

Short communication

Identification of *Edwardsiella ictaluri* from diseased freshwater catfish, *Pangasius hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam

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Hawke (1979) reported the first isolation of *Edwardsiella ictaluri* from farmed channel catfish, *Ictalurus punctatus* (Rafinesque). The bacterium produces an acute septicaemic condition known in the USA as enteric septicaemia of catfish (ESC) and which results in high mortality, notably in channel catfish (Austin & Austin 1999). The blue catfish, *Ictalurus furcatus* (Valenciennes), and the white catfish, *Ameiurus* (= *Ictalurus*) *catus* (L.), are also susceptible (Hawke, McWhorter, Steigerwalt & Brenner 1981; Plumb & Sanchez 1983). Non-ictalurid fish species show no susceptibility as determined by mortality, clinical disease or established infection following experimental challenge (Plumb & Sanchez 1983). However, Kasornchandra, Rogers & Plumb (1987) recovered and identified *E. ictaluri* as the causative agent of a bacterial disease in walking catfish, *Clarias batrachus* (L.), in Thailand. To date, there is no published description of the isolation or identification of *E. ictaluri* from diseased *Pangasius* spp.

The freshwater catfish, *Pangasius hypophthalmus* (Sauvage), is indigenous to the Mekong Delta in Vietnam. It has been cultured traditionally for decades in earthen ponds and more recently in river-based cages.

A newly recognized disease was recently reported in farmed Vietnamese *Pangasius* sp. (Ferguson, Turnbull, Shinn, Thompson, Dung & Crumlish 2001). Post-mortem appearance of this condition, 'bacillary necrosis of *Pangasius*', was typified by multifocal irregular white lesions of varying sizes on several organs including liver, spleen and kidney. Histopathologically, the lesions were acute to sub-acute multifocal areas of necrosis and pyogranulomatous inflammation. Associated with these lesions were several species of parasites, but common to all affected fish were variable numbers of large bacilli, usually seen at the margins of lesions. The pathological findings strongly suggested that the disease had a bacterial aetiology. Several species of bacteria were isolated from affected fish, including a novel species of *Bacillus*. These preliminary results formed the basis for a more detailed study into the bacteriology of 'bacillary necrosis'. In the results presented here, bacteria biochemically typical of *E. ictaluri* were recovered in large numbers from all of the fish sampled with gross and histopathological signs typical of 'bacillary necrosis of *Pangasius*'.

In total, 17 *P. hypophthalmus* with ($n = 12$) and without ($n = 5$) clinical and gross signs of disease were sampled from three farm sites located in two provinces in the Mekong Delta, Vietnam. Two farm sites were in An Giang province and one farm, which was sampled on two occasions, was located in CanTho Province. Where possible, healthy and diseased fish were sampled from the same cage or pond on each farm. Samples were taken from all three farms in September 2001 and again from one

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farm in March 2002. Tissues from all the fish were fixed in 10% buffered formalin, subsequently processed for routine histopathology and sections stained with haematoxylin and eosin (Drury & Wallington 1980).

Bacterial isolates recovered from the liver of fish sampled in September 2001 were grown only on tryptone soya agar (TSA, Oxoid, Basingstoke, UK) whereas isolates from the fish sampled in March 2002 were recovered on TSA as well as on agar selective for bacteria belonging to Enterobacteriaceae (EMB, Merck, UK). The plates were sealed using Nesco film, incubated at 28 °C, and colony growth observed at both 24 and 48 h post-inoculation. Two type strains of *Edwardsiella* were obtained from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, UK) and used as standards during the characterization tests. The two NCIMB isolates were 13272 (*E. ictaluri*) and 2034 (*E. tarda*). Isolates recovered from the clinically sick *P. hypophthalmus* were inoculated onto TSA, EMB and *E. ictaluri* agar (EIA) (Shotts & Waltman 1990). TSA and EMB were used during sampling at the farm sites, whereas EIA was used subsequently in the laboratory.

All the tests were performed following the protocols described in Frerichs & Millar (1993). Pure cultures of isolates were produced on TSA and then single colonies were selected for Gram stains. The oxidation/fermentation (O/F) (Hugh & Leifson 1953) reaction was tested using O/F basal media purchased from Difco, UK. Cytochrome oxidase was tested using oxidase strips (Oxoid). The catalase reaction was determined following the method of Gagnon, Hunting & Esselen (1959), which used 3% (v/v) hydrogen peroxide. A single bacterial colony was selected and incubated in tryptone soya broth (TSB, Oxoid) at 28 and 37 °C and the motility was checked after 24 h using the hanging drop method. The biochemical profiles were determined using the API 20E kit (Bio-Mérieux, UK). Tolerance to salt was measured by placing two to three colonies into 5 mL of TSB with 0.5, 1, 1.5, 2, 2.5 and 3% NaCl added. These suspensions were incubated at 28 °C and checked for growth 7 days post-inoculation. Temperature sensitivity was determined by inoculating TSA plates with each of the isolates, then incubating the plates at 15, 22, 28 and 37 °C; colony growth was checked and recorded daily for 7 days.

Two isolates from Farm 1 (CanTho province, September 2001) and Farm 2 (An Giang province,

September 2001) were sent for concise alignment of 500 base pairs and 16S rRNA Genbank analysis by MIDI Laboratory (Newark, USA). This allowed identification of the organisms to species level. All of the isolates were tested for antibiotic sensitivity to furazolidone (FR, 50 and 100 µg), ciprofloxacin (CFC, 5 µg), nitrofurantoin (NFT, 50 and 100 µg), norfloxacin (NFC, 5 and 100 µg), gentamicin (GMC, 30 and 120 µg), oxolinic acid (OA, 2 µg), oxytetracycline (OT, 30 µg), potentiated sulphonamide (SXT, 25 µg), enrofloxacin (ENR, 5 µg), florfenicol (FFC, 30 µg) and amoxicillin (AML, 10 µg). The antibiotics selected were a combination of those routinely screened in the diagnostic laboratory at the Institute of Aquaculture, Stirling University, and those used by Vietnamese fish farmers (T.T. Dung, personal communication). The paper multi-discs impregnated with the antibiotics were all obtained from Oxoid except FFC, which was from Mast Diagnostics (Liverpool, UK). A bacterial lawn on TSA was produced from a suspension of two to three colonies emulsified in 5 mL of sterile saline using the spread plate method and the multi-discs were placed on the lawn. Results were interpreted as sensitive (≥ 16 mm), partially sensitive (12–15 mm) or resistant (< 11 mm) based on the zone diameters of growth inhibition.

The gross and histopathological lesions of the clinically affected fish were entirely consistent with those already described for 'bacillary necrosis of *Pangasius*' (Ferguson *et al.* 2001). Large bacilli were still visible at the margins of typical lesions, and there were virtually no complications because of parasites or other concurrent disease. The isolates recovered on TSA from fish with clinical signs of disease were predominantly pure. Only three fish sampled onto TSA in September 2001 produced more than one bacterial species, but growth was still dominated by colonies later identified as *E. ictaluri*. All of the isolates were incubated at 28 °C, and after 48 h on TSA these produced pinpoint to small sized colonies (average 0.14 ± 0.03 mm, $n = 12$) that appeared off-white and translucent with an irregular surface and edge. The colony morphology was very similar for the 12 Vietnamese isolates and NCIMB 13272 (*E. ictaluri*). The fish samples taken in March 2002 on EMB yielded pure growth, and all of the isolates grew well on both EMB and EIA, producing similar colony shape and size, observed as pinpoint, round, raised, translucent and pale-coloured. Forty-eight hours at 28 °C was required on all solid agars

before individual colonies were clearly visible to the naked eye.

The bacterial isolates recovered from the clinically sick *P. hypophthalmus* were typical of *E. ictaluri*. All cultures comprised Gram-negative short or variable length fermentative, non-motile rods that were catalase positive but not oxidase positive. All of the Vietnamese isolates tested positive for lysine decarboxylase and glucose. However, one isolate from Farm 1 also utilized citrate while two isolates from Farm 3 gave a positive response for urease; this was the same for the NCIMB 13272 (*E. ictaluri*) isolate but not the NCIMB 2034 (*E. tarda*) isolate, which was much more biochemically reactive compared with the other isolates tested. All bacterial cultures produced growth in TSB at all salt concentrations tested. The optimal growth temperature was determined to be 28 °C, although all isolates grew slowly at 22 and 37 °C, while only the NCIMB 2034 isolate produced any growth at 15 °C.

The two isolates sent to MIDI Laboratories were identified to species level as *E. ictaluri* with 99.91% confidence, using 530 bp match and 16S rRNA analysis (MIDI Laboratory, USA). All of the Vietnamese isolates showed either partial or full resistance to OT, SXT and OA compared with the two type strains, which were sensitive to all of the antibiotics tested. Two isolates from Farm 2 were resistant to OA only, and one isolate from Farm 1 was resistant to OT and SXT only. All of the isolates were sensitive to the other antibiotics tested.

The disease identified as 'bacillary necrosis', recently described from farmed *P. hypophthalmus* (Ferguson *et al.* 2001), was first observed in 1999 in Vietnam, where farmers reported high mortality in fish that had white spots on their internal organs (T.T. Dung, personal communication). In the present study, bacteria were recovered mostly in pure culture, from 12 clinically diseased *P. hypophthalmus* with typical 'bacillary necrosis' lesions. All these isolates have been identified as *E. ictaluri*. These results provide further evidence to support the suggestion that 'bacillary necrosis of *Pangasius*' has a bacterial aetiology. Infections in freshwater *P. hypophthalmus* by *E. ictaluri* have not previously been reported.

Although the Vietnamese isolates were typical of *E. ictaluri*, there were some differences. Normally, *E. ictaluri* is described as a small straight rod, which measures 1 µm × 2–3 µm (Plumb 1993). However, the Vietnamese isolates showed much greater

variability in length and width, often with very large rods clearly visible. This variation in size was consistently observed in all 12 isolates recovered from clinically diseased *P. hypophthalmus*. Mixed sized rods have not been commonly described in the literature but they have been observed in histopathology sections from experimental infections in channel catfish (J. Newton, personal communication). Biochemically, *E. ictaluri* has been described as a homogeneous organism with fewer positive reactions than *E. tarda* (Waltman, Shotts & Hsu 1986). The Vietnamese isolates were biochemically compatible with *E. ictaluri* and two of the isolates were identical to the NCIMB *E. ictaluri*. The remaining 10 cultures were identical to each other and differed by only one reaction from the NCIMB strain.

On-farm control of ESC outbreaks in the USA has relied heavily on in-feed antibiotics, particularly oxytetracycline and potentiated sulphonamides (Bowser, Munson, Francis-Floyd & Stiles 1986; Waltman & Shotts 1986). Disturbingly, the majority of Vietnamese isolates (nine out of 12) were resistant to these antibiotics, although they were found to be sensitive to the remaining eight antibiotics tested. Despite this *in vitro* sensitivity, albeit somewhat limited, Vietnamese farmers have reported little success combating 'bacillary necrosis' using antibiotics, so alternative methods of prevention and control will need to be investigated.

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