Antimicrobial Susceptibility Pattern of *Edwardsiella ictaluri* Isolates from Natural Outbreaks of Bacillary Necrosis of *Pangasianodon hypophthalmus* in Vietnam

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The purpose of this study was to assess the *in vitro* susceptibility of 64 Vietnamese isolates of *Edwardsiella ictaluri*, the causal agent of the infectious disease Bacillary Necrosis Pangasius in *Pangasianodon hypophthalmus*, using the agar dilution technique. All isolates originated from different farms and were collected between 2002 and 2005. None of the isolates displayed acquired resistance to amoxicillin, amoxicillin–clavulanic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, neomycin, and nitrofurantoin. Acquired resistance to streptomycin was detected in 83%, to oxytetracycline in 81%, and to trimethoprim in 71% of the isolates, as indicated by a bimodal distribution of the minimal inhibitory concentrations (MICs) of these antimicrobials. The MICs of enrofloxacin displayed a monomodal distribution with tailing toward the higher MIC values, possibly indicating reduced susceptibility of a minority of isolates (3 out of the 64). For the quinolone antimicrobial agents flumequin and oxolinic acid, acquired resistance was encountered in 8% and 6% of the strains, respectively. All strains were intrinsically resistant to the polypeptide antimicrobial agent colistin. Seventy-three percent of the isolates were shown to have acquired resistance to at least three antimicrobial agents. The results of this study emphasize the strict need to control both the prophylactic and curative use of antimicrobial agents in Vietnamese aquaculture.

**Introduction**

Total aquaculture production in Vietnam topped 1 million tons in 2003 and is expected to reach over 2 million tons by 2010.29 Mekong Delta is the main producer, being responsible for over 80% of the total Vietnamese production. The freshwater catfish *Pangasianodon hypophthalmus* is the most commonly cultured edible fish species in this region. Currently, *P. hypophthalmus* has become the second most important Vietnamese food commodity produced and exported after rice.

Of the several infectious diseases diagnosed in this fish species, Bacillary Necrosis, caused by *Edwardsiella ictaluri*, is the most frequently occurring.11,17 This increasingly important disease occurs in production systems of fish of all ages, although especially fingerlings and juvenile fish seem to be affected. This results in severe economic losses through decreased production, expense of treatment, and fish mortality.

Besides the Vietnamese freshwater production, the American channel catfish (*Ictalurus punctatus*) industry also suffers massively from *E. ictaluri* infections that have been termed “enteric septicemia of catfish” (ESC). ESC accounts for approximately 60% of all mortality in farmed channel catfish, which results in approximately 50 million dollars in annual losses.30 Recently, *E. ictaluri* was also identified as the cause of disease in *P. hypophthalmus* in Indonesia.45 To treat bacterial infections, antimicrobial agents are widely used in Vietnamese aquaculture, both on a preventive and curative basis.11,15,33 In most of the cases, the amount of drugs to be administered merely is an estimation, leading to incorrect dosage.40 In view of this, besides antimicrobial sensitivity monitoring being necessary for choosing effective antimicrobial agents, susceptibility testing is also crucial to monitor antimicrobial resistance development.

Hitherto, to our knowledge, only a handful of studies have investigated the antimicrobial susceptibility of *E. ictaluri*, always including American isolates. Hawke19 was the first to test the antimicrobial susceptibility of 10 *E. ictaluri* isolates. Later, Waltman and Shotts42 screened 118 *E. ictaluri* isolates retrieved in the United States for susceptibility to 37 antimicrobials using the disc sensitivity test. They found that the majority of isolates were susceptible to most agents active against Gram-negative bacteria, but resistance was observed against colistin and sulfonamides in more than 90% of isolates. Reger et al.34 likewise tested the antimicrobial susceptibility of American *E. ictaluri* isolates and found full
susceptibility to enrofloxacin, gentamicin, and doxycycline. Half a decade ago, Stock and Wiedemann\(^8\) studied the antimicrobial susceptibility of 41 *E. ictaluri* strains to 71 antibiotics. All these isolates originated from American channel catfish, and hardly any acquired resistance was detected.

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

### Materials and Methods

#### Bacterial strains

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

Presumptive identification was done using the API 20E commercial kit (Microbank\(^\text{TM}\); PRO-LAB Diagnostics, West- pond, UK).

The identity of *E. ictaluri* was confirmed by the tDNA-PCR technique.\(^5\) Briefly, genomic DNA was extracted by suspending one colony of a bacterial culture in 20 μl lysis buffer (0.25% sodium dodecyl sulfate, 0.05 M NaOH). After heating at 95°C for 5 minutes and centrifugation for 20 seconds at 13,000 rpm, 180 μl sterile distilled water was added and centrifugation was done at 13,000 rpm for 5 minutes. The spacers in between the tRNA genes were amplified using the primer TSB (5′-AGTCCGGTGTCTACTAACACTGAG3′) and fluorescently labeled primer T3B (5′-AGTCCGGTGGTCTCGAACATCC3′). PCR mixtures and cycle conditions were the same as described before.\(^5\) Capillary electrophoresis was carried out using the ABI Prism\(^\text{TM}\) 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Foster City, CA). Electropherograms obtained with the Genescan software were compared to the database using in-house software. Edwardsiella tarda (LMG 27937) and *E. ictaluri* (CCUG 18764) were additionally included in this assay.

*Escherichia coli* (ATCC 25922) was included as a reference strain in the agar dilution tests.\(^9\)

#### Antibacterial agents

The following antimicrobial agents were obtained as laboratory standard powders: amoxicillin, amoxicillin + clavulanic acid, chloramphenicol, florfenicol, gentamicin, kanamycin, streptomycin, neomycin, enrofloxacin, oxylinic acid, flumequin, oxytetacycline, trimethoprim, nitrofurantoin, and colistin. These antimicrobial agents were purchased from Sigma Aldrich N.V. (Bornem, Belgium), except enrofloxacin, which was obtained from Bayer AG (Brussels, Belgium); streptomycin from Certa (L’Alleud, Belgium); and colistin from VMD (Arendonk, Belgium). They were dissolved in appropriate solvents to make stock solutions and then further diluted in sterile distilled water according to the methods recommended by the Clinical and Laboratory Standards Institute (CLSI M49-A).\(^9\)

#### Susceptibility testing

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

All the Edwardsiella isolates were inoculated on Columbia blood agar (Difco, Wesel, Germany) and incubated for 48 hours at 26°C. The *E. coli* reference strain ATCC 25922 was likewise inoculated on Columbia blood agar, but incubation occurred for 24 hours at 37°C. Inocula were prepared by suspending overnight cultures in phosphate-buffered saline (PBS) to a density of 0.5 on the McFarland scale. Consequently, 1/10 dilutions in PBS were prepared. Approximately 1 × 10⁸ colony forming units of the strains were then inoculated on the antibiotic-containing plates and on antibiotic-free control plates by means of a Denley multiwell inoculator (Mast, Sussex, UK), after which plates were incubated aerobically at 26°C. For Edwardsiella isolates, MICs were recorded after 48 hours and for the *E. coli* reference strain after both 24 and 48 hours’ incubation. The MIC was defined as the lowest concentration of the antimicrobial agent with no visible bacterial growth. For interpretation of MIC results, the microbiological criterion (epidemiological or wild-type cut-off value) was used.\(^9\)

### Results

MIC endpoints of the *E. coli* reference strain did not differ when plates were incubated during 24 or 48 hours and for the antimicrobial agents included in Clinical and Laboratory Standards Institute document M49-A; they fell within acceptable quality ranges.\(^9\)

An overview of the MIC values for the different *E. ictaluri* isolates is shown in Table 1. The MIC₅₀ and MIC₉₀ values and percentages of isolates considered to have acquired resistance are likewise presented.

For the β-lactam antimicrobial agents amoxicillin and the combination of amoxicillin–clavulanic acid, as well as for chloramphenicol, florfenicol, nitrofurantoin, and the aminoglycoside antibiotics gentamicin, kanamycin, and neomycin, a monomodal distribution of MICs was noted, indicating absence of acquired resistance. In contrast, for streptomycin, oxolinic acid, flumequin, oxytetacycline, and trimethoprim, the MICs showed a bimodal distribution. According to the microbiological criterion, isolates in the higher range of MICs should be considered to have acquired resistance.\(^3⁹\) For enrofloxacin, tailing toward the higher MIC values was observed, possibly indicating reduced susceptibility in three isolates.

All *E. ictaluri* isolates were intrinsically resistant to the polypeptide antimicrobial agent colistin with MIC values equal to or above 64 μg/ml.

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

### Discussion

Different criteria may be used for the interpretation of MIC results. For several terrestrial animals, interpretive criteria or breakpoints have been established by the Clinical and Laboratory Standards Institute (CLSI document M31-A3) so that the results of the tests can be interpreted as susceptible, intermediate, or resistant and reported as such to veterinarians.\(^1⁰\) For selection of these breakpoints, a range of para-
Table 1. Distribution of Minimal Inhibitory Concentration (MIC) of Various Antimicrobial Agents on 64 Edwardsiella ictaluri Isolates from Pangasianodon hypophthalmus in Vietnam

| Antimicrobial agent          | ≤0.12 | 0.25 | 0.5 | 1   | 2   | 4   | 8   | 16  | 32  | 64  | ≥128 | M50 | M90 | % Resistance  
|-----------------------------|------|------|-----|-----|-----|-----|-----|-----|-----|-----|------|-----|-----|---------------
| Amoxicillin                 | 3    | 19   | 42  |     |     |     |     |     |     |     |      | 0.5 | 0.5 | 0             
| Amoxicillin + clavulanic acid| 42   | 22   |     |     |     |     |     |     |     |     |      | 0.12 | 0.25 | 0              
| Chloramphenicol             | 13   | 28   | 23  |     |     |     |     |     |     |     |      | 0.25 | 0.5 | 0              
| Florfenicol                 | 31   | 33   |     |     |     |     |     |     |     |     |      | 0.25 | 0.25 | 0              
| Gentamicin                  | 15   | 34   | 15  |     |     |     |     |     |     |     |      | 2    | 4   | 0              
| Kanamycin                   | 18   | 39   | 7   |     |     |     |     |     |     |     |      | 4    | 8   | 0              
| Streptomycin                | 2    | 9    | 1   | 1   | 51  |     |     |     |     |     | ≥128  | 128  | 82.8 | 82.8          
| Neomycin                    | 2    | 52   | 10  |     |     |     |     |     |     |     |      | 4    | 8   | 0              
| Oxolinic Acid               | 9    | 27   | 14  | 3   |     |     |     |     |     |     |      | 0.25 | 1   | 4.7            
| Flumequin                   | 1    | 3    | 9   | 46  |     |     |     |     |     |     |      | 2    | 3   | 7.8            
| Oxytetracycline             | 2    | 8    | 1   | 1   | 3   | 26  | 23  |     |     |     |      | 32   | 64  | 81.3           
| Trimethoprim                | 9    |     | 8   |     | 13  | 34  |     |     |     |     |      | ≥128 | ≥128 | 73.4          
| Nitrofurantoin              | 8    | 8    | 5   | 34  | 9   |     |     |     |     |     |      | 2    | 4   | 0              
| Colistin                    | 3    | 61   | ≥128| ≥128|     |     |     |     |     |     |      |     |     | Intrinsically resistant 

| SM, streptomycin; OTC, oxytetracycline; TMP, trimethoprim; FM, flumequin; OXO, oxolinic acid; ENR, enrofloxacin. 

Table 2. Resistance Phenotypes of 64 Edwardsiella ictaluri Isolates from Pangasianodon hypophthalmus in Vietnam

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>Number (%) of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM</td>
<td>4 (6.3)</td>
</tr>
<tr>
<td>OTC</td>
<td>2 (3.1)</td>
</tr>
<tr>
<td>SM + OTC</td>
<td>2 (3.1)</td>
</tr>
<tr>
<td>OTC + TMP</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>SM + OTC + TMP</td>
<td>42 (65.6)</td>
</tr>
<tr>
<td>SM + OTC + TMP + FM</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>SM + OTC + OXO + FM</td>
<td>1 (1.6)</td>
</tr>
<tr>
<td>SM + OTC + TMP + OXO + FM + ENR</td>
<td>3 (4.7)</td>
</tr>
<tr>
<td>No acquired resistance</td>
<td>8 (12.5)</td>
</tr>
</tbody>
</table>

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

Chloramphenicol has been strictly prohibited for animal use in most countries, including Vietnam, because of its severe toxicity and causing depression of the bone marrow.29,31 Its fluorinated derivative, florfenicol, has instead become available and rapidly became popular in several animal industries, including aquaculture.14,18,28,44 All E. ictaluri isolates included in the present study were highly susceptible to florfenicol, with an MIC ≤0.25 μg/ml. McGinnis likewise tested E. ictaluri isolates from American channel catfish and found full susceptibility.26 In contrast, Ho et al.23 isolated E. tarda, Aeromonas hydrophila, Pseudomonas fluorescens, Vibrio cholerae, and Salmonella spp. isolates from aquatic animals in Taiwan that were less susceptible to florfenicol. This finding emphasizes the need to be vigilant and control the use of florfenicol in the sense that it should not be used indiscriminately.

In the present study, acquired resistance to the aminoglycoside antibiotics gentamicin, kanamycin, and neomycin was not detected in the E. ictaluri isolates. In contrast, the MIC values for streptomycin showed a bimodal distribution, with 83% of the isolates displaying an MIC value in the higher range and thus having acquired resistance. Previous studies showed full susceptibility of E. ictaluri and E. tarda to streptomycin,19,32,38,42 rendering this study the first to report high levels of resistance to this antibiotic.

All isolates in our study clearly displayed intrinsic resistance to the polypeptide antimicrobial agent colistin. This
Finding correlates with the results from a previous study performed by Muyembe et al.,32 who found that all 35 human isolates of *E. tarda* were resistant to colistin. Waltman and Shotts42 screened 118 *E. ictaluri* isolates retrieved in the United States for susceptibility to 37 antimicrobials using the disc sensitivity test. The study found that the majority of isolates were susceptible to most agents active against Gram-negative bacteria, but resistance was observed against colistin in more than 90% of isolates. The findings suggested that colistin could be used as an additive to generate a selective medium for the isolation of *Edwardsiella* species, including *E. ictaluri*.

In the present study, three strains displayed reduced sensitivity to enrofloxacin, for which an MIC value of 2 μg/ml was noted. Fluoroquinolones inhibit the activity of DNA gyrase, and in most bacterial species resistance is due to mutations in the gyrase or topoisomerase genes.6,37,43 In *Enterobacteriaceae* resistance to quinolones is most commonly acquired in two steps. One mutation in the *gyrA* genes mediate full resistance to first-generation quinolones, such as nalidixic acid and flumequin, and reduced susceptibility to other fluoroquinolones. A second mutation in either *gyrA* or *gyrB* genes mediates full resistance to fluoroquinolones.16,41 In the present study, the three isolates with reduced sensitivity to enrofloxacin indeed displayed resistance toward flumequin. Further research will be needed to elucidate the mechanisms of enrofloxacin resistance found in these isolates.

For the antimicrobial agents flumequin and oxolinic acid, clear bimodal distributions of MICs were evident and acquired resistance was encountered in 8% and 6% of the strains, respectively. As far as we know, this is the first report of resistance toward these antimicrobial agents in *E. ictaluri*. Stock and Wiedemann38 did not find any resistance against *E. ictaluri* of resistance toward these antimicrobial agents in 8% and 6% of the isolates, respectively. As far as we know, this is the first report of resistance toward these antimicrobial agents in *E. ictaluri*.

In this study, the distribution of MIC values of oxytetracycline was clearly bimodal, with the majority of strains falling in the higher range (≥16 μg/ml), indicating acquired resistance in 81% of the isolates. The high percentage of oxytetracycline resistance encountered in this study most likely is related to the wide and sometimes improper use of this antimicrobial agent in aquaculture.1,2,12,13,35,46 Several authors have done research on the distribution of let genes in tetracycline-resistant fish pathogens, focusing on *E. tarda*.4,23,24,36 Unfortunately, information on genotypic susceptibility of *E. ictaluri* is frequently lacking and hence hitherto poorly understood. In view of the high resistance percentages noted, this certainly merits further investigation.

Similarly to oxytetracycline, 73% of the isolates had acquired resistance to trimethoprim. According to Hawke19 and Waltman and Shotts,42 oxytetracycline and trimethoprim are used for treatment of ESC caused by *E. ictaluri* in industrial catfish cultured in the United States. Likewise, oxytetracycline and trimethoprim were denoted as being the most commonly used antimicrobial agents in fish and shrimp in Vietnam.5,25,33,42

The present study pointed out that 73.4% of the isolates had acquired resistance to at least three antimicrobial agents. These isolates, when causing disease, may be especially difficult to control. This phenomenon may even become more cumbersome when one considers the fact that multiple resistance transfer by plasmids does occur and even is considered a problem of major concern in aquatic antimicrobial therapy.3 Plasmid-mediated resistance to chloramphenicol, trimethoprim, sulphonamides, and tetracyclines has indeed been identified in fish pathogens.27 Plasmids transferring resistance to as many as five antimicrobials have been identified from marine and freshwater fish pathogens—for example, *Vibrio anguillarum*, *Vibrio salmonicida*, *Aeromonas salmonicida*, *A. hydrophila*, *E. tarda*, and *Yersinia ruckeri*.4,10,11

The high number of isolates simultaneously displaying resistance to streptomycin, oxytetracycline, and trimethoprim may suggest the presence of an epidemic clone in the area from which our isolates were obtained. Alternatively, it may indicate a high prevalence of plasmids carrying resistance genes to these three antimicrobial agents in non-clonal isolates.

Several researchers have pointed toward hygienic shortcomings in fish-rearing methods. This, in combination with increased fish population densities, crowding of farming sites, and lack of sanitation barriers, has facilitated the rapid spread of infectious agents leading to the prophylactic use of antimicrobial agents, often with the misplaced goal of forestalling these sanitary shortcomings.8,37,20 Overuse of antimicrobial agents favors spread of antimicrobial-resistant bacteria in the aquaculture environment and the emergence of antimicrobial resistance in fish pathogens as demonstrated in this study. This may result in the transfer of these resistance determinants to bacteria of terrestrial animals and human pathogens, and in alterations of the bacterial microbiota both in sediments and in the water column.7,21,22,25

Further studies are necessary to elucidate the genetic mechanisms of the encountered resistance. In the meantime, there is a strict need to control both the prophylactic and curative use of antimicrobial agents in Vietnamese aquaculture.

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**Disclosure Statement**

No competing financial interests exist.

**References**

1. Akinbowale, O.L., H. Peng, P. Grant, and M.D. Barton. 2007. Antibiotic and heavy metal resistance in motile *Aeromonas* and *Pseudomonas* from rainbow trout (*Oncorhynchus masou*).


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