diseased channel catfish (*Ictalurus punctatus* Rafinesque) (Holt et al. 1994).

Morphology, isolation and identification

Edwardsiella ictaluri has been described as a small straight rod, measuring $1\mu m \ge 2-3\mu m$ (Figure 1) (Plumb 1993). However, Vietnamese isolates may be more heterogenous in length and width, often forming very large rods (Crumlish et al. 2002) (Figure 2). It is weakly motile with peritrichous flagellation.

The biochemical characteristics of *E. ictaluri* were studied by Waltman et al. (1986), who tested 119 isolates of *E. ictaluri* and found that most of the isolates were positive for methyl red, nitrate reductase, lysine decarboxylase, ornithine decarboxylase and catalase. In addition, 100% of the isolates were negative for citrate, malonate, Voges-Proskauer, phenylalanine, indole production, arginine dihydrolase, cytochrome oxidase, β -galactosidase, and many carbohydrates (Table 1). *E. ictaluri* was originally characterized as urease negative, but Booth (2006) recently described an acid activated urease gene that is involved in pathogenesis in the catfish host. Other important features are non reaction on most of sugars and susceptibility to NaCl concentrations higher than 1.5% in culture medium (Shotts and Teska 1989).

Colonies of *E. ictaluri* are smooth, of circular shape and do not produce pigment (Figure 3). It has been reported that bacteria grow on brain heart infusion (BHI) agar at a slow rate. They require 48 hours at 28°C to produce white pin-point colonies of approximately 2 mm in diameter. Optimal growth occurs between 25-30°C, but not at higher temperature (Hawke et al. 1981; Shotts and Teska 1989). On primary culture, the colonies are easily overgrown by contaminating bacteria (Francis-Froyd 1987).

In order to diminish the number of contaminating bacteria, and in this way to enhance *E. ictaluri* isolation, a selective medium can be used, more specifically *E. ictaluri* agar (EIA) (Shotts and Waltman 1990). It contains tryptone 1%, yeast 1%, phenylalanine 0.125%, ferric ammonium citrate 0.12%, bile salts 0.1%, bromothymol blue 0.003% and agar 1.5%. The pH is adjusted to 7.0. After autoclaving and cooling to 50°C, 0.35% (v/v) filter sterilized mannitol and 10 μ g/ml colistin sulphate are added. The organism forms small, translucent, greenish colonies on EIA.

Numerous studies reported that *E. ictaluri* appears to be a rather homogeneous species biophysically, biochemically and serologically. Almost no differences in these characteristics were found among many isolates of *E. ictaluri* from a variety of fish species from different geographical regions (Waltman et al. 1986; Plumb and Vinitnanthanrat 1989; Rogers 1981; Plumb and Klesius 1988; Bertolini et al. 1990; Vinitnanthanrat and Plumb 1993). Chen and Light (1994) reported no cross reactivity of *E. ictaluri* specific antibodies with nine other fish bacterial pathogens, nor did fish immunized with these nine pathogens develop antibody titres to *E. ictaluri*. In contrast, Lobb and Rhoades (1987) reported some serological and plasmid differences in various strains of *E. ictaluri*. This was later confirmed by Lobb et al. (1993), particularly in isolates from non-ictalurid fishes.

Characteristic		
Motility at:		
25°C	+	
35°C	-	
Growth at 40°C	-	
Tolerance of NaCl:		
1.5%	+	
4.0%	-	
Cytochrome oxydase	-	
Indole	-	
Methyl red	-	
Citrate (Christensen's)	-	
H2S production on:		
Triple sugar iron	-	
Peptone iron sugar	-	
Lysine decarboxylase	+	
Ornithine decarboxylase	+	
Malonate utilization	-	
Gas from glucose	+	
Acid production from:		
D-mannose, maltose	+	
D-mannitol, sucrose	-	
Trehalose	-	
L-Arabinose	-	
Jordan's tartrate	-	
Nitrite from nitrate	+	
Mol % G + C of DNA	56 - 57	

Table 1. Biochemical characteristics of Edwardsiella ictaluri



Figure 1. *E. ictaluri* isolated from American catfish (Gram stain x 1,000).

Figure 2. Vietnamese isolates of *E. ictaluri* show much higher variability in length and width, often with very large rods (Gram stain x 1,000).



Figure 3. Colonies of *E. ictaluri* are smooth and of circular shape on Tryptic Soya Agar after 48 hours incubation at 28°C.

1.1.2. Prevalence and epidemiology

In Vietnam, BNP in freshwater catfish (*Pangasianodon hypophthalmus*) may occur in fish of all ages, although especially fingerlings and juvenile fish seem to be affected. Disease occurs mainly during the rainy season when water temperatures are in the range of 23-30°C. In general, crowding, mixing and adverse climatic conditions are accounted as risk factors for development and spread of the BNP (Crumlish et al. 2002; Yuasa et al. 2003).

In the USA, ESC in channel catfish (*Ictalurus punctatus*) which is also caused by *E. ictaluri*, primarily occurs in the autumn and spring when water temperatures are between 18 and 28°C (Francis-Floyd et al. 1987). ESC accounts for approximately 60% of all mortality in farmed channel catfish, which results in approximately 50 million dollars in annual losses (Mitchell 1997).

Epizootics associated with acute septicemia from *E. ictaluri* have only been observed in channel catfish, although several authors described natural infections with this pathogen in other species including white catfish (*Ameiurus catus*) (Plumb and Sanchez 1983); Japanese eel (*Anguilla japonica*) (Chung and Kou 1983); striped catfish (*Pagasius hypophthalmus*) (Crumlish et al. 2002, Yuasa et al. 2003); walking catfish (*Clarias batrachus*) (Kasornchandra et al. 1987); green knifefish (*Eigemannia virescens*) (Kent and Lyons 1982); the Bengal danio (*Danio devario*) (Waltman et al. 1985); Madtom (*Noturus gyrinus*) (Klesius et al. 2003) and rainbow trout (*Oncorhynchus mykiss*) (Keskin et al. 2004). Furthermore, fish species that have been experimentally infected include blue catfish (*I. furcatus*) (Plumb 1999), white catfish (*Ameiurus catus*), brown bullhead (*A. nebulosus*) (Plumb and Sanchez 1983; Iwanowicz 2006) and zebrafish (*Danio rerio*) (Petrie-Hanson et al. 2007).

Originally, Hawke (1979) suggested that *E. ictaluri* is an obligate pathogen not surviving in water for longer than eight days. However, another study found that the organism could survive for up to 90 days at 18°C and 25°C when inoculated into sterilized pond-bottom mud (Plumb and Quinlan 1986). Earlix (1995), on the other hand, demonstrated that *E. ictaluri* does not survive well in water or mud containing other bacteria, due to microbial competition.

A main transmission route of *E. ictaluri* is by transfer of infected fish. Fish that survived an *E. ictaluri* infection may carry this bacterium during prolonged periods of time and may serve as reservoirs of the pathogen (Mqolomba and Plumb 1992; Klesius 1992). In ponds where the fish died from an *E. ictaluri* infection, high *E. ictaluri* counts were found in the water in the vicinity of dead fish (Earlix 1995). These carcasses, as well as infected fish, can also be transported between ponds or fish farms by fish-eating birds and terrestrial animals. Other infection sources

include seines and nets that are not disinfected or thoroughly air-dried. Vertical transmission from brood fish to offspring during spawning is likely to occur but as yet unproved.

Various environmental stress factors such as poor water quality, low chloride concentration and temperature fluctuations, as well as stress induced by handling, close confinement and improper diet may all enhance susceptibility of catfish to infection (Ciembor et al. 1995; Mqolomba and Plumb 1992; Plumb and Sanchez 1983; Wise et al. 1993a).

Severity of an outbreak is variable and is influenced by several factors including water temperature and feeding regime. Mortality among catfish is the highest when temperatures range from 18°C to 28°C (Plumb 1999) and there is a direct relationship between feeding frequency and the severity of disease (Wise and Johnson 1998).

1.1.3. Clinical signs and lesions

When BNP was first observed in 1999 in farmed *Pangasius hypophthalmus* in Vietnam, farmers reported high mortality in fish that had white spots on their internal organs (Figure 4). Lesions associated with bacillary necrosis of *Pangasius*, are indeed characterized by multifocal irregular white spots of varying sizes on several organs including liver, spleen and kidney (Ferguson et al. 2001). Externally, diseased fish seem to be normal, however, as compared to healthy fish, the body color of diseased fish may be pale (Figure 5).

Changes in fish behavior may be observed, including reduced appetite and immediately before death, fish may swim slowly at the surface of the water.

ESC in channel catfish may occur in an acute form (Figure 6), which is characterized by enteritis and septicemia with rapid mortality as early as 2 days post-exposure, and a chronic form, which is characterized by meningoencephalitis with "hole-in-the-head" lesions (Figure 7), seen at 3-4 weeks post-exposure (MacMillan 1985; Miyazaki and Plumb 1985; Shotts et al. 1986; Newton et al. 1989). This chronic form may also be characterized by exophthalmia. Subacute infections may be seen as well, with lower mortality rates than during acute infections. Disease progress depends on fish condition, water quality and especially environmental temperature. Fish less than 1 year old are more susceptible than older fish. Disease usually is more acute within the optimal temperature range of 22°C to 28°C and more chronic outside that range (MacMillan 1985; Francis-Floyd et al. 1987).

Natural outbreaks of ESC in channel catfish have been described by Jacrboe et al. (1984) and Blazer et al. (1985). Some clinical signs of ESC are virtually pathognomonic. Affected fish demonstrate reduced feeding activity. They may swim erratically in tight circles or hang listlessly in the water column in a head up and tail down position. This is usually rapidly followed by death.

External lesions of ESC include small red and white ulcers covering the skin, petechial haemorrhage around the mouth, base of fins, or ventral and lateral surfaces, pale and swollen gills, exophthalmia, and a swollen abdomen due to the accumulation of ascitic fluids (Areechon and Plumb 1983; Jacrboe et al. 1984; MacMillan 1985; Hawke et al. 1998). A blood tinged or clear yellowish ascitic fluid is indeed a hallmark of acute septicemia. The intestine contains clear red fluid and is partially filled with gas. Other internal lesions include petechial haemorrhage in the muscles, intestine, fat and liver. The liver is friable with characteristic pale foci of tissue necrosis, and there is massive necrosis in the spleen and kidney (Hawke 1979; Areechon and Plumb 1983; Jacrboe et al. 1985; Matman et al. 1985).



Figure 4. White spots in internal organs of fingerling *Pangasianodon* catfish with BNP.

Figure 5. External clinical signs of BNP. A fingerling *Pangasianodon* catfish seems normal, but exhibits a pale body colour.

Figure 6. Petechial haemorrhages and ulcers on the ventral abdomen of a channel catfish with ESC.

Figure 7. Open lesion on the cranial region and exophthalmia typical of a chronic infection of channel catfish with ESC.

1.1.4. Pathogenesis

Although *E. ictaluri* is the leading cause of mortality in both channel and *Pangasianodon* catfish culture, until now little information exists relating to the pathogenesis of *E. ictaluri* infections. Most publications deal with the pathogenesis of ESC and information concerning the pathogenesis of BNP is almost completely lacking.

Portal of entry

E. ictaluri may enter the host via various routes and the portal of entry may influence the course of the disease. Morrison and Plumb (1994) described that exposure of the olfactory organ of fish to *E. ictaluri* may initiate chronic ESC. In a study of Nusbaum and Morrison (1996), acute disease appeared 5-7 days post immersion exposure of channel catfish and in this case gills were thought to be the portal of entry. The authors reported that very few or no bacteria were found to accumulate in the nares or the gut, while large numbers of bacteria were consistently found associated with gill tissue.

Various other research groups confirmed the gill as a primary site for *E. ictaluri* invasion (Ciembor et al. 1995; Nusbaum and Morrison 2002). Gills are highly vascularized with a large number of blood capillaries for respiratory functions. This may make these organs good entry sites from where bacteria may be disseminated throughout the body. In other fish pathogens, such as *Vibrio anguillarum* and *E. tarda*, gills have also been reported as a site of entry (Baudin-Laurencin and German 1987; Ling et al. 2001).

As for other bacteria belonging to the *Enterobacteriaceae*, the gastrointestinal tract may also be a port of entry for *E. ictaluri* (Newton et al. 1989; Baldwin and Newton 1993).

Virulence factors

Extracellular products

Extracellular products probably play a major role in the pathogenesis of ESC and BNP (Stanley et al. 1994).

Waltman et al. (1985) reported that most *E. ictaluri* isolates have hemolytic activity over a wide range of temperatures. The hemolysin is an extracellular toxic protein which may be involved in iron acquisition. It may also aid in penetration of mucosal layers, allow intracellular survival and assist the bacterium in spreading through the host. Production of hemolysin by *E. ictaluri* may contribute to reduced hematocrit, hemoglobin, plasma protein, and plasma glucose associated with ESC infections (Waltman et al. 1986). However, the correlation between hemolytic activity and virulence is not consistently found in *E. ictaluri*

isolates (Stanley et al. 1994; Williams and Lawrence 2005). Two hemolysin genes of *E. ictaluri*, designated *eihA* and *eihB* were identified by William and Lawrence (2005). These genes have high identity to the iron regulated two-component hemolysin genes of *E. tarda*. The hemolysin of *E. ictaluri* is a member of the *Serratia* family of two-component hemolysins (William and Lawrence 2005) which include shlB, secretion/activation proteins and shlA, cytolysin proteins.

Chondroitinase activity is known as a possible contributor to virulence of several fish pathogens. Waltman et al. (1985) demonstrated that all *E. ictaluri* isolates tested were capable of degrading chondroitin sulfate, a main component of cartilage. Hence, chondroitinase may play a role in the formation of the "hole-in-the-head lesion" in affected fish (Shotts et al. 1986). Actually, according to Stanley et al. (1994), virulent *E. ictaluri* isolates showed a higher ability to degrade chondroitin sulfate than avirulent isolates. This was also noted by Cooper et al. (1996), who used transposon mutagenesis to create stable *E. ictaluri* transposon mutants, that are deficient in chondroitinase activity. Channel catfish injected with these mutant strains did not develop disease signs, while injection of channel catfish with the virulent parent strain resulted in severe disease and high mortality.

Lipopolysaccharides, adherence and invasion

Lipopolysaccharides, major components of the outer membrane of Gram-negative bacteria, act as endotoxin. Their O-polysaccharide chains are involved in resistance to complementmediated killing (Allen et al. 1998; Amaro et al. 1997; Merino et al. 2000) and opsonization/killing by phagocytes (Burns & Hull 1999; Engels et al. 1985; Price et al. 1990).

E. ictaluri O-polysaccharide may play a role in adherence to host mucosal surfaces as well (Lawrence et al. 2003). It contains N-acetylgalactosamine and galactose in a 2:1 ratio. *E. ictaluri* is agglutinated by a galactose-specific lectin derived from *Ricinus communis* (Ainsworth, 1993). This agglutination is prevented by preincubation of the lectin with galactose. These findings indicate that galactose on the bacterial cell surface may play a significant role in adherence *in vivo*. Moreover, preincubation of catfish olfactory mucosa with soluble D-galactose significantly reduced adhesion of *E. ictaluri* to cell surfaces (Wolfe et al. 1998).

Wong et al. (1989) described two different hemagglutinins in *Edwardsiella* species. One was inhibited by D-mannose (mannose-sensitive hemagglutination-MSHA) and detected in *E. tarda, E. hoshinae* and *E. ictaluri*. A second type of hemagglutinin was not inhibited by this sugar but blocked by the glycol-protein fetuin (mannose-resistant hemagglutination-MRHA). It was

mainly associated with *E. tarda* and only few of the *E. ictaluri* strains demonstrated MRHA activity. Skirpstunas and Baldwin (2003) described four major outer membrane proteins in *E. ictaluri* with molecular weights of 22, 31, 59 and 72 kilodaltons (kDa), which may be involved in initial bacterial-host cell interactions. The exact role of these proteins and of the hemagglutinins mentioned above in pathogenesis of ESC and BNP remains, however, to be elucidated.

Silva (1998) reported that *E. ictaluri* is able to invade a channel catfish ovary cell line and to replicate in these cells. Skirpstunas and Baldwin (2002) demonstrated that *E. ictaluri* isolates from ESC may invade IEC-6 (rat small intestinal epithelium) cells, Henle 407 (human embryonic intestinal epithelium) cells and FHM (fat head minnow) cells.

Booth et al. (2006) stated that an acid-inducible urease enzyme of *E. ictaluri* may play a role in the bacterium's capacity to intracellularly survive and replicate in channel catfish macrophages. In a signature-tagged mutagenesis study, Booth et al. (2006) confirmed the role of urease activity in the pathogenesis of *E. ictaluri* infections. Disruption of *ureG*, an essential gene for urease activity, resulted in loss of the ability to colonize or to cause pathology in channel catfish.

Acid resistance

Acid resistance mechanisms have been reported to play a role in survival of several bacterial species in an environment with acid pH such as the stomach and intracellularly in phagocytes.

In *E. coli*, different systems have been described that play a role in acid resistance. One of these, acid resistance system 3, utilizes the inducible form of arginine decarboxylase (AdiA) to decarboxylate arginine to agmatine in a process that also consumes intracellular protons (Gong et al. 2003). Interestingly, agmatinase converts agmatine to putrescine and urea (Salas et al. 2002). Thune et al. (2007) reported that AdiA may be involved in the *de novo* synthesis of urea, which is metabolized to ammonia by the urease enzyme, resulting in an increase in environmental pH. The acid resistance system 3 is also functional in *E. ictaluri* but its exact role in pathogenesis of ESC and BNP is not yet clear.

Plasmids and type III secretion system

E. ictaluri strains from channel catfish may harbor several plasmids but their involvement in virulence is not exactly known (Lobb and Rhoades 1987; Newton et al. 1988; Reid and Boyle 1989; Lobb et al. 1993). Fernandez et al. (2000) showed that two small plasmids, pEI1 and pEI2 carry genes encoding proteins with leucine-rich repeat motifs. Such proteins normally play a role in type III secretion systems (TTSS) associated with the transfer of effectors

proteins directly from bacteria to the cytosol of target host cells, often with associated invasion of the host cell by the pathogen (Hueck 1998). Thune et al. (2007) identified a 41,205-bp fragment in the *E. ictaluri* genome containing a 26,135-bp pathogenicity island with 33 genes encoding a TTSS similar to the TTSS encoded on the *Salmonella* Pathogenicity Island 2 which is involved in intracellular survival and replication of *Salmonella* (Cirillo et al. 1998; Hensel M. 2000; Hensel et al. 1998).

1.1.5. Diagnosis

E. ictaluri does not require special nutrients but its growth rate is rather slow. Therefore, in mixed cultures, *E. ictaluri* can be overgrown by more rapidly growing bacteria.

Although brain-heart infusion agar (BHI) or tryptic soya agar (TSA) may be used for isolation of *E. ictaluri* from infected fish, the use of *E. ictaluri* agar (EIA) is advisable if samples are taken from heavily contaminated environments (Shotts and Waltman 1990). The latter medium inhibits Gram-positive and most Gram-negative contaminating organisms. Optimal temperature for incubation is 28-30°C. On EIA, *E. ictaluri* forms small, translucent, greenish colonies, while colonies of *E. tarda* have black centres, *Aeromonas hydrophila* colonies are brownish and larger, and *Pseudomonas fluorescens* colonies are blackish and punctuate.

For identification of *E. ictaluri*, the API 20E system is often used although it is not as accurate for *E. ictaluri* as for some other fish pathogens (Taylor et al. 1995; Topic Popovic et al. 2007).

Specific monoclonal antibody or polyclonal antisera produced in rabbits (Ainsworth et al. 1986; Klesius and Horst 1991; Rogers 1981) may be useful for identification of *E. ictaluri* (Bertolini et al. 1990; Plumb and Klesius 1988; Saeed and Plumb 1987). Slide agglutination, fluorescent antibody techniques (FATs), enzyme-linked immunostaining and enzyme-linked immunosorbent assays (ELISAs) have all been used to provide confirmatory diagnosis.

For identification of *E. ictaluri* isolates or direct demonstration of the agent's DNA in tissue of affected fish, molecular techniques such as the polymerase chain reaction (PCR) may be used. PCR approaches using the 16S rDNA as target gene, coupled with restriction enzyme analysis of the amplified fragment, have been proved to be highly specific and sensitive for the detection of *E. ictaluri* DNA in fish tissues and in blood (Bilodeau et al. 2003; Panangala et al. 2005; Zhang and Arias 2007). In 2003, a real-time PCR for rapid detection of *E. ictaluri* was developed (Bilodeau et al. 2003). It is a promising technique for rapid diagnosis since it is capable of detecting the equivalent of as few as 2.5 cells of the pathogen in less than 5 hours from the time of sample collection. Recently, a multiplex PCR was developed allowing

simultaneous detection of 3 important fish pathogens in channel catfish aquaculture industry, namely *Flavobacterium columnare*, *E. ictaluri* and *A. hydrophila* (Panangala et al. 2007).

1.1.6. Treatment and disease control

Control of *E. ictaluri* disease in catfish is mainly accomplished by management actions, treatment and vaccination.

Management measures to control BNP and ESC include reduction of stress in fish and cessation of feeding when BNP or ESC induced losses are detected (Wise and Johnson 1998). It has been demonstrated that some channel catfish strains are less susceptible to ESC. Channel catfish x blue catfish hybrids are also less susceptible and might be introduced in farms where *E. ictaluri* is endemic (Wolters and Johnson 1994; Wolters et al. 1996, Camp et al. 2000). In 1995, Paripatananont and Lovell demonstrated that supplementation of diet with zinc or vitamins may alter susceptibility of channel catfish to *E. ictaluri*. Channel catfish fed no zinc had 100% mortality, compared with 25-30% mortality in animals receiving 15-30 mg of zinc kg-1 of fish.

Both BNP and ESC may be controlled by oral administration of antimicrobial agents. The most common antimicrobial treatments used are oral application of sulfadimethoxine-ormethoprim or oxytetracycline (Waltman and Shotts 1986; Plumb et al. 1987; Crumlish et al. 2002; Sarter et al. 2007). Florfenicol has also been used for treatment of ESC (Gaikowski et al. 2003; Gaunt et al. 2004; McGinnis 2003). Antimicrobial treatments are expensive and usually only effective if given early in a BNP or ESC outbreak, as sick fish do not eat. An additional disadvantage of the excessive use of antimicrobials is that it favours spread of antimicrobial resistance in fish-associated and aquatic environmental bacteria (Waltman et al. 1989; Cooper et al. 1993).

In 1991, a commercial whole-cell bacterin was provisionally licensed in the USA for control of ESC. However, several studies demonstrated that bacterins are not very efficacious and they do not induce immunity of long duration against *E. ictaluri* infection (Shoemaker and Klesius 1997; Thune et al. 1997). Therefore, this vaccine is no longer marketed. Attenuated vaccines may induce more effective immune responses (Klesius and Shoemaker 1999; Lawrence et al. 1997; Thune et al. 1999; Wise and Terhune 2001).

Age-related factors and the induction of a cellular immune response could be of critical importance in inducing strong anti-*E. ictaluri* defences. In 1999, Petrie-Hanson and Ainsworth found that catfish fry under 3 weeks of age are immunologically unresponsive to

E. ictaluri. This is also thought to be due to poorly developed lymphoid organs in the young fish (Petrie-Hanson and Ainsworth 2001).

1.2. Antimicrobial resistance with emphasis on fish-associated and aquaculture environmental bacteria

For more than 50 years, antimicrobial agents have been used to control bacterial infections in humans, animals, and plants. They were already used in the 1950s for controlling bacterial diseases in fish. In the early days of antimicrobial chemotherapy, antimicrobial resistance was not considered an important problem, since new highly effective antimicrobial agents of different classes were regularly discovered. However, it generally took not longer than 1 to 5 years after introduction of an antimicrobial agent before the first resistant target bacteria were detected (Schmit et al. 2001). Nowadays, antimicrobial agents are among the most frequently used therapeutics in human and veterinary medicine and acquired antimicrobial resistance has become a huge problem (Schwarz and Chaslus- Dancla 2001).

1.2.1. General mechanisms of antimicrobial resistance

For an antimicrobial agent to be effective against a given micro-organism, three conditions must be met: (i) a vital target susceptible to a low concentration of the antimicrobial agent must exist in the bacterium, (ii) the antimicrobial agent must be able to penetrate the bacterium surface and to reach the target in sufficient quantity and (iii) the antimicrobial agent must not be inactivated or extruded before binding to the target. Bacteria can evade antimicrobial action and hence develop resistance by means of five mechanisms: (i) enzymatic inactivation or modification of the drug before or after entering the cell, (ii) alteration of the envelope, making the bacterial cell less permeable, (iii) increased efflux of the drug, (iv) modification of the target resulting in less avidly binding with the antimicrobial compound, (v) bypassing the target by acquisition of a novel metabolic pathway (Struelens 2003).

1.2.2. Intrinsic and acquired resistance

Resistance of bacteria to antimicrobial agents may be intrinsic or acquired. Intrinsic or innate resistance means that each member of an entire bacterial species is resistant without any additional genetic alteration. *Mycoplasma* species, devoid of a cell wall, are for example naturally insensitive to β -lactam antibiotics which operating mode is the inhibition of the cell wall synthesis. By contrast, resistance may be acquired by some strains within a species usually susceptible to the antimicrobial agent under consideration. Acquired antimicrobial

resistance occurs either by mutations within indigenous genes or by horizontal transfer of resistance genes.

A quantitative measurement of bacterial sensitivity to a specific antimicrobial agent can be made by determining minimal inhibitory concentrations (MICs). Unfortunately, antimicrobial activity *in vitro* is not always reflected *in vivo*. The resulting clinical resistance relates to many confounding factors such as the inaccessibility of the infection site for the antimicrobial agent and thus can not always be fully predicted from the susceptibility *in vitro* (Normark et al. 2002; Struelens 2003).

1.2.3. Genetic basis of acquired resistance

Mutational resistance

Mutation of chromosomal genes involves deletion, substitution, or addition of one or a few base pairs, causing slight changes in the amino acid sequence of the resulting peptide. These sequence alterations usually have little or no influence on the biological activity, but they may result in a gene product with reduced affinity for the antimicrobial agent. Mutational resistance may also involve regulatory regions, leading to overproduction of detoxifying systems. Single step mutations leading to full resistance occur, but are clinically not as important as resistance that is gradually built up by several successive mutations.

Although the basal rate of mutation is low in bacterial genomes, the mutation frequencies to resistance may vary depending on the mechanism of resistance and efficiency of error correcting DNA repair systems of the organism. Strains with a so-called mutator phenotype exhibit a much higher mutation frequency to resistance against a number of antimicrobial agents than normal for that species.

Resistance due to mutations may result in a fitness cost: resistant strains, selected during exposure to antimicrobial agent, usually show decreased fitness for competing with the wild type ancestor in the absence of the selecting antimicrobial agent. However, fitness cost may be compensated by secondary mutations but not necessarily affecting the target protein, thereby ensuring the persistence of the mutation (Normark et al. 2002; Struelens 2003).

Transferable resistance

Horizontal spread of resistance genes may occur by three different mechanisms: conjugation, transformation and transduction. The acquisition of resistance by transduction, DNA transfer via bacteriophages, is rare in nature. Only a few bacterial species are naturally competent for

transformation, meaning that they are able to take up and integrate exogenous free DNA from the environment originating from lysed donor bacteria (Struelens 2003). Conjugation is thought to be the most important way of exchanging DNA. This route of resistance transfer is mediated by self-transmissible genetic elements that are transported from a donor bacterium to an acceptor bacterium via a protein tunnel (Davies 1994). There are a number of different DNA elements described transferring antimicrobial resistance: plasmids, transposons and gene cassettes/integrons. These types of elements are composed of double-stranded DNA, but differ distinctly in size, structure, biological property as well as way of spreading (Schwarz and Chaslus-Dancla 2001).

Conjugation may occur between bacterial strains of the same species, within species of the same genus, or even between species belonging to different genera or families. Some conjugative transposons of Gram-positive bacteria are known to be promiscuous and will transfer to many other types of Gram-positive and even Gram-negative bacteria. Bacteria that are resistant to one antimicrobial agent are more likely to become resistant to other antimicrobial agents. The reason for this is not clear. It might be related to the presence of mutational defects in DNA mismatch repair mechanisms, making these strains more prone both to mutation and to promiscuous exchange of DNA between species (Haesebrouck et al. 2002).

1.2.4. Plasmids, transposons, gene cassettes and integrons

Plasmids

Plasmids are extra-chromosomal circular fragments of DNA that replicate autonomously in a host cell. They are present in nearly all bacterial species and vary in size from a few to more than several hundred kilobase pairs (kb) (Schwarz and Chaslus-Dancla 2001). Plasmids appear to increase bacterial genetic diversity, acquiring and losing genes, and can be horizontally exchanged among bacterial populations by conjugation or mobilization. A plasmid replicates independently of the chromosome, and the replicated copies are usually distributed among the daughter cells when the "mother" cell divides. The number of copies varies among plasmids and bacterial cells may harbor more than one plasmid type as long as they belong to different incompatibility groups (Schwarz and Chaslus-Dancla 2001).

Two decades ago, Novick (1987) described a formal scheme of plasmid classification based on incompatibility (Inc) groups. The procedure for incompatibility grouping is based on the introduction, by conjugation or transformation, of a plasmid of an "unknown" Inc group into a strain carrying a plasmid of a known Inc group. If the resident plasmid is eliminated in the progeny, the incoming plasmid is assigned to its same Inc group (Datta and Hedges 1971). Plasmids with the same replication control are "incompatible", whereas plasmids with different replication controls are "compatible". On this basis two plasmids belonging to the same Inc group cannot be propagated in the same cell line (Datta and Hughes 1983; Couturier et al. 1988). Inc group identification has been frequently used to classify plasmids.

Bacterial plasmids can be divided in self-transmissible and non-transmissible plasmids. Nontransmissible plasmids can be subdivided into mobilizable and non-mobilizable plasmids. Self-transmissible or conjugative plasmids carry *tra* genes required for transfer. These plasmids are usually quite large, because of the large number of genes needed for conjugation. The *tra* gene products are involved in DNA metabolism, DNA transport and cell-cell interaction.

Non-transmissible plasmids, lacking the genes required for transfer, which co-reside in the same host cell, may use the transfer apparatus provided by the conjugative element, as long as they have the origin of transfer (*oriT*) of the self-transmissible plasmid. This process is known as mobilization. Mobilizable plasmids possess *mob* genes, which are analogous to *tra* genes. Their products increase the range of self-transmissible plasmids by which their plasmid can be mobilized (Snyder and Champness 1997).

Non-mobilizable plasmids do not have an oriT side from a self-transmissible plasmids and can not be transferred via conjugation. These plasmids apply transformation as mechanism of transfer (Sneyder and Champness 1997).

Transposons

Transposons are mobile genetic elements capable of mediating the transfer of DNA by removing or inserting themselves into the host chromosomal and plasmid DNA within one bacterial cell. Transposons comprise genes that encode transposases, the enzymes that promote transposon movement or transposition, and genes that regulate the transposition. In addition to these genes, bacterial transposons may contain inverted repeats on each end. In contrast to plasmids, transposons do not possess a replication system and therefore must integrate for their stable maintenance into a vector molecule such as chromosomal DNA or plasmids in the cell. Some transposons can only transpose within one cell or they can enter other cells via conjugation using the transfer system of conjugative plasmids (Schwarz and Noble 1999).

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Transposons also vary in size and structure. The smallest transposons, also known as insertion sequences, encode little more than transposase enzymes. Larger transposons usually carry one or more additional genes, most of which code for antimicrobial resistance properties (Schwarz and Chaslus-Dancla 2001).

Gene cassettes and integrons

Gene cassettes are the smallest known type of mobile elements and generally consist of only a specific recombination site and a single gene which is in most known cases an antimicrobial resistance gene. Gene cassettes differ from plasmids by the lack of replication systems. They move by site-specific recombination. In general, gene cassettes are integrated into a larger genetic structure known as an integron (Schwarz and Chaslus-Dancla 2001).

Integrons commonly consist of a 5' and a 3' conserved region, bracketing the integrated gene cassettes. The 5' conserved region codes for the integrase that is responsible for the expression of the gene cassettes-borne gene (Schwarz and Chaslus-Dancla 2001). Integrons are widespread among diverse Gram-negative species and also have been reported in Grampositive bacteria. They are found associated with transposons and conjugative plasmids (Struelens 2003).

1.2.5. Resistance to tetracyclines

According to Levy (1984), about 50 years ago, tetracycline resistance was rare in pathogenic bacteria and bacteria belonging to the normal microflora. It has been recorded that only 2% of a collection of 433 *Enterobacteriaceae* isolated between 1917 and 1954 were resistant to tetracycline. Nowadays, resistance towards tetracyclines frequently occurs in these bacteria (Chopra and Roberts 2001).

Tetracyclines are a family of antibiotics that inhibit protein synthesis by preventing the attachment of aminoacyl tRNA to ribosomal acceptor (A) site (Chopra and Roberts 2001). They are broad-spectrum agents that include tetracycline, chlortetracycline, doxycycline and minocycline. Due to its broad-spectrum activity, low toxicity and low cost, oxytetracycline is widely used against various diseases caused by Gram-negative and Gram-positive bacteria. Particularly, since the Food and Drug Administration (FDA) approved the use of oxytetracycline (OTC) for humans and aquatic animals, the use of tetracyclines for the treatment of fish diseases in aquatic farms has increased and has contributed to the spread of tetracycline resistance in bacteria that are pathogenic for fish (DePaola et al. 1988; Schmidt et al. 2001; Miranda et al. 2003).

The first report on acquired antibacterial resistance in fish pathogenic bacteria dealt with sulfathiazole and tetracycline resistance in *Aeromonas salmonicida* isolated from brook trout in the United States in 1959 (Ewing et al. 1961).

Tetracycline resistance determinants from different bacterial species isolated from aquaculture and aquatic environments have been characterized in different geographical areas in the world (Aoki and Takahashi 1987; Andersen and Sandaa 1994; DePaola and Roberts 1995; Adams et al. 1998; Schmidt et al. 2001; Miranda et al. 2003; Kim et al. 2004; Dang et al. 2006; Jacobs and Chenia 2007).

E. ictaluri isolates obtained from diseased channel catfish (*I. punctatus*) in the southern United States were tested for antimicrobial resistance. None of the 10 strains were found to have acquired resistance (Reger et al. 1993).

Mechanisms of acquired resistance to tetracyclines

Acquired resistance to tetracycline is mediated mainly by two mechanisms: the protection of ribosomes by large cytoplasmic proteins, and the energy-dependent efflux of tetracyclines (Roberts 1996). Tetracycline resistance is most often due to the acquisition of new genes (Chopra and Roberts 2001). So far, there have been 23 efflux genes, which code for energy-dependent efflux of tetracyclines; 11 ribosomal protection genes, which code for a protein that protects bacterial ribosomes; 3 genes that code for enzymes that modify and inactivate the tetracycline molecule, and 1 gene [*tet* (U)] that specifies tetracycline resistance by an unknown mechanism (Chopra and Roberts 2001; Roberts 2001; Roberts 2005)

Energy-dependent efflux

The efflux gene group is one of the major groups that contain *tet* (A)-(E), (G), (H), (K), (L), (Z) and probably (I), (J), (30), (34) and (35) which have been found in both Gram-negative and Gram-positive species (Tauch et al. 2000, Chopra and Roberts 2001; Claudio et al. 2003). However, most of these determinants are not uniformly distributed, and they are often associated with specific bacterial genera and species. *tet*(A)–(E) and (G), are found primarily in Gram-negative bacteria (Chopra and Roberts 2001; Claudio et al. 2003) such as *Vibrio* species from fish (Aoki et al. 1987; Zhao and Aoki 1992) and *E. tarda* (Jun et al. 2004).

Dissemination of the proton-dependent tetracycline efflux protein in aquaculture environments has been well-documented by several authors (Aoki et al. 1987; DePaola et al. 1993; DePaola and Roberts 1995; DePaola et al. 1988; Kim et al. 1994; Rhodes et al. 2000; Schmidt et al. 2001). Furthermore, the relevant genes have been identified by using DNA hybridization or PCR methods (Andersen and Sandaa 1994; DePaola et al. 1993; Depaola et al.

al. 1995; Depaola et al. 1988; Kim and Aoki 1994; Rhodes et al. 2000; Schmidt et al. 2001; Jun et al. 2004; Carattoli et al. 2005; Akinbowale et al. 2007). Transfer of resistance-encoding plasmids carrying *tet* (A) between aquaculture environments and humans has been demonstrated (Rhodes et al. 2000). Several authors reported the wide distribution of the *tet*(A) gene among different bacterial species from different sources. The most prevalent type of tetracycline-resistant determinant in fish pathogens is indeed *tet* (A). Analysis of *E. tarda* isolates obtained from a catfish pond, showed an incidence of approximately 56% of *tet* (A) after oligonucleotide hybridization for three types of *tet* genes, *tet* (A), (B) and (C) (Depaola et al. 1993). Jun et al. (2004) demonstrated the presence of *tet* (A), *tet* (B), *tet* (D) and *tet* (G) genes in *E. tarda* isolates from diseased fish from aquatic farms in Korea. Jacobs and Chenia (2007) reported the presence of *tet*(A), *tet*(B) and *tet*(D) genes were also found in aquaculture systems. The *tet*(A), *tet*(B) and *tet*(D) genes were also found in aquaculture farms in different regions of the world (Aoki and Takahashi 1987; Furushita et al. 2003; Miranda et al. 2003). Generally, most of the authors found that the occurrence of more than one tetracycline gene was common.

Ribosomal protection

Ribosomal protection determinants, although not yet completely elucidated, are abundant and widely distributed in nature (Taylor and Chau 1996). To date, ten classes of determinants encoding ribosomal protection have been identified: tet(M), tet(O), tet(Q), tet(S), tet(W), tet(T), otr(A), tet(32), tet(36) and mosaic tet genes.

In general, *tet* (M) gene, has the widest host range of all *tet* genes. This gene is located on conjugative transposons, such as *Tn*916 (Flannagan et al. 1994; Salyers et al. 1995; Chopra and Roberts 2001).

Kobayashi et al. (2007) detected the tet(M), tet(S), and tet(W) genes in samples from river and channel sediments of the Mekong Delta in Vietnam and this suggests that the Delta is a potential source of tetracycline resistance genes. In a *A. hydrophila* isolate from channel catfish in the southern United States, both tet(A) and tet(E) determinants were found.

Enzymatic inactivation

Enzymatic inactivation of tetracyclines is mediated by three genes (Schwarz et al. 2006). The first described gene is the tet(X) gene. This gene encodes an NADPH-requiring oxidoreductase, which modifies and inactivates the tetracycline molecule in the presence of oxygen (Chopra and Roberts 2001). The tet(37) gene was cloned from the oral cavity of humans and no specific bacteria were identified carrying the gene. It also requires oxygen to

function but is unrelated to *tet* (X) gene (Diaz-Torres et al. 2003). According to Nonaka and Suzuki (2002) a third gene, *tet*(34), encodes an enzyme which inactivates tetracycline and is similar to the xanthine-guanine phosphoribosyl transferase gene of *Vibrio cholerae*.

1.2.6. Exchange of antimicrobial resistant bacteria or their resistance genes between fish, the environment and humans

Recently, public health agencies have raised concerns worldwide about the impact of antimicrobial use in aquaculture on environmental bacteria and, potentially, on human pathogens (FAO 2003; Huys et al. 2005). The widespread use of antimicrobial agents for treating bacterial disease in aquaculture has been associated with increased antimicrobial resistance in Aeromonas hydrophila (Akashi and Aoki 1986; Aoki et al. 1971), A. salmonicida (Aoki et al. 1971; Aoki et al. 1986), E. tarda (Aoki et al. 1977; Waltman and Shotts 1986), E. ictaluri (Waltman and Shotts 1986), Vibrio anguillarum (Aoki et al. 1987), V. salmonicida (Husevag et al. 1991), Photobacterium damselae subsp. piscicida (previously known as Pasteurella piscicida) (Aoki and Kitao 1985) and Yersinia ruckeri (De Grandis and Stevenson 1985). In addition, water, sediments, wild fish and other biological systems are exposed to the antimicrobial drug in a direct manner because the drug is dissolved in the water or spread through particles transported in the water during treatment of fish (Aoki 1975; Samuelsen et al. 1992; Sandaa et al. 1992; Depaola et al. 1995; Le et al. 2005; Kobayashi et al. 2007; Sarter et al. 2007). Particularly, the sediments beneath the fish where feed and fecal material aggregate, function like a potential incubator for the exchange of genes between fish pathogens and the environment (Sørum 2006).

Many researchers found that resistant commensal bacteria may act as a reservoir for resistance genes, which may spread to other bacteria. Transfer of these resistance genes to pathogenic bacteria may cause therapy failure. Some fish associated Gram-negative bacteria such as *A*. *hydrophila* and *E. tarda* are opportunistic human pathogens but they rarely cause infections except in chronically ill individuals (Shotts 1987). However, these bacteria can survive in or colonize human intestines, potentially transferring antimicrobial resistance determinants to the normal microflora or to ingested pathogens.

During the last few years, many studies demonstrated that determinants of antimicrobial resistance emerged in the aquatic environment, and had the potential of being transferred horizontally to bacteria of the terrestrial environment, including human and animal pathogens (Rhodes et al. 2000; Sørum and Sunde 2001; Sørum 2006; Cabello 2006). Transfer of resistant

bacteria or resistance genes from animals to humans may also occur through the food chain (Aoki 1975; Teuber 2001; Cabello 2006).

Mechanisms involved in exchanging resistance determinants between aquatic and terrestrial bacteria include conjugation and conjugative transposition (Agerso and Guardabassi 2005; Casas et al. 2005). Several studies demonstrated that fish pathogens such as *Aeromonas* can transmit and share determinants for antimicrobial resistance with *Escherichia coli* (Rhodes et al. 2000, Sørum and Sunde 2001; Sørum 2006).

IncU plasmids with determinants for tetracycline resistance on *Tn1721* have been described in *A. salmonicida*, *A. hydrophila*, *A. caviae* and *E. coli* isolates obtained from different geographical locations in Europe (Rhodes et al. 2000). Similarly, plasmids containing class 1 integrons and resistance determinants for trimethoprim, sulfonamide and streptomycin have been demonstrated in *A. salmonicida* isolates from fish. These plasmids can be transferred with high frequency to *E. coli* and *Salmonella enterica* (Sørum and L'Abée 2002; Sørum 2006). The sulfonamide-resistance determinant *sul1* has been found on plasmids in *A. salmonicida* and in bacteria of other niches including *Erwinia* (a plant pathogen), *Vibrio cholerae* and *E. coli*, thereby suggesting the transfer of genetic information between bacteria of the terrestrial and aquatic environment (Sørum 2006).

In conclusion, studies mentioned above indicate that fish-associated bacteria as well as bacteria from the aquaculture environment may represent reservoirs of antimicrobial resistance genes for other bacteria, including micro-organisms which are potentially harmful for humans (Sørum 2006). It is therefore crucial that alternative control strategies for bacterial diseases of fish are developed, including vaccination and optimal management routine. This should allow decreasing the use of antimicrobial agents in this animal species.

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2. AIMS OF THE PRESENT STUDIES

Aims of the present studies

The general aims of this thesis consist of studying the antimicrobial resistance of *E. ictaluri* and investigating the pathogenesis of *E. ictaluri* infections in *Pangasianodon* catfish with emphasis on the early stages of the bacterium-host interaction.

The specific aims of the present studies may be defined as follows:

- To investigate the antimicrobial *in vitro* susceptibility pattern of *E. ictaluri* isolates from *P. hypohthalmus* in South-East Asia, more specifically Vietnam, by means of the agar dilution test.
- To investigate the genetic determinants responsible for tetracycline resistance among these *E. ictaluri* isolates, to assess its transferability and to characterize the genes encoding resistance to other antimicrobial agents which are co-transferred during *in vitro* conjugation experiments.
- To study the early stages of the *E. ictaluri-P. hypohthalmus* interaction using an *in vivo* immersion infection model and to assess the *in vitro* ability of *E. ictaluri* isolates to invade Chinook salmon embryo, fathead minnow epithelial, and rainbow trout liver cell lines.

3. EXPERIMENTAL STUDIES

3.1. Antimicrobial susceptibility pattern of *Edwardsiella ictaluri* isolates from natural outbreaks of bacillary necrosis of *Pangasianodon hypophthalmus* in Vietnam

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ABSTRACT

The purpose of this study was to assess the *in vitro* susceptibility of 64 Vietnamese isolates of *Edwardsiella ictaluri*, the causal agent of the infectious disease Bacillus Necrosis Pangasius (BNP) in *Pangasianodon hypophthalmus*, using the agar dilution technique. None of the isolates displayed acquired resistance to amoxicillin, amoxicillin-clavulanic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, neomycin and nitrofurantoin. Acquired resistance to streptomycin was detected in 83%, to oxytetracycline in 81% and to trimethoprim in 71% of the isolates, as indicated by a bimodal distribution of the minimal concentrations (MICs) of these antimicrobials. The MICs of enrofloxacin displayed a gents flumequin and oxolinic acid, acquired resistance was encountered in 8 and 6% of the strains, respectively. All strains were intrinsically resistant to the polypeptide antimicrobial agents three antimicrobial agents. The results of this study emphasize the strict need to control both the prophylactic and curative use of antimicrobial agents in Vietnamese aquaculture.

Keywords: antimicrobial susceptibility testing, Edwardsiella ictaluri, Pangasianodon hypophthalmus, Vietnam.

INTRODUCTION

Total aquaculture production in Vietnam topped one million tonnes in 2003 and is expected to reach over two million tonnes by 2010 (Ministry of Fisheries. 2005). Mekong Delta is the main producer, being responsible for over 80% of the total Vietnamese production. The freshwater catfish *Pangasianodon hypophthalmus* is the most commonly cultured edible fish species in this region. Currently, *P. hypophthalmus* has become the second most important Vietnamese food commodity produced and exported after rice.

Of the several infectious diseases diagnosed in this fish species, Bacillary Necrosis, caused by *Edwardsiella ictaluri*, is the most frequently occurring (Ferguson et al. 2001; Crumlish et al. 2002). This increasingly important disease occurs in production systems of fish of all ages, although especially fingerlings and juvenile fish seem to be affected. This results in severe economic losses through decreased production, expense of treatment and fish mortality.

Besides the Vietnamese freshwater production, the American channel catfish (*Ictalurus punctatus*) industry also suffers massively from *E. ictaluri* infections which have been termed "enteric septicemia of catfish" (ESC). ESC accounts for approximately 60% of all mortality in farmed channel catfish, which results in approximately 50 million dollars in annual losses (Mitchell 1997). Recently, *E. ictaluri* was also identified as the cause of disease in *P. hypophthalmus* in Indonesia (Yuasa et al. 2003). To treat bacterial infections, antimicrobial agents are widely used in Vietnamese aquaculture, both on a preventive and curative basis (Crumlish et al. 2002; Dung et al. 1997; Phuong et al. 2005). In most of the cases, the amount of drugs to be administered merely is an estimation, leading to incorrect dosage (Van 2005). In view of this, besides antimicrobial sensitivity monitoring being necessary for rendering treatment schemes effective, susceptibility testing is also crucial to seize the unavoidable risk associated with antimicrobial misuse, that is, resistance development.

Hitherto, to our knowledge, only a handful of studies have investigated the antimicrobial susceptibility of *E. ictaluri*, always including American isolates. Hawke, (1979) was the first to test the antimicrobial susceptibility of ten *E. ictaluri* isolates. Later on, Waltman and Shotts (1986) screened 118 *E. ictaluri* isolates retrieved in the USA for susceptibility to 37 antimicrobials using the disc sensitivity test. They found that the majority of isolates were susceptible to most agents active against Gram-negative bacteria but resistance was observed against colistin and sulfonamides in more than 90% of isolates. Reger et al. (1993) likewise tested the antimicrobial susceptibility of American *E. ictaluri* isolates and found full susceptibility to enrofloxacin, gentamicin and doxycycline. Half a decade ago, Stock et al. (2001) studied the antimicrobial susceptibility of 41 *E. ictaluri* strains to 71 antibiotics. All

these isolates originated from American channel catfish and hardly any acquired resistance was detected.

The aim of the present study was to investigate the antimicrobial susceptibility pattern of *E*. *ictaluri* isolates from *P. hypohthalmus* in South-East Asia, more specifically Vietnam, by means of the agar dilution test.

MATERIALS AND METHODS

Bacterial strains

Sixty-four *E. ictaluri* isolates from natural disease outbreaks of Bacillary Necrosis in *P. hypophthalmus* in the Mekong Delta, Vietnam were included in this study. The isolates all originated from different farms and were collected in 2002 (37 isolates), 2003 (10 isolates), 2004 (14 isolates) and 2005 (3 isolates).

Presumptive identification was done using the API 20E commercial kit (MicrobankTM, PRO-LAB Diagnostics, UK).

The identity of *E. ictaluri* was confirmed by the tDNA-PCR technique (Baele et al. 2000). Briefly, genomic DNA was extracted by suspending one colony of a bacterial culture in 20 μ l lysis buffer (0.25% SDS, 0.05 M NaOH). After heating at 95°C during 5 minutes and centrifugation for 20 seconds at 13,000 rpm, 180 μ l sterile distilled water was added and centrifugation was done at 13,000 rpm for 5 min. The spacers in between the tRNA genes were amplified using the primer T5B (5'AGTCCGGTGCTCTAACCAACTGAG3') and fluorescently labelled primer T3B (5'AGGTCGCGGGGTTCGAATCC3'). PCR mixtures and cycle conditions were the same as described before (Baele et al. 2000). Capillary electrophoresis was carried out using the ABI Prism TM 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, California). Electropherograms obtained with the Genescan software were compared to the database using in-house software (Baele et al. 2000). *E. tarda* (LMG 2793T) and *E. ictaluri* (CCUG 18764) were additionally included in this assay.

Escherichia coli (ATCC 25922) was included as a reference strain in the agar dilution tests (Clinical and Laboratory Standards Institute, 2006).

Antibacterial agents

The following antimicrobial agents were obtained as laboratory standard powders: amoxicillin, amoxicillin + clavulanic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, streptomycin, neomycin, enrofloxacin, oxolinic acid, flumequin, oxytetracycline, trimethoprim, nitrofurantoin and colistin. These antimicrobial agents were purchased from Sigma Aldrich N.V, Bornem, Belgium, except enrofloxacin which was obtained from Bayer AG, Brussels, Belgium; streptomycin from Certa, L'Alleud, Belgium and colistin from VMD, Arendonk, Belgium. They were dissolved in appropriate solvents to make stock solutions and then further diluted in sterile distilled water according to the methods recommended by the Clinical and Laboratory Standards Institute (2008).

Susceptibility testing

Minimal inhibitory concentration (MIC) tests were carried out on Mueller-Hinton II agar (Becton Dickinson, Cokeysville, USA) containing doubling dilutions of the antimicrobials. Final concentrations of 0.12 μ g/ml to 128 μ g/ml were tested for all antibacterial agents, and antibiotic-free agar plates were included as a control for normal growth.

All the *Edwardsiella* isolates were inoculated on Columbia blood agar (Difco, Wesel, Germany) and incubated for 48 hours at 26°C. The *E. coli* reference strain ATCC 25922 was likewise inoculated on Columbia blood agar but incubation occurred for 24 hours at 37°C. Inocula were prepared by suspending overnight cultures in phosphate-buffered saline (PBS) to a density of 0.5 on the McFarland scale. Consequently, 1/10 dilutions in PBS were prepared. Approximately 1 x 10^5 colony forming units of the strains were then inoculated on the antibiotic containing plates as well as on antibiotic-free control plates by means of a Denley multipoint inoculator (Mast, Sussex, U.K), after which plates were incubated aerobically at 26°C. For *Edwardsiella* isolates, MICs were recorded after 48 hours and for the *E. coli* reference strain after both 24 hours and 48 hours incubation. The MIC was defined as the lowest concentration of the antimicrobial agent with no visible bacterial growth. For interpretation of MIC results, the microbiological criterion (epidemiological or wild-type cut off value) was used (Turnidge et al. 2007).

RESULTS

MIC endpoints of the *E. coli* reference strain did not differ when plates were incubated during 24 or 48 hours and for the antimicrobial agents included in CLSI document M49-A, they fell within acceptable quality ranges (former NCCLS M31-A2)) (Anonymous 2002).

An overview of the MIC values for the different *E. ictaluri* isolates is shown in Table 1. The MIC_{50} and MIC_{90} values and percentages of isolates considered to have acquired resistance are likewise presented.

Table 1. Distribution of minimal inhibitory concentration (MIC) of various antimicrobialagents on 64 Edwardsiella ictaluri isolates from Pangasianodon hyphthalmus in Vietnam

Antimicrobial agent	Number of isolates with MIC (µg/ml) of								MIC	MIC	% Resis-			
	≤0.12	0.25	0.5	1	2	4	8	16	32	64	≥128	50	90	tance*
Amoxicillin	3	19	42									0.5	0.5	0
Amox + Clav	42	22										0.12	0.25	0
Chloramphenicol	13	28	23									0.25	0.5	0
Florfenicol	31	33										0.25	0.25	0
Gentamicin				15	34	15						2	4	0
Kanamycin					18	39	7					4	8	0
Streptomycin						2	9		1	1	51	≥128	≥128	82.8
Neomycin					2	52	10					4	8	0
Enrofloxacin	9	27	11	14	3							0.25	1	4.7
Oxolinic Acid				1	9	21	27	2		4		8	8	6.3
Flumequin		1		3	9	46			2	3		4	4	7.8
Oxytetracyline	2		8	1	1			3	26	23		32	64	81.3
Trimethoprim	9				8					13	34	≥128	≥128	73.4
Nitrofurantoin		8	8	5	34	9						2	4	0
Colistin										3	61	≥128	≥128	IR

**Edwardsiella ictaluri* considered to have acquired resistance according to the microbiological criterion are represented in bold

IR: intrinsically resistant

For the β -lactam antimicrobial agents amoxicillin and the combination of amoxicillinclavulanic acid, as well as for chloramphenicol, florfenicol, nitrofurantoin and the aminoglycoside antibiotics gentamicin, kanamycin and neomycin, a monomodal distribution of MICs was noted, indicating absence of acquired resistance. In contrast, for streptomycin, oxolinic acid, flumequin, oxytetracycline and trimethoprim, the MICs showed a bimodal distribution. According to the microbiological criterion, isolates in the higher range of MICs should be considered to have acquired resistance (Turnidge et al. 2007). For enrofloxacin, tailing towards the higher MIC values was observed, indicating reduced susceptibility in 3 isolates.

All *E. ictaluri* isolates were intrinsically resistant to the polypeptide antimicrobial agent colistin with MIC values equal to or above 128 µg/ml.

Resistance phenotypes of the *E. ictaluri* isolates are presented in Table 2.

Table 2. Resistance phenotypes of 64 *Edwardsiella ictaluri* isolates from*Pangasianodon hypophthalmus* in Vietnam

Resistance phenotype*	Number (%) of isolates
SM	4 (6.3)
OTC	2 (3.1)
SM + OTC	2 (3.1)
OTC+ TMP	1 (1.6)
SM + OTC + TMP	42 (65.6)
SM + OTC + TMP + FM	1 (1.6)
SM + OTC + OXO + FM	1 (1.6)
SM + OTC + TMP + OXO + FM + ENR	3 (4.7)
No acquired resistance	8 (12.5)

* SM: streptomycin, OTC: oxytetracycline, TMP: trimethoprim, FM: flumequin, OXO: oxolinic acid, ENR: enrofloxacin

DISCUSSION

Different criteria may be used for the interpretation of MIC results. For several terrestrial animals, interpretive criteria or breakpoints have been established by the Clinical and Laboratory Standards Institute (CLSI document M31-A3) so that the results of the tests can be interpreted as susceptible, intermediate or resistant and reported as such to clinicians (Clinical and Laboratory Standards Institute (CLSI) 2008). For selection of these breakpoints a range of parameters are used, including results of in vitro susceptibility tests on a large number of isolates and pharmacokinetic/pharmacodynamic data analyzing susceptibility data in relation to pharmacokinetics of the antimicrobial agent from normal dosing regimens. Other parameters used for establishing interpretive criteria of MIC results are in vitro resistance markers, both phenotypic and genotypic, as well as clinical and bacteriological outcome data from clinical studies (Turnidge et al. 2007). For aquatic pathogens, however, most of these data are lacking and CLSI breakpoints are not available (former NCCLS M31-A2)) (Anonymous 2002). Therefore, in the present study, the microbiological criterion was used for interpretation of MIC results. This parameter allows distinguishing wild-type populations of bacteria from those with acquired resistance. Using this criterion, interpretation of MIC results was straightforward for most antimicrobial agents tested here, since MIC distributions were either monomodal, indicating absence of acquired resistance, or bimodal, indicating acquired resistance in isolates in the higher range of MIC values. Only for enrofloxacin, MICs rather showed an extended frequency distribution range and the division between isolates with or without acquired resistance was more difficult to establish.

The microbiological criterion refers to direct *in vitro* interactions between the antimicrobial agents and the *E. ictaluri* isolates and does not necessarily predict how the patient will respond to therapy. However, for most antimicrobial agents tested here, MIC values were at least 10 times higher for isolates with acquired resistance. The likelihood that fish infected with these isolates will respond well to treatment with this antimicrobial agent should be considered to be low.

Chloramphenicol has been strictly prohibited for animal use in most countries including Vietnam, because of its severe toxicity and causing depression of the bone marrow (Ministry of Fisheries 2005). Its florinated derivative, florfenicol, has instead become available and rapidly became popular in several animal industries, including aquaculture (Dowling 2006; Gaunt et al. 2003; Michel et al. 2003; Wrzesinsk et al. 2005). All *E. ictaluri* isolates included in the present study were highly susceptible to florfenicol, with an MIC $\leq 0.25 \mu \text{g/ml}$. McGinnis likewise tested *E. ictaluri* isolates from American channel catfish and found full

susceptibility (McGinnis et al. 2003). In contrast, Ho et al (2000) isolated *Edwardsiella tarda*, *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Vibrio cholerae* and *Salmonella* spp isolates from aquatic animals in Taiwan which were less susceptible to florfenicol. This finding emphasizes on the need to be vigilant and control the use of florfenicol in the sense that it should not be used indiscriminately.

In the present study, acquired resistance to the aminoglycoside antibiotics gentamicin, kanamycin and neomycin was not detected in the *E. ictaluri* isolates. In contrast, the MIC values for streptomycin showed a bimodal distribution, with 83% of the isolates displaying an MIC value in the higher range and thus having acquired resistance. Previous studies showed full susceptibility of *E. ictaluri* and *E. tarda* to streptomycin (Hawke 1979; Muyembe et al. 1973; Stock et al. 2001; Waltman et al. 1986), rendering this study the first to report high levels of resistance to this antibiotic.

All isolates in our study clearly displayed intrinsic resistance to the polypeptide antimicrobial agent colistin. This finding correlates with the results from a previous study performed by Muyembe et al. (1973) who found that all 35 human isolates of *E. tarda* were resistant to colistin. Waltman et al. (1986) screened 118 *E. ictaluri* isolates retrieved in the USA for susceptibility to 37 antimicrobials using the disc sensitivity test. The study found that the majority of isolates were susceptible to most agents active against Gram-negative bacteria but resistance was observed against colistin in more than 90% of isolates. The findings suggested that colistin could be used as an additive to generate a selective medium for the isolation of *Edwardsiella* species, including *E. ictaluri*.

In the present study, three strains displayed reduced sensitivity to enrofloxacin, for which an MIC value of 2 μ g/ml was noted. To our knowledge, this is the first report of reduced sensitivity towards enrofloxacin in *E. ictaluri*. Fluoroquinolones inhibit the activity of DNA gyrase and in most bacterial species resistance is due to mutations in the gyrase or topoisomerase genes (Barnes et al. 1991; Sørum 2006). In *Enterobacteriaceae* resistance to quinolones is most commonly acquired in two steps. One mutation in the *gyrA* genes mediates full resistance to first generation quinolones such as nalidixic acid and flumequin and reduced susceptibility to other fluoroquinolones. A second mutation in either *gyrA* or *gyrB* genes mediates full resistance to fluoroquinolones (European Medicines Agency. Veteritary Medicines and Inspections (EMEA 2006). In the present study, the three isolates with reduced sensitivity to enrofloxacin indeed displayed resistance towards flumequin. Further research will be needed to elucidate the mechanisms of enrofloxacin resistance found in these isolates.

For the antimicrobial agents flumequin and oxolinic acid, clear bimodal distributions of MICs were evident and acquired resistance was encountered in 8 and 6% of the strains, respectively. As far as we know, this is the first report of resistance towards these antimicrobial agents in *E. ictaluri*. Stock et al. (2001) did not find any resistance against quinolone agents among the *Edwardsiella* species tested, including *E. ictaluri* (Waltman et al. 1986). Presently, only oxolinic acid is allowed for use in Vietnamese aquaculture. The newer quinolones such as enrofloxacin are strictly banned (Muyembe et al. 1973). Presently, EMEA (2006) reported that (fluoro) quinolones are among the most important antimicrobial agents for treatment of severe and invasive infections in human and animals. The use of fluoroquinolones in aquaculture should, therefore, always be carefully considered and controlled by the food and drug national authority.

In this study, the distribution of MIC values of oxytetracycline was clearly bimodal, with the majority of strains falling in the higher range ($\geq 16 \ \mu g/ml$) indicating acquired resistance in 81% of the isolates. The high percentage of oxytetracycline resistance encountered in this study most likely is related to the wide and sometimes improper use of this antimicrobial agent in aquaculture (Akinbowale et al. 2007; Alderman et al. 1998; DePaola et al. 1988; Reimschuessel et al. 2006; Van 2005). Several authors have done research on the distribution of *tet* genes in tetracycline resistant fish pathogens, focusing on *Edwardsiella tarda* (Aoki et al. 1987; Jun et al. 2004; Kim et al. 2004; Roberts 2005). Unfortunately, information on genotypic susceptibility of *E. ictaluri* is frequently lacking and hence hitherto poorly understood. In view of the high resistance percentages noted, this certainly merits further investigation.

Similarly to oxytetracycline, seventy-three % of the isolates had acquired resistance to trimethoprim. According to Hawke (1979) and Waltman et al. (1986), oxytetracycline and trimethoprim are used for treatment of enteric septicemia of catfish caused by *Edwardsiella ictaluri* in industrial catfish cultured in the US. Likewise, oxytetracycline and trimethoprim were denoted as being the most commonly used antimicrobial agents in fish and shrimp in Vietnam (Dung et al. 1997; Le et al. 2005; Phuong et al. 2005; Waltman et al. 1986).

The present study pointed out that 73.4 % of the isolates had acquired resistance to at least three antimicrobial agents. These isolates, when causing disease, may be especially difficult to control. This phenomenon may even become more cumbersome when one considers the fact that multiple resistance transfered by plasmids does occur and even is considered a problem of major concern in aquatic antimicrobial therapy (Aoki 1988). Plasmid-mediated resistance to chloramphenicol, trimethoprim, sulphonamides and tetracyclines has indeed been identified

in fish pathogens (McPhearson et al. 1991). Plasmids transferring resistance to as many as five antimicrobials have been identified from marine and freshwater fish pathogens, e.g: *Vibrio anguillarum*, *V. salmonicida*, *Aeromonas salmonicida*, *A. hydrophila*, *Edwarsiella tarda* and *Yersinia ruckeri* (Aoki et al. 1987; Crumlish et al. 2002; Clinical and Laboratory Standards Institute (CLSI). 2008).

Several researchers have pointed towards hygienic shortcomings in fish rearing methods. This in combination with increased fish population densities, crowding of farming sites and lack of sanitation barriers has facilitated the rapid spread of infectious agents leading to the prophylactic use of antibiotics, often with the misplaced goal of forestalling these sanitary shortcomings (Cabello 2006; Sørum 2006; Van 2005). This process has entrained several disadvantageous facts: the emergence of antimicrobial resistant bacteria in the aquaculture environment and the emergence of antimicrobial resistance in fish pathogens as demonstrated in this study. This may result in the transfer of these resistance determinants to bacteria of terrestrial animals and human pathogens, and in alterations of the bacterial microbiota both in sediments and in the water column (Cabello 2004; Huys et al. 2001; Holmström et al. 2003; Le et al. 2005).

To seize the potential risk of the findings in this study, further studies are necessary to elucidate the genetic mechanisms of the encountered resistance. In the meantime, there is a strict need to control both the prophylactic and curative use of antimicrobial agents in Vietnamese aquaculture.

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3.2. Inc*K* plasmid-mediated tetracycline resistance in *Edwardsiella ictaluri* isolates from diseased freshwater catfish in Vietnam

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ABSTRACT

Eight tetracycline resistant Edwardsiella ictaluri isolates obtained from diseased freshwater catfish (Pangasianodon hypophthalmus) in Vietnam, and showing different resistance phenotypes to other antimicrobial agents, were studied. The tet genes were determined using PCR. Conjugation experiments were performed to assess transferability of the tetracycline resistance determinant and the size and incompatibility group (Inc) of each tet-carrying plasmid were determined. PCR and sequencing were used for characterization of the cotransferred resistance genes. A tetA gene was demonstrated in the E. ictaluri isolates and for all of them, E. coli transconjugants were obtained. All transconjugants contained highmolecular weight *tetA*-carrying plasmids (~140 kb) belonging to the incK group, as was shown with the PCR-based replicon typing method. The strA-strB, dhfr1 and sul 2 genes were detected on the *tetA*-carrying plasmids of the transconjugants showing resistance to streptomycin, trimethoprim and sulfonamides, respectively. The *dhfr1* gene was found to be located in a class 1 integron as determined by PCR and sequencing. Interestingly, the 3' CS region of class 1 integrons was not detected by PCR. This study shows the presence of incK plasmid-mediated tetracycline resistance among E. ictaluri isolates from diseased freshwater catfish in Vietnam.

INTRODUCTION

Industrial aquaculture is a rapidly growing industry in many developed and developing countries, as Vietnam, where the freshwater catfish *Pangasianodon hypophthalmus* has grown into a global giant faster than any other aquaculture species in history. This indigenous fish species is high in demand from global consumers. With the rapid expansion and intensification of the freshwater industry, infectious diseases often break out. Bacillary necrosis caused by *Edwardsiella ictaluri*, is responsible for serious economical damage in Vietnamese catfish farms and for control of this disease, antimicrobial agents are often used both prophylatically and therapeutically (Crumlish et al. 2002; Ferguson et al. 2001). This may favor the spread of antimicrobial resistance genes in fish-associated and environmental aquatic bacteria.

In a recent study, acquired resistance to oxytetracycline was demonstrated in 52 of 64 Vietnamese *E. ictaluri* isolates tested and the majority of these isolates also showed acquired resistance to other antimicrobial agents, including streptomycin, sulphonamides and trimethoprim (Dung et al. 2008). Different mechanisms of resistance to tetracyclines have been described with ribosomal protection, efflux and enzymatic inactivation of the antibiotic as major modes of action. Most tetracycline resistant bacteria carry one or more of the 40 different tetracycline resistance genes described so far (Brown et al. 2008; Chopra et al. 2001; Robert 2005).

The aim of the present study was to determine the genetic determinants of tetracycline resistance among *E. ictaluri* isolates from Vietnamese freshwater catfish and to assess its transferability. Genes encoding resistance to other antimicrobial agents which were co-transferred during conjugation experiments were also characterized.

MARTERIALS AND METHODS

Bacterial isolates and determination of tet genes

Eight of the 52 tetracycline resistant *E. ictaluri* isolates obtained during a previous study and showing the three most prevalent antimicrobial resistance phenotypes (Dung et al. 2008), were selected (Table 1). All selected isolates were obtained from the kidney of diseased catfish (*Pangasianodon hypophthalmus*) during different outbreaks of bacillary necrosis in Vietnam. For the determination of the *tet* genes, PCR was performed (Cauwerts et al. 2006; Jun et al. 2004). Total DNA (genomic and plasmid DNA) and PCR mixtures were prepared as described previously (Baele et al. 2000; Martel et al. 2001).

Conjugation experiments

Conjugation experiments were carried out in Luria Broth medium with *E. coli* J5, resistant to rifampicin, used as the recipient strain. Tests were performed overnight at 37°C with a donor/recipient ratio of 0.2. Transconjugants were selected on MacConkey agar plates (Oxoid LTD, Basingstoke, Hampshire, England) supplemented with tetracycline (25 mg/L) and rifampicin (250 mg/L) (Bertrand et al. 2006). The transfer frequency was estimated by dividing the number of transconjugants per milliliter by the number of recipients per milliliter.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of the *E. coli* transconjugants was determined by the Kirby Bauer disk diffusion test (Neo-sensitabs, Rosco Diagnostica, Taastrup, Denmark) as described previously (document M31-A3) (Table 1) (NCCLS, 2008; Smet et al., 2008). Clinical Laboratory Standards Institute (CLSI) guidelines were followed for inoculum standardization, medium and incubation conditions, and internal quality control organisms (*E. coli* ATCC 25922).

Plasmid analysis

Plasmid profiles were determined for the *E. ictaluri* isolates and their *E. coli* transconjugants (Kado and Liu 1981). The molecular size of each *tet*-carrying plasmid was estimated by using a BAC Tracker Supercoiled DNA ladder (Epicentre Biotechnologies, Madison, Wisconsin). Plasmid DNA of the *E. coli* transconjugants was obtained as described by Takahashi and Nagano (1984). The incompatibility (Inc) group of each *tet*-carrying plasmid was determined by the PCR-based replicon typing (PBRT) method (Carattoli et al. 2005).

Molecular characterization of co-transferred resistance genes

The characterization of the co-transferred resistance determinants on the *tet*-carrying plasmids were performed by PCR and sequencing on plasmid DNA of the *E. coli* transconjugants as described in previous reports (Bertrand et al. 2006; Costa et al. 2008; Huys et al. 2005; Schmidt et al. 2007; Zhang et al. 2004).

Table 1

Characteristics of the E. ictaluri strains and the tetA-carrying plasmids analysed in this study

Isolate number	Year of isolation	Antimicrobial resistance ^a (parental strains)	co-transferred resistance	Antimicrobial resistance genes on the plasmid	Transfer frequency
E18	2002	TET, TMP	TMP	tetA, dhfr1	1.35 X 10 ⁻⁶
E29	2002	TET, TMP	TMP	tetA, dhfr1	2.05 X10 ⁻⁶
LO2	2002	TET, TMP, SULF, STR	TMP, SULF, STR	tetA, dhfr1, sul2, strA-strB	3.65 X 10 ⁻⁶
QO2	2002	TET, TMP, SULF, STR	TMP, SULF, STR	tetA, dhfr1, sul2, strA-strB	1.27 X 10 ⁻⁵
198	2002	TET, TMP, SULF, STR, Flum	TMP, SULF, STR	tetA, dhfr1, sul2, strA-strB	3.42 X 10 ⁻⁶
192	2002	TET, TMP, SULF, STR, Flum	TMP, SULF, STR	tetA, dhfr1, sul2, strA-strB	3.65 X 10 ⁻⁷
E136	2005	TET, TMP, SULF, STR	TMP, SULF, STR	tetA, dhfr1, sul2, strA-strB	1.03 X 10 ⁻⁷
E137	2005	TET, TMP, SULF, STR	TMP, SULF, STR	tetA, dhfr1, sul2, strA-strB	3.90 X 10 ⁻⁶

^a Antimicrobial drugs used were the following: flumequine (Flum), tetracycline (TET), trimethoprim (TMP), streptomycin (STR), sulfonamides (SULF)

RESULTS

PCR, with primers specific for different tetracycline resistance genes, demonstrated the presence of a *tetA* gene among all selected isolates.

E. coli transconjugants were obtained for all isolates. The characteristics of the *E. ictaluri* strains and their *tet*A-carrying plasmids are shown in Table 1. Transfer frequency was approximately 2.54×10^{-6} . Antimicrobial susceptibility testing of the *E. coli* transconjugants revealed that all other resistance determinants, with the exception of flumequine resistance, were cotransferred with the tetracycline resistance determinant. Plasmid analysis showed a strong band of approximately 140 kb indicating the presence of a high-molecular weight *tet*A-carrying plasmid for all transconjugants (data not shown). The PBRT method applied on plasmid DNA of the transconjugants showed that all plasmids carrying the *tet*A gene belonged to the inc*K* group.

Characterization of the co-transferred resistance determinants was performed by PCR on plasmid DNA with primers specific for trimethoprim, sulfonamide and streptomycin resistance genes. The *strA-strB* genes were detected on the *tetA*-carrying plasmids of the transconjugants that showed resistance to streptomycin. The *aadA* gene, another streptomycin resistance determinant, was not found. The *dhfr1* and *sul2* genes were found on the *tetA*-carrying plasmids of the transconjugants showing resistance to trimethoprim and sulphonamides, respectively. Often the trimethoprim resistance determinant is located in an integron. Therefore, PCR with primers specific for the class 1 (*int11*) and class 2 (*int12*) integrase was perfomed (Zhang et al. 2004)). Only the *int11* gene was identified on all *tetA*-carrying plasmids indicating the presence of class 1 integrons. Characterization of the variable region of class 1 integrons normally possess a 5' conserved segment (3'CS) separated by a variable region. However, the 3'CS, containing the *qacAE1* and *sul1* genes and an open reading frame *orf5*, was not detected by PCR for all class 1 integrons located on the *tetA*-carrying plasmids.
DISCUSSION

Recently, public health agencies have raised a worldwide concern about the impact of antimicrobial use in the aquaculture environment (Huys et al. 2005). The emergence of antimicrobial resistance among fish pathogens undermines the effectiveness of antimicrobial therapy in aquaculture. It also increases the possibilities for transfer of resistance determinants from aquatic bacteria to bacteria of terrestrial animals and human beings, including pathogens (Costa et al. 2008; Huys et al. 2005; Schmidt et al. 2001; Sun et al. 2009). Therefore, the exchange of antimicrobial resistance genes between bacteria in the aquaculture environment is of great concern. Very few information is available about plasmid-borne resistance genes among *E. ictaluri* isolates (Welch et al. 2009). Therefore, the genetic determinants of tetracycline resistance and its transferability were studied.

A *tetA* gene was demonstrated in the *E. ictaluri* isolates. This gene, as well as other tetracycline resistance determinants, has also been described in other fish pathogens (Aoki et al. 1987; Crumlish et al. 2002; Miranda et al. 2003; Schmidt et al. 2001; Sun et al. 2009). Several studies have investigated the genetic support of the *tetA* gene and found a Tn1721-like transposon to be involved in its mobility (Ojo et al. 2003; Rhodes et al. 2000; Sorum et al. 2003). The *tetA* gene present in our isolates may be carried by a Tn1721-like transposon, but this needs further investigation.

All transconjugants contained high-molecular weight *tetA*-carrying plasmids (~140 kb) belonging to the IncK group, as was shown with the PBRT method. To our knowledge, this is the first description of the *tetA* gene on incK plasmids in *E. ictaluri*. In a recent study an IncA/C plasmid, containing genes encoding tetracycline resistance, was demonstrated in an *Edwardsiella ictaluri* strain (Welch et al. 2009). The *tetA* gene was also found to be located on a smaller plasmid in an *Aeromonas salmonicida* isolate (Schmidt et al. 2001; Sørum et al. 2003). These findings might indicate that this gene is circulating among different plasmids. Further characterization of our incK plasmids and comparison with other plasmids, containing tetracycline resistance determinants, may help to explain the spread of this gene among several plasmids.

The linked *strA-strB* gene pair was detected on the *tetA*-carrying plasmids of the transconjugants showing resistance to streptomycin. This gene pair is widely disseminated among diverse gram-negative bacteria and has also been detected in other bacteria isolated from farmed fish (L'Abée-Lund and Sørum 2000; Sunde and Norström 2005). A study in Norway characterized a small plasmid from the fish pathogen *Aeromonas salmonicida* and showed that the *strA-strB* genes were carried by a Tn*5393*-like transposon (L'Abée-Lund and

Sørum 2000). The genetic support of the linked *strA-strB* gene pair on our *tetA*-carrying plasmids remains unknown and needs further investigation.

The *dhfr1* gene, encoding resistance to trimethoprim, was found to be located in a class 1 integron as determined by PCR and sequencing. This gene cassette was also found in class 1 integrons associated with plasmids in clinical *Aeromonas salmonicida* isolates (Schmidt et al. 2001). Interestingly, the 3'CS was not detected in our class 1 integrons by PCR. This may indicate that the priming site in the 3'CS is missing. The presence of these 3'CS-lacking integrons has also been reported in bacteria from an aquatic environment and at low frequencies in *E. coli* recovered from humans and animals (Rosser and Young 1999; Saenz et al. 2004; Vinué et al. 2008).

CONCLUSION

This study shows the presence of incK plasmids carrying tetracycline, streptomycin, trimethoprim and sulphonamide resistance genes among *E. ictaluri* isolates from diseased freshwater catfish. It further strengthens the need for prudent use of antimicrobial agents in catfish production.

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3.3. Early interactions of *Edwardsiella ictaluri*, the causal agent of bacillary necrosis, with *Pangasianodon* catfish

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ABSTRACT

The purpose of the study was to investigate the initial stage of the bacterium - host interactions following immersion challenge using two *E. ictaluri* isolates and to compare the retrieved data with the invasion ability of these isolates in various fish cell lines. For that purpose, first of all, 6 groups of 22 *Pangasianodon hypophthalmus* fingerlings (\pm 21 grams) were placed in water with approximately 10⁸ cfu/ml of *E. ictaluri* isolate HO2 (group 1, 2) or isolate 223 (group 3, 4); groups 5 and 6 served as negative controls. At different time points post inoculation, two fish of each group were sacrificed and samples of the gills and internal organs were taken for bacteriological, histological and immunohistochemical evaluation. These two and four addtional *E. ictaluri* isolates were also tested for their ability to invade a Chinook salmon embryo cell line (CHSE-214), a fat head minnow cell line (FHM) and a rainbow trout liver cell line (R1 214).

With regard to the *in vivo* study, the bacterial load was higher for fish of groups 1 and 2 (HO2 inoculated) compared to those of groups 3 and 4 (223 inoculated). The same difference was also noted upon immunohistochemical examination. Bacteria especially were abundantly present in the gills and intestinal tract of fish inoculated with isolate HO2. Histological analysis revealed multifocal necrotic areas in kidney, spleen and liver of several fish inoculated with the HO2 isolate. For the 223 isolate, no necrosis was found.

The *in vitro* results showed that all tested *E. ictaluri* isolates were invasive in the three cell lines albeit at different degrees. Isolate HO2 was highly invasive in all three cell lines tested and displayed a significantly higher invasion capacity than isolate 223 in cell line CHSE.

In conclusion, this study demonstrates that the adopted immersion infection model can be successfully used to reproduce BNP. Based on the results of this study, we are urged to suggest that both the gastrointestinal tract and gills may act as a portal of entry of *E. ictaluri* in *Pangasianodon* catfish. The highly virulent isolate HO2 was more invasive than the low virulent isolate 223 although this was only evident in cell line CHSE. This may point towards a correlation between *in vivo* virulence and *in vitro* invasiveness although further studies are needed to confirm this hypothesis.

INTRODUCTION

The freshwater catfish *Pangasianodon hypophthalmus* has grown into a global giant faster than any other aquaculture species in history. *Pangasianodon* production was set to produce more than one million metric tons in 2007, a target originally set for 2010 (FAO Globefish 2007). It is widely recognized that culture intensification is often accompanied by disease problems. Commercial *Pangasianodon* catfish production is heavily impacted by a severe disease known as Bacillary Necrosis of *Pangasianodon* (BNP) caused by a Gram negative bacterium *Edwardsiella ictaluri*, which is also the etiological agent of enteric septicemia in channel catfish *Ictalurus punctatus* (ESC). Both diseases are responsible for great economic losses in the catfish industry across the world (Plumb, 1999; Wagner et al., 2002; Crumlish et al., 2002).

In spite of the increasing significance of the disease, until now little information exists in the literature relating to the pathogenesis of *E. ictaluri* infections in *Pangasianodon* catfish. Ferguson et al., (2001) was the first to describe *E. ictaluri* as a pathogen of *Pangasianodon* catfish causing multifocal white lesions on liver, spleen and kidney. Hitherto, to our knowledge, no studies on the bacterium-host interactions of BNP were published. Most publications indeed deal with the study of the pathogenesis of ESC. ESC is characterized by enteritis and septicemia with red and white ulcers covering the skin and petechial hemorrhages around the mouth and base of fins (Areechon and Plumb, 1983; Jacrboe et al., 1984; MacMillan, 1985; Hawke et al., 1998). The etiological agent is able to enter into the host through the nares (Morrison and Plumb, 1994), across the gut (Shotts et al., 1986; Baldwin and Newton, 1993; Klesius, 1994) and through the gills (Nusbaum and Morrison, 1996).

Several studies evaluated differences in pathogenesis between *E. ictaluri* strains after experimental infection studies in channel catfish (Areechon and Plumb, 1983; Baldwin and Newton, 1993; Ciembor et al., 1995). The majority of researchers inoculated channel catfish by intraperitoneal or intramuscular injection. Using an immersion infection model, a few authors were able to reproduce the disease experimentally in channel catfish (Newton et al., 1989; Nusbaum and Morrison, 2002). In contrast to ESC, no experimental models for BNP have been established. Likewise, *in vitro* studies using cell lines have only been carried out adopting *E. ictaluri* strains retrieved from outbreaks of ESC and not BNP (Skirpstunas and Baldwin, 2002; 2003).

The aim of this study was to investigate the early stages of the *E. ictaluri* – *P. hypophthalmus* interaction using an immersion infection model. Furthermore, *in vitro* studies were carried out

assessing the ability of *E. ictaluri* isolates from *P. hypophthalmus* to invade Chinook salmon embryo, fathead minnow epithelial and rainbow trout liver cell lines.

MARTERIALS AND METHODS

Bacterial isolates

Six *E. ictaluri* isolates obtained from Vietnamese catfish *P. hypophthalmus* were used in this study (Table 1). All six *E. ictaluri* isolates were used in the *in vitro* experiments; type strain CCUG 18764 was additionally included as a positive control. Two out of the six isolates were used in the *in vivo* experiments namely isolate 223 and HO2. Presumptive identification of the retrieved isolates was done using the API 20E commercial kit (MicrobankTM, PRO-LAB Diagnostics, UK). The identity of *E. ictaluri* was confirmed by the tDNA-PCR technique (Baele et al., 2000).

Stock suspensions of the isolates were stored at -80°C in lyophilization medium LYM (6 g glucose (Merck, UK), 20g Brain Heart Infusion (BHI, Oxoid, Basingstoke UK), 60 ml sterile horse serum (Gibco Life Technologies, Paisly, Scotland). After thawing, the bacteria were grown on Columbia agar (Gibco Life Technologies) with 5% sheep blood for 48 h at 26°C. Colonies were picked up from the agar plates and grown 18 h at 26°C in brain heart infusion broth (BHIB, Becton Dickinson, Cokeysville, USA) with gentle shaking. The cultures were checked for purity and the number of colony forming units (CFU) per ml was determined by plating tenfold dilutions on Columbia agar plates.

Isolate	Geographical location	Fish species and organs from which the bacteria were isolated
E31	Angiang province, Vietnam	Pangasianodon catfish, liver, kidney
HO2	Angiang province, Vietnam	Pangasianodon catfish, liver, kidney
223	Angiang province, Vietnam	Pangasianodon catfish, liver, kidney
246	Angiang province, Vietnam	Pangasianodon catfish, liver, kidney
E124	Cantho province, Vietnam	Pangasianodon catfish, liver, kidney
E136	Cantho province, Vietnam	Pangasianodon catfish, liver, kidney

Table 1. Edwardsiella ictaluri field isolates used in the in vivo and in vitro experiments

Experimental infections

Fish. The experimental infections were carried out at the College of Aquaculture and Fisheries (CAF), Cantho University, Vietnam. One hundred thirty two *P. hypophthalmus* fingerlings of 21 ± 4 grams were obtained from a local hatchery. Fish were divided over 6 different 40L tanks with a stocking density of 22 fish/tank. The fish were kept in a non-flow through system,

and oxygen was supplied by air stones. The tanks were maintained at a temperature of 26 - 28°C. Other water parameters were daily checked on a regular basis (pH=7-7.5, dissolved oxygen=4-5 ppm, total hardness=100 ppm, NH3<0.004 ppm, NO2< 0.01ppm).

Before bacterial challenge, the fish were checked for external parasites and the presence of *E. ictaluri*. For that purpose, in total twelve fish (two fish per aquarium) were sacrificed using an overdose of tricaine methanesulphonate (MS222, Sigma chemical). Wet mount preparations were made from the skin and gills and examined microscopically. Samples of the liver and posterior kidney were taken and streaked on Eosin Methylene-Blue Lactose sucrose Agar (EMB, Merck) and Brain-Heart Infusion Agar (BHIA). Swabs from skin, gills and feces were also streaked on *Edwardsiella ictaluri* agar, which is used for the selective isolation of *E. ictaluri* (EIA, Shotts and Waltman 1990). Plates were incubated at 26°C for 48 h. The fish were found to be free of external parasites. *E. ictaluri* was not isolated from any of the collected samples.

Fish were fed daily with catfish-fingerling standard feed (Uni-President Vietnam Co., LTD, Aquatic Feed, Binh Duong, Vietnam). Feed was provided ad-libitum during the acclimatization period. After challenge, feeding was stopped.

The experiment was performed with the approval of the Ethical Committee of the Cantho University, Vietnam.

Immersion challenge procedure. Following an acclimatization period of 35 days, 4 groups of 20 fish were challenged with *E. ictaluri.* Groups 1 and 2 were infected with isolate HO2 and groups 3 and 4 with isolate 223. For that purpose, the cultured BHIB of isolates 223 or HO2 was added to a separate tank to give a final concentration of approximately 10^8 colony forming units (CFU)/ml, a number which was confirmed by plate counting on BHIA. The remaining two groups of 20 fish (group 5 and 6) were not infected and served as negative controls. To each negative control tank the same volume of sterile BHIB without *E. ictaluri* was added. After 1 hour of contact exposure, the fish were transferred to their original tanks.

Clinical signs and necropsy. After infection, the fish were clinically observed on a regular basis. At 1, 2, 4, 6, 9, 12, 24, 48, 72 and 96 h post infection, from each group, two fish were sacrificed using an overdose of MS222 and necropsied; samples were taken for bacteriological and immunohistochemical examination as described below.

Bacteriological examination. Firstly, a skin sample was taken by streaking a sterile cotton swab once along the entire sideline of the left side in caudal direction. The sample was inoculated onto EIA. Secondly, the left operculum was clipped off, the first gill arch was removed and cut into two equally sized parts. One half of the first gill arch was also streaked on EIA. All plates were incubated at 26°C for 48h. The quantitation of bacterial growth on the

agar plates was standardized as follows: absent = no colonies observed (score 0); few = < 10 colonies/plate (score 1); moderate = 10-50 colonies/plate (score 2); heavy = > 100 colonies/plate (score 3) (Decostere et al. 1999).

The internal organs (heart, liver, spleen, kidney and part of the anterior section of the intestine) were placed individually in sterile tubes and then homogenized in phosphate-buffered saline (PBS) as 10 % (w/v) suspensions. The number of CFU per gram of tissue was counted by plating ten-fold dilutions of the suspension on EMB agar except for the gut suspension which was inoculated on EIA. For each dilution, three plates were used. All plates were incubated at 26°C and viable counts were made after 48 h. Consequently, the average number of CFU of the three replicate inoculations was calculated.

E. ictaluri was identified on the basis of typical shape, size and color of the colony (Shotts and Waltman, 1990; Crumlish et al., 2002). The morphology of the individual bacteria and API 20E biochemical profile was also used for identification (Topic Popovic et al., 2007).

Histological and immunohistochemical examination (IHC). The other half of the first gill arch, a sample of the spleen, liver, kidney and the remaining anterior part of intestine were fixed in 4 % phosphate buffered formaldehyde for 24 h. The tissues were paraffin-embedded, sectioned at 5 μ m and stained with Haematoxylin & Eosin (H & E) and Giemsa.

For immunohistochemistry, sections of paraffin-embedded tissues were placed on SuperFrost slides (Sigma-Aldrich, Bornem, Belgium), deparaffinized with xylene, rehydrated and pretreated using the microwave pressure technique. Thereafter, endogenous peroxidase was blocked using H_2O_2 (manufacturer) and slides were washed once with PBS. For immunolabelling, slides then underwent sequential application of 30% goat serum, primary rabbit anti-*Edwardsiella ictaluri* antibodies (1/1200) (rabbit anti-*Edwardsiella ictaluri* serum provided by Dr Kim Thompson, Institute of Aquaculture, University of Stirling, UK) and secondary biotinylated goat anti-rabbit antibodies (1/500) (DakoCytomation, Heverlee, Belgium). The staining was finalized using StreptABComplex / horseradish peroxidase (DakoCytomation) and 3, 3'-diamino benzidine tetrahydrochloride (Sigma-Aldrich, Bornem, Belgium). Sections were counterstained by use of an aqueous-based haematoxylin staining and mounted.

In vitro experiments

Cell lines and culture conditions. Three different fish cell lines were used in the present study: Chinook salmon embryo cell line (CHSE-214; IZSLER, Italy), fathead minnow epithelial cell line (FHM; ECACC, England) and rainbow trout liver cell line (R1 214; DSMZ,

Germany). Both CHSE-214 and FHM were grown in Eagle's Minimum Essential Medium (EMEM; Gibco Life Technologies, Paisley, Scotland) containing 2 mM L-glutamine, 1% non essential amino acids (NEAA), 1% kanamycin, 1% penicillin/streptomycin and 10% fetal calf serum (FCS) at 20°C. CHSE-214 was kept in a 5% CO₂ incubator. The cells were subcultured every 2-3 days after detachment with 10% trypsin at room temperature and dilution at 1:10 in fresh medium. R1 cells were maintained in medium 199 with Earle's salts with L-glutamine (Gibco Life Technologies, Paisley, Scotland) supplemented with 1% kanamycin, 1% penicillin/streptomycin and 10% FCS at 20°C. The cells were subcultured every 2 weeks after detachment with TNE (50 mM Tris, 0.1 M NaCl, 5 mM EDTA, pH 7.5) at 4°C for 10 min.

Bacteria. Bacterial cells were harvested from the cultured BHIB by centrifugation at $2500 \times g$ for 10 min at 4°C and washed three times in PBS.

Cell viability and counts. The viability of cells was determined by 0.4 % trypan blue exclusion before and after bacterial exposure. The number of cells per well was estimated before each assay by counting cells in two wells of the culture plate using a calibrated grid and an inverted light microscope.

Invasion assays. Invasion assays were performed using standard in vitro methodology that was described by Skirpstunas and Baldwin, (2002) with minor modifications. CHSE, FHM, and R1cell were seeded in 24 well tissue culture flat plates (Greiner, Frickenhausen, Germany) at a density of approximately 10⁵ cells per well and were allowed to attach for at least 2 h. An adjusted inoculum of the E. ictaluri isolates was added to the cells at a multiplicity of infection (MOI; bacteria-to-cell ratio) of approximately 10:1 (approximately 10⁶ CFU/well). The inoculated cells were incubated for two hours at 20 °C in a 5 % CO₂ incubator. To enhance the initial contact between cells and bacteria, plates were centrifuged (1500 rpm, 10min, 20°C) and additionally incubated for one hour at 20°C. Cells inoculated with PBS without bacteria served as negative controls. Following incubation, the cells were washed three times with PBS (pH 7.6) and MEM supplemented with gentamicin (Gibco, Life Technologies, Paisley, Scotland) (100µg/mL) was added in order to kill extracellular bacteria. After one hour of incubation at 20°C, monolayers were washed three times with PBS. To assess invasion, one milliliter of 1% Triton X-100 (Sigma-Aldrich, Bornem, Belgium) in aqua dest was added to each well. After 10 min incubation with gently shaking the plates, an additional 100 µl PBS per well was added. The number of viable intracellular bacteria was determined by plating ten-fold serial dilutions on Columbia blood agar and incubating for 36- 48 hours at 26°C. All invasion studies were performed in triplicate and experiments were repeated three times at different points in time. The invasiveness was expressed as the percentage of bacteria that were re-isolated in

comparison to the number of bacteria that were inoculated. In each experiment, wells containing only cells and only bacteria were used as controls.

Statistical analysis

For the *in vivo* experiments, the bacteriological scores of the skin and gills were statistically analyzed using the chi-square test of independence (computer program STATISTIX 5.0, Analytical Software). A significance level of 0.01 was used.

With respect to the internal organ colonization, a student's t-test was used to compare the number of bacteria (log transformed) between groups (computer program SPPS 17.0). Probabilities of <0.05 were considered significant.

For the *in vitro* tests, the percentages of invasion were divided by mean of three experiments, to lower inter-experimental variations. The data were analyzed using one-way ANOVA and Duncan multiple range test. Significant differences were determined at P<0.05.

RESULTS

In vivo experiments

Clinical presentation and gross pathology

Clinical signs and mortality were not observed in control animals and in animals inoculated with isolate 223. The earliest clinical sign, seen at 4 h post inoculation with isolate HO2, consisted of more than half of the fish slowly swimming near the water surface, gasping for air. At 48 h post inoculation, half of the fish exhibited an accelerated breathing rate and a pale skin. One fish died and three moribund fish were observed at 72 h post inoculation. Upon necropsy, the gills of most fish were pale and numerous white pin-points (< 1 mm diameter) were seen scattered throughout the kidney and spleen and to a lesser extent in the liver (Figure 1).



Figure 1. Fingerling *Pangasianodon* catfish. Post-mortem examination at 72h post-inoculation with *E. ictaluri* HO2. Multifocal renal (large arrow) and hepatic (small arrow) necrosis.

Bacteriological examination

E. ictaluri was not isolated from any of the control fish. The results of the bacteriological examination of the skin and gill are given in Table 2. The bacteriological scores for the skin and gill tissue were significantly (P<0.01) higher for isolate HO2 than for 223.

At 1 h after infection, bacteria were detected in all sampled parts (liver, trunk kidney, spleen, heart and intestine) of the fish inoculated with HO2 with the highest number of CFU in the anterior section of the intestine (8.5 (\pm 1.7) x 10⁵ CFU/g). For the fish inoculated with isolate 223, the mean number of CFU (5.9x10²) was significantly lower than the mean number of CFU in fish for which HO2 was used for inoculation (1.3x10⁴).

Histological and immunohistological examinations

Upon examination of the control fish, no lesions nor immunoperoxidase positive bacteria were observed. In the fish inoculated with isolate 223, no lesions were observed and only in the gills could immunoperoxidase bacteria very rarely be encountered.

In HO2 inoculated animals, at 1h post inoculation, fusion of the secondary lamellae and hyperplasia were observed in the gills. In addition, necrotic lesions were found in liver, trunk kidney and the spleen at 1h and 6h after inoculation. In these organs, a mild multifocal necrosis with infiltration of macrophages and occasional heterophils was found (Figure 2). Immunohistochemical examination revealed *E. ictaluri* bacteria in the secondary lamellae of the gills at 1 h post infection, in hepatic Kupffer cells after 12 h and in sinusoids of spleen and liver at all time points. Several immunoperoxidase positive extracellular bacteria were found in renal and hepatic vessels between 3-48 h post inoculation in samples of the majority of fish.

Giemsa stained samples from HO2 inoculated animals revealed single or occasionally clustered short rod-shaped bacteria in the cytoplasm of macrophages in necrotic areas in kidney, liver and spleen at 1 h, 6 h and 72 h post inoculation. These bacteria were positively immunolabelled using anti *E. ictaluri* antibodies (Figure 2, 3).

Isolate	Hours post-challenge	Number of <i>E. ictaluri</i> colonies ^a	
		Skin	Gill
HO2			
	1	3/3 ^b	3/3
	2	3/3	3/3
	4	2/2	2/3
	6	1/2	2/3
	9	2/3	2/2
	12	2/2	2/3
	24	1/1	3/2
	48	2/1	2/2
	72	2/2	2/1
	96	1/2	2/2
223			
	1	1/3	2/3
	2	2/3	2/2
	4	1/2	2/1
	6	2/1	1/1
	9	2/2	2/1
	12	1/1	ND
	24	1/1	1/2
	48	1/0	1/1
	72	1/1	1/0
	96	1/0	0/1

Table 2. Results of the bacteriological examination of the skin and gill	ls of fish
inoculated with E. ictaluri isolate HO2 and 223	

^a: Score 0: no colonies observed; score 1: 1- 10 colonies/plate; score 2: 11-100 colonies/plate; score 3: >100 colonies/plate ^b: At each time point, two fish were sacrificed. Results of both animals are

presented.

ND: Not determined



Figure 2. Fingerling *Pangasianodon* catfish at 1h post inoculation with *E. ictaluri* isolate HO2. The kidney exhibits focal necrosis with intralesional bacteria. Immunostaining. Bar = $20 \ \mu m$



Figure 3. The liver of fingerling *Pangasianodon* catfish at 1h post inoculation with *E. ictaluri* isolate HO2 showing necrosis with intralesional clustered *E. ictaluri*. Immunostaining. Bar = $20 \,\mu\text{m}$

In vitro experiments.

The results of the invasion tests of the seven *E. ictaluri* isolates including the type strain in FHM, CHSE and R1 cell lines are given in Table 3.

All the *E. ictaluri* isolates were invasive in the three cell lines albeit at different degrees (Table 3). The invasion of type strain *E. ictaluri* CCUG 18764 and isolate E31 was significantly (P < 0.05) higher than the invasion of all other isolates with the exception of isolate HO2 for cell line CHSE. In addition, the invasion of isolate HO2 which constituted approximately 4.2% was significantly (P < 0.05) higher than the invasion of isolate 223 which was about 0.2% in the CHSE-214 cell line. Generally, the highest invasiveness values were observed in cell line R1 for all strains while the lowest invasiveness values were seen in CHSE-214 when invasion was compared among the three different cell lines.

Table 3. Invasion of *E. ictaluri* in cell lines FHM, CHSE and R1. Values followed by different superscript letters are significantly different from one another (P<0.05).

Isolate	Percent (%) of intracellular CFU (mean±standard error)			
Isolate	FHM	CHSE	R1	
E31	$6.3 (\pm 1.4)^{a}$	$7.4 (\pm 4.1)^{a}$	9.4 (±3.5) ^a	
HO2	$4.2 (\pm 2.0)^{\rm b}$	$4.2 (\pm 4.7)^{a}$	$4.9 (\pm 1.6)^{b}$	
223	$2.3 (\pm 1.3)^{b}$	$0.2 (\pm 0.1)^{\rm b}$	$6.1 (\pm 2.5)^{b}$	
246	$1.8 (\pm 0.8)^{\rm b}$	$0.2 (\pm 0.1)^{\rm b}$	$4.8 (\pm 2.7)^{\rm b}$	
E124	$1.6 (\pm 0.8)^{b}$	$0.2 (\pm 0.1)^{\rm b}$	$4.4 (\pm 4.2)^{b}$	
E136	$3.6 (\pm 2.2)^{\rm b}$	0.1 (±0.1)	$3.8 (\pm 0.5)^{\mathrm{b}}$	
CCUG18764	$12.4 (\pm 8.8)^{a}$	$11.8 (\pm 8.5)^{a}$	$14.4 (\pm 0.9)^{a}$	

DISCUSSION

Hitherto, as far as we know, no experimental infection model has been described for BNP. In this study, BNP was reproduced for the first time using immersion challenge, an exposure route imitating the natural way of infection. This infection model has been successfully used in the past by the author adopting various isolates, amongst which isolate HO2 and 223. Isolate HO2 proved to be highly virulent whereas isolate 223 was of low virulence (unpublished data). Likewise, in this study, clinical signs were only seen in fish inoculated with isolate HO2, with the earliest clinical signs appearing at 4 h after challenge. Fish inoculated with isolate 223 did not exhibit clinical signs nor mortality. Furthermore, lesions were not detected in the fish challenged with isolate 223 group and statistically lower numbers of bacteria in the various tested organs except for the heart were observed. This again confirms the differences in virulence between isolate HO2 and 223.

In this study, the early stages of the bacterium-host interactions were studied, partly aimed at elucidating the portal of entry of *E. ictaluri* in *Pangasianodon* catfish. Several fish pathogens including *Pseudomonas piscicida*, *Vibrio anguillarum*, *Yersinia ruckeri* and *E. tarda*, are reported to use the gastrointestinal tract as an entry site (Ross et al., 1966; Olsson et al., 1996; Ling et al., 2000; 2001; Tobback et al., 2009). Likewise, for *E. ictaluri*, the gastrointestinal tract is believed to be primary portal of entry in channel catfish in ESC (Newton et al., 1989; Baldwin and Newton, 1993).

In the present study, high numbers of *E. ictaluri* were detected in the anterior section of intestine for isolate HO2 at 1 h post challenge. Booth, (2005) has reported that *E. ictaluri* may pass through the gastrointestinal tract because of its acid resistance mechanisms, including urease production. *E. ictaluri* is indeed capable of hydrolyzing urea in order to enhance growth and replication at acid pH, although the bacterium is determined a "urease negative" organism in traditional biochemical tests (Holt et al., 1994; Waltman et al., 1986), suggesting that a pre-existing urease is activated by low environmental pH. Generally, gastrointestinal tract may be a portal of entry of *E. ictaluri* for BNP.

The number of colonies isolated from the skin and gill tissue was significantly higher for isolate HO2 than for 223. Several bacteria were found in the gill of fish inoculated with isolate HO2 at the sites of secondary lamellae fusion and hyperplasia. Similarly, in ESC the presence of bacteria intracellularly in the secondary lamellae of the gills of infected channel catfish appointed the gills as an entry route for *E. ictaluri* (Ciembor et al., 1995; Nusbaum and Morrison, 1996). When evaluating the entry of ³⁵S labeled *E. ictaluri* into channel catfish, Nusbaum and Morrison, (1996) indicated that bacteria rapidly became associated with the gills

at 2 h post infection. Moreover, the findings of Nusbaum and Morrison, (2002) showing the appearance of *E. ictaluri* bacteria in the circulation of channel catfish indeed suggest entry through the gills within 5 minutes following inoculation. Consequently, it may be speculated that gills may also act as a portal of entry of *E. ictaluri* in *Pangasianodon* catfish as is the case for ESC.

BNP and ESC are both caused by *E. ictaluri* but they differ in the clinical picture and gross lesions they induce. BNP reveals relatively few external signs: a pale gill and skin color often is the only noted abnormality (Ferguson et al., 2001). ESC reveals a more marked clinical picture in that petechial hemorrhages and inflammation in the skin, around the mouth and at the base of fins are observed (Areechon and Plumb, 1983; Jacrboe et al., 1984; MacMillan, 1985; Hawke et al., 1998). Internally, BNP is characterized by white spots throughout the spleen, liver and kidney. This in contrast to ESC, which elicits abdominal distention with ascites and a swollen kidney and spleen, a liver motted with congestion and a septicemic intestine (Areechon and Plumb, 1983; Jacrboe et al., 1984; Blazer et al., 1985; Miyazaki and Plumb, 1985). These differences may be due to variation in *E. ictaluri* strains or it may be related to differences in susceptibility between *Pangasianodon* catfish and channel catfish. It might be interesting to study the course of an infection with *E. ictaluri* strains isolated from ESC in *Pangasianodon* catfish, using the infection model presented in the present studies.

Although the disease signs and macroscopic lesions associated with ESC and BNP may differ, there are similarities in histopathological lesions. The histopathological picture of ESC is characterized by severe necrosis of the trunk kidney and spleen as well as edema and necrosis of the liver (Areechon and Plum, 1983). BNP likewise reveals multifocal necrosis in multiple internal organs, including renal interstitium, the parenchyma in liver and spleen (Ferguson et al., 2001; Ferguson, 2006), hence the name of the disease BNP "Bacillary Necrosis of Pangasianodon catfish". Several studies demonstrated proliferation of the interlammelar tissue of the gills in ESC. Similarly, the present study shows the fusion of the secondary lamellae and hyperplasia in the gills. In ESC, necrosis and hemorrhagic ulcerative lesions of the head as well as mild focal infiltration and granulomatous inflammation in the underlying musculature of skin have also been reported (Jarboe et al., 1984; Miyazaki and Plum, 1985; Shott et al., 1986). In contrast, lesions on the head, skin or muscles were not recorded in BNP. In the present studies, large numbers of E. ictaluri were found in the kidney and spleen inside macrophages of several fish inoculated with the isolate HO2. Booth et al., (2006) showed that E. ictaluri is able to invade, survive and replicate in channel catfish macrophages. Several other fish pathogenic bacteria have developed mechanisms through which they can escape killing by the host macrophages: Aeromonas salmonicida, Renibacterium salmoninarum,

Vibrio anguillarum and *F. psychrophilum* (Garduno et al., 1993; Gutenberger et al., 1997; Boesen et al., 2001; Decostere et al., 2001). Further studies in greater depth are needed to enable us to confirm or reject the hypothesis of intracellular survival of *E. ictaluri in Pangasianodon* catfish macrophages.

In the second part of this study, the invasion of *E. ictaluri* isolates from *Pangasianodon* catfish was examined using cultured fish cells (FHM, CHSE-214 and R1). Tissue culture cells have played a crucial role in investigating bacteria–host interactions because cultured cells are easy to work with, can be maintained under controlled conditions, and may be relevant to the diseases under study (Quinn et al., 1997).

In several earlier studies, epithelial cell lines were used because it is likely to be the first cell type that a fish pathogen would encounter *in vivo* before establishing infection. Many fish pathogens, including *Aeromonas hydrophila*, *Vibrio anguillarum*, *Photobacterium damselae* and *Edwardsiella tarda* have been shown in *in vitro* tests to be capable of invading epithelial cells (Tan et al., 1998; Wang et al., 1998; López-Dóriga et al., 2000; Ling et al., 2000). Skirpstunas and Baldwin, (2002) also described the FHM cell line as a functional *in vitro* system for the study of the pathogenesis of *E. ictaluri* infections and showed that *E. ictaluri* isolates with the best invasion capabilities (in FHM cell lines) were isolated from fish during natural outbreaks of ESC.

The present study demonstrates that all tested *E. ictaluri* isolates from *Pangasianodon* catfish were invasive in all cell lines albeit at different degrees. The invasion in the FHM and CHSE-214 cell line was on the whole markedly lower than that in the rainbow trout liver cell line (R1).

Isolate HO2 was highly invasive in all three cell lines tested and displayed a significantly higher invasion capacity than isolate 223 in cell line CHSE. Skirpstunas and Baldwin, (2002; 2003) stated that the majority of the moderately invasive *E. ictaluri* isolates retrieved from cases of ESC were included in the high virulence category. Further studies are needed to pinpoint a possible correlation between invasion *in vitro* and the virulence *in vivo* and elucidate the actual factors responsible for invasion.

CONCLUSION

This study demonstrates that the adopted immersion infection model can be successfully used to reproduce BNP. Based on the results of this study, we are urged to suggest that both the gastrointestinal tract and gills may act as a portal of entry of *E. ictaluri* in *Pangasianodon* catfish. The highly virulent isolate HO2 was more invasive than the low virulent isolate 223

although this was only evident in cell line CHSE. This may point towards a correlation between *in vivo* virulence and *in vitro* invasiveness although further studies are needed to confirm this hypothesis.

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4. GENERAL DISCUSSION

Vietnamese E. ictaluri isolates are often multi-resistant

In Vietnam, with rapid expansion and intensification of the freshwater industry, infectious diseases break out frequently, leading to serious economical damage in *Pangasianodon* farms. Therefore, antimicrobial agents are often used both prophylactically and therapeutically. A serious awkward side effect of the abundant use of antimicrobials is that it favours the spread of antimicrobial resistance in fish-associated bacteria as well as in bacteria from the aquaculture environment (Sorum 2006). This may not only jeopardize treatment of bacterial diseases in fish but may also represent a public health hazard. Indeed these bacteria may act as reservoirs of antimicrobial resistance genes for other bacteria, including micro-organisms that are potentially harmful for humans. Results of the studies presented in this thesis clearly demonstrate that, at least in the fish pathogen *E. ictaluri*, acquired antimicrobial resistance to several antimicrobial agents is widespread. Vietnamese *E. ictaluri* strains often carry different resistance genes on the same plasmid, which may favour spread of multi-resistance to other bacteria.

Currently, two different types of criteria are used for interpretation of antimicrobial susceptibility testing data: clinical breakpoints and epidemiological cut-off values (Bywater et al. 2006; Schwarz et al. 2009). Clinical breakpoints are intended to guide a therapeutic approach and aim to predict which antimicrobial agents are most likely to lead to therapeutic success. In this thesis, in vitro susceptibility tests were carried out and for several isolates resistance genes were studied as well. However, for selection of clinical breakpoints, a range of other parameters are also required. These include pharmacokinetic and pharmacodynamic parameters in the respective animal species as well as results of clinical efficacy studies. For E. ictaluri and for many other aquatic pathogens, these data are lacking. In some studies clinical breakpoints are used that have been approved for other bacteria, other animal species or other disease conditions, as can be found for instance in Clinical and Laboratory Standard Institute documents. This is, however, unacceptable (Schwarz et al. 2009). Therefore, in our studies epidemiological cut-off values were used, allowing to distinguish wild-type from non wild-type bacterial populations. Bacteria belonging to the non wild-type population have a higher MIC for an antimicrobial agent which may be due to mutations or to the acquisition of antimicrobial resistance genes, as shown in chapter 3.2 of this thesis. These cut-off values do not necessarily predict the therapeutic outcome. As a rule, epidemiological cut-off values should therefore only be used to describe MIC distributions of bacteria without clinical context (Schwarz et al. 2009). However, for most antimicrobial agents tested in chapter 3.1 of this thesis, MIC values were at least 10 times higher for bacteria not belonging to the wildtype population. The likelihood that fish infected with these bacteria will respond well to

treatment with this antimicrobial agent should be considered to be low but further studies are necessary to confirm this statement. These may include efficacy studies in experimentally infected fish, using the immersion challenge model developed in chapter 3.3.

Comparing resistance percentages between published studies is only possible if the same interpretive criteria have been used by the different authors (Schwarz et al. 2009). Interpretive criteria may change over time, for instance if new parameters become available. In the present studies, not only MIC50, MIC90 and percentages of resistance are presented, but also full MIC distributions. This allows re-analysing of our data by other authors.

Multi-resistance exclusively refers to acquired resistance properties and not to intrinsic resistance. Although there is no universally accepted definition of "multi-resistance", it usually refers to resistance to at least three classes or sub-groups of antimicrobial agents mediated by different mechanisms (Schwarz et al. 2009). If a single resistance mechanism affects different classes of antimicrobial agents, this should not be considered as multi-resistance. Applying this criterion, it can be concluded that more than 70% of our isolates appeared to be multi-resistant. For some isolates phenotypic susceptibility testing data were supplemented with molecular analysis for resistance genes. In several of these isolates, the presence of four resistance genes borne on the same plasmid and affecting different antimicrobial classes or sub-groups was detected, confirming multi-resistance in these isolates.

The results of our study demonstrate that *E. ictaluri* is intrinsically resistant to colistin. Colistin and other polymyxins are cationic agents that bind to the anionic bacterial outer membrane of Gram negative bacteria, leading to a detergent effect that disrupts membrane integrity. In particular, polymyxins show a high affinity for the lipid moiety of lipopolysaccharides and preferentially displace Mg^{2+} and Ca^{2+} from cationic binding sites, leading to cytoplasmic leakage (Landman et al. 2008). The exact mechanism of intrinsic resistance to colistin in *E. ictaluri* is not known, but most probably it is due to alterations in lipid A, as has been shown for other intrinsically resistant bacteria including *Proteus mirabilis, Burkholderia cepacia* and *Chromobacterium violaceum* (Landman et al. 2008; Cox and Wilkinson 1991; Hase and Rietschel 1977; Haesebrouck and Devriese 1994).

The high occurrence of acquired antimicrobial resistance in *E. ictaluri* most probably primarily relates to antimicrobial usage in aquaculture. Indeed, an increase in the frequency of oxytetracyline resistance following the use of this agent has been detected in many fish production systems throughout the world (Austin 1985; Suggita et al. 1998). DePaola and Roberts (1995) and DePaola et al. (1995) described a direct relationship between the

administration of oxytetracycline-medicated feed to catfish and the frequency of oxytetracycline resistance in the intestinal and aquatic micro-flora. It has been mentioned that even in the absence of antimicrobial treatment, tetracycline resistance genes may persist in aquatic populations to protect bacteria from tetracycline produced by other micro-flora in their environmental niche (Schmidt et al. 2001).

Several years ago, fluoroquinolones were suggested as a treatment for clinical *E. ictaluri* infections (Crumlish et al. 2002). Results of our studies indicate reduced sensitivity to the newer fluoroquinolones such as enrofloxacin in a limited number of strains. For the first generation quinolone antimicrobial agents flumequin and oxolinic acid, acquired resistance was encountered in 8% and 6% of the *E. ictaluri* strains tested, respectively. Presently, only oxolinic acid is allowed for use in aquaculture in Vietnam and the newer quinolones are now strictly banned. Fluoroquinolones are among the most important antimicrobial agents for treatment of severe and invasive infections in human and animals and they should be used with caution (Mofi 2004; EMEA 2006).

In chapter 3.2 of this thesis, the tet(A) resistance gene was found in *E. ictaluri* isolates with oxytetracycline MICs of 16 µg/ml or higher. However, 3 isolates were also shown to carry this gene without displaying phenotypic tetracycline resistance (unpublished data). This lack of correlation between resistance phenotype and genotype may be due to defective expression of the resistance gene as described by Martineau et al. (2000). Turnidge and Paterson (2007) pointed out that these may represent examples of "hidden resistance" whereby resistance mechanisms are detected genotypically in organisms with MICs within the "susceptible" range. Such mechanisms may become phenotypically apparent after an antimicrobial agent has been used clinically during prolonged periods of time.

In recent years, the role of integrons and gene cassettes in the spread of antimicrobial resistance has been well established (Hall and Collis 1998; Rowe-Magnus and Mazel 2001) and a strong relation between the presence of an integron and multi-resistance phenotypes has been observed (Leverstein-van-Hall et al. 2002). While not mobile, integrons tend to be associated with transposons and/or conjugative plasmids which can serve as vehicles for their transmission (Ploy et al. 2000). In particular, aminoglycoside and sulfonamide resistance are significantly related to integron carriage in *Enterobacteriaceae*. In our studies, a genetic linkage in *E. ictaluri* isolates was demonstrated between *tet*(A), *dhfr1*, *sul2* and *strA-strB* genes and the *dhfr1* gene, encoding resistance to trimethoprim, was shown to be located in a class 1 integron. Schmidt et al. (2001) also found this gene cassette in class 1 integrons associated with plasmids in clinical *Aeromonas salmonicida* isolates. Remarkably, the *sul1*

gene was not detected in our class 1 integron by PCR, although this gene has been described to be located in the 3' CS region of class 1 integrons (Zhang et al. 2004). The reasons for this remain unclear. However, full sequencing of our plasmids should be carried out to determine if the *sul1* gene is missing or if it is interrupted.

Studying host-pathogen interactions as a first step for development of alternative control measures

The studies mentioned above demonstrate that acquired antimicrobial resistance is widespread in *E. ictaluri* isolates. It is therefore crucial that alternative control strategies for BNP are developed. These may include vaccination. In the past, development of vaccines was rather empirical. Progress in microbiology, molecular biology, immunology and bioinformatics has led to a more rational approach to the development of vaccines. A most valuable strategy for the selection of protective antigens is by studying host-pathogen interactions. It is obvious that a comprehensive understanding of the pathobiology of infections and the molecular basis of pathogenicity is required for rational design of antibacterial vaccines. Bacterial virulence attributes involved in colonisation, resistance to host-defence mechanisms and damage to the host need to be identified (Haesebrouck et al. 2004). Therefore, in chapter 3.3 of this thesis, the early interactions of *E. ictaluri* with its host were studied.

When we started our studies, no experimental infection model had been described allowing reproducing BNP. With the immersion infection model developed here, we were not only able to reproduce the disease, but also to demonstrate variation in virulence between different *E. ictaluri* isolates. This infection model has indeed successfully been used by the author adopting various isolates, amongst which isolate HO2 and 223. Isolate HO2 proved to be highly virulent whereas isolate 223 was of low virulence. Only in the group infected with HO2 mortality was seen and only fish belonging to this group developed clinical signs and lesions. Another advantage of the model developed here, is that the exposure route imitates the natural way of infection. The immersion infection model is not only suitable to carry out pathogenesis studies, but it may also be a valuable tool for determining the efficacy of various treatment procedures and control measures, including vaccination.

The severity of outbreaks of BNP in the field is variable and is influenced by several environmental factors such as poor water quality, low chloride concentration and temperature fluctuations. Stress induced by handling, close confinement and improper diet may also enhance susceptibility of catfish to infection (Ciembor et al. 1995; Mqolomba and Plumb 1992; Plumb and Sanchez 1983; Wise et al. 1993). Besides these environmental and

management factors, the intrinsic virulence of the circulating *E. ictaluri* isolate is most probably also an important cause for this variation.

The basis of the observed difference in virulence between isolates HO2 and 223 is unknown and further studies comparing the characteristics of both isolates may help to obtain better insights in the virulence factors of *E. ictaluri*. Such studies should include assays comparing bacterium-macrophage interactions for both isolates. Indeed, results described in chapter 3.3 of this thesis indicate that the highly virulent isolate is able to multiply in macrophages in *Pangasianodon* catfish, confirming the results of Booth (2006) who demonstrated invasion, survival and replication of *E. ictaluri* in macrophages of channel catfish. An acid-inducible urease enzyme may help the bacterium to tolerate the acid conditions in the host macrophages (Booth 2005). Expression studies of the urease gene in both isolates might reveal interesting results.

Comparison of the highly virulent isolate HO2 and the low virulent isolate 223 should also be done at the genomic level. Whole genome sequencing of both isolates would most probably also allow identifying candidate virulence factors.

In all *E. ictaluri* isolates tested, high numbers of the micro-organism were detected in the anterior section of the intestine as early as at 1 h post challenge. In addition, bacteria were found in the gills of fish inoculated with isolate HO2 at the sites of secondary lamellae fusion and hyperplasia. Consequently, it may be speculated that both the gastrointestinal tract and gills may act as a portal of entry of *E. ictaluri* in *Pangasianodon* catfish. This is in agreement with studies dealing with the pathogenesis of ESC in channel catfish. It has indeed been described that *E. ictaluri* in channel catfish is able to enter into the host through the nares (Morrison and Plumb 1994), across the gut (Shotts et al. 1986; Baldwin and Newton 1993; Klesius 1994), and through the gills (Nusbaum and Morrison 1996). Although apparently *E. ictaluri* uses similar routes of entry in *Pangasianodon* and channel catfish, BNP and ESC differ in the clinical picture and gross lesions they induce. It is not clear if these differences are due to variation in *E. ictaluri* strains or to differences in susceptibility between both hosts. Further experimental infection studies in *Pangasianodon* and channel catfish with different *E. ictaluri* strains should help to clarify this matter.

All tested *E. ictaluri* isolates were found to be invasive in the three cell-lines used in this thesis (FHM, CSHE- 214, and R1-214) albeit at different degrees of infectivity. The invasion efficiency also differed between the cell-lines. The invasion in the FHM and CHSE-214 cell-line was on the whole markedly lower than that in the rainbow trout liver cell-line (R1). This may indicate differences in surface-exposed receptors (quantitative or qualitative). *In vivo*

studies showed that *E. ictaluri* is rapidly internalized through epithelial-lined membranes and no damage to mucosal epithelial cells was seen. This supports the hypothesis that *E. ictaluri* may express ligands at his surface that recognize host cell receptors to facilitate internalization (Skirpstunas and Baldwin 2003). However, the specific mechanisms used by *E. ictaluri* to invade different cell types remain to be defined.

Conclusions

In conclusion, acquired antimicrobial resistance is widespread in *E. ictaluri* isolates from Vietnam and is often carried on mobile elements. This may not only jeopardize efficacy of antimicrobial treatment in aquaculture, but may also represent a human health hazard as antimicrobial resistance genes may be transferred horizontally to bacteria of the terrestrial environment, including human and animal pathogens (Rhodes et al. 2000; Sørum and Sunde 2001; Sørum 2006; Cabello 2006). This high occurrence of acquired antimicrobial resistance in *E. ictaluri* most probably primarily relates to antimicrobial usage in aquaculture. Therefore, the strict control of both prophylactic and curative use of antimicrobial agents in aquaculture is of vital importance. Moreover, it is crucial that alternative control strategies for bacterial diseases of fish are developed, including vaccination and optimal management routine. The immersion infection model developed here is a valuable tool for evaluating the efficacy of these control measures.

This model may also be used to carry out further experimental infection studies aiming to unravel the pathogenesis of BNP, which is a prerequisite for rational design of vaccines. Comparison of *E. ictaluri* strains of different virulence, both at a phenotypic and genotypic level should help to obtain better insights in the virulence factors of this micro-organism.
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SUMMARY

One of the serious conditions affecting *Pangasianodon* catfish is Bacillary necrosis (BNP), caused by Edwardsiella ictaluri. To the present day, control of this disease mainly relies upon the administration of antimicrobial agents. Hitherto, no studies investigating the antimicrobial susceptibility of E. ictaluri isolates from P. hypophthalmus have been carried out, hindering the underpinned choice of an effective antimicrobial agent. Additionally, one needs to keep in mind that the problems associated with the use of antimicrobial agents are numerous and include difficulties with administration, the high cost of antibiotic treatment, the risk of antibiotic residues in the fish products and the emergence of resistant strains. In view of the above, a preventive approach to combat *E. ictaluri* infections should be adopted. This requires an understanding of how the pathogen causes disease in *P. hypophthalmus* which hitherto to a great extent is unknown. A summary of the relevant literature concerning the pathogen, E. *ictaluri* and the disease it causes is given in Chapter 1, highlighting the issue of antimicrobial resistance and the available data on the pathogenesis of BNP. Following, the general aims of this study are listed, which consist of studying the antimicrobial resistance of E. ictaluri and investigating the pathogenesis of BNP with emphasis on the early stages of the bacterium host interaction. The specific aims of the thesis can be found in Chapter 2.

In Chapter 3, the various experimental studies are listed. To be able to choose effective antimicrobial agents for treatment and monitor antimicrobial resistance development, first of all, the antimicrobial susceptibility pattern of *E. ictaluri* in South-East Asia, more specifically Vietnam, was determined using the agar dilution technique. Sixty-four Vietnamese E. ictaluri isolates from P. hypophthalmus collected between 2002 and 2005 were included. Low minimal inhibitory concentration (MIC) values were found for amoxicillin, amoxicillinclavulanic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, neomycin and nitrofurantoin, indicating absence of acquired resistance. On the contrary, acquired resistance to streptomycin, oxytetracycline and trimethoprim was detected in 83%, 81% and 71% of the isolates respectively, as indicated by a bimodal distribution of the MICs of these antimicrobials. For enrofloxacin, a monomodal distribution was noted, tailing towards the higher MIC values, indicating reduced susceptibility in a minority of isolates. For the quinolone antimicrobial agents flumequin and oxolinic acid, acquired resistance was encountered in 8 and 6% of the strains, respectively. All strains were intrinsically resistant to the polypeptide antimicrobial agent colistin. Seventy-three percent of the isolates were shown to have acquired resistance to at least three antimicrobial agents. To be able to fully grasp the significance of these findings, further studies are necessary to elucidate the genetic

mechanisms of the encountered resistance. In the meantime, there is a need to control both the prophylactic and curative use of antimicrobial agents in Vietnamese aquaculture.

The second experimental study meets the above cited need for elucidating the genetic background of tetracycline resistance among E. ictaluri isolates from Vietnamese freshwater catfish and for assessing its transferability. In this study, genes encoding resistance to other antimicrobial agents which were cotransferred during conjugation experiments were also characterized. For this purpose, 8 distinct tetracycline resistant E. ictaluri isolates and showing different resistance phenotypes to other antimicrobial agents were studied. The tet genes were determined using PCR. Conjugation experiments were performed to assess transferability of the tetracycline resistance determinant and the size and incompatibility group (Inc) of each tet-carrying plasmid. PCR and sequencing were used for characterization of the co-transferred resistance genes. A *tetA* gene was demonstrated in the *E. ictaluri* isolates and for all of them, E. coli transconjugants were obtained. All transconjugants contained highmolecular weight *tetA*-carrying plasmids (~140 kb) belonging to the incK group, as was shown with the PCR-based replicon typing method. To our knowledge, this is the first description of the tetA gene on incK plasmids in E. ictaluri. The strA-strB, dhfr1 and sul 2 genes were detected on the *tetA*-carrying plasmids of the transconjugants showing resistance to streptomycin, trimethoprim and sulfonamides, respectively. The *dhfr1* gene encoding resistance to trimethoprim, was found to be located in a class 1 integron as determined by PCR and sequencing. Interestingly, the typical 3' CS was not detected by PCR in our class I integrons by PCR. In conclusion, this study shows the presence of incK plasmids carrying tetracycline, streptomycin, trimethoprim and sulphonamide resistance genes among E. ictaluri isolates from diseased freshwater catfish in Vietnam. These findings suggest efficient coselection and spread of multiple resistance in E. ictaluri, and further strengthen the need for prudent use of antimicrobial agents in catfish production.

The above studies indicate the undeniable need for preventive measure to combat BNP. The main way this can be achieved, is by understanding the mechanism through which the bacterium interacts with its host and causes disease. However, very little is known about the factors involved in the pathogenesis of *E. ictaluri* infections in *Pangasianodon* catfish. The last study of this thesis meets this need for more data. In this study, the initial stage of the bacterium-host interactions following immersion challenge were determined using two clinical *E. ictaluri* isolates; the retrieved data were compared with the invasion ability of these isolates in various fish cell lines. For that purpose, first of all, 6 groups of 22 *P. hypophthalmus* fingerlings (± 21 grams) were placed in water with approximately 1×10^8

cfu/ml of *E. ictaluri* isolate HO2 (group 1, 2) or isolate 223 (group 3, 4); groups 5 and 6 served as negative controls. At different points post inoculation (1, 2, 4, 6, 9, 12, 24, 48, 72 and 96 h), two fish of each group were sacrificed and samples of the gills and internal organs were taken for bacteriological, histological and immunohistochemical evaluation. These two and four additional *E. ictaluri* isolates were also tested for their ability to invade a Chinook salmon embryo cell line (CHSE-214), a fat head minnow cell line (FHM) and a rainbow trout liver cell line (R1 214). With regard to the *in vivo* study, the number of bacteria per gram of tissue was higher for fish of groups 1 and 2 compared to those of groups 3 and 4. The same difference was also noted upon immunohistochemical examination. Histological analysis revealed multifocal necrotic areas in kidney, spleen and liver as well as hyperplasia in the gills of several fish inoculated with the HO2 isolate. For the 223 isolate, no necrosis was found. The *in vitro* results showed the invasive capability of all *E. ictaluri* isolate 223 although this was only evident in cell line CHSE.

This study demonstrates that the adopted immersion model can be successfully used to reproduce BNP. Based on the results of this study, we are urged to suggest that both the gastrointestinal tract and gills may act as a portal of entry of *E. ictaluri* in *Pangasianodon* catfish. The highly virulent isolate HO2 was more invasive than the low virulent isolate 223 although this was only evident in cell line CHSE. This may point towards a correlation between the *in vitro* invasive ability and *in vivo* virulence of isolates tested although further studies are needed to pinpoint this possible correlation and elucidate the actual factors responsible for invasion.

In Chapter 4 of the thesis (General discussion), the findings of all the studies are summarized and discussed. Perspectives for further studies are also noted.

SAMENVATTING

"Bacillary necrosis" (BNP) veroorzaakt door Edwardsiella ictaluri is één van de ernstige aandoeningen die Pangasianodon katvissen aantast. Het bestrijden van deze ziekte gebeurt momenteel grotendeels door het toedienen van antimicrobiële agentia. Tot op heden zijn er geen studies beschikbaar die de antimicrobiële gevoeligheid van E. ictaluri isolaten van P. hypophthalmus onderzocht hebben, wat de gefundeerde keuze van een werkzaam antimicrobieel agens in de weg staat. Bovendien moet men indachtig zijn dat de problemen geassocieerd met het gebruik van antimicrobiële agentia talrijk zijn: moeilijkheden bij de toediening, de hoge kosten van antimicrobiële behandeling, het risico op antibiotica residuen in de afgeleide producten van vissen en het opduiken van resistente stammen. Daarom is het belangrijk om een preventieve aanpak te hanteren bij het bestrijden van E. ictaluri infecties. Dit veronderstelt een inzicht in de manier waarop het pathogeen agens ziekte veroorzaakt bij P. hypophthalmus wat tot op heden grotendeels ongekend is. Een samenvatting van de relevante literatuur betreffende het pathogeen agens, E. ictaluri, en de ziekte die het veroorzaakt, is te vinden in Hoofdstuk 1, waar het item antimicrobiële resistentie en de beschikbare data over de pathogenese van BNP zijn weergegeven. Vervolgens worden de algemene doelstellingen van deze thesis opgelijst, met name het bestuderen van de antimicrobiële resistentie van E. ictaluri en het onderzoeken van de pathogenese van BNP met nadruk op de vroegtijdige stadia van de bacterie-gastheer interactie. De specifieke doelstellingen van de thesis kunnen gevonden worden in Hoofdstuk 2.

In Hoofdstuk 3 worden de verschillende experimentele studies opgelijst. In de eerste studie werd het antimicrobieel gevoeligheidspatroon van E. ictaluri in Zuid-Oost Azië met name Vietnam bepaald door middel van de agar dilutie methode; dit had als bedoeling doeltreffende antimicrobiële agentia voor een behandeling te kunnen selecteren en de ontwikkeling van antimicrobiële resistentie te kunnen monitoren. Vierenzestig Vietnamese E. ictaluri isolaten van P. hypophthalmus werden verzameld tussen 2002 en 2005. Lage minimale inhibitorische concentraties (MIC) werden gevonden voor amoxicilline, amoxicilline-clavulaanzuur, chlooramfenicol, florfenicol, gentamicine, kanamycine, neomycine en nitrofurantoïne, wat de afwezigheid van verworven resistentie aangeeft. Verworven resistentie tegenover streptomycine, oxytetracycline en trimethoprim werd daarentegen gevonden bii respectievelijk 83%, 81% en 71% van de isolaten, wat zich uitte door een bimodale verdeling van de MICs van deze antimicrobiële agentia. Voor enrofloxacine werd een monomodale verdeling gezien, met een uitloper naar de hogere MIC waarden toe, wat een verminderde gevoeligheid van een minderheid van isolaten aangeeft. Voor de quinolone agentia flumequine en oxolinezuur werd verworven resistentie opgemerkt bij respectievelijk 8 en 6% van de isolaten. Alle isolaten waren intrinsiek resistent tegenover het polypeptide antimicrobieel agens colistine. Drieënzeventig procent van de isolaten vertoonden verworven resistentie tegenover ten minste drie antimicrobiële agentia. Bijkomende studies zijn noodzakelijk om de genetische mechanismen van de opgemerkte resistentie op te helderen, wat zal toelaten om de betekenis van deze resultaten ten volle te kunnen omvatten. Intussen is het noodzakelijk om zowel het profylactische als curatieve gebruik van antimicrobiële agentia in de Vietnamese aquacultuur te controleren.

De tweede experimentele studie komt tegemoet aan de bovenvermelde nood om de genetische achtergrond van de tetracycline resistentie bij E. ictaluri isolaten van Vietnamese zoetwater katvissen op te helderen en om de overdraagbaarheid van deze resistentie te kunnen inschatten. In deze studie werden tevens de genen die voor resistentie coderen tegenover andere antimicrobiële agentia en die mede werden overgedragen bij conjugatie experimenten Hiertoe werden 8 tetracycline resistente E. ictaluri isolaten met gekarakteriseerd. verschillende resistentie fenotypes tegenover andere antimicrobiële agentia onderzocht. De tet genen werden bepaald door middel van PCR. Er werden conjugatie experimenten uitgevoerd om de overdraagbaarheid van de tetracycline resistentie determinant en de grootte en incompatibiliteitsgroep (Inc) van ieder tet-dragend plasmide na te gaan. PCR en sequeneren werden gebruikt om de mede overgedragen resistentie genen te karakteriseren. Er werd een tetA gen aangetoond in de E. ictaluri isolaten en voor al deze werden E. coli transconjuganten bekomen. Alle transconjuganten bevatten tetA-dragende plasmiden met een hoog moleculair gewicht (~140 kb) die tot de incK groep behoren, wat werd aangetoond aan de hand van de op PCR gebaseerde replicon typerende methode. Voor zover wij weten, is dit de eerste beschrijving van het tetA gen op incK plasmiden bij E. ictaluri. De strA-strB, dhfrl en sul 2 genen werden gedetecteerd op de tetA-dragende plasmiden van de transconjuganten die resistentie vertoonden tegenover respectievelijk streptomycine, trimethoprim en sulfonamides. Het dhfrl gen wat resistentie codeert tegenover trimethoprim, werd gelocaliseerd op een klasse 1 integron door middel van PCR en sequenering. Een interessante bevinding was het feit dat het typische 3' CS niet gedetecteerd werd via PCR op onze klasse I integrons. Als conclusie kan gesteld worden dat deze studie de aanwezigheid van incK plasmiden aantoont die dragers zijn van tetracycline, streptomycine, trimethoprim en sulfonamide resistentie genen bij E. ictaluri isolaten van zieke zoetwater katvissen in Vietnam. Deze bevindingen suggereren een efficiënte co-selectie en spreiding van meervoudige resistentie bij E. ictaluri en benadrukken nogmaals de noodzaak voor het voorzichtig gebruik van antimicrobiële agentia bij de productie van katvissen.

De hierboven vermelde studies tonen de onmiskenbare nood aan van preventieve maatregelen om BNP te bestrijden. De belangrijkste manier waarop men dit kan bekomen, is door het mechanisme te begrijpen waarmee de bacterie interageert met haar gastheer en zieke veroorzaakt. Er is echter bijzonder weinig gekend over de factoren die betrokken zijn bij de pathogenese van E. ictaluri infecties bij Pangasianodon katvissen. De laatste studie van deze thesis speelt in op deze nood aan meer gegevens. In deze studie werden de bacterie-gastheer interacties na immersie challenge bestudeerd waarbij gebruik werd gemaakt van twee klinische E. ictaluri isolaten; de bekomen gegevens werden vergeleken met de invasie capaciteit van deze isolaten in verschillende cellijnen afkomstig van vissen. Hiertoe werden eerst en vooral 6 groepen van 22 P. hypophthalmus vissen (±21 gram) geplaatst in water met ongeveer 1x10⁸ cfu/ml van *E. ictaluri* isolaat HO2 (groep 1, 2) of isolaat 223 (groep 3, 4); groepen 5 en 6 fungeerden als negatieve controles. Op verschillende tijdstippen na de inoculatie (1, 2, 4, 6, 9, 12, 24, 48, 72 en 96 u), werden twee vissen van elke groep opgeofferd en werden stalen van de kieuwen en de interne organen genomen voor bacteriologisch, histologisch en immunohistochemisch onderzoek. Deze twee en vier bijkomende E. ictaluri isolaten werden eveneens getest op hun capaciteit om een "salmon embryo cell line" (CHSE-214), een "fat head minnow cell line" (FHM) en een "rainbow trout liver cell line" (R1 214) te invaderen. Inzake de *in vivo* studie, was het aantal bacteriën per gram weefsel hoger voor vissen van groep 1 en 2 in vergelijking met deze van groep 3 en 4. Hetzelfde verschil werd opgemerkt bij immunohistochemisch onderzoek. Histologisch onderzoek toonde multifocale necrotische gebieden aan in de nier, milt en lever en hyperplasie van de kieuwen bij verschillende vissen die geïnoculeerd werden met het isolaat HO2. Bij het isolaat 223 werd geen necrose aangetroffen. De in vitro resultaten toonden aan dat alle E. ictaluri isolaten konden invaderen in de drie cellijnen, zij het met verschillende gradaties. Het isolaat HO2 vertoonde een significant hogere invasie capaciteit in vergelijking met het isolaat 223; dit was evenwel enkel zichtbaar in de cellijn CHSE.

Deze studie toont aan dat het aangewende immersie model succesvol kan gebruikt worden om BNP te reproduceren. Op basis van de bevindingen van deze studie zijn wij sterk geneigd te stellen dat zowel de gastro-intestinale tractus als de kieuwen fungeren als intredepoort voor *E. ictaluri* bij *Pangasianodon* katvissen. Het hoogvirulente isolaat HO2 was meer invasief dan het laagvirulente isolaat 223 alhoewel dit enkel duidelijk was in de cellijn CHSE. Dit kan wijzen op een correlatie tussen de invasie capaciteit *in vitro* en de *in vivo* virulentie van de geteste isolaten; bijkomende studies zijn evenwel noodzakelijk om deze mogelijke correlatie verder uit te diepen en de mogelijke factoren verantwoordelijk voor invasie op te helderen. In Hoofdstuk 4 van de thesis (Algemene discussie) worden de resulaten van alle studies samengevat en besproken. Perspectieven voor verdere studies worden eveneens aangegeven.

TÓM TẮT

Một trong những yếu tố ảnh hưởng nghiêm trọng đến cá tra nuôi (*Pangasianodon hypophthalmus*) là bệnh mủ gan gây ra bởi vi khuẩn *Edwardsiella ictaluri*. Hiện nay, việc kiểm soát và khống chế bệnh vi khuẩn kể cả bệnh truyền nhiễm do vi khuẩn *E. Ictaluri* chủ yếu dựa vào thuốc kháng sinh . Thực tế cho đến nay chưa có nghiên cứu nào về tính kháng thuốc của các chủng vi khuẩn *E. ictaluri* phân lập từ cá tra (*Pangasianodon hypophthalmus*), điều này cản trở việc lựa chọn những loại kháng sinh hiệu quả trong phòng và trị bệnh. Hơn thế nữa, việc sử dụng kháng sinh không kiểm soát sẽ mang lại hàng loạt tác động xấu như: rất khó để kiểm soát và quản lý thuốc kháng sinh sử dụng, chi phí cho việc điều trị bằng thuốc kháng sinh rất tốn kém, nguy cơ tồn dư lượng kháng sinh trong sản phẩm, sự lan truyền và phát triển của những chủng vi khuẩn kháng thuốc.

Do vậy, biện pháp ngăn chặn sự lây nhiễm của bệnh do *E.ictaluri* gây ra cần phải được thực hiện. Để thực hiện điều đó, đòi hỏi cần phải có những kiến thức về phương thức xâm nhập và gây bệnh cho cá tra mà cho đến nay vẫn chưa được làm sáng tỏ. Trong phần giới thiệu chung, thông tin về tác nhân gây bệnh *E. ictaluri* và bệnh mà nó gây ra được đề cập ở chương 1, nhằm xác định tính kháng kháng sinh của vi khuẩn và cung cấp thông tin sẵn có về quá trình phát sinh bệnh gan thận mủ. Theo sau đó, mục tiêu chung của nghiên cứu sẽ được đề cập, bao gồm: nghiên cứu về tính kháng kháng sinh của vi khuẩn *E. ictaluri*, tìm hiểu về con đường lan truyền và phát sinh bệnh của vi khuẩn thông qua nghiên cứu về mối tương tác qua lại giữa tác nhân và vật chủ ở giai đoạn sớm của bệnh. Mục tiêu của nghiên cứu này được đề cập ở chương 2.

Những nghiên cứu khác nhau đã được trình bày trong Chương 3. Để có thể chọn ra loại kháng sinh hữu hiệu cho việc điều trị bệnh và kiểm soát sự gia tăng mức độ kháng kháng sinh. Trước hết, kiểu hình kháng kháng sinh của vi khuẩn *E. ictaluri* ở khu vực Đông Nam Á, đặc biệt ở Việt Nam được xác định dựa vào phương pháp pha loãng thuốc kháng sinh trong môi trường thạch. Kết quả nghiên cứu trên 64 chủng vi khuẩn *E. ictaluri* phân lập từ 2002 đến 2005 trên cá tra *P. hypophthalmus*, đã cho thấy không có sự kháng thuốc kháng sinh: amoxicillin, amoxicillin-clavulanic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, neomycin và nitrofurantoin.

Tuy nhiên, đa số vi khuẩn *E. ictaluri* đã kháng thuốc đáp ứng với streptomycin (83% chủng vi khuẩn), oxytetracycline (81%), trimethoprim (71%), một số ít chủng đã giảm tính nhạy với các loại kháng sinh trên. Đối với nhóm quinolone, tính kháng đáp ứng cũng được ghi nhận trên một số loại kháng sinh như: flumequin (8%), oxolinic acid (6%) và enrofloxacin (5%). Trong khi đó, hầu hết vi khuẩn đã kháng thuốc tự nhiên đối với khánh sinh colistin

(polypeptide). Đặc biệt, kết quả đã xác định trên 73% chủng vi khuẩn *E. ictaluri* biểu hiện sự đa kháng thuốc (kháng ít nhất 3 loại thuốc). Để có thể hiểu sâu hơn, những nghiên cứu tiếp theo cần thực hiện, nhằm làm sáng tỏ cơ chế kháng đáp ứng của vi khuẩn. Trong khi chờ đợi kết quả của những nghiên cứu kế tiếp, cần thiết phải kiểm soát nghiêm ngặt việc dùng thuốc kháng sinh để phòng trị bệnh trong nuôi trồng thủy sản ở Việt Nam.

Nghiên cứu thứ hai đã đáp ứng vêu cầu làm sáng tỏ đặc điểm cơ bản của tính di truyền và đánh giá sư truyền các gen kháng tetracycline của vi khuẩn *E. ictaluri*, gây bênh trên da trơn nước ngọt ở Việt Nam. Trong nghiên cứu này, các gen mã hóa kháng với nhiều loại kháng sinh, đồng chuyển giao gen kháng thuốc trong thí nghiêm tiếp hợp cũng được mô tả. Để thực hiên nghiên cứu này 8 chủng E. ictaluri kháng tetracyclin có các kiểu kháng thuốc khác nhau đối với các loại kháng sinh khác nhau. Các gen kháng tetracycline (tet genes) đã xác định bằng phương pháp PCR. Thí nghiệm tiếp hợp được thực hiện nhằm đánh giá khả năng lan truyền của các yếu tố di truyền mang gen kháng tetracyline, xác định kích thước và kiểm tra nhóm plasmid không tương hợp (Inc: Incompatibility) của plasmid mang gene kháng tetracycline. Đặc điểm sự đồng chuyển giao các genes kháng thuốc được xác đinh bằng kỹ thuật PCR và giải trình tự DNA. Gen tetA đã được tìm thấy ở tất cả chủng vi khuẩn E. ictaluri và các chủng vi khuẩn này đều tham gia tiếp hợp. Tất cả chủng lại có plamid mang *tetA* với trong lượng phân cao (~140 kb) và thuộc nhóm incK thực hiện bằng phương pháp PCR dựa trên bản sao (the PCR-based replicon typing method). Như được biết, đây là mô tả đầu tiên về tetA trên incK plasmids của vi khuẩn E. ictaluri. gen strA-strB, dhfr1 và sul 2 được phát hiện trên plasmid mang tetA minh chứng vi khuẩn này tương ứng kháng với streptomycin, trimethoprim and sulfonamides. Với kỹ thuật PCR và giải trình tự DNA đã tìm thấy gen dhfr1 định vị trên class 1 integron. Điều thú vị là, vùng 3' CS của class 1 integron lại không được phát hiện thông qua kỹ thuật PCR. Nhìn chung, kết quả nghiên cứu này chỉ ra sự hiện diện của plasmids nhóm K không tương thích (incK) mang gen kháng thuốc tetracycline, streptomycin, trimethoprim và sulphonamide ở những chủng vi khuẩn E. ictaluri gây bệnh trên cá do trơn nước ngọt. Ở Việt nam, khám phá này chỉ ra sự phối hợp có chon lọc hiệu quả và sư lan tràn những gen đa kháng của vi khuẩn E. ictluri. Vì thế việc sử dung thân trong các loại thuốc kháng sinh trong nuôi cá tra là rất cần thiết.

Nhìn chung, những nghiên cứu trên đã chỉ ra yêu cầu cần thiết cho những biện pháp ngăn chặn bệnh gan thận mủ. Phương thức trên có thể đạt được thông qua việc nắm rõ về cơ chế mà tác nhân tác động vào vật chủ và gây bệnh trên đối tượng đó. Tuy nhiên có rất ít thông tin về những yếu tố liên quan đến quá trình phát sinh bệnh của vi khuẩn *E. ictaluri* đối với cá da trơn *Pangasianodon*. Thí nghiêm cuối cùng trong luận án đã đáp ứng yêu cầu cung cấp thông tin cho vấn đề trên. Trong nghiên cứu này giai đoạn đầu sự xâm nhập của vi khuẩn vào cơ thể

vật chủ (*in vivo*) thông qua gây cảm nhiễm bằng phương pháp ngâm với 2 chủng vi khuẩn *E. ictaluri* để so sánh với khả năng xâm nhập của vi khuẩn vào các loại tế bào cá khác nhau. Từ mục tiêu trên, 6 nhóm cá, mỗi nhóm 27 con cá tra giống cỡ (\pm 21 grams) được ngâm trong dung dịch vi khuẩn *E. ictaluri* có mật độ vi khuẩn 1x10⁸ cfu/ml với chủng HO2 (thuộc nhóm 1, 2) hoặc chủng 223 (nhóm 3, 4) nhóm 5, 6 là lô đối chứng. Ở những thời điểm khác nhau (2, 4, 6, 9, 12, 24, 48. 72 và 96 h) sau khi gây cảm nhiễm, 2 cá mỗi bể được thu mẫu ở mang và các cơ quan nội tạng để nghiên cứu đánh giá về vi sinh, mô học và hóa mô. Đồng thời, kiểm tra khả năng xâm nhập của 6 chủng *E. ictaluri* vào các loại tế bào khác nhau như: tế bào chinook salmon embryo (CHSE-214), fat head minnow cell line (FHM) và tế bào rainbow trout liver (R1 214).

Trong nghiên cứu *in vivo*, kết quả cho thấy số lượng vi khuẩn trên 1 gram mẫu mô (gan, thận, lá lách và ruột sau) ở cá nhóm 1, 2 cao hơn so với cá nhóm 3 và 4. Kiểm tra hóa-mô cũng thấy có sự khác nhau đáng kể trên cá thí nghiệm của 2 chủng vi khuẩn này. Về mô học, ở đa số cá cảm nhiễm với chủng HO2 đã tìm thấy nhiều vùng hoại tử sơ đa nhân ở gan, lá lách và thận. Ở cá cảm nhiễm với chủng 223 không phát hiện những dấu hiệu hoại tử này.

Kết quả nghiên cứu *in vitro*, cho thấy khả năng xâm nhập của tất cả các chủng vào 3 loại tế bào ở những mức độ khác nhau. Chủng vi khuẩn HO2 có khả năng xâm nhập cao hơn một cách có ý nghĩa so với chủng vi khuẩn 223 mặc dù chỉ ghi nhận trên tế bào CHSE.

Kết luận chung, vi khuẩn *E. ictaluri* trong phương thức gây cảm nhiễm bằng phương pháp ngâm có ảnh hưởng mẫn cảm trên cá tra giống. Ngoài ra, có thể hệ tiêu hóa và mang là đường xâm nhập của vi khuẩn *E. ictaluri* vào cơ thể cá da trơn *Pangasianodon*. Chủng vi khuẩn HO2 có độc lực cao mức trung bình và chủng vi khuẩn 223 có độc lực thấp thể hiện sự tương quan trong nghiên cứu *in vivo* và *in vitro* mặc dù những nghiên cứu tiếp theo cần được thự hiện nhầm chỉ rõ mối thương quan đó và làm sáng tỏ yếu tố tác động đến quá trình xâm nhập của vi khuẩn vào vật chủ.

Trong chương 4 của luận án (thảo luận chung), những thông tin của tất cả các nghiên cứu được tóm tắt và thảo luận, làm tiền đề cho những nghiên cứu tiếp theo.

Curriculum vitae

Tu Thanh Dung was born in the province of Cantho, Vietnam, on August 08, 1962.

She graduated as a Bachelor of Science in Aquaculture in 1986 at Cantho University.

In 1995, TT Dung was awarded a scholarship from the Asian Institute of Technology (AIT) to follow the course "Master of Science in aquaculture" at AIT Bangkok, Thailand. She completed her M.Sc. in August 1996 with her thesis "In vitro and vivo study of the effect of selected herb extracts on bacterial isolates from hybrid catfish".

Afterwards, TT Dung returned to Vietnam and worked as senior scientist and lecturer in fish disease courses (3rd and 4th year undergraduate students) at Cantho University. She spent seven years working as project manager in several bacterial fish disease projects in co-operation with Stirling University, Scotland. These projects were funded by the Department for International Development, UK. Results obtained during these projects were published in peer reviewed journals. TT Dung is co-author of 3 of these articles.

In 2003, she followed the "Advanced Course for post-graduate in theory and laboratory techniques in pathology, histopathology and microbial diseases" for 6 months at the Institute of Aquaculture, Stirling University, Scotland, UK.

In 2004, she spent 3 months for internship in a research project in biotechnology, including diagnosis of aquatic animal diseases and serological and molecular assays at Michigan State University, USA.

In 2005, she was able to enroll for a 3 year sandwich doctoral study program in Veterinary Sciences at the Faculty of Veterinary Medicine, Ghent University, Belgium. In 2006, she spent one month in the Laboratory of Genome Science at Tokyo University Marine Science and Technology, Japan.

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