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PATHOGEN ENTRANCE AND DEVELOPMENT OF DISEASE DURING INFECTION OF THE AMERICAN CHANNEL CATFISH *Ictalurus punctatus* BY THE ENTEROBACTERIUM *Edwardsiella ictaluri*

By

Simon Menanteau-Ledouble

A Dissertation
Submitted to the Faculty of Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in Veterinary Medical Science
in the College of Veterinary Medicine.

Mississippi State, Mississippi

December 2009

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PATHOGEN ENTRANCE AND DEVELOPMENT OF DISEASE DURING INFECTION OF THE AMERICAN CHANNEL CATFISH *Ictalurus Punctatus* BY THE ENTEROBACTERIUM *Edwardsiella Ictaluri*

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Title of Study: PATHOGEN ENTRANCE AND DEVELOPMENT OF DISEASE DURING INFECTION OF THE AMERICAN CHANNEL CATFISH *Ictalurus Punctatus* BY THE ENTEROBACTERIUM *Edwardsiella Ictaluri*

Pages in Study: 98

Candidate for Degree of Doctor of Philosophy

Since being first reported in the late 1980ies, the Enterobacterium *Edwardsiella ictaluri* has rose in prevalence to become one of the two most damaging pathogens affecting the channel catfish industry. Despite this significance of the pathogen, understanding of the development of the disease, especially its route of entry into the host and the earlier stages of the infection, is still incomplete.

A series of challenges were conducted using bioluminescent *E. ictaluri* either by infecting fish through immersion or topical application of the bacteria directly on the intact or abraded epithelium. This showed that abraded fish developed septicemia and died faster than non-abraded ones. Furthermore, results from a co-habitation challenge suggested that the bacterium induced septicemia through the skin instead of becoming water-borne. Finally, a histological technique was developed allowing the determination that the bacteria radiated from the initial skin infection site and penetrated deeper into

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the tissue as the challenge progressed. These results all suggest that site of abrasion on the skin can act as a route of entrance for the pathogen into the fish, a fact never previously reported.

Transposon mutagenesis was also performed to construct a library of 1728 mutants. Screening of this library allowed us to identify 16 genes which inactivation lead to a decrease in the bacterium ability to colonize the epithelium or cause mortality. Sequencing of these genes allowed the identification of RstA/B, a regulator of invasion genes in *Salmonella enterica* Typhimurium, a putative ribonuclease, similar to a *Shigella* protein regulating the expression of adhesin and a protein that constitutes the second member of a newly discovered adhesin family.

Finally, to investigate the development of the infection, fish were infected by bioluminescent *E. ictaluri* and sampled at various time points. At each time point, nine organs (gills, muscles, intestine, spleen, liver, stomach, heart, head kidney and trunk kidney) were sampled, and their bioluminescence was measured and half of these organs were homogenized, serial diluted, and plate counts determined. This allowed confirmation of a complex disease pathogenesis during ESC involving a period of intense reproduction in the spleen, anterior and posterior kidneys followed by a sharp increase in the levels of bacteria in the blood.

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DEDICATION

Parce qu'elle m'à appris qu'amour et famille triomphe de tout à la fin. Parce que, lorsque tout est dit, c'est la plus importante des leçons, je dédie cette recherche à ma mère, Catherine Ledouble.

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ACKNOWLEDGMENTS

I would like to express my most sincere gratitude to the people whose time and effort allowed this dissertation to happen. Most specifically, I would like to extend my thankful acknowledgement to my major professor, Doctor Mark Lawrence for his optimism and continuous guidance and support during these past years as well as to Doctor Petrie-Hanson and the other members of my dissertation committee, Dr. Lanie Bilodeau, Karen S. Coats, Jerald A. Ainsworth and Scott Willard for their input and direction.

Furthermore, I would like to express my gratitude toward Larry Hanson for his mentoring in the aquatic diagnostic lab.

I would also like to acknowledge the assistance of Michelle M. Banes for her patience and her invaluable assistance in the lab as well as Maryvonne Leroux and Sulagna Saha for helping conducting part of the experiments. Furthermore, the contribution of Dr. Attila Karsi, who constructed the pAKLux1 used at many point during the research, Timothy Brown, who assisted during the development of the immunohistochemistry protocol, and Dr. Michele Williams who assisted during the analysis of the *Edwardsiella ictaluri* genome, cannot be overstated.

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Finally, I would like to acknowledge Dr. Claudia Hohn and the personnel of the fish hatchery at the College of Veterinary Medicine for providing the fish used in this study.

Without these many persons, the present work could not have been conducted, thank you.

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CHAPTER I

INTRODUCTION

Context of the industry

Brief overview of the fish farming industry in the USA

The U.S. aquaculture industry produced a total of over 360,000 metric tons in 2006, accounting for a value of above 1.244 billion dollars (National Marine Fisheries Service 2008). Among these, channel catfish (*Ictalurus punctatus*) was the dominating product with almost 257 thousand metric tons produced in 2006 for a value just under 500 million dollars for total production (National Marine Fisheries Service 2008). Other species of aquacultural importance included the salmonids, with a production of 37,313 metric tons corresponding to a value of 105 million dollars, and crawfish, with a production volume of 36 thousand tons for a total value of 96 million dollars. About 96% of the catfish production in the U.S occurs within four southeastern states (Arkansas, Alabama, Louisiana, and Mississippi).

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Economic environment of the catfish industry

The average price of catfish reached its lowest in 2002. At this point, it fell under production cost for the majority of operations, resulting in the closing of a large number of catfish producing operations. As a consequence, the number of catfish producing operations has been in steady decline since 2002 (Figure 1.1), with the number of operations dropping from 753 in 2006 to 680 in 2008 (National Agricultural Statistics Service) (Table 1.1 and Table 1.2).

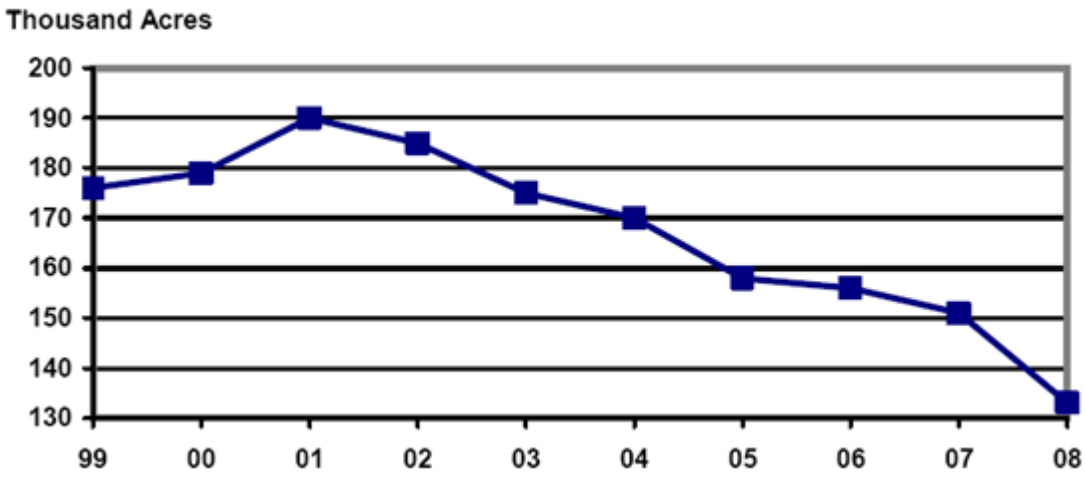

Figure 1.1. Total pond surface acres devoted to catfish production in the four main producing states during the last ten years.

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Table 1.1. Number of catfish operations in the four main producing states during the last three years

	2006	2007	2008
Alabama	217	199	185
Arkansas	132	137	128
Louisiana	24	20	17
Mississippi	380	370	350
Total in the four states:	753	726	680

Table 1.2. Total pond surface acres devoted to catfish production in the four main producing states during the last three years

	2006	2007	2008
Alabama	24300	23000	22000
Arkansas	30000	30400	29900
Louisiana	5700	5500	5500
Mississippi	96000	92500	87300
Total in the four States:	156000	151400	144700

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This reduction in catfish operations impacted the volume of catfish produced, which has decreased regularly since 2001 only to stabilize between 2007 and 2008 (Figure 1.2).

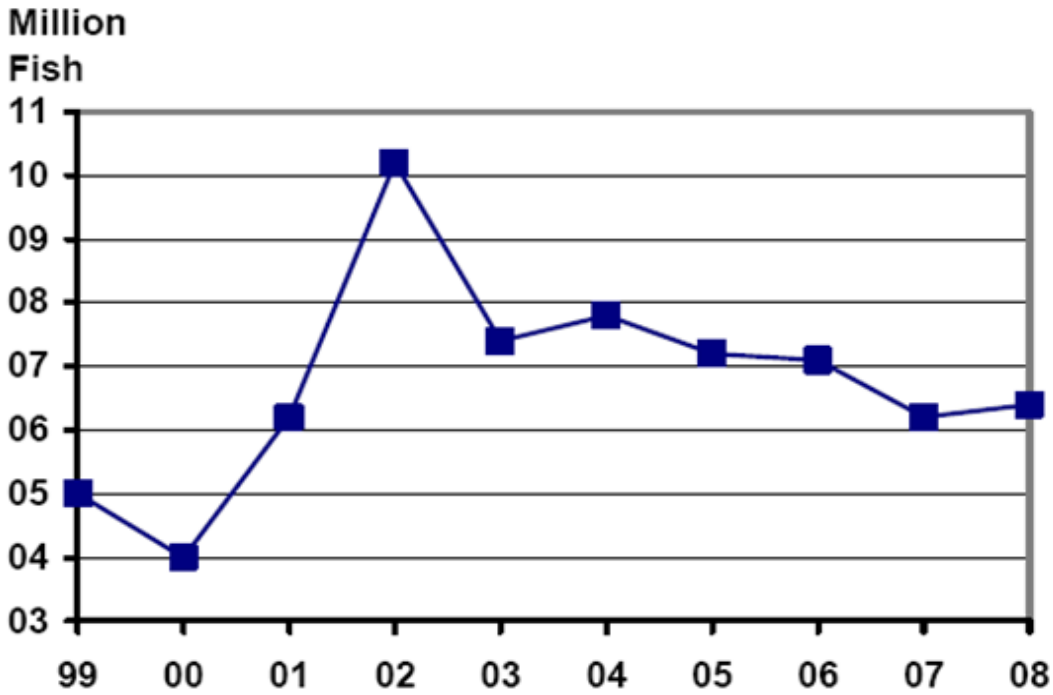

Figure 1.2. Total number of large food-size catfish produced during the last ten years.

This drop in supply of catfish was one of the contributing factors in the stabilization of the average price for catfish that returned production to profitability (Figure 1.3). However, part of this increase in sale income was absorbed by the increase in production costs in recent years due to increased energy and food costs. Furthermore, part of the decrease in supply has been replaced with other fish species,

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most notably tilapia (both U.S. produced and imported) and pangasid catfish (imported from Vietnam) (Harvey 2004).

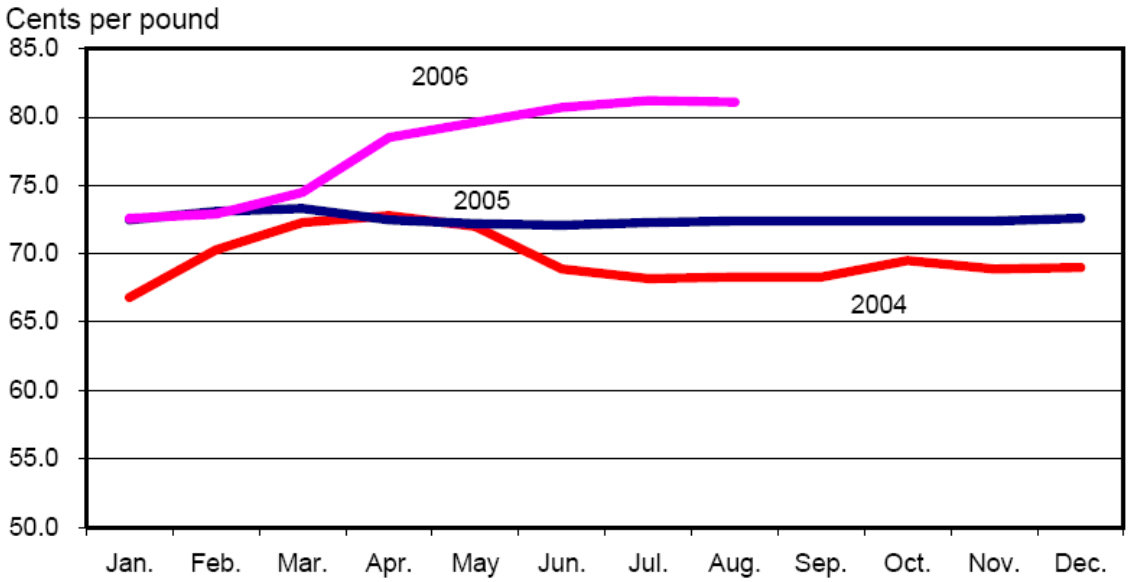

Figure 1.3. Average selling price per pound of catfish during the last three years on record.

As a consequence of this low profit margin and despite notable improvement compared to the beginning of the decade, the stability of the catfish aquaculture industry remains fragile and can ill afford an increase in its production cost due to disease outbreaks.

Ictalurus punctatus biology and farming practices

Channel catfish can be sexually differentiated at about 6 months of age. However, they only reach reproductive maturity in their second or third year.

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Reproduction occurs during the spring months, generally from April to June, when the temperature rises above 21°C. The eggs are large (2.4-3.0 mm), very adhesive, and usually laid in a large egg mass. The number of eggs a female will carry varies widely, depending mainly on its age and size, and will generally be between 3,000 and 50,000 eggs per female. Females reach their reproductive optimum, in term of number of eggs produced, size of the eggs, and egg fertility, between their third to fifth year of age.

Catfish have an elaborate breeding behavior where the male is usually in charge of incubating the eggs. The incubation period of *Ictalurus punctatus* lasts 100 to 150 degree days (as calculated by multiplying the number of days by the average temperature during this day, in order to account for the polythermy of the eggs and the impact of the ambient temperature on their metabolic levels) with an optimal incubation temperature being between 25° to 27°C. After hatching, the young catfish absorb their yolk sacs and begin swimming within 3 to 4 days, and predatory behavior appears with the fish readily accepting artificial diet.

After their first winter, either spent in indoor tanks or in outdoor ponds, catfish are transferred to outdoor earthen ponds at the beginning of the spring, where they are kept until being harvested, generally during the summer of the following year.

Main disease problems affecting the industry

During the production cycle, catfish are subject to bacterial, viral, and parasitic infections. The most important are discussed in below.

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Columnaris disease. Among the most significant pathogens is *Flavobacterium columnare*, a yellow-pigmented filamentous gram-negative bacterium in the family *Flavobacteriaceae*. This pathogen is considered to be ubiquitous in the aquaculture environment and is mainly considered an opportunistic pathogen, requiring stressors such as low oxygen, high nitrite, high ammonia, high water temperature, rough handling, mechanical injury, or crowding to cause disease (Durborow et al. 1998). Recent work has highlighted the high degree of variability in virulence between different strains of the bacterium (Alvarado et al. 2005).

Outbreaks of *Columnaris* occur when the water temperatures are in the upper part of the preferred temperature range for catfish, 25 to 32°C, which corresponds to spring, summer, and fall. *F. columnare* causes an acute to chronic infection of the gills and epidermis, including the fins. External lesions occur on the gills, oropharynx, and skin and are characterized by presence of a characteristic yellowish-brown color that is visible with the naked eye (Durborow et al. 1998).

Transmission can be direct between fish or through the water and, in its more lethal form, the bacterium's route of infection occurs primarily by attaching to the gill surface. Bacteria then expand along the gill tissue in spreading patches until covering the entirety of an individual gill filament. As disease progresses, portions of the gills are degraded by protein and cartilage degrading enzymes. Another classical site of attachment is the dorsum of the fish, just behind the dorsal fin, leading to the classical 'saddleback lesion'. Even if columnaris disease starts as an external infection (Welker et al. 2005), disease can quickly progress to a septicemia. In one study, it was shown that,

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40% of the fish where it was present externally, *F. columnare* could also be isolated (Durborow et al. 1998).

Once established, columnaris disease can spread rapidly and cause a high rate of mortality. Columnaris disease can predispose other opportunistic diseases such as infection by *Saprolegnia* fungus. Diagnosis is often based on clinical signs and a wet mount of the mucus. Long slender rods are clearly visible at a magnification of 100 to 400 times, classically forming 'haystack' structures. Diagnosis can be confirmed by culture (Durborow et al. 1998).

Proliferative gill disease. The etiological agent of proliferative gill disease is the myxosporean myxozoan parasite *Henneguya ictaluri* (Pote et al. 2003). The parasite has a two-host life cycle (Whitaker et al. 2001) that is similar to that of other myxozoan life cycles previously described, including that of *Myxobolus cerebralis* (Belem & Pote 2001). The precise route of entry of *Henneguya ictaluri* in the fish host is, as yet, unknown (Belem & Pote 2001). However, small parasite stages can be observed in the mucosa and submucosa of the stomach 24 hours post-exposure as well as in the skin and buccal cavity epithelium. They are also found in the blood vessels of the heart and liver and in the parenchyma of spleen and head and trunk kidney. From 24 to 72 h post-infection, the number of the organisms decreases in all locations except in the gills and the gastric glands of the stomach. This trend is even more marked by 96 hours post-infection; most of the parasites have disappeared from the fish except in the gills and the stroma

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(Belem & Pote 2001). After reaching the gills, the parasite develops into its final microspore stages.

The early clinical signs of this infection include anorexia and listlessness. Infected fish often try to compensate for the damage sustained to their respiratory apparatus by moving toward regions where dissolved oxygen concentration is higher, near aeration devices or in shallow areas of the pond (Pote et al.). Gross lesions are characterized by swelling and fragility of the gills as well as by a mottled red and white appearance, which has led to the condition's nickname: "hamburger gill disease" (Whitaker et al. 2001).

Mortalities can range from 1 to 65% of the fish in affected ponds (Pote et al. 2003). These mortalities are often severe in the earliest stages of the disease and decrease after the first 2 weeks (Pote et al. 2003). Fingerlings are generally more susceptible than adult food fish. Clinical lesions are generally limited to the gills, which are mottled, fragile, and bleed easily. The disease progresses with fractures of the cartilage and, in the more chronic form of the disease, misshaped gill filaments (Pote et al. 2003).

Histologically, the condition is characterized by the presence of granulomatous branchitis. Gill filaments can be congested and hemorrhagic with a mixed infiltration of inflammatory cells (Belem & Pote 2001). Epithelial hyperplasia and lamellar fusion are frequently observed (Pote et al. 2003). Intensely basophilic parasitic trophozoites can sometime be seen as well as characteristic lysis and fractures of the cartilages (Figure 1.4). When gill filaments regenerate, they often remain misshapen (Figure 1.5).

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Interestingly, the apparent severity of lesions does not always correlate well with mortalities (OIE 2006).

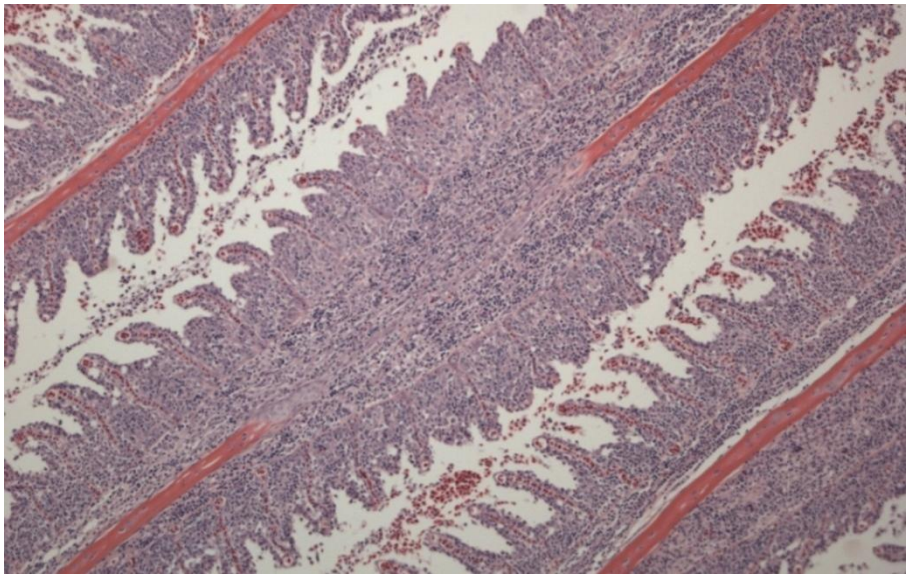

Figure 1.4. Broken cartilage, characteristic of an active infection by the myxozoan parasite *Henneguya ictaluri* (200X).

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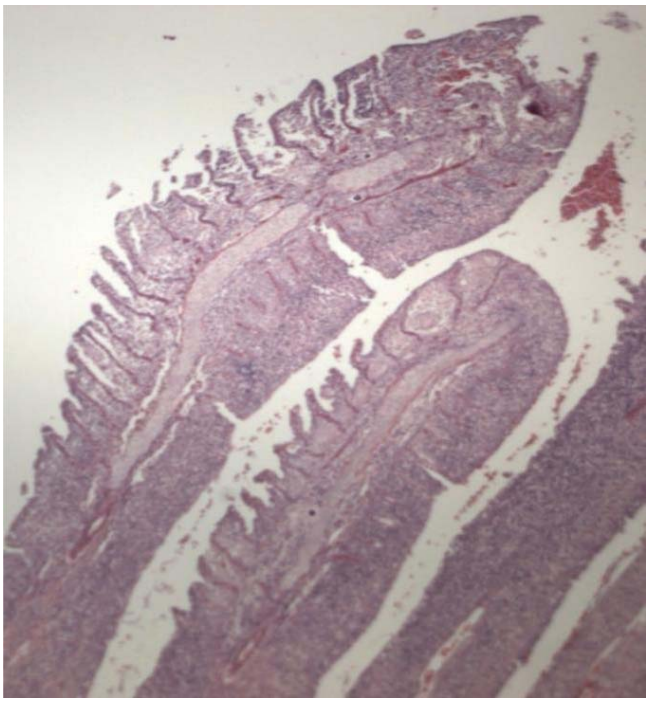

Figure 1.5. Misshapen cartilage in a fish in the early recovery stages from an infection by the myxozoan parasite *Henneguya ictaluri* (200X).

Channel catfish virus disease. The causative agent of channel catfish virus disease (CCVD) was identified in 2006 as *Ictalurid herpesvirus* I by the International committee on Taxonomy of Viruses. However, it is commonly known as Channel Catfish Virus (Smail & Munroe 2001). Like the other herpesviruses, Channel Catfish Virus (CCV) is an enveloped virus, and its genome is linear double stranded DNA. It also shares the gross morphology of herpesviruses, especially with the alpha subfamily of herpesviruses (Booy et al. 1996). However, its protein sequence is distinct from other herpesviruses (Fijan 1998), and it has been suggested that it represents a new subfamily (OIE 2006).

The only known hosts for CCV are the channel catfish (*Ictalurus punctatus*) and the blue catfish (*Ictalurus furcatus*), with some variability in susceptibility depending on

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the fish strain (OIE 2006). The age of the fish is one of the main factors determining severity of infection; most outbreaks occur in fish less than one year old, and typically outbreaks occur in fish less than 4 months old. Surviving fish usually have strong protective immunity with production of circulating antibodies; however, survivors also have a covert latent state (Smail & Munroe 2001). Adults appear to be mainly resistant, although even adult survivors often show impaired growth.

Outbreaks occur when pond temperatures are highest (from May to September) and are more common in years when the temperatures are elevated. Experimentally, catfish fry fail to develop the disease at temperatures below 15˚C, and moving infected catfish into cooler water temperature (from 19˚C to 28˚C) was shown to reduce mortality (Gray et al. 1999).

In addition to the acute form, there appears to be a latent form of the disease where the virus is present but not actively replicating. The conditions that cause virus reactivation are unknown, but stress, especially associated with elevated (>27˚C) temperature, is often associated with outbreaks (Wolf & Darlington 1971). Because this latent form is difficult to detect, the exact prevalence of this form of disease is difficult to determine. However, CCV is considered to be endemic in channel catfish in most parts of the USA where commercial aquaculture is active. In addition, CCVD has been reported from Latin America and Russia following introduction of catfish from the USA. Interestingly, the high virulence that the virus exhibits in channel catfish is uncharacteristic of herpesviruses and might suggest the virus is actually exotic in

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channel catfish, possibly being introduced from another catfish species (Smail & Munroe 2001).

Typically, the first sign of a CCVD outbreak is an increase in mortality and morbidity in ponds at temperatures above 25˚C. Most mortalities occur within 10 days. Mortality rate can be variable, but commonly it is between 40 to 50%. Mortalities generally cease within 2 to 3 weeks (Venable et al. 2000).

Gross lesions associated with CCVD are nonspecific but are typical of an agent targeting the kidney. Therefore, clinical signs can be mistaken for infections by *Edwardsiella ictaluri* or *Bolbophorus* (Stingley et al. 2003). Externally, clinical signs are characterized by mortality, lethargy (some fish can be found in a characteristic head up position), and erratic spiral swimming. Diseased fish also demonstrate ascites, exophthalmia, and protruding vent associated with the perturbation of osmoregulatory function. Hemorrhages can also be seen in the fins, internal organs, and the musculature (Smail & Munroe 2001). Gills are generally pale dues to loss of blood (Fijan 1998). Internally, the spleen is congested and dark. A yellowish or slightly reddish mucoid material is found in the digestive tract (Fijan 1998).

Secondary infection, mainly with *Flavobacterium columnare* or *Aeromonas* spp. (OIE 2006), often occur in chronic cases. Histology reveals extensive necrosis in posterior kidney, mainly in the interstitial tissue, although renal tubules can be involved as well. Liver, intestine, and spleen also display edema and necrosis (Terhune et al. 2003). However, in the most acute cases, lesions may not have time to develop prior to death of the fish.

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Bolbophorus damnificus. *Bolbophorus damnificus* is a digenetic trematode endemic in white pelican populations. The presence of the bird, which is the primary host, appears to be the main factor influencing prevalence of the parasite. *B. damnificus* has a complex life cycle that has only recently been determined (Yost et al. 2007) and involves one definitive and two intermediate hosts (Figure 1.6). Direct transmission of the pathogen is not possible.

Unlike other digenetic trematodes that have been reported to have several different potential hosts, *B. damnificus* appears to be very host specific. The only definitive host identified is the American white pelican (*Pelecanus erythrorhynchos*), which sheds *Bolbophorus* eggs into ponds when defecating (Yost 2008). Intensity of shedding is irregular (Terhune et al. 2003). Eggs hatch to produce miracidiae that can infest the first intermediate host, the ram's horn snail (*Planorbella trivolvis*), where it matures and is released as a cercaria (Yost 2008). *P. trivolvi* are present all year and can shed up to 1400 cercariae a day (Overstreet et al. 2002). Shedding from infected snails is temperature dependent; when water temperature decreased from 25 to 15˚C, the number of cercariae shed from snails decreased from 345 to 1.6 cercarie per ml. Shedding of cercariae reestablishes when temperature was raised.

Cercariae infect and encyst in the fish host, usually just under the skin (Terhune et al. 2003) and form metacercariae. The parasite remains encysted in the fish until the life cycle is completed when a pelican ingests the fish (Overstreet et al. 2002). Infection of the pelican is rapid, occurring in 30 hours in nestling pelicans. The parasite will reach

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sexual maturity and produce eggs by day 3 in experimentally infected adult pelicans. Adult parasites can survive for up to five months in their host (Terhune et al. 2003).

Infected catfish have small (0.1 to 0.2 cm) cysts that can be seen anywhere in the body but appear more often in the tail region (Avery et al. 2001). Generally, these cysts appear as a raised bump under the skin or deeper in the muscle tissue (Avery et al. 2001). In severe infections in smaller fish, infections can cause massive damage to the kidneys and liver. Gross lesions are non-specific and can seem similar to that of other pathogens, including channel catfish virus and *Edwardsiella ictaluri*, that cause perturbation of the osmoregulatory system. In particular, ascites, which can cause abdominal distension, and exophtalmia occur (Terhune et al. 2003; Terhune et al. 2003).

Larger fish appear less susceptible to *Bolbophorus* infections with lesions limited to the skin and fins. However, even large fish are likely to feed poorly and appear emaciated (Venable et al. 2000). Even if the fish does survive infection, the small white cysts drastically reduce marketable value of fillets (Avery et al. 2001). The impact of these infections is variable, ranging from no visible effect to extensive mortalities (Yost 2008).

Trematodes generally are not very pathogenic to their host. Therefore, *B. damnificus* is an exception (Singleton 2004). Channel catfish may be an abnormal host for *B. damnificus*, which would explain the high virulence as well as the poor performance of affected individuals. Despite the virulence of this pathogen, the impact of the disease is mostly indirect, affecting the industry less in terms of fish loss and more

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in terms of decreased performance and increased cost. As a result, the economic impact is difficult to evaluate.

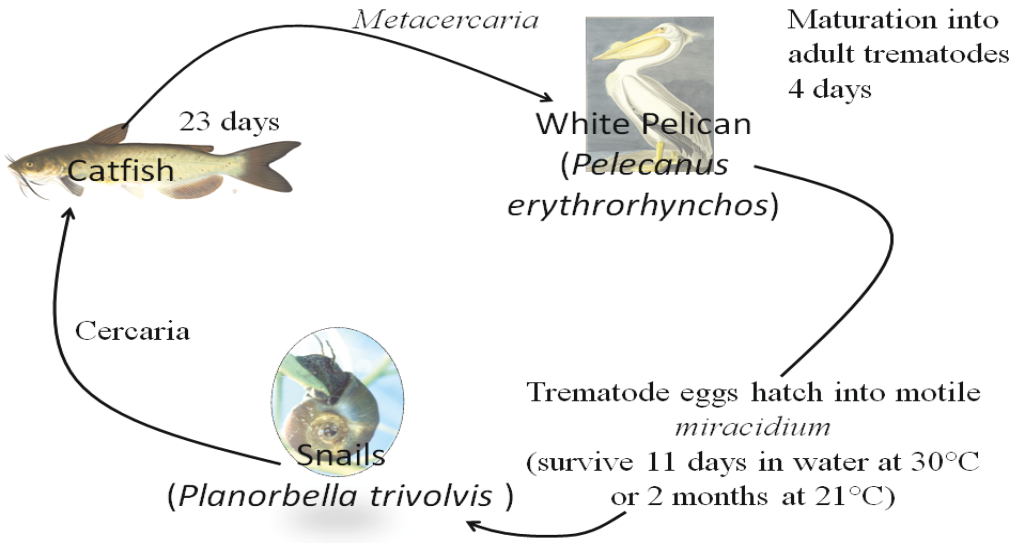

Figure 1.6. The life cycle of *Bolbophorus damnificus* (Adapted from Yost et al. 2007).

Edwardsiella ictaluri

General features of *Edwardsiella ictaluri*

Edwardsiella ictaluri, the causative agent of Enteric Septicemia of Catfish (ESC), is a small (0.75 X 1.5-2.5 µm) pleiomorphic gram-negative rod in the family *Enterobacteriaceae*. *E. ictaluri* has peritrichous flagella, and fimbriae have been detected by electron microscopy. It survives in sterilized pond-bottom mud for over 90 days at 25˚C (Lawrence et al. 1997) and is weakly motile at 25 to 30˚C but not at higher temperature.

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E. ictaluri is indole, lactose, and cytochrome oxidase negative, biochemical tests helpful for its identification. It ferments glucose and is catalase positive. On Tryptic Soy Infusion (TSI) agar, it produces an alkaline slant and acid butt without H_2S. It is non-fermentative for other sugars and does not grow on salinity higher than 1.5%. It is a rather homogenous species biophysically, biochemically, and serologically (Fernandez et al. 2001).

All *E. ictaluri* isolates harbor two native plasmids that appear to be similar to *E. coli* plasmids (Fernandez et al. 2001). These plasmids were termed pCL1 and pCL2, and their sizes are 5.7 kb and 4.9 kb, respectively (Plumb 1993). Additional plasmids have been inconsistently reported.

Known virulence factors of the bacterium

A variety of virulence factors have been identified in *Edwardsiella ictaluri*. Lipopolysaccharide plays a role in virulence (Lawrence et al. 1997; Williams et al. 2003; Thune et al. 2007). Its surface harbors sialic acid (Ourth & Chung 2004) that mediates complement resistance and can prevent formation of membrane attack complex (Thune et al. 2007). Two *E. ictaluri* outer membrane proteins, 193UV and 233PR, appear similar to adhesion proteins from *Salmonella* (Skirpstunas & Baldwin 2002) and are likely to play a role in invasion of epithelial cells (Williams et al. 2008). The genome sequence revealed fimbrial genes and multiple flagella genes (Thune et al. 2007).

E. ictaluri possesses a type III secretion system similar to that of *Salmonella* (Williams & Lawrence 2005). It also produces a secreted hemolysin (Lawrence et al.

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1997) and at least two types of chondroitinase. It does not appear to produce siderophores, but it does have several heme binding proteins (Thune et al. 2007). The genome sequence also revealed pathogenicity islands similar to *Salmonella*. Pathogenicity islands are short regions of the chromosome that harbor a high number of co-regulated virulence genes (Fergusson et al. 2001, Crumlish et al. 2002).

Distribution of enteric septicemia of channel catfish

Channel catfish (*Ictalurus punctatus*) are the main species affected by *E. ictaluri*, but sensitive species include the white catfish (*Ameiurus catus*), the blue catfish (*Ictalurus furcatus*), and, less commonly, the brown bullhead (*Ameirurus nebulosus*). Blue catfish tend to be more resistant, and the European catfish (*Silurus glanis*) is only slightly susceptible. The disease has recently been identified in the Vietnamese catfish (Plumb 1998). The majority of clinical cases are from the USA, but it is also present in Thailand, Australia (Fergusson et al. 2001, Crumlish et al. 2002), and Vietnam (Plumb 1998).

The disease pattern of ESC is seasonal. Outbreaks occur in spring, early summer, and fall when the water temperature is between 18° to 28˚C, with maximum virulence at 25˚C. However, an increase in the number of cases occurring in summer and winter may be evidence that the pathogen is broadening its temperature range (Plumb 1998). Interestingly, the disease is mainly one of the farming industry. Stress seems to increase susceptibility and severity of ESC, with a reported increase from 77 to 97% mortalities when fish are stressed (Hawke et al. 1998). Reports of natural fish kills caused by

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Edwardsiella ictaluri are rare (Hawke et al. 1998), suggesting that the stress related to farming practices is of great importance in the development of the disease.

Clinical signs of ESC

Diseased fish are often described as swimming erratically in circles (Plumb 1998). They can be listless and hang on the surface with 'head-up tail down posture' (Hawke et al. 1998). Anorexia is one of the first signs of disease (Venable et al. 2000). Gross lesions are somewhat nonspecific and can be confused with those caused by other catfish pathogens such as *Bolbophorus damnificus* or channel catfish virus (Plumb 1998). Fish display petechial hemorrhages, especially in the skin under the jaw, on the operculum and the belly, and at the base of the fins (Plumb 1998). Small, white depigmented areas of 1 to 3 mm wide appear on the skin and can progress to cutaneous ulcers. In chronically ill fish, an open lesion can develop between the frontal bones of the skull posterior to or between the eyes. This lesion has resulted in the common name 'hole-in-the-head' disease. However, this lesion is not pathognomonic; other bacteria like *Aeromonas hydrophila* may cause similar lesions (Plumb 1998) and not all fish with ESC have this lesion.

Internal lesions include ascites, which can be cloudy and bloody and rarely clear yellow. The kidney and spleen are hypertrophied, while the spleen is dark red. Inflammation occurs in adipose tissue, peritoneum, and intestine, and the liver can be pale or very congested (Roberts 2001a). The disease is characterized by septicemia and the presence multifocal necrosis on the spleen, kidneys, and liver with infiltration of

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immunologic cells and the development of true abscesses. Multiple hemorrhagic foci can be observed in most organs, and melanomacrophage centers are often destroyed. There is also a high frequency of melanin-containing lymphocytes in the engorged vessels (Vedemeyer 1996, Plumb 1998).

Diagnostic, prophylaxis and treatment

The clinical signs of ESC, such as petechial hemorrhages, compromised osmoregulation, and infected ulcers, are often nonspecific. Therefore, presumptive diagnosis is often based on clinical signs and history (presence of younger catfish and water temperature in the 18° to 28°C range) (Roberts 2001b). Definitive diagnostic is based on isolation of the pathogen from infected fish. Because *E. ictaluri* can be overgrown by other bacteria (Collins & Thune 1996), use of selective media such as *Edwardsiella ictaluri* medium can improve consistency of isolation (Roberts 2001b). *E. ictaluri* requires up to 48 hours to form 1mm wide colonies on BHI agar at 26°C (Wise & Johnson 1998), therefore treatment is often started before definitive confirmation has been achieved.

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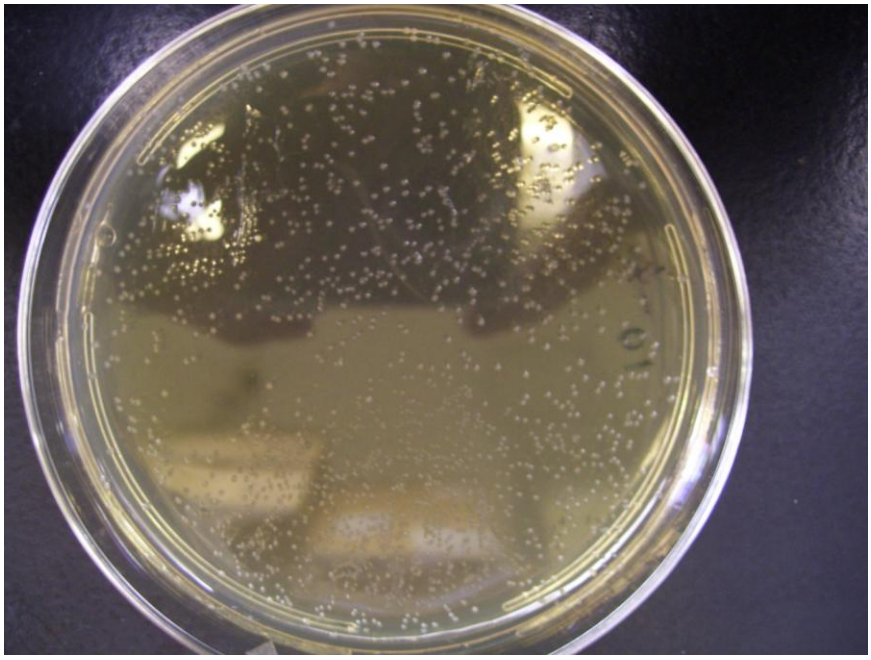

Figure 1.7. Appearance of *Edwardsiella ictaluri* colonies on Brain-Heart-Infusion agar.

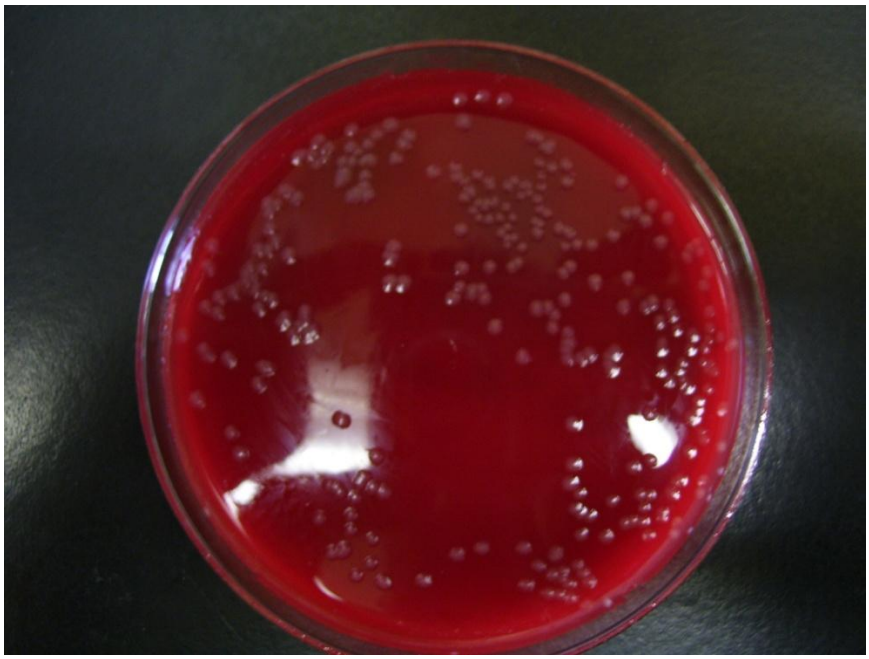

Figure 1.8. Appearance of *Edwardsiella ictaluri* colonies on Tryptic Soy Agar plates enriched with 5% sheep blood.

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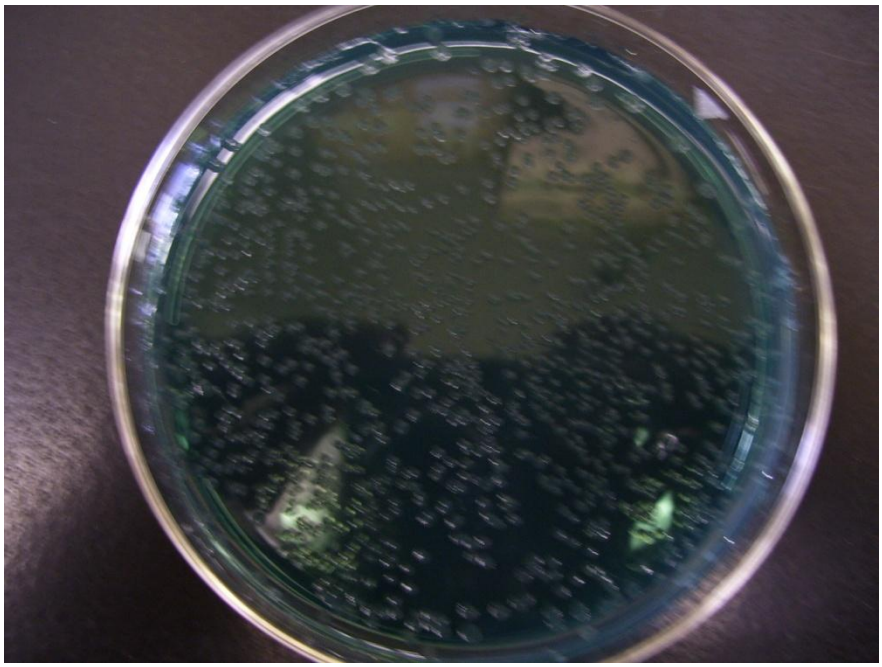

Figure 1.9. Appearance of *Edwardsiella ictaluri* colonies on *Edwardsiella ictaluri* medium agar.

Traditionally, ESC is treated with a feed additive sulfonamide antibiotic sulfadimethoxine along with the synergistic dihydrofolate reductase inhibitor trimethroprim, which is available commercially as Romet® (McGinnis et al. 2003).

More recently, florphenicol was approved by the FDA for use in the treatment of *E. ictaluri* and *F. columnare* in channel catfish under the brand name Aquaflor® (Cooper et al. 1993, Welch et al. 2009).

However, resistance to both of these antibiotics has been reported in clinical isolates of the bacterium (Wise & Johnson 1998)

Interestingly, it was also found that totally withdrawing feed from the fish mitigates the impact of the disease efficiently (Plumb & Vinitnantharat 1993, Plumb

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1998). However, this method is not desirable because it leads to a significant decrease in the pond's production levels and its profitability.

Prophylactic methods have been researched almost since the first isolation of the bacterium in the 1980. *E. ictaluri* has long been considered a good candidate for the development of vaccine due to its biochemical and serological homogeneity as well as its ability to induce a strong immune response (Gudding et al. 1999). Killed bacterin have been used successfully to control other fish pathogens such as the *Aeromonads*, *Vibrio, Yersinia ruckerii* (Plumb & Vinitnantharat 1993). One common method for the production of a killed vaccine is by exposure to 0.8% formalin (Gudding et al. 1999). However, initial attempts to vaccinate catfish using formalin killed *E. ictaluri* produced mixed results. While the vaccines did confer some level of protection, it was limited and transient (Nusbaum & Morrisson 1996).

There are several possible reasons for this lack of protection by killed bacteria (bacterin). For example, it was shown that inactivated bacterial particles are unable to cross the epithelial barrier to be presented to the circulating immune system (Gudding et al. 1999), which would limit the effectiveness of bacterin delivery to catfish by bath immersion or oral ingestion. Furthermore, killed bacteria are not as effective at stimulating cell-mediated immunity, which is particularly important for an intracellular pathogen such as *E. ictaluri* (Antonio & Hedrick 1994). In *E. ictaluri*, there is a poor correlation between host resistance and the levels of circulating antibodies (Klesius & Sealey 1995; Wise et al. 2000), confirming the importance of *E. ictaluri's* ability to

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survive intracellularly and the need for the development of a strong cell-mediated immunity.

As a result, most of the recent *E. ictaluri* vaccine research has focused on developing a live attenuated vaccine. The current commercially available live attenuated vaccine was developed by selection for rifampicin resistance (Wise et al. 2000) and it has shown potential for vaccination in eggs and fry (Baldwin & Newton 1993; Shoemaker et al. 2006).

Mechanisms of tissue colonization in prokaryotes

Because adhesion to the host cell is the indispensible first step to any colonization process, bacterial pathogens have evolved a wide array of mechanisms to bind to host cells. The variety displayed by these mechanisms illustrates the number of unrelated evolutionary pathways that have been, at one time or another co-opted in the so-called arm-race between host and pathogen. The most significant among these adhesion mechanisms are discussed below.

Fimbriae

Fimbriae are among the most well-known mechanisms of bacterial adhesion. They consist of a linear sequence of protein subunits (termed pilin) anchored to the bacterial cell envelope (Kisiela et al. 2006). Several types of fimbriae have been described, based on their function, morphology, and mode of assembly.

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Among the fimbrial types, type I is probably the most common, especially among gram–negative species. They are an important virulence factor in several *Enterobacteriaceae*, such as *E. coli* or *Salmonella enterica* Typhimurium. *S.* Typhimurium possesses several fimbrial genes and chief among them is a type I fimbriae (Kisiela et al. 2006). Most of the type I fimbriae is composed of a series of FimA subunits connecting to their anchoring point in the outer membrane. The distal end of type I fimbriae is composed of several proteins, most notably FimH, which is a lectin-like mannose binding molecule (Lambert & Smith 2008) that acts as an adhesin (Singleton 2004; Kisiela et al. 2006) to oligomannosidic molecules (Singleton 2004).

Another fimbrial type of importance is the type IV (also termed bundle forming pili). These fimbriae are present in a variety of distantly related pathogens including *Vibrio*, *E. coli* (Kisiela et al. 2006), *Salmonella enterica* (Barnhart et al. 2002), and *Neisseria gonorrhoeae* (Singleton 2004). They are secreted through the type 2 secretion system and anchored to the cytoplasmic membrane. These fimbriae are unique due to their ability to retract, allowing the bacteria to gain a 'twitching motility' (Barnhart et al. 2002).

Adhesion and the 'zipper mechanism'

Many common adhesion molecules are described under the term 'adhesin' and bind specific receptor moieties (Boyle & Finlay 2003). Often the receptor is a host adhesion molecule on the host cell surface (Barnhart et al. 2002; Boyle & Finlay 2003),

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such as integrins, cadherins, and members of the immunoglobulin super-family (Boyle & Finlay 2003).

A well characterized example of adhesin is the molecule invasin, described in *Yersinia enterolitica* and *Yersinia pseudotuberculosis* (Cossart & Sansonetti 2004). This molecule is structurally similar to the RGD domain of fibronectin that normally interacts with integrin receptors. These integrin receptors harbour β_1 chains that are normally involved in cell adherence to the extracellular matrix (Young et al. 1992); (Eitel et al. 2005). In addition to invasin, *Yersinia* also possesses another adhesin termed YadA that interacts with the β_1-integrins (Van Nhieu & Sansonetti 1999). Similarly, in *Shigella,* the IpaB/B/D molecules form a complex that binds $\alpha_5\beta_1$ integrin and CD44 on the host cell membrane (Berndt et al. 2009).

The adhesion mechanisms of *Salmonella* include a variety of adhesion proteins able not only to bind receptors on the host cells, but also to adhere to a variety of molecules from the extracellular matrix such as fibronectin, laminin, or collagen (Lambert & Smith 2008). For example, PagN, a protein with 54% similarity to the known *E. coli* adhesins Hek and Tia, was recently linked to adhesion (Vazquez-Torres & Fang 2000), and an adhesin targeting β2-integrin of CD18-expressing cells was also identified (Boyle & Finlay 2003). The gastric pathogen *Helicobacter pylori* also has two adhesins: BabA, which initiates the initial attachment to mucosa, and a second one targeting Lex, a selectin ligand that is upregulated during gastritis (Cossart & Sansonetti 2004).

Adhesins are not an exclusive feature of gram-negative species. Members of the genus *Listeria* possess internalin A, which has a leucine-rich region that interacts with

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the first ectodomain of human E-cadherin. E-cadherin is a transmembrane glycoprotein at adherens junctions in polarized epithelial cells (Schubert et al. 2002) that mediates adhesion to phagocytes (Berndt et al. 2009). Another adhesin in *Listeria*, internalin B, also belongs to the LRR family of proteins and is loosely attached the lipoteichoic acids on the bacterial surface. It has three different cell ligands, the most important of which is Met, which is a transmembrane receptor that normally reacts to the presence of hepatocyte growth factor (HGF).

In addition to their binding properties, many adhesins are known to affect gene regulation in the pathogen or its host. For example, exposure of *S. Enteritidis* and Invantis to fibronectin, laminin or Matrigel Basement Matrix was shown to up-regulate the expression of *fli*C, a gene involved in invasion of epithelial cells (Cossart & Sansonetti 2004).

The binding of an adhesin to the cell receptor often induces a signaling cascade leading to the recruitment of additional receptors to the area as well as reorganization of the actin filaments, which causes membrane extension and pathogen uptake. This process is termed the "zipper" mechanism (Van Nhieu & Sansonetti 1999). For example, the IpaB/C/D complex of *Shigella* appears to activate Cdc42 and Rac, which are GTPases. Gdc42 is involved in the formation of microspikes, while Rac is involved in filopodial extensions (Cossart & Sansonetti 2004). Similarly, the *Yersinia* outer membrane protein invasin most likely uses focal adhesion kinase; mutations in this protein impair *Yersinia* uptake (Cossart & Sansonetti 2004). In the case of *Listeria*, the binding of InlB to Met probably activate the Rac-WAVE-ARP$_2$β and the Rac-PAK-LIM-kinase-cofilin cascades

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(Bierne & Cossart 2002, Cossart & Sansonetti 2004) which induce the recruitment and rearrangement of the actin filaments (Bierne et al. 2001) leading to a ruffling of the host cell membrane and the phagocytosis of the bacterium (Cossart & Sansonetti 2004).

Type III secretion system and the 'trigger mechanism'.

A third major way for bacteria to gain entry into host cells is by the injection of bacterial effectors through the type III secretion system, which induces a rearrangement of the actin filaments leading to the formation of filopodial and lamellipodial structures (Van Nhieu & Sansonetti 1999). For example, *Shigella* produces at least fifteen effector proteins and a plasmid encoded Mxi/Spa type-III secretor system that induce macropinocytic uptake by M-cells (Vazquez-Torres & Fang 2000). *Salmonella* also possesses a similar type III secretion system that causes cytoskeletal membrane ruffling (Yen et al. 2002).

Two-partner secretion system

In addition to these three major mechanisms, a variety of other molecules mediate bacterial adherence.

Most interesting among them is the two-partner transport system that was initially described in *Serratia marcescens* (Talà et al. 2008) before being identified in a variety of gram-negative pathogens, including *Bordetella pertussis*, *Haemophilus influenzae* (Schmitt et al. 2007) and about 97% of the virulent isolates of *Neisseria meningitidis* (Gentle et al. 2005). This secretion system is composed of two proteins:

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TpsA, which is an effector protein, and TpsB, which transports TpsA across the bacterial outer membrane.

While the activity of the TpsB molecules is centered around a well-conserved β-barrel structure similar to that of other members of the Omp85 family of outer-membrane channel proteins (Yen et al. 2002), more heterogeneity exists among the TpsA effector proteins. For example, in *S. marcescens*, the TpsA corresponds to ShlA, a hemolysin, secreted extracellularly from the periplasm (Schmitt et al. 2007). In *B. pertussis*, the effector protein is a filamentous hemagglutinin, sometimes termed FHA, that constitutes an important virulence protein and is the target of the recombinant pertussis vaccine (Schmitt et al. 2007). In the *Neisseriaceae*, the two-partner secretion system is sometimes referred to as HrpA and HrpB. It has been demonstrated that the secreted protein HrpA undergoes proteolytic processing during its translocation across the membrane and has a cell-type specific role in adhesion, especially in the rough strain of the bacterium (Talà et al. 2008). HrpA also appears to have a significant role at a later stage of infection during escape from the endocytic vacuoles (Plumb 1998, Austin & A. 1999, OIE 2003).

Rationale and significance

Despite three decades of research, there is still debate as to how *E. ictaluri* gains entry into its host. The best characterized route of entry is through the stomach and intestinal wall (Plumb 1998). Another possible route of infection is through the nares, followed by migration along the olfactory nerve. This route would account for the

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behavioral symptoms often associated with the disease, and it was suggested that this route would be associated with the chronic form of the disease (Plumb 1998). It also was hypothesized that *E. ictaluri* could use the gills as a portal of entry.

On the other hand, *Edwardsiella tarda*, a close relative of *E. ictaluri*, has often been linked to myonecrosis in human hosts (Nusbaum & Morrisson 1996, Wise et al. 1997). Furthermore, *E. ictaluri* can often be found in the capillaries of the skin (Skirpstunas & Baldwin 2002). Furthermore, the pathogen produces two different chondroitinases, which are enzymes that have been involved in increasing the permeability of the epithelium by degradation of the basal membrane (Smith et al. 1997). During infection by *E. ictaluri*, these enzymes are probably responsible of the 'hole in the head' lesion that often forms in the chronic form of the disease. In addition, it has been demonstrated that *E. ictaluri* was able to invade a wide variety of eukaryotic cells, including epithelial cells (Slaven et al. 2001).

Therefore, it seems likely that *E. ictaluri*, like many aquatic pathogens, is able to take advantage of zones of skin abrasion as a primary site of attachment and entry into the host fish. Therefore, our first study was designed to use bioluminescent *E. ictaluri* to determine if skin abrasion provided a site of colonization, and if skin abrasion can alter the development of septicemia and increase severity of the disease.

The attachment and colonization of epithelial cells by *E. ictaluri* could occur through a number of mechanisms, including an adhesin-like protein and/or fimbriae. Therefore, the next step in our research was to identify mechanisms of attachment by using transposon mutagenesis to inactivate random genes in the bacterium. Mutants

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were then screened to determine which ones displayed a decrease in their ability to colonize the epithelium.

Finally, it is known that *E. ictaluri* has a complex infection cycle comparable in many respects to that of *Salmonella enterica* Typhi. ESC appears to involve a primary site of amplification before the bacteria reenters the blood in larger numbers. However, no systematic study has yet been conducted to determine changes in the distribution of *E. ictaluri* during the development of ESC. The final step in our research was therefore to use bioluminescence and plate counts to investigate the presence of the bacteria at different time points. This lead to a better understanding of the disease process which may, in turn, allows for a better targeting of treatment or assist in the development of vaccines against the disease.

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CHAPTER II

IMPORTANCE OF SKIN ABRASION AS A PRIMARY SITE OF ADHESION FOR *Edwardsiella ictaluri* AND IN THE SUBSEQUENT DEVELOPMENT OF ENTERIC SEPTICEMIA IN CHANNEL CATFISH *Ictalurus punctatus*

Abstract

The route of entry of *Edwardsiella ictaluri* into its catfish host has been a subject of investigation since the pathogen was first discovered. There are evidences to support entry through the intestinal tract, the nares, and the gills. Here, we evaluated the role of skin abrasion through a series of experimental challenges using bioluminescent *E. ictaluri* carrying the plasmid pAKLux1. Our results show that *E. ictaluri* is able to colonize abrasion sites on catfish skin and that catfish with abrasions developed septicemia faster. We also found that abrasions are associated with significantly increased mortalities following experimental immersion exposure. Finally, a protocol was developed during this study that allowed for immunohistochemical examination of the tissue layers underneath the abrasion sites, confirming the presence of *E. ictaluri* in subdermal tissues from abrasion sites. This study constitutes the first report on the role of abrasion sites on the channel catfish skin as a portal of entry for *E. ictaluri* and further illustrates how versatile this pathogen can be in its mechanisms of entry.

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Introduction

The mucus and skin barriers are the body's first line of defense against pathogens. Indeed, by its physical nature, the mucus impedes bacterial attachment because it is composed of a polymeric high-molecular weight mucin that is continually sloughing away (Aranishi and Nakane, 1997; Magnadottir, 2006). Additionally, an indigenous flora compete with potential invaders and a variety of antimicrobial compounds impede bacterial invaders, including lectins (Shepard, 1994), lysozyme (Fast, Sims *et al.*, 2002), proteases (including cathepsins L and B-like proteases, serine protease, and aminopeptidase (Ellis, 2001), alkaline phosphatase (Fast, Sims *et al.*, 2002), and antibodies (Shepard, 1994). Similarly, epithelial cells themselves form a tight impermeable physical barrier that contains several distinct proteases (Aranishi and Nakane, 1997) and is lined with lysozyme. Below the epithelial layer reside many immune cells including plasma cells and macrophages (Booy, Trus *et al.*, 1996; Lin, Davidson *et al.*, 1998).

It is, therefore, not surprising that many fish pathogens, including *Flavobacterium columnare* (Bader *et al.*, 2003), *Aeromonas* in salmonids, and *Edwardsiella tarda* (Plumb, 1998) take advantage of skin abrasion to establish infection. In the specific case of *Edwardsiella ictaluri*, the recurrent presence of skin ulcerations in the chronic form of disease (Plumb, 1998) suggests an affinity of the pathogen for skin. Additionally, *E. ictaluri* was shown to invade a wide range of epithelial cells cultures *in vitro* (Skirpstunas and Baldwin, 2002).

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We found evidence suggesting that *E. ictaluri* has a predilection for sites of skin damage (Karsi, Menanteau-Ledouble *et al.*, 2006). The theory that *E. ictaluri* is able to take advantage of skin lesions would be consistent with the increase in ESC prevalence in the early 1980s, a period marked with an intensification of farming practices and an increase in catfish stocking densities and hence in an increase in fish wounds due to territorial behaviors. However, to the best of our knowledge, the effect of skin abrasions on the pathogenesis of *E. ictaluri* infection has not been thoroughly investigated. Therefore, we designed a set of experiments to determine the plausibility of skin abrasion as an initial site of entry for *E. ictaluri* and subsequent development of septicemia.

Materials and Methods

Bacterial strains

E. ictaluri strain 93-146 was originally isolated from a clinical outbreak of ESC in a commercial aquaculture pond. Plasmid pAK*lux*1 was transferred into this strain from *Escherichia coli* strain SM10 by conjugation as described (Karsi, Menanteau-Ledouble *et al.*, 2006), which resulted in constitutive expression of the bacterial luciferase operon in 93-146. The resulting constitutive luminescence allows for detection and quantification of bacteria using the IVIS Imaging System (Xenogen; USA).

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Fish origin

Juvenile channel catfish were obtained from the specific pathogen free (SPF) hatchery at the College of Veterinary Medicine, Mississippi State University.

All fish were used according to the university policy for animal care as ratified by IACUC protocol.

Immersion challenge

In the first experiment, 30 SPF channel catfish (approximately 4 months of age and 10 cm long) were randomly allocated between two treatment groups: abraded and non-abraded. Fish were sedated using Finkel-MS222 (Argent; USA), and fish from the abraded group received three ~4 cm abrasions on the right lateral abdomen (penetrating to the subdermis) using a 20 gauge syringe needle (Figure 2.1). Abraded and non-abraded fish were experimentally infected with bioluminescent *E. ictaluri* by bath immersion in water containing approximately 6.0 x 10^6 colony forming units (CFU) per liter.

At predetermined time points, disease progression was quantified in each of the 15 fish for each treatment by measuring bioluminescence with an IVIS Living Image system as described (Karsi, Menanteau-Ledouble *et al.*, 2006). Quantitative luminescence measurements were collected from both the whole body of each fish (representing septicemic infection) and from the abdomen (representing bacterial attachment to abrasion sites). A standard area was applied to all the fish at each time point to extract luminescence data from both whole body and abdomen. Individual fish

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were identified using fin clips to allow monitoring of disease progression for each individual.

To determine the effect of treatment on the bacterial loads at each time point, statistical analysis was performed on the mean bioluminescence from each treatment by performing an analysis of variance (ANOVA) using the proc GLM procedure of version 9.1 of SAS (SAS Institute, Cary, NC).

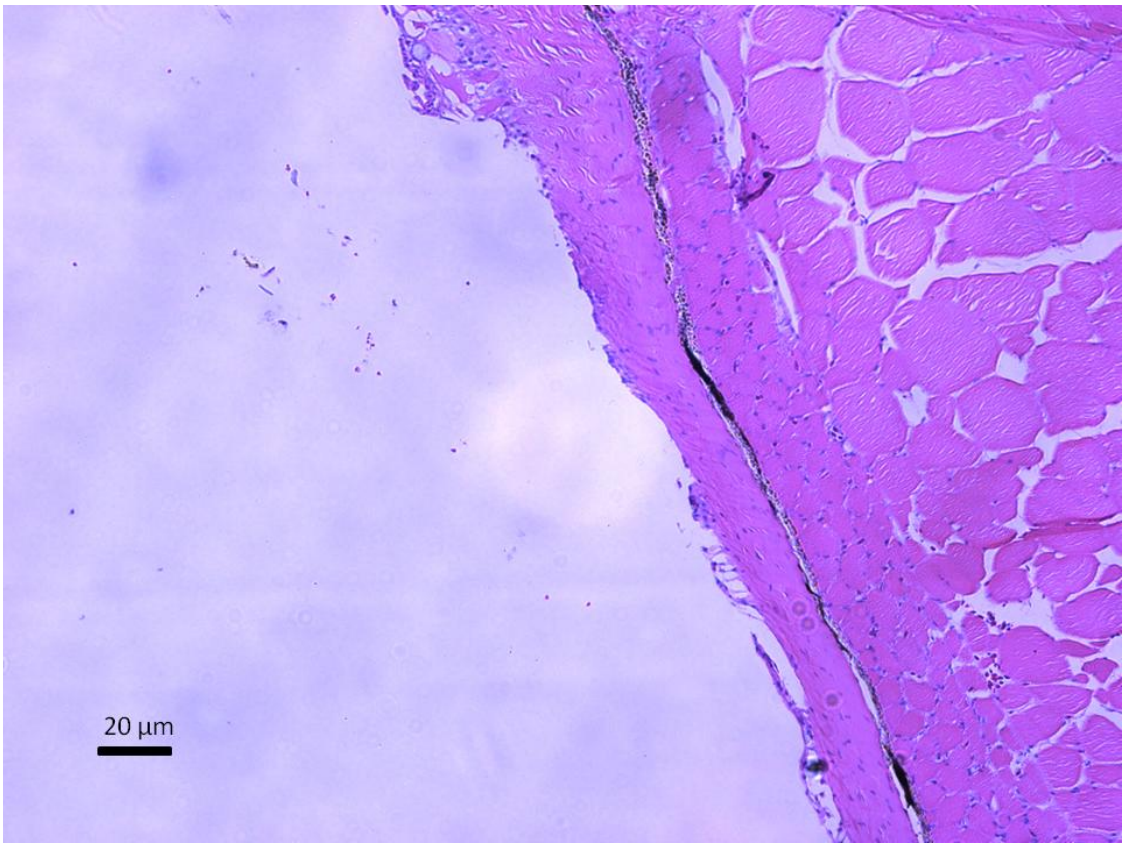

Figure 2.1. Micrograph of an abrasion site, illustrating the depth of the abrasion (60X).

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Percent mortality from immersion challenge

To investigate more specifically how skin abrasion affects mortality, 300 fish of approximately 7 months of age (10 to 15 cm long) were evenly allocated between three treatments: abraded, non-abraded, and non-challenged control. Four tanks were allocated to each treatment, for a total of 12 tanks. Immersion challenge in water containing 9.0 x 10^6 CFU/ml of bioluminescent *E. ictaluri* was conducted using the same method described for the first challenge experiment.

Mortalities were recorded daily to calculate the mean percent survival per tank. Dead fish were necropsied, and spleen and head kidney were sampled, homogenized and 100µl of the homogenate was plated on Brain Heart Infusion (BHI) agar to confirm the cause of death as bioluminescent *E. ictaluri*. At the end of the study, the risk ratio correlated to abrasion was calculated using Fisher's exact test.

Skin challenge

Six tanks were used in this third challenge. For each tank, one fish was abraded on the abdomen using the same method described for the immersion challenge. A second fish in each tank served as a non-abraded control. A bacterial suspension containing approximately 6.0 x 10^8 CFU *E. ictaluri* pAKlux1 was applied directly to the lateral abdomen of abraded and non-abraded fish using a cotton swab. One non-exposed fish was also added to each tank of skin-challenged fish to determine the potential of developing ESC through tank water by co-habitation with infected fish. Luminescence was quantified from the whole body of each fish, and statistical analysis

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was performed on the mean bioluminescence for each treatment as during the first challenge.

Histological analysis of abrasion sites

In this final challenge, 22 abraded fish were topically inoculated with 6.0 x 10^6 CFU of bioluminescent *E. ictaluri* (quantified by plate counts) as described for the skin challenge, and three fish were sampled at 1 h, 6 h, 12 h, 24 h, 48 h, 96 h. At each time point, bioluminescence was recorded using the IVIS system, and tissue samples of a homogenous 3x1.5cm size, were collected from the zones of abrasion and processed histologically. In addition to standard H&E staining, serial sections also underwent indirect immunohistochemistry using Ed9 (Ainsworth and Chen, 1990) and FITC labeled goat anti-mouse immunoglobulin (Southern Biotech) as described below. Sections were then observed using a Olympus BX51 microscope and Picture Frame software (MicroFire). For each slide, the total number of bacteria present on the slide was recorded along with their distance from the epithelial surface. Because some slides did not appear to harbor bacteria, the statistical comparison (using the proc Mixed procedure in SAS) was performed both by using all the slides and by omitting the ones where no bacteria were found.

Immunohistochemistry

To visualize *E. ictaluri* on histological sections, an indirect fluorescent immunohistochemistry (FIHC) protocol was developed for this study. Briefly, organs

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were immediately fixed after sampling by two hour immersion in a 1% formalin solution. To prevent excessive crosslinking, the cassettes were transferred into 50% ethanol until paraffin embedding. This formalin fixative procedure was used because it allowed to greatly reduce tissue auto-fluorescence.

Five 5µm sections were put on slides and dewaxed by two 5 minute immersions in clear-rite, two quick dips in 100% ethanol, one immersion in 70% ethanol, and a prolonged immersion for several minutes in running tap water. Sections were then rehydrated for 5 minutes in PBS and blocked in darkness for 30 minutes in PBS containing 4% mouse serum, 0.4% Triton-X 100, and 1.0% BSA. Sections were then incubated for two hours in a 1:100 dilution of Ed9 monoclonal antibody (Ainsworth, Capley *et al.*, 1986) in blocking solution.

Slides were then rinsed four times (ten minutes per rinse) in PBS before being incubated for two hours in secondary antibody (1:500 dilution of FitC labeled goat anti-mouse antibody in blocking solution) in an opaque moist chamber at room temperature. Slides were rinsed three times in bicarbonate buffered saline, rinsed briefly in deionized water, and air dried in the dark prior to being mounted with aqueous mounting medium (permafluor, Lab Vision). Slides were stored in the dark to limit bleaching of fluorescence.

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Results

Immersion challenge

The immersion study illustrated that *E. ictaluri* was able to bind and colonize the muscles at abrasion sites where the epithelium was absent (Figure 2.2). Additionally, it showed that the total number of bacteria in the fish, both in the abrasion region and in the whole body, was significantly higher at the 95% level of confidence in abraded fish than in non-abraded ($p<0.05$) at each time point up to 84 hours post-infection (Figure 2.3). The mean luminescence over the entire experiment was also significantly higher in abraded than non-abraded fish ($5.717x10^{08}$ and $1.913x10^{08}$ photons/sec/cm^2/steradian, respectively). Finally, survival was significantly reduced ($P= 0.022$) in abraded when compared to non-abraded fish, with a mean survival duration of 86. 67 hours (±6.2 hours) instead of 121 hours (±3.4 hours) (Figure 2.4).

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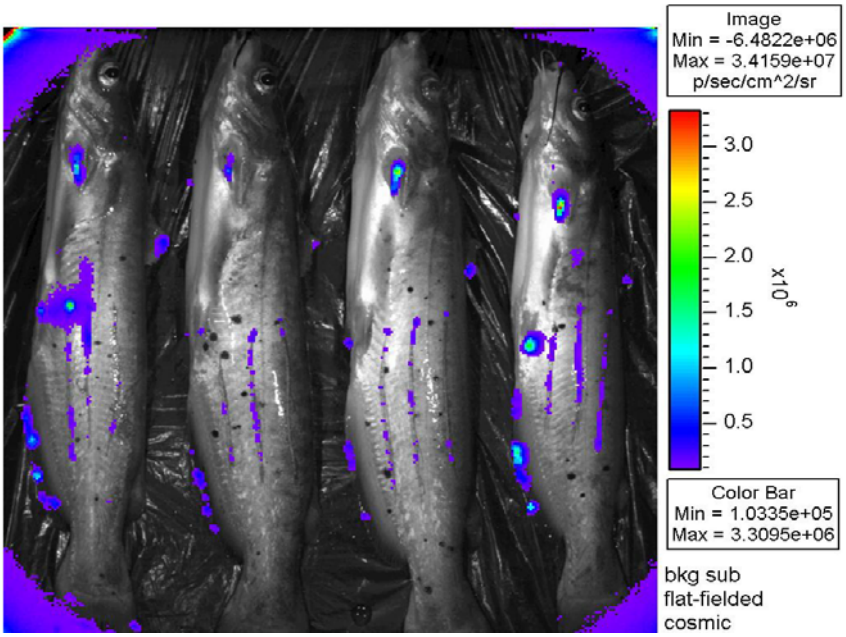

Figure 2.2. Immersion-exposed channel catfish at 12 hours post-infection illustrating the attachment of bioluminescent *E. ictaluri* on the lines of abrasion. Image was collected using an IVIS Imaging System (Caliper Life Sciences).

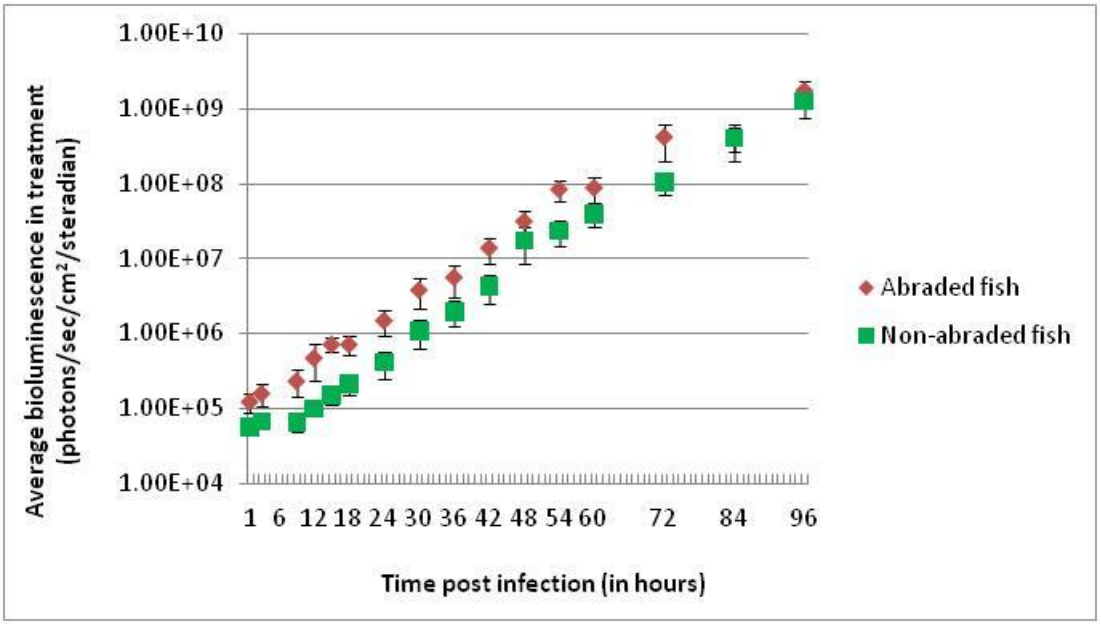

Figure 2.3. Total luminescence in the whole body of abraded and non-abraded fish during the immersion challenge (error bars indicate 95% confidence intervals).

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Percent mortalities from immersion challenge

In the immersion mortality challenge, 44 out of the 100 abraded fish died over a course of 18 days, while no mortalities occurred among the non-abraded or the control fish ($P < 0.0001$ using Fisher's exact test).

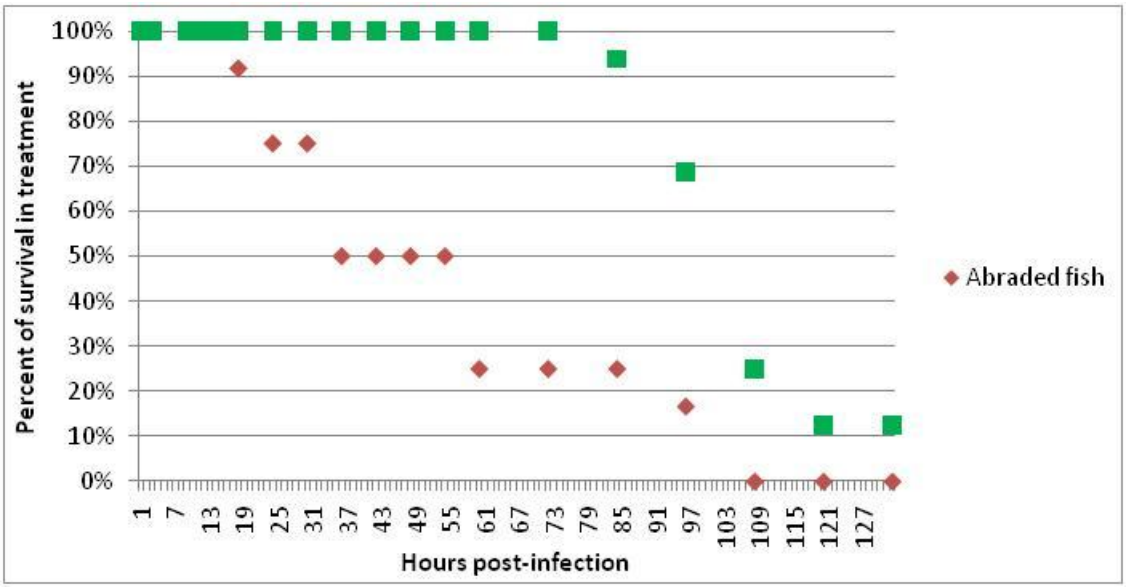

Figure 2.4. Percent survival for abraded and non-abraded fish over time during the immersion challenge.

Skin challenge

During the skin challenge, bacterial load was higher and developed quicker in the abraded fish than in the non-abraded (Figure 2.5), both in the abrasion region and in the whole body. This difference was statistically significant at the 95% level of confidence for each time point up to 156 hours post-infection. The mean luminescence over the entire experiment was also significantly higher in the abraded than the non-abraded fish

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($1.848x10^{08}$ p/s/cm^2/sr and $3.664x10^{07}$ p/s/cm^2/sr, respectively). By 144 hours post-infection, all the abraded fish had died, and 80% of the non-abraded fish died by 180 hours post-infection (Figure 2.6). Similarly, mean survival time was longer in non-abraded fish (196.8 hours compared to 99.4 hours in abraded fish). No mortalities occurred in the control (co-habitation) fish, but they had a transient infection (Figure 2.5).

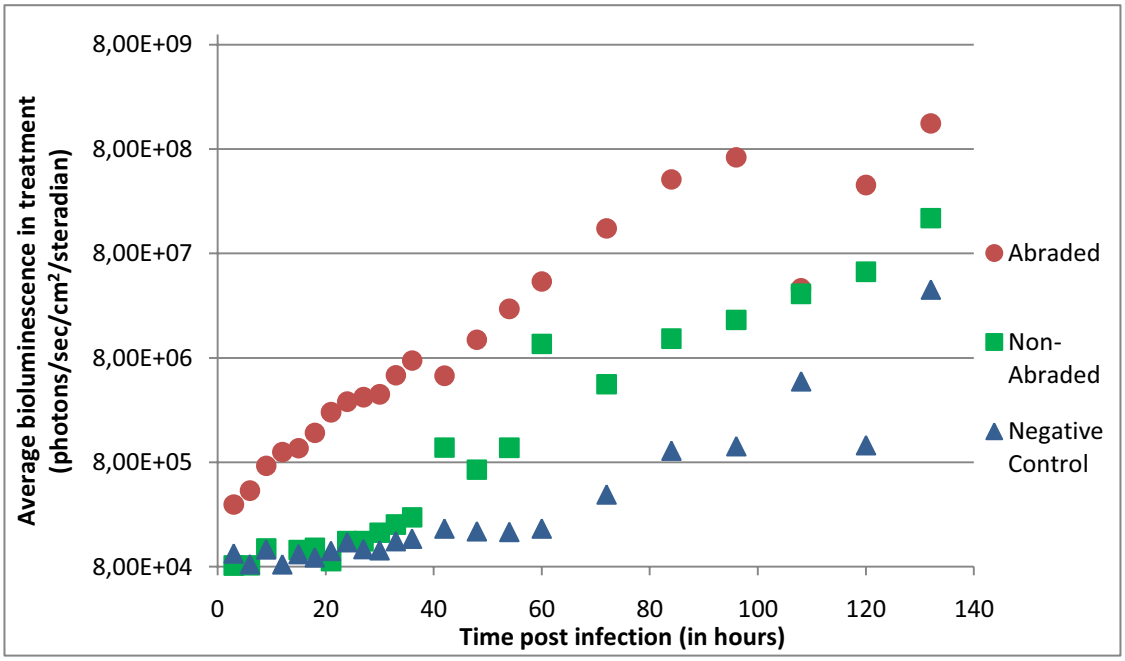

Figure 2.5. Total luminescence in the whole body of abraded, non-abraded, and control (co-habitation) fish during the skin challenge (error bars indicate 95% confidence intervals).

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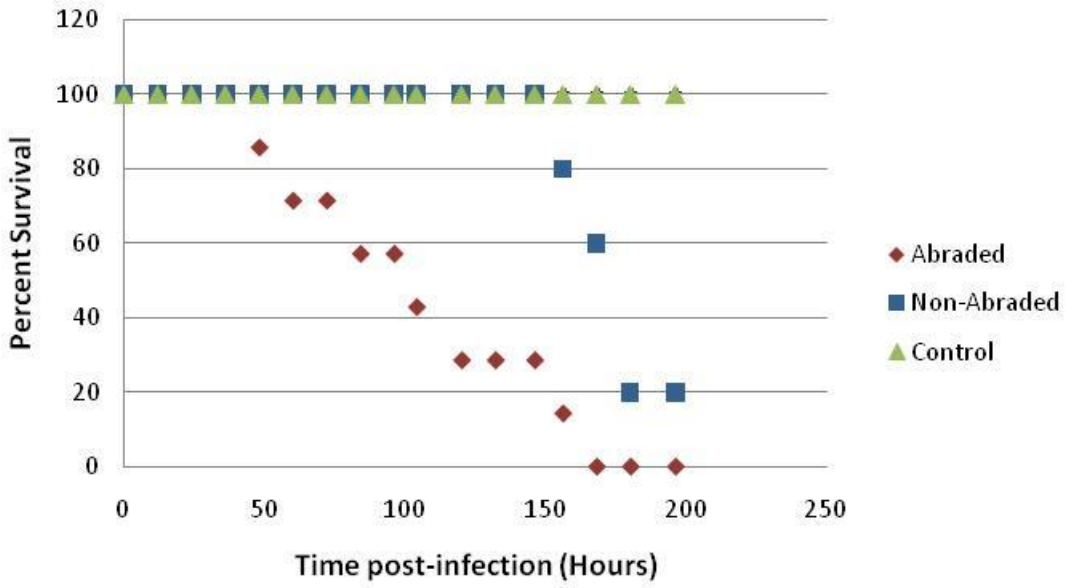

Figure 2.6. Percent survival for abraded, non-abraded, and control (co-habitation) fish over time during the third challenge.

Histological analysis of abrasion sites

In the histological analysis, bacteria were only visible in 49 percent of the slides (Figure 2.7). When present, most of these bacteria were present in the stroma of the stratum spongiosum and only occasionally were they located intracellularly within the muscle fibers. The histology was very consistent and was composed of muscle fibres and epithelium with no sign of inflammation. Where bacteria were present, the number of bacteria steadily increased up to 24 hours post-infection, as did the average and maximum distance of the bacteria from the surface of the fish. After 24 hours, these numbers slowly decreased (Figure 2.8). However, only in the case of the maximum distance from the surface was this variation statistically significant ($p= 0.0150$ for distance; $p= 0.06$ for average distance and $p=0.074$ for number of bacteria).

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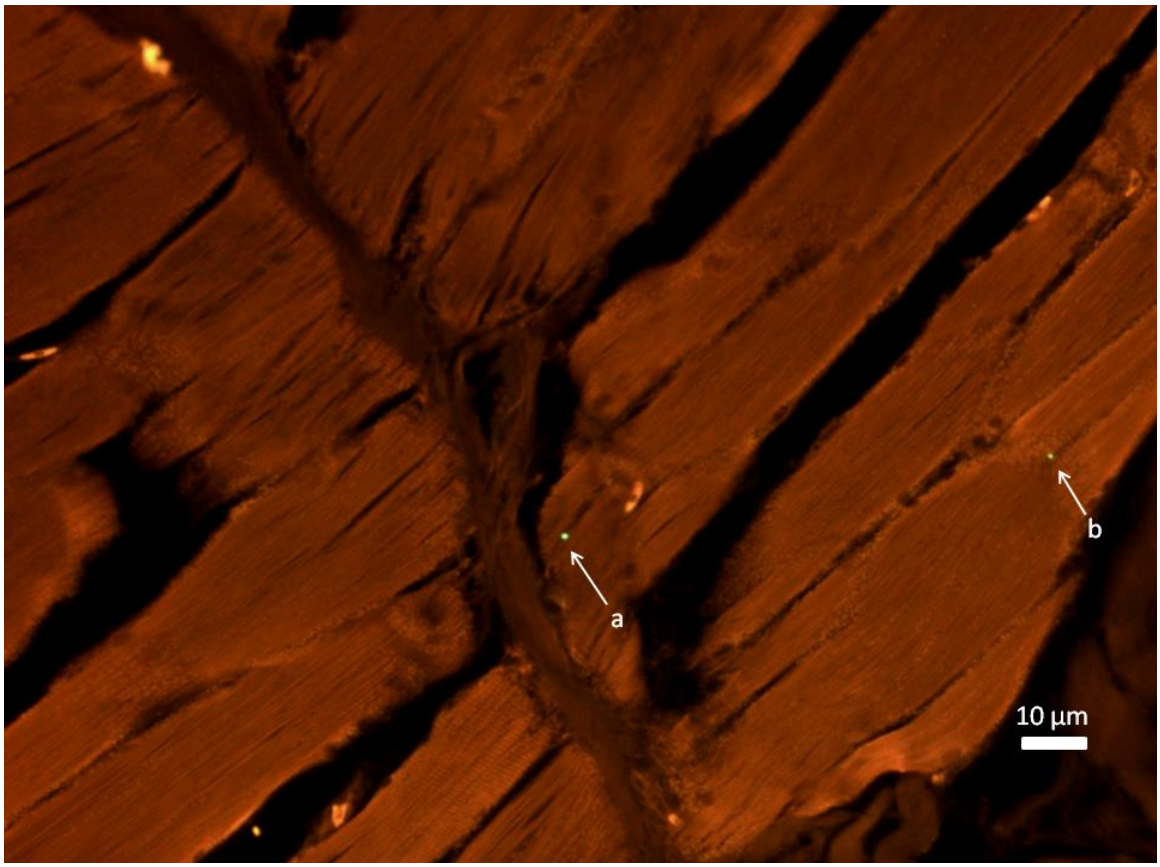

Figure 2.7. Micrograph of a muscle sections showing two *E. ictaluri* (arrows a and b) (200x).

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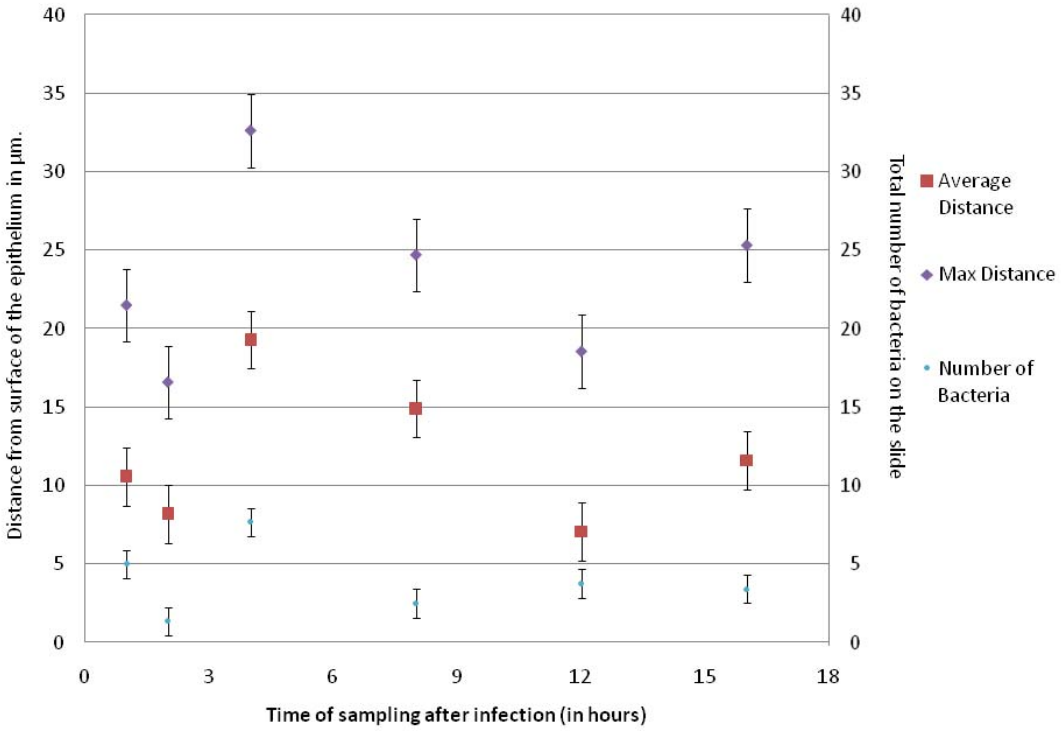

Figure 2.8. Maximum distance and average distance of bacteria from the epithelial surface and average number of bacteria per slide as observed histologically during the fourth challenge.

Discussion

Several routes of entry for *E. ictaluri* into channel catfish have been determined. The intestine was the first route of entry confirmed by experimental infection using immunostaining as well as electron microscopy within the anterior and middle portion of the intestine as early as half an hour post-infection following gastric intubation (Baldwin, 1992; Baldwin and Newton, 1993). However, Nusbaum and Morrison (1996) showed only a low level of the pathogen in the intestine relatively late during the

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infection process. In addition, it is not known how accurately gastric intubation mimics natural infection.

Entry through nares was implicated in early investigations of pathogenesis (Newton, Grizzle *et al.*, 1989), and this route has been experimentally confirmed by inoculation directly into the olfactory sinus (Morrison and Plumb 1994). With this route of entry, the bacterium is able to cause degeneration of epithelial cells, and infection can spread from this primary site of infection to involve the cranial vault. Ulceration of the skull can result in the characteristic "hole in the head" lesion (Miyazaki and Plumb, 1985; Morrison and Plumb, 1994). In addition, infection can progress up the olfactory nerves and cause neurological signs.

E. ictaluri was detected in the gills as early as two hours post-infection (Nusbaum and Morrisson, 1996), and it has been hypothesized that the bacterium can enter the blood circulation through gill macrophages. From there, it would promptly be transported to the head kidney, where it would be absorbed within the resident macrophage population. High bacterial loads have been detected consistently within the gills following experimental infection by bath immersion (Karsi, Menanteau-Ledouble *et al.*, 2006). Interestingly, if gills are used by the bacteria as a route of entry, this entry does not appear to correlate with damage in the gill tissues during acute infections as such damage are only observed later in the infection process at a time when all of the fish vascular system has been compromised, as illustrated by the widespread hemorrhages (Jack; personal communication).

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Results from our immersion challenge experiments in the current study show for the first time that *E. ictaluri* has the ability to use sites of skin abrasion as a preferential site for adhesion and multiplication. Our findings also suggested that these abrasions help the bacteria in developing a septicemia and inducing host death.

The abraded skin challenge showed that topical application of *E. ictaluri* was followed by colonization of the underlying musculature and development of septicemia. Comparison of the bacterial loads in abraded and non-abraded fish showed that abrasion of the epithelium facilitated and quickened the progression of disease. Skin abrasion, however, was not required for the development of septicemia (Figure 2.5). Additionally, none of the control co-habitation fish died, suggesting that bacteria induced septicemia through the muscle instead of becoming water-borne prior to penetrating the fish through intestine, nares, or gills. Therefore, intact epithelium constitutes a potential route of invasion for *E. ictaluri* into channel catfish. This route of entry had, to the best of our knowledge, never been documented previously.

During both the immersion and skin challenges, bacterial loads were significantly different between abraded and non-abraded fish during the early time points in infection, but this difference became less statistically significant as the challenge progressed (Figure 2.2 and 2.4). This can be explained by the fact that the abraded fish, especially the ones showing the highest bacterial loads, generally died earlier. This caused the average bacterial loads between the two treatments to grow closer as the challenge progressed. Additionally, each death decreased the sample size in the abraded fish and increased the confidence interval. Thus, the loss of statistical

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significance at later time points is probably not due to a true decrease in the difference between treatments.

Finally, during the last challenge, we were able to confirm the presence of *E. ictaluri* in abrasions using a specifically designed indirect fluorescent immunohistochemistry protocol. The choice of an indirect immunohistochemistry was imposed upon us by the absence of labeled antibodies against *E. ictaluri*. However, it had the added advantage of being more sensitive than a direct immunohistochemistry. The choice of the fluorescent FitC probe for the secondary antibody allowed a clear and unambiguous identification of the bacteria compared to a colorometric probe.

Bacterial numbers by immunohistochemistry were not as high as expected, possibly due to this colonization site being a transient location. Both the average and the maximum distance of the bacteria from the surface peaked at 24 h post-infection, which also suggests that colonization of skin abrasion sites is a transient occurrence prior to development of septicemia. In summary, our results indicate that, in addition to intestinal epithelium, olfactory epithelium, and gill epithelium, *E. ictaluri* is able to invade catfish through the muscle layers and that the route of entry used during infection is more a matter of opportunity in terms of which tissue it encounters first rather than the route being due to actual tissue specificity. Furthermore, our results demonstrate that skin abrasion makes catfish more susceptible to ESC and increases the rate at which disease progresses.

These results have practical implications for catfish production. Specifically, our current findings provide another good reason for avoiding procedures such as seining

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that may cause skin abrasions during the ESC temperature window of 22-28 ºC, and they provide yet another reason to improve the dispersal of predatory birds.

Acknowledgements

The authors wish to recognize the technical assistance of Michelle Banes as well as Dr. Petrie-Hanson, Dr. Eels and Timothy Brown for their assistance developing and performing the immunohistochemistry. We also thank Dr. Claudia Hohn, who provided the SPF catfish used during this study. Furthermore, we acknowledge Drs. Carolyn Boyle, Richard Minnis, and Qiqi Lu for invaluable assistance with statistical analysis. This project was funded by USDA CRIS project #MISV-0801310 and by the Mississippi State University College of Veterinary Medicine.

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CHAPTER III

USE OF HIGH THROUGHPUT MUTANT SCREENING FOR *IN VIVO* INVESTIGATION OF THE INVASION OF CHANNEL CATFISH (*Ictalurus punctatus*) EPITHELIUM BY THE ENTEROBACTERIUM *Edwardsiella ictaluri*.

Abstract

Initial invasion of the host is the first and vital part of any infection process. For this reason, bacterial pathogens have evolved a wide array of mechanisms to perform this function, including fimbriae, adhesins, and effectors that are delivered through the type III secretion system. There is compelling evidence that the catfish pathogen *Edwardsiella ictaluri* is invasive to non-phagocytic cells, but no systematic investigation has yet been conducted to determine the precise mechanisms involved. This information is especially important because *E. ictaluri* is an intracellular pathogen, and vaccines have had limited success. Therefore, a mutant library was constructed by random insertion of the Mar2xT7 transposon into the chromosome of *E. ictaluri* harboring the bioluminescence plasmid pAKGFPLux. This library was then screened, through a series of three nested challenges, for mutants showing a decreased ability to colonize the catfish epithelium. Twenty mutants were identified that have decreased adhesion and virulence. The regions surrounding the insertion sites were then

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sequenced to determine what genes were mutated. One mutant had an insertion in the gene encoding RstA/B, which is known to play a role in regulation of the expression of invasion genes in *Salmonella enterica* Typhimurium. A second mutant was lacking a putative ribonuclease that is similar to a *Shigella* protein that regulates the expression of adhesin. A third mutant was defective in a protein similar to a *Brucella* protein that was initially identified as a transporter, but actually is the second member of a newly discovered adhesin family. Results from this study could enable development of a new strategy for blocking *E. ictaluri* invasion at the initial adherence stage.

Introduction

Edwardsiella ictaluri (Hawke *et al.* 1981) is a small bacillus in the *Enterobacteriaceae* family. It is a major pathogen affecting the channel catfish industry in the southeastern USA (Thad Cochran National Warmwater Aquaculture Center 2007) and has been associated with mortalities up to 80% in younger fish (Plumb 1998). Outbreaks occur mainly during the spring and fall when temperature is between 18 and 28°C, with the optimum at 25°C (Plumb 1998). The disease is characterized by a complex infection cycle during which multifocal necrosis in the hematopoietic organs and liver is the predominant feature (Roberts 2001).

Attachment and invasion of host cells is an important part of the virulence process in a variety of bacterial pathogens. Adhesion mechanisms include adhesins such as internalin A of *Listeria* (Cossart & Sansonetti 2004), the molecule invasin present in *Yersinia enterolitica* and *Yersinia pseudotuberculosis* (Boyle & Finlay 2003), or the

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Shigella IpaB/B/D complex (Van Nhieu & Sansonetti 1999). In addition, fimbriae (such as the type I fimbriae in *Salmonella*) (Kisiela et al. 2006) mediate adherence as well as effector proteins secreted through the type III secretion system (Vazquez-Torres & Fang 2000).

In ESC, bacterial adherence to the host is particularly important in initiating infection because the aquatic environment provides a natural physical mechanism for washing away bacteria. In addition, *E. ictaluri* is an intracellular pathogen that is known to survive phagocytosis (Ainsworth & Chen 1990, Booth et al. 2006, Karsi & Lawrence 2007). It also has the ability to invade non-professional phagocytic cells such as epithelial cells (Skirpstunas & Baldwin 2002). This ability to invade and survive in an intracellular location is an important aspect of ESC because it allows protection from the catfish humoral response, as evidenced by the limited protection provided by killed *E. ictaluri* vaccines and the lack of correlation between antibody levels and protection (Klesius & Sealey 1995).

Possible mechanisms for *E. ictaluri* adherence have been identified. Extracellular fibrillar material resembling fimbriae is present (Williams et al. 2003). In addition, two outer membrane proteins that possibly mediate adherence to epithelial cells were identified: *193UV* and *233PR* (Thune et al. 2007). A type-III secretion system similar to the one used by *Salmonella* and *Shigella* (Van Nhieu & Sansonetti 1999, Vazquez-Torres & Fang 2000) has been reported, and it has been associated with virulence (Thune et al. 2007).

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However, no *E. ictaluri* proteins have been definitively linked with adherence. We have shown that *E. ictaluri* attaches to site of skin abrasions on catfish, and we developed an immersion infection model that allows consistent detection of *E. ictaluri* attachment to skin abrasions. In the present study, we utilized this model system to identify random *E. ictaluri* mutants that are defective in host adherence. Identification of the mutated genes allowed us to discover mechanisms responsible for the intracellular invasiveness of *E. ictaluri*.

Materials and Methods

Bacterial strains and transposon mutagenesis

E. ictaluri strain 93-146 is a clinical isolate that has been routinely used to investigate mechanisms of ESC in our laboratory. This strain was transformed with pAKGFPLux, which allowed constitutive expression of the bacterial luciferase operon (Karsi et al. 2006, Karsi & Lawrence 2007). *E. ictaluri* was cultivated at 28 ˚C in brain heart infusion (BHI) broth and agar plates, and *E. coli* was grown at 37 ˚C in Luria-Bertani (LB) broth and agar plates. Ampicillin at 100 mg/l was used to maintain pAKGFPLux.

Transposon mutagenesis was conducted using pMar2xT7, a plasmid harboring the Mar2xT7 transposon, which was derived from the *mariner* family initially isolated from *Pseudomonas aeruginosa* strain PA14 (Liberati et al. 2006). This transposon is randomly inserted in the host's genome, and because the transposase is located outside of the transposed sequence, no secondary transposition can take place.

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Transformation with pMar2xT7 occurred by conjugation (Karsi et al. 2006). Briefly, the plasmid was first transferred into *E. coli* SM10 λ*pir* by electroporation. Then, both the transformed *E. coli* and *E. ictaluri* 93-146 pAKGFPLux were cultivated overnight in broth medium, and the strains were mixed and collected on 0.45µm filters. Filters were placed onto BHI agar for 24 hours to allow time for conjugation to take place. At this point, bacteria were washed from the filter and spread on a BHI plate containing 100mg/l ampicillin, 12.5 mg/l gentamycin, and 25 mg/l colistin. *E. ictaluri* is naturally resistant to colistin (Waltman & Shotts 1986, Dung et al. 2008), so it provides convenient counterselection against donor strain. Ampicillin ensures maintenance of pAKGFPLux, and gentamycin selects for *E. ictaluri Mar2xT7* transposon mutants.

Fish

Juvenile channel catfish were obtained from the specific-pathogen-free (SPF) laboratory at the Mississippi State University College of Veterinary Medicine. These fish were raised in a pathogen free environment with respect to *E. ictaluri* until the time of the challenge. For all challenge studies, fish were transferred into 10 gallon tanks supplied with flow-through dechlorinated municipal water. Temperature was maintained at 27°c +/- 1°C throughout the studies.

Mutant screening

Random *E. ictaluri* mutants (1728 total) were individually picked and grown overnight in 96 well plates containing 240µl of BHI-ampicillin. At this point,

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bioluminescence of the plates was confirmed using the Fluorchem imaging system (Alpha Innotech; USA), and 60µl of sterile 50% glycerol were added to the plates before freezing at -80°C.

Mutant screening was conducted using a skin abrasion model previously developed (Chapter 2) and occurred in three phases. In the first round of screening, mutants were thawed in the 96-well plates, used to inoculate fresh 96-well plates in BHI-ampicillin, and cultivated overnight. Then, the plates were imaged using the Fluorchem imaging system to confirm that the luciferase operon was active.

Thirteen month old SPF channel catfish were transferred into challenge tanks (6 fish per tank) and abraded three times on each side. A different *E. ictaluri* mutant was applied topically on each specific abrasion line (six mutants per fish). Then, following a 72 hour incubation period, fish were imaged using the IVIS Imaging System (Caliper Life Sciences) to quantify the amount of bacterial colonization of the epithelium. Each mutant was also imaged in 96-well plates to ensure they were still bioluminescent. Wild type strain 93-146 pAKGFPLux was used as a control for comparison.

Two hundred four mutant strains that displayed decrease between their luminescence on the plate and their ability to colonize the epithelium were selected for the second round of screening. Each mutant was cultivated overnight and visualized using the Fluorchem imaging system. In this screening, one year old fish were transferred 3 per tank in challenge tanks, but each fish was only abraded once on each side. A single mutant was applied on each abrasion site (two per fish). Bioluminescence

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was quantified using the same method as the first screening round, and eighteen mutants were selected.

These eighteen mutants were then tested a third time. In this screening, three fish were stocked per tank and abraded once on each side. Each mutant was applied to both abrasion sites on all the fish in an individual tank (one mutant per tank). A tank of three fish was exposed to wild type strain 93-146 pAKGFPLux for comparison. Because each tank contained a single mutant, cross contamination was not possible in this third screening. Bioluminescence was quantified using the IVIS Imaging System, and mortalities were recorded.

Finally, a challenge was similarly performed, using each one of the mutants individually on 42 fish as described for the previous challenge. Mortality was then recorded twice daily for a period of three weeks.

Mutant identifications

Genomic DNA was isolated from each of the eighteen adherence-deficient mutants using the DNEasy kit protocol (Qiagen). Then, the DNA sequence flanking each transposon insertion site was identified by single primer PCR and sequencing as described by Karlyshev *et al*. (Karlyshev et al. 2000) and modified by Karsi (Karsi et al. 2009). Sequences were identified by BLAST search against the *E. ictaluri* genome (GenBank #CP001600) (Williams et al. 2008).

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Results

Of the 1728 mutants constructed, 204 appeared to show a decrease in adherence during the first round of screening. This decreased invasiveness was confirmed for eighteen of these mutants during the second and third rounds of screening.

When compared to wild type strain 93-146, the amount of bacterial colonization as quantified by bioluminescence was lower in every one of these eighteen mutants (average bioluminescence of $3.146x10^{04}$±53,000 photon/cm^2/second/steradians (p/cm²/s/sr) for all mutants) the wild type strain ($3,314x10^{05}$±47,000 p/cm²/s/sr). These differences were statistically significant at the 95% confidence level for all but one strain: 9-4 (Figure 1). Mortalities were also decreased when compared to the wild type as illustrated in figure 2.

Sequencing of the regions surrounding the insertion sites allowed the identification of the mutated genes. For two mutants (1-58 and 3-67), the insertion events occurred at the same place in the bacterial genome. Therefore, the screening process independently identified the same mutant twice. All of the identified genes are presented in Table 1. Furthermore, in two more cases, 14-33 and 14-34, the insertion occurred independently at different places but within the same gene. Seven of these genes were identified as coding for hypothetical proteins with no known function; identifying their role and function will require additional research to be performed.

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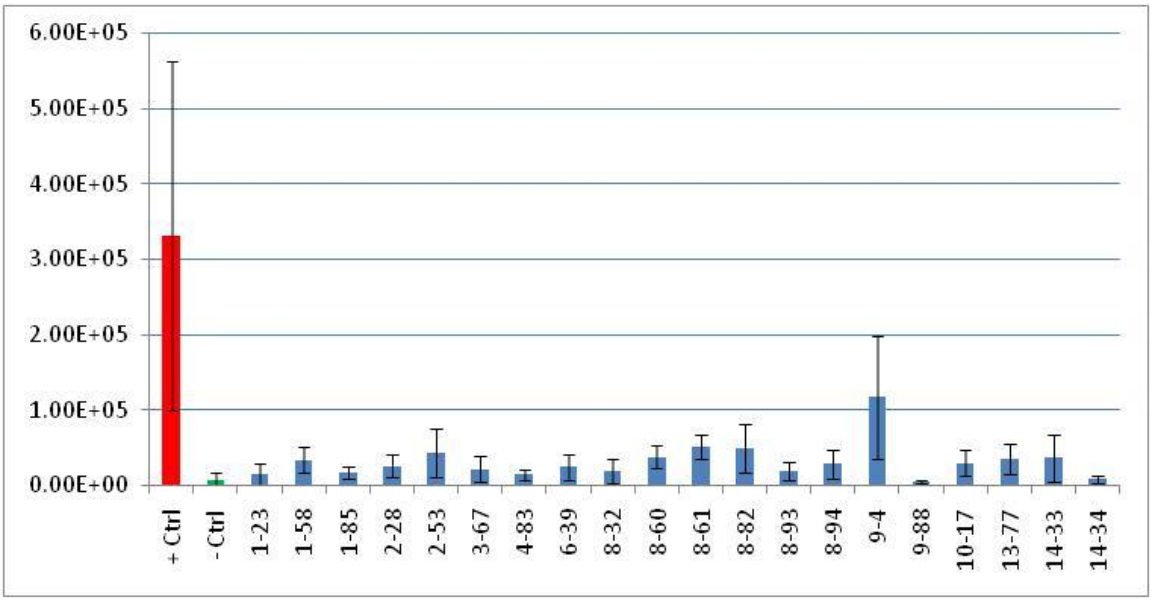

Figure 3.1. Mean bioluminescence at the abrasion sites during the last round of challenge as measure in photon/cm^2/second/steradians using IVIS (Xenogen).

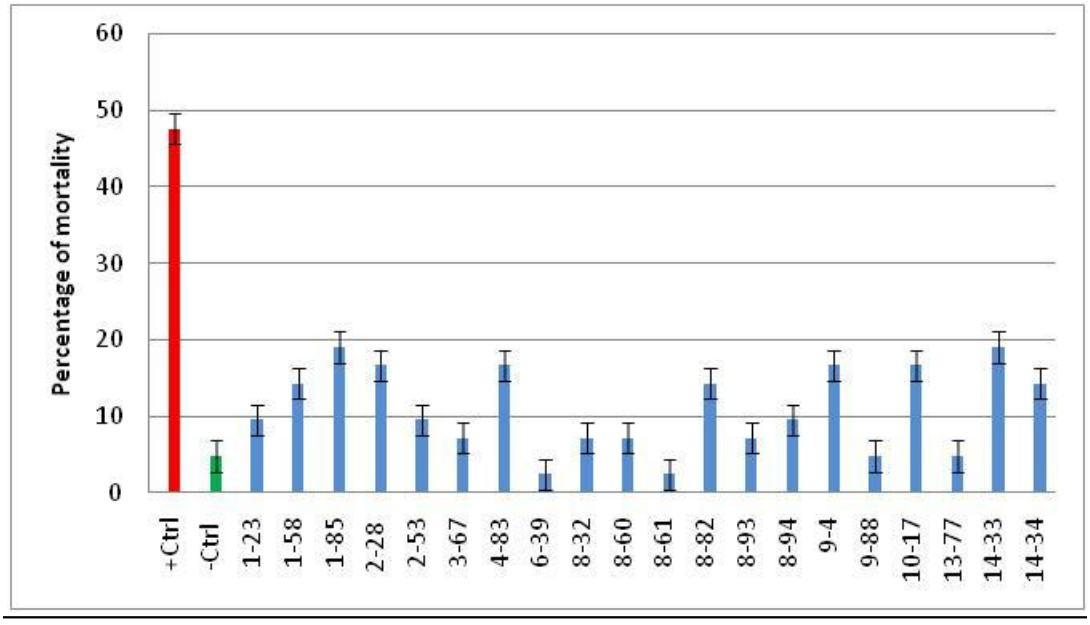

Figure 3.2. Mortalities measured during the last round of challenge.

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Table 3.1. List of the genes mutated in this experiment.

Mutant identified:	Corresponding gene:
Plate 1; well 23	Sensor protein RstB
Plate 1; well 58	Periplasmic dipeptide transport protein (DBP)
Plate 1; well 85	Peptidase, U61 family
Plate 2; well 28	Septum site-determining protein MinC, putative
Plate 2; well 53	N-acetylmuramoyl-L-alanine amidase AmiD
Plate 3; well 67	Periplasmic dipeptide transport protein (DBP)
Plate 4; well 83	Glycerol-3-phosphate transporter
Plate 6; well 39	Dimethyladenosine transferase, putative
Plate 8; well 32	5-methyltetrahydropteroyltriglutamate-homocysteine, putative
Plate 8; well 60	Hypothetical protein (Type 2)
Plate 8; well 61	Valine-pyruvate aminotransferase
Plate 8; well 82	Hypothetical protein (Type 1)
Plate 9; well 4	Hypothetical protein (Type 3)
Plate 9; well 88	Hypothetical protein (Type 7)
Plate 10; well 17	Ribonuclease R, putative
Plate 13; well 77	Hypothetical protein (Type 4)
Plate 14; well 33	Hypothetical protein (Type 6)
Plate 14; well 34	Hypothetical protein (Type 5)

Discussion

During this study, a library of 1728 mutants was constructed and screened in a high throughput fashion. This allowed for high efficiency during the screening process, but it may have resulted in cross-contamination. However, the risk of some mutants falsely appearing to display impaired adherence was eliminated by the use of multiple rounds of challenge. Indeed, results obtained were consistent between the second and third round. Actually, it is likely that some deficient mutants were not detected during this study, and a slower screening process would have been required to identify all of the mutants of interest within the library.

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In 17 of the 18 mutants, the transposition event significantly impaired the ability of the bacteria to colonize the infection site and reduced the virulence of the bacteria. Therefore, this study allowed the identification of 16 genes involved in the colonization of fish epithelium by *E. ictaluri*.

The gene mutated in mutant strain 1-23 encodes sensor protein RstB. RstB is part of the RstBA regulation network in *Escherichia coli* and is induced through PhoQP in a Mg^{2+} limited environment (Ogasawara et al. 2007). Interestingly, among the genes under its regulation are the acid shock RNA *asr*, which is associated with resistance to low pH and colonization of mouse intestine by *E. coli* (Armalyte et al. 2008), and *csgD*, which regulates the expression of curli fimbriae .

Mutants 1-58 and 3-67 both had insertions in a gene coding for a dipeptide binding protein (DBP). DBPs include the protein DppA identified in *E. coli* and *Salmonella typhimurium* (Abouhamad et al. 1991). DppA mediates chemotaxis to dipeptides through the Tap chemoreceptor as well as binding to heme. It is upregulated during invasion of the murine urinary tract by uropathogenic *E. coli (Haugen et al. 2007)*. DppA is similar to the dipeptide ABC transporter DpsA and oligopeptide permease OppA1-F that were implicated in *Streptococcus agalactiae* adherence to epithelial cells and fibronectin and promoted the expression of the fibronectin-binding protein FbsA (Samen et al. 2004).

Mutant 1-85 had an insertion in a gene encoding a member of the U-61 family of peptidases, which includes the serine peptidases LD-carboxypeptidase and muramoyltetrapeptide carboxypeptidase (Korza & Bochtler 2005). These proteins are

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involved in the processing of nascent peptidoglycan and their incorporation in the bacterial cell wall (Bardin et al. 1984). This gene also appeared to be part of a large operon of seven proteins, including another cell-wall peptidase and five proteins of unknown function; therefore, it is possible that the mutation effects were due to a polar event disturbing expression of downstream genes in the operon.

Mutant 2-28 had a mutation in a putative MinC protein. This gene is the first in a short operon of four genes. The second and third proteins encode unidentified proteins, and the last one encodes a universal stress protein. Because genes within bacterial operons are co-regulated, they generally have a similar function (Snyder & Champness 2003). Stress response proteins are often involved in the survival of pathogens during the infection process and have routinely been involved in the regulation of virulence genes (Haugen et al. 2007).

Mutant 2-53 had an insertion in a gene homologous to the N-acetylmuramoyl-L-alanine amidase AmiD, an outer membrane anchored lipoprotein involved in the recycling of peptidoglycan (Pennartz et al. 2009). It has previously been shown in *Listeria monocytogenes* that Ami deficient mutants were 5 to 10 times less adherent than the wild type strain to a variety of cell type (Milohanic et al. 2001). Similar results were also achieved with Auto, another cell-wall peptidoglycan hydrolase of *L. monocytogenes (Maike et al. 2009)*.

Mutant 4-83 was perhaps the most interesting find of this experiment. The mutated gene is annotated in *E. ictaluri* as a glycerol-3-phosphate transporter. However, this *E. ictaluri* protein is similar to a *Brucella* protein termed SP41 that also appeared

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paralogous to the glycerol-3-phosphate transporter family (Castañeda-Roldán et al. 2006). SP41 was further identified as playing a role in the adhesion and invasion of the bacterium into HeLA cells through a mechanism that most probably involves siliac acid. To our knowledge, the present study represents only the second reported instance of a member of this new adhesin family being identified.

Mutant 6-39 has an insertion in a gene encoding a putative dimethyladenosine transferase, a protein known to catalyze the dimethylation of two adjacent adenosines in the loop of a conserved hairpin near the 3'-end of 18S rRNA of the small ribosomal subunit. Interestingly, when analyzed through BlastX for similarity with known proteins, the sequence is similar to KsgA, a dimethyl-transferase isolated from *Shigella bodyii*. KsgA was found through signature tagged mutagenesis to be necessary for the survival of *Yersinia pseudotuberculosis* in the intestinal tract of mice (Mecsas et al. 2001).

Mutant 8-32 was inactivated in a gene encoding MetE. MetE is part of an important regulon that allows synthesis of methionine and regulates the synthesis of biofilms in *Salmonella enterica* serovar Enteritidis (Dong et al. 2008).

Mutant 8-60 is inactivated in a gene of unknown function. However, using BlastX, it was found very homologous (score of 85.1) with the 4-hydroxybenzoate octaprenyltransferase enzyme in *Yersinia mollaretii*, which is part of the ubiquinone biosynthesis pathway (Young et al. 1972). Perhaps more interesting was the presence of a gene encoding superoxide dismutase downstream from the insertion site. It is possible that the observed attenuation is due to a polar effect on this gene.

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Mutant 8-61 was affected in a gene encoding valine-pyruvate aminotransferase, an enzyme involved in valine, leucine and isoleucine biosynthesis. Interestingly, upstream from the insertion site is a gene coding for the outer membrane lipoprotein LolB, which is generally required for LolA-dependent localization of lipoproteins (including LolB itself) to the outer membrane.

The insertion in mutant 8-82 occurred in a gene encoding a short unidentified protein. However, this gene was located just upstream from UDP-N-acetylenolpyruvoylglucosamine reductase, an enzyme known to be involved in the synthesis of peptidoglycan (Real & Henriques 2006). Mutant 9-4 was affected in a gene encoding another unidentified protein. However, it was found to be highly similar (83%) with a N-6 DNA methylase of the proteobacteria *Tolumonas auensis* that was isolated from the sediment of a freshwater lake in 1996. In Mutant 9-88, the insertion event occurred in a short (644 base-pair) gene coding for a hypothetical protein. The other protein in the operon was similarly short and also unidentified.

In mutant strain 10-17, the insertion was located in a gene encoding putative ribonuclease R. Ribonucleases are known regulatory genes. Most notable among them is *vacA* in *Shigella flexneri*, which is required for the expression of a number of virulence genes, including the IpaB/C/D complex that binds $\alpha_5\beta_1$ integrins and CD44 on the host cell (Van Nhieu & Sansonetti 1999). IpaB/C/D also includes its associated secretion apparatus (Tobe et al. 1992).

Mutant 13-77 also had an insertion in a gene encoding a hypothetical protein. However, its sequence appeared homologous to a transcriptional regulating protein,

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especially to an Acr regulator of *Edwardsiella tarda* (74% homology) or a *Serratia proteamaculans* regulator of the TetR family (57% homology). Both Acr and TetR belong to the same superfamily and are linked to the regulation of virulence factors in a number of organisms including *Salmonella* enteric (Chiu et al. 2005) and *Vibrio cholerae* (Cerda-Maira et al. 2008).

Finally, in both the mutants 14-33 and 14-34 the insertion event occurred independently in the same gene. This gene was identified by homology as a possible glutamate dehydrogenase. Glutamate levels have been linked to the ability of *Pseudomonas aeruginosa* to grow under iron restricted conditions (Somerville et al. 1999). Furthermore, upstream from this glutamate dehydrogenase gene was located another lolB lipoprotein, similar to the gene described for mutant 8-61.

In conclusion, this study allowed for the identification of genes required for *E. ictaluri* colonization of the catfish epithelium. Some of the genes encode proteins that have been linked with adherence in other bacterial pathogens. However, several of the identified genes will require further characterization to determine their function and role in adherence/colonization. Future studies to characterize the identified mutants include mutant complementation and generation of antibodies against the genes' products to confirm if competitive inhibition of these products in the wild-type lead to a decrease of adherence. Preliminary mortality data from this study also indicates that these mutants are attenuated in catfish. Further experimental infections will be necessary to confirm that these genes are indeed required for virulence.

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CHAPTER IV

TISSUE CONCENTRATIONS OF E*dwardsiella ictaluri* IN CHANNEL CATFISH FOLLOWING EXPERIMENTAL INFECTION BY BATH IMMERSION

Abstract

This study was conducted to evaluate the potential of bioluminescence as a marker for quantifying *Edwardsiella ictaluri* in catfish tissues and to investigate the infection cycle during enteric septicemia of channel catfish. Channel catfish (*Ictalurus punctatus*) were challenged by immersion in a solution containing *E. ictaluri* expressing bacterial luciferase and sampled at various time points. At each time point, nine organs (gills, muscles, intestine, spleen, liver, stomach, heart, head kidney and trunk kidney) were sampled, and their bioluminescence was measured using the IVIS Imaging System. After that, half of these organs were homogenized, serial diluted, and spread on ampicillin selective BHI plates and plate counts were performed. This allowed confirmation of a complex disease pathogenesis during ESC involving two distinct steps in the development of the bacteremia, separated by a period of intense reproduction in the anterior kidney. We also found that bioluminescent imaging was much more sensitive compared to the more classical technique of serial dilution and plate counting.

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Introduction

Since its first description in 1979 as the causative agent of enteric septicemia of channel catfish (ESC), *Edwardsiella ictaluri* (Hawke, 1979) has become recognized as one of the main pathogens affecting the channel catfish (*Ictalurus punctatus*) industry. Disease outbreaks caused by this pathogen generally occur at temperatures ranging between 20 and 30°C, with an optimum temperature between 25 and 28°C (Plumb 1998, Roberts 2001), and can causes mortalities up to 85% (Kocabas et al. 2002) . Furthermore, *E. ictaluri* was involved in more than 30% of all cases diagnosed by the Aquatic Diagnostic Laboratory in Stoneville, Mississippi in 2007 (Thad Cochran National Warmwater Aquaculture Center 2007).

Despite *E. ictaluri*'s impact on the industry and the progresses made in understanding the disease, much is still to be discovered about the pathogenesis of ESC (Nusbaum & Morrison 2002, Thune et al. 2007). Several studies suggest a complex infectious cycle involving multiple organs similar to that already well described in *Salmonella* and *Yersinia* (Baldwin 1992). For example, after an experimental infection by immersion in water containing S^{35} labeled *E. ictaluri*, Nusbaum and Morrison (2002) first detected bacteria in the gills and liver. The bacteria then quickly spread to the digestive tract and kidney. Perhaps due to the low temperature at which this challenge was conducted (20°C), the bacterium was quickly cleared. Nonetheless, the authors reported a sharp increase in bacterial numbers in the gills and liver between 30 and 40 hours post-infection. This increase may correlate with a similarly abrupt rise in bacterial

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levels described in the peripheral blood of infected fish between 24 and 72 hours post-infection (Wise et al. 1997).

In a previous study, we found that bacterial numbers were highest in the spleen and head kidney by 6 hours post infection following immersion exposure. However, by 20 hours post-infection, the trunk kidney was also heavily infected (Karsi et al. 2006). Following infection by gastric intubation, Baldwin and Newton (1993) also reported colonization of the trunk kidney within 48 hours of the challenge. Following this stage, bacteria multiplied and were disseminated by the circulating leukocytes along the vascular system to a variety of organs (Baldwin & Newton 1993).

In the current study, our objective was to utilize the bioluminescence model we developed for *E. ictaluri* to provide a thorough description of ESC pathogenesis following experimental immersion challenge. Using this technique, we evaluated bacterial concentrations in several organs during the course of infection. We also sought to compare bacterial quantification in tissues using two different methods: bioluminescence imaging and serial dilutions and colony counts. Our results offer new insights into the pathogenesis of ESC and demonstrate the effectiveness of using bioluminescence to improve experimental bacterial infection models in animals.

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Materials and Methods

Fish

Juvenile channel catfish were obtained from the specific-pathogen-free (SPF) laboratory at the Mississippi State University College of Veterinary Medicine. These fish were raised in a pathogen free environment with respect to *E. ictaluri* until the time of the challenge, when they had reached the age of four months and a size of roughly 10cm in length.

Bacteria

E. ictaluri strain 93-146, which was originally isolated from an outbreak of ESC in a commercial catfish pond in Louisiana, was cultivated in brain heart infusion broth and on agar plates at 28 ˚C. For experimental infections, broth cultures were grown with rotary aeration for approximately 16 h. For bioluminescence labeling, pAKlux1 was transferred into strain 93-146 by conjugation as described (Karsi & Lawrence 2007). *E. ictaluri* carrying pAKlux1 was grown in the presence of 100 µg/ml ampicillin.

Experimental infection

Fish were transferred into ten 10 gallon tanks (12 fish per tank) supplied with flow-through dechlorinated municipal water. Temperature was maintained at 27°C +/- 1°C throughout the study. After three days of acclimation, fish were experimentally infected as described in chapter two by bath immersion in water containing 10^9 CFU per

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liter of *E. ictaluri* 93-146 pAKlux1. Bacteria were gradually removed by restoring water flow after 1 h.

Sampling

At regular time points (1, 6, 16, 24, 36, 48, 60, 72, 96, 110, 125 and 175 hours post-infection), six fish were randomly collected and euthanized by immersion in water containing a lethal concentration of MS222. For each fish, the following organs were collected aseptically: gills, muscles, intestine, spleen, liver, stomach, heart, head kidney, and trunk kidney. Each organ was weighed, and photon emission from each individual organ was collected for 30 seconds using an IVIS Imaging System (Caliper Life Science). Bioluminescence was quantified using Living Image software in photons/ s/cm^2/ steradian using a custom area selected area for each organ. Organs from half of the fish (three per time point) were manually homogenized in 0.1 ml of sterile water before being serially diluted in triplicate and spread on BHI agar plates containing ampicillin. To monitor the development of bacteremia, 75 µl of blood were sampled from each fish, and serial diluted and the 10^{-5}, 10^{-6} and 10^{-7} dilutions were spread on BHI agar plates containing ampicillin. Because of the rapid clotting of fish blood, it was not possible to analyze these blood samples by luminescence.

Mean bioluminescence per g of tissue from six replicates for each organ at each time point was calculated. Mean CFU per g of tissue from three replicates for each organ at each time point was calculated. Statistical analysis was performed using the

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Student T-test. Correlations between bioluminescence/g tissue and CFU/g tissue were calculated using regression analysis.

Results

The levels of correlation between the luminescence and the plate counts were weak, as illustrated by the high error type reported in Table 4.1.

Bacterial levels in the various organs sampled as measured using plate counts are reported in figure 4.1; the same results obtained by measurement of bioluminescence are reported in figure 4.2.

Table 4.1. Correlations between luminescence in photons/ s/ cm^2/ steradian and number of CFU/ g by colony counting in each organ type.

Organs :	value of y.	Error type
All organs:	6,8458E-06	6,06E+01
Gills:	-1,65E-06	8,68E+01
Skeletal muscle	8,00E-03	3,63E+01
Intestine:	4,77E-04	2,36E+01
Spleen:	2,29E-04	1,99E+01
Liver:	5,15E-06	5,00E+01
Stomach:	6,67E-01	1,96E+01
Heart:	-2,88E-06	2,85E+01
Posterior kidney:	2,48E-04	5,79E+01
Anterior kidney:	1,51E-04	4,65E+01

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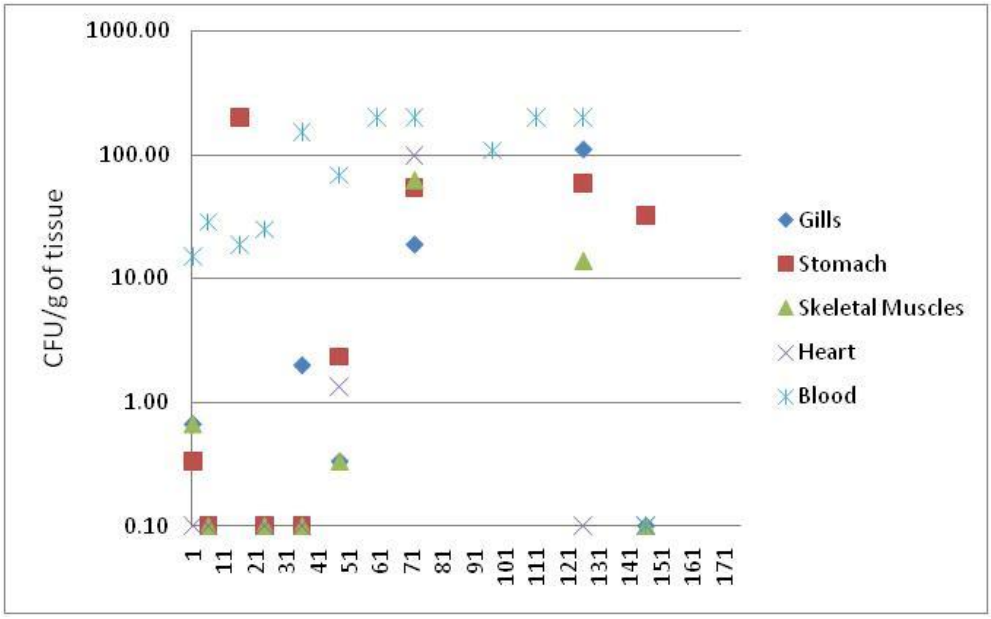

Figure 4.1. Average numbers of bacteria in blood, gills, muscles, stomach and heart as measured at different time points using plate counts and serial dilution.

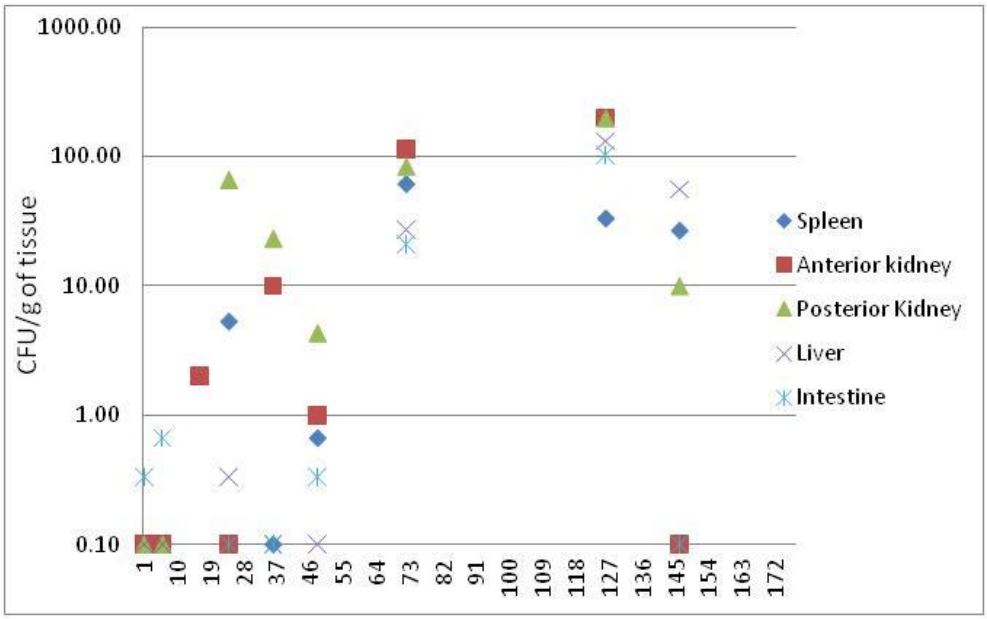

Figure 4.2. Average numbers of bacteria in the intestine, spleen, liver, posterior and anterior kidneys, as measured at different time points using plate counts and serial dilution.

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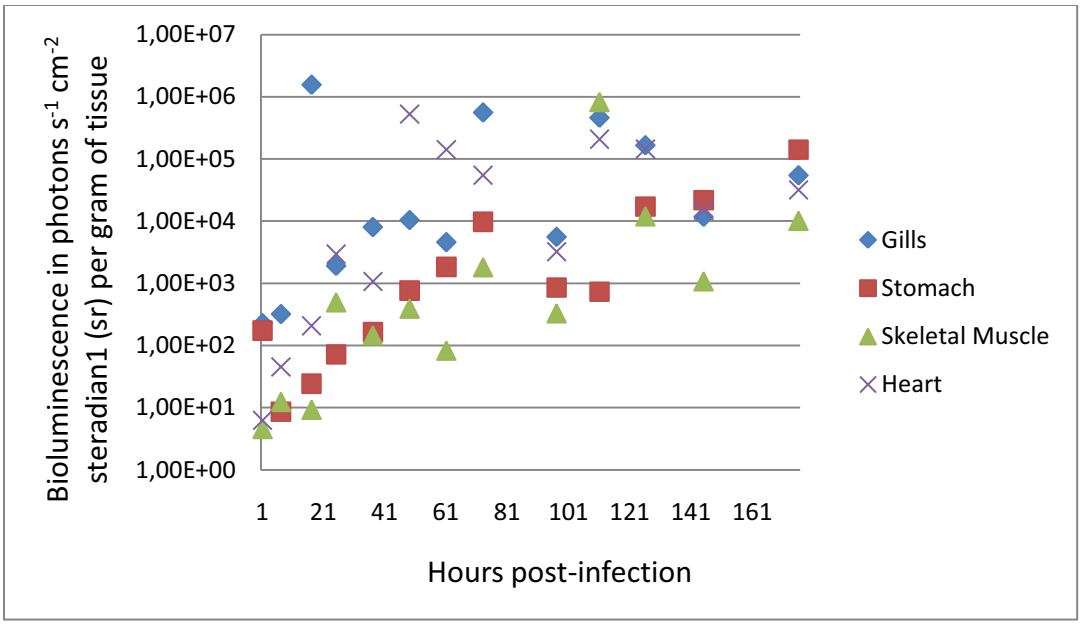

Figure 4.3. Average numbers of bacteria in blood, gills, muscles, stomach and heart as measured at different time points using bioluminescence measurement from the IVIS imaging system.

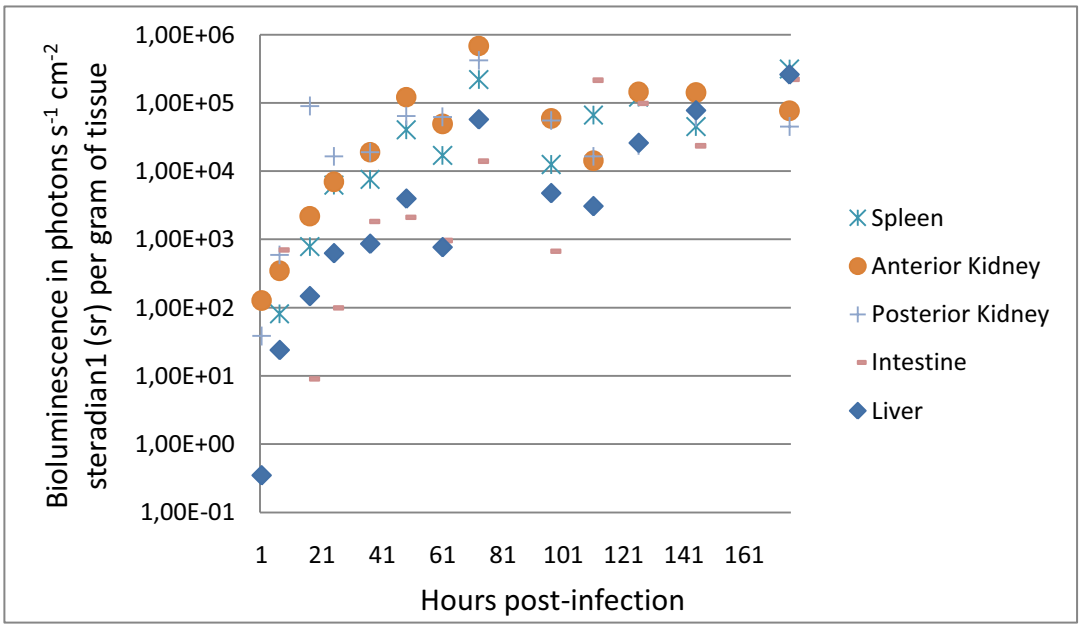

Figure 4.4. Average numbers of bacteria in the intestine, spleen, liver, posterior and anterior kidneys, as measured at different time points using bioluminescence measurement from the IVIS imaging system.

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When performed on the blood samples, dilution and plate counts revealed that bacterial numbers rose quickly until reaching an average number of 151.5 (±97) CFU /g at 36 hours post-infection before dropping slightly to 68.2 (±37) CFU/g and then gradually increasing and stabilizing at a value of approximately 200 CFU/g by the end of the experiment.

The bacterial levels in the gills, as determined by the dilution and plate count method, fluctuated in the beginning of the infection until 48 hours post infection when it rose quickly from an average value of 0.33 (±0.57) CFU/g to a final value of above 10000 (±14000) CFU/g at the end of the experiment. When determined through measurement of bioluminescence, the number of bacteria in the gills appeared to rise steadily (excluding outliers) from 2.27E+02 (±3.41E+00) photons s^{-1} $(cm^2)^{-1}$ steradian^{-1} (sr) to to 1.04E+04 photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at 48 h post-infection. Bacterial concentrations fluctuated between 5.60E+05 (±3.18E+04) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} and 5.58E+03 (±1.44E+02) for the remainder of the study.

Bacterial levels in the muscle, as measured by the dilution and plate count method, remained low until 48 hours post-infection, after which point they rose from 0.33 (±0.57) CFU/g to 74.5 (±6,36)CFU/g at 96 hours post-infection. At later times, these bacterial levels slowly decreased until the end of the experiment. When determined by measurement of bioluminescence, the bacterial levels in the muscles increased more steadily from 4.53E+00 (±1.12E-01) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at one hour post-infection to a value of 8.26E+05 (±5.18E+04) at 110 hours post-infection, after which the bacteria

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slowly cleared, with their number decreasing to 1.08E+03 (±4.57E+01) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at the end of the challenge.

The bacterial loads in the stomach, as observed by dilution and plate counts, quickly peaked in the first hours of infection, rising from 0.33 (±0.58) CFU/g to 200 CFU/g at 24 hours post-infection. These numbers then dropped below the detection level by 48 hours post-infection before rising again over the course of the infection, reaching a value of 59 CFU/g by 96 hours post-infection. When determined through bioluminescence, the bacterial levels rose from a value of 1.74E+02 (±6.94E+00) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} to 9.77E+03 (±4.63E+01) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at 72 hours post-infection before progressively dropping to 8.52E+02 (±1.79E+01) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at 96 hours post-infection. It then progressively rose again, reaching 1.41E+05 photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at the last sampling point.

Bacterial loads in the heart remained stable for the first hours of infection, then at 48 hours post-infection, they started rising from a value of 1.33 (±1.53) CFU/g to a value of 98 CFU/g by 110 hours post-infection. When determined by bioluminescence, the bacterial numbers rose until 48 hours post infection when they reached a value of 5.27E+05 (±2.96E+04) photons s^{-1} $(cm^2)^{-1}$ sr^{-1}, but there was a large amount of variability with only some fish having very high numbers in the heart at this time point. Bioluminescence remained between approximately 2.08E+05 (±1.29+04) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} and 1.5E+04 photons s^{-1} $(cm^2)^{-1}$ sr^{-1} for the remainder of the study.

Bacterial levels in the intestine, as measured by dilution and plate count, remained low in the first hours of the infection before rising abruptly from 0.33 (±0.58)

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CFU/g at 48 hours post-infection to 102 (±137.89) CFU/g at 125 hours post-infection. A similar pattern was observed through bioluminescence with the bacteria slowly increasing in number until reaching 1.40E+04 (±1.27E+02) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} by 72 hours post-infection, at which point these numbers dropped, as observed in many organs, to 6.70E+02 (±1.44E+01) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} by 96 hours post-infection. After this point, they started rising again, reaching 2,21E+05 photons s^{-1} $(cm^2)^{-1}$ sr^{-1} by the end of the experiment.

In the liver, the pattern was simple, with bacterial levels rising steadily over the duration of the experiment from below detection level to 39 CFU/g by 96 h post-infection. Bioluminescence showed a similar pattern with numbers rising from background levels to 5.73E+04 (±1.40E+03) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at 72 hours post-infection to 2,61E+05 photons s^{-1} $(cm^2)^{-1}$ sr^{-1} by the end of the experiment.

Interestingly, the pattern of bacterial concentration in the spleen and kidneys was somewhat similar. When measured using dilution and plate counts, the bacterial levels in the spleen, at first, appeared to undergo an initial increase in the first hours of the infection, rising from below detection levels to a value of 5.33 (±9.24) CFU/g at 24 hours post-infection. Counts then dropped to 0.66 (±0.58) CFU/g by 48 hours post-infection before rising again to reach a value of 75 CFU/g at 96 hours post-infection. Tissue concentrations dropped below detection levels by the last time points. When measured through bioluminescence, bacterial numbers appeared to rise from below detection levels to a value of 2.20E+05 (±3.88E+03) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at 72 hours post-infection. They then dropped slightly at 96 hours post-infection, reaching a value of

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1.25E+04 (±3.34E+02) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} before rising again, reaching 3,14E+05 photons s^{-1} $(cm^2)^{-1}$ sr^{-1} by 102 hours post-infection.

In the posterior kidney, the bacterial concentration rose until 24 hours postinfection when they reached 66.67 (±115.47) CFU/g. At this point, bacterial concentration dropped to 4.33 (±7.51) CFU/g at 48 hours post-infection before rising again to reach 55 CFU/g by 96 hours post-infection. Then concentrations dropped below detection level. Using bioluminescence, a steady increase of bacterial loads occurred from 3.83E+01 (±5.59E-01) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at one hour post-infection until 72 hours post infection when they peaked at a value of 4.19E+05 (±4.21E+03) photons s^{-1} $(cm^2)^{-1}$ sr^{-1}. Luminescence dropped slightly to 5.50E+04 (±1.05E+03) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at 96 hours post infection, at which point it started rising again, reaching 4,51E+04 photons s^{-1} $(cm^2)^{-1}$ sr^{-1} by the end of the experiment.

Applying the dilution and plate count technique to the anterior kidney showed that the bacterial concentrations rose until 36 hours post-infection when they reached a level of 10 (±16.46) CFU/g, then they dropped to an average value of 1 CFU/g at 48 hours post-infection before rising again, reaching 200 CFU/g by 125 hours post-infection. Concentrations dropped by the end of the experiment. When determined through bioluminescence, the bacterial concentration in this organ appeared similar to that in the spleen and posterior kidney with the bacterial levels rising steadily from 1.27E+02 (±6.38E+00) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at one hour post infection to 6.82E+05 (±1.48E+04) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} at 72 hours post-infection. After this point they dropped slightly, reaching 5.91E+04 (±1.33E+03) photons s^{-1} $(cm^2)^{-1}$ sr^{-1} by 102 hours

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post-infection before rising slightly and stabilizing around 1.40E+05 photons s^{-1} $(cm^2)^{-1}$ sr^{-1} in the last time points.

As mentioned, the concentrations of bacteria were quite similar between the spleen, anterior and posterior kidneys. Indeed, bacterial loads were only statistically significantly different between the spleen and posterior kidney at one hour post-infection (P=0.00155) and between the spleen and anterior kidney at 36 hours post-infection (P=0.01999).

Discussion

We previously showed that bioluminescence is an accurate measure of bacterial concentrations *in vitro*. Using *E. ictaluri* carrying pAKLux1, we found that the amount of luminescence measured is proportional to the amount of bacteria present (Karsi et al. 2006). We also showed that bioluminescence may potentially serve as a method for monitoring bacterial concentrations in vivo. However, it is also known that *in vivo* results will be affected because part of the light produced by the bacteria will be diffused and absorbed by surrounding tissues. For example, we found that bioluminescence was almost 10-fold higher in albino catfish ($46.00E10^8$ photons s^{-1} $(cm^2)^{-1}$ sr^{-1}) than in non-albino catfish ($6.19E10^7$ photons s^{-1} $(cm^2)^{-1}$ sr^{-1}), likely due to the difference in absorption between the pigmented and non-pigmented skin. Therefore, this study was conducted to assess the usefulness of bioluminescence assay to measure bacterial concentrations in vivo. We were also aiming to describe the pathogenesis of

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ESC following experimental immersion exposure by providing a thorough description of changes in *E. ictaluri* concentrations in catfish tissues over time.

Over the course of this study, we were able to monitor bacterial concentrations in multiple fish organs with great accuracy using bioluminescence. We observed a first peak of bacterial concentrations in the gills in the first few hours of infection until 16 hours post-infection. This was promptly followed by a parallel rise in bacterial concentrations in both the blood and the heart, suggesting that the gills acted as the point of passage of the bacteria into the bloodstream. This result is similar to a result already described by Nusbaum and Morrison (1996).

At this point, bacterial concentrations started to rise in the spleen and kidneys. Most likely the bacteria establish infection in these organs following filtration from the bloodstream by the resident macrophages or carriage into the melanomacrophage centers following their phagocytosis by circulating leukocytes (Glenney & Petrie-Hanson 2006). At this point, the bacteria likely cause damage to the renal tissue, which would explain the perturbation of osmoregulatory function and subsequent clinical signs such as ascites and exophthalmia.

Meanwhile, the bacterial levels build up in the stomach until 72 hours post-infection, when the bioluminescence starts to decrease in this organ. *This timing is identical to the pattern described by* Baldwin and Newton (1993) who monitored pathogenesis of ESC following gastric intubation. These authors suggested that the bacterium uses the resident macrophages as a means to gain entrance through the digestive tract. This hypothesis is consistent with our observations.

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At this point, the bacterial concentrations decrease in the intestine, spleen, and kidneys but increase sharply in the blood, suggesting that the bacteria might be leaving the organs to reenter the circulation, possibly being released from the resident macrophages in these organs. This second episode of bacteremia would correspond to the sharp increase in the level of bacteria in the peripheral blood observed by Wise *et* al. (1997) at 72 hours post-infection.

This second bacteremia allows for the pathogen to disseminate further. Indeed, at the following time point, we observe a rise in the bacterial levels in every organ sampled with the exception of the skeletal muscles. This rise is especially noticeable in the intestine, which changes from the least to most densely colonized organ at that point. This could be an efficient means for dissemination of the bacterium into the environment. At this point the fish immune system is likely overrun by the pathogen, and death follows quickly.

When compared to the results obtained by the monitoring of luciferase activity, the results obtained by the more traditional method of plate counts are much more less sensitive, possibly due to the high levels of dilution used in the course of this study, and it is only after the sharp increase in bacterial level at 48 hours post-infection that bacteria can be reliably detected on the plates.

Furthermore, bacterial concentrations as measured using bioluminescence appear more consistent. While it must be noted that the sample size using bioluminescence was twice the size of the sample size using the dilution and plate count technique, it is also worth noting that the amount of labor involved in serial dilution and

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plate counting puts a practical limit on the sample size that can be achieved by this method. It can therefore be concluded that not only is bioluminescence an effective method for the monitoring of bacterial concentrations in tissues during infection, it is also much more sensitive and consistent and allows the detection of fluctuation that would normally not be detectable through the method of plate counts.

Importantly, our results describe accurately, for the first time, the occurrence of two bacteremias during the pathogenesis of ESC. The first occurs shortly after entry in the host, and the second occurs following a period of infection in hematopoietic tissues. In addition, our results support both gills and intestinal tract as points of entry, with gills allowing early entry and intestinal tract entry peaking at 72 h post-infection. In summary, our results confirm results from previous studies on the pathogenesis of ESC, in particular the presence of bacteria in the gills during the early stages of infection (Nusbaum & Morrisson 1996) and the sharp increase in the level of bacteremia after 72 hours post-infection (Wise et al. 1997). In addition, these results further extend our understanding of events occurring in catfish tissues during this important disease which may lead to a better targeting of our treatment and vaccine strategies in the future.

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CHAPTER V

CONCLUSION

The unifying thread of this study was the investigation of the early stages of infection by *Edwardsiella ictaluri*. In the first chapter, we used the recently developed luciferase plasmid to investigate the role of abrasion in the fish epithelium as a contributing factor in the development of the disease. This technique allowed us to detect that the bacteria readily adhered to these abrasion sites. Furthermore, we were able to demonstrate that such abrasions were a contributing factor to infection. Infection developed faster to develop and mortality levels were higher when fish were abraded, and the bacteria progressively migrated from this initial adhesion site, colonizing progressively deeper tissues. This was the first report of the role of such abrasions concerning *E. ictaluri*.

In the following phase of research, random insertion events were used to detect genes involved in this epithelial colonization. Twenty such mutants were discovered with a decreased ability to colonize and induce mortality. Most notable among the genes identified were RstBA, a regulator linked to the regulation of the formation of curli fimbriae or amyloid fibers in *Escherichia coli*, as well as a heat-shock protein, presenting strong homology to one often associated with flagellar synthesis as well as its associated proton pump.

Finally, the last study focused on the dynamics of infection within the fish. This was investigated by challenging fish with bioluminescent *E. ictaluri* prior to sampling at given time

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points post-infection. The bacterial loads within the tissues were then quantified using serial dilution and plate counts as well as bioluminescence. This study showed the much higher sensitivity of bioluminescence techniques compared to plate counts. In addition, it allowed us to trace the complex cycle of infection of *E. ictaluri* that starts by a first episode of bacteremia when the bacteria first reach the bloodstream, mainly from the stomach and gills. The bacteria then establish infection in the kidneys and spleen, most likely carried there by the circulating leukocytes that phagocytosed them. There, they multiply actively before reentering the blood stream causing a sharp increase in the level of septicemia and disseminating the infection thorough the fish' organs, soon followed by death of the fish host.

One feature setting *E. ictaluri* apart from its relatives, *Edwardsiella tarda, Edwardsiella hoshinae*, and, more distantly, *Salmonella*, is how thoroughly it seems adapted to its host of choice, the American channel catfish (*Ictalurus punctatus*). Despite this apparent adaptation, most aspects of *E. ictaluri*'s virulence, including the ones explored in this study, seem to rely mostly on non-specific, well conserved aspects. This is the case for the bacterium's ability to invade a wide type of epithelial cells, as illustrated by its ability to use skin abrasion sites, gills, nares, and intestinal epithelium as portals of entry. Similarly, the genes discovered through our use of transposon mutagenesis are common virulence factors among the *Enterobacteriaceae*, and the bacterial accumulation in the melanomacrophage centers in the early stages of infection is reminiscent of *Salmonella* Typhi's infection of the Peyer's patches in higher mammals. *E. ictaluri* localization in melanomacrophage centers is likely to be an effect of the fish immune system rather than a unique feature of the bacterium.

It would, therefore, be interesting to investigate further the reasons for the bacterium's narrow host range. Similarly, the evolution of the *Edwardsiella* genus and its divergence from its *Salmonellae* relative would be a fascinating line of study.

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