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Short communication

A novel 'skinny pot-belly' disease in Asian seabass fry, *Lates calcarifer* (Bloch)

S Gibson-Kueh[1], M Crumlish[2] and H W Ferguson[2]

1 Animal and Plant Health Laboratory Division, AgriFood and Veterinary Authority of Singapore, Singapore
2 Institute of Aquaculture, University of Stirling, Stirling, UK

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In Singapore, viral nervous necrosis (VNN) is considered to be the most serious disease of farmed Asian seabass fry, *Lates calcarifer* (Bloch) (barramundi), causing mortalities of 80–100% and heavy economic loss (Lim, Chong & Kueh 1997; Kasornchandra 2002). When some 'skinny pot-bellied' seabass fry were presented to the Aquatic Animal Health Laboratory early in 2004, with a history that the disease was usually first observed at around 3 weeks of age, VNN was the prime suspect. Instead of central nervous lesions, however (Munday, Langdon, Hyatt & Humphrey 1992), histopathological examination showed the presence of a severe necrotizing and ulcerative enteritis, progressing to perforation and peritonitis, with disseminated multifocal granulomas. Associated with these lesions were numerous bacterial colonies. This preliminary report presents a description of this novel systemic bacterial disease of young seabass, typified by emaciation and abdominal distension, and associated with high mortality. Immunohistochemistry suggests an association with *Edwardsiella ictaluri*.

Live moribund fish samples were submitted in 2004 to the Aquatic Animal Health Laboratory of the AgriFood and Veterinary Authority of Singapore, for disease investigation and diagnosis. Fish were transported in plastic bags with clean sea water and air in the ratio of 1:2. A total of 435 specimens from four distinct batches of fish were submitted (Table 1) and examined for parasites (10 fish) and by histopathology (165 fish).

Freshly killed fish were examined for ectoparasites by wet-mount microscopic examination of skin scrapes and gill biopsies of fish greater than 1–2 cm body length or squashed whole fry. For histopathology, tissues were fixed in 10% phosphate-buffered formalin for at least 24 h, and then dehydrated in a graded alcohol series before routine processing and embedding in paraffin wax. Formalin-fixed bony tissues were demineralized overnight with formic acid citrate solution before processing through an alcohol series. Five micrometer sections were routinely stained with haematoxylin and eosin (H&E), while selected sections were also stained with Gram and Ziehl-Neelsen stains.

Immunohistochemistry was performed according to Adams & de Mateo (1994). Briefly, tissue sections were dewaxed and rehydrated before being placed in a humid chamber and fixed with methanol containing 10% (v/v) hydrogen peroxidase (H_2O_2). The slides were washed three times with Tris-buffered saline (TBS) at pH 7.2 (Sigma, Haverhill, UK) then incubated with normal goat serum (Sigma) diluted in TBS at 1/10. The goat serum was removed and rabbit anti-*E. ictaluri* serum (produced at the Institute of Aquaculture using the National Collection of Industrial and Marine Bacteria isolate 13272) and rabbit anti-*E. tarda* (kindly donated by Dr J. Newton, Auburn University) were added to the tissue sections at 1/100 and 1/1000 (diluted in TBS). All sections were incubated for 1 h in a moist

Correspondence *H W Ferguson, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK (e-mail: hwf1@stir.ac.uk)*

Table 1 Brief history of 'skinny pot-belly' seabass fry disease cases

Case ref.	Body length of affected fish (mm); age (days)	History/clinical signs	Percentage mortality
Batch A	10–15; 38	Abnormal body darkening, poor feeding, emaciation, abdominal distension or 'pot-belly', red swollen anus with trailing faecal strands/casts. Abnormal swimming behaviour	2% daily since 20 days old
Batch B	50–70; 51	Episode of high mortality at 20 days old apparently responsive to oxytetracycline bath (100 ppm × 1 h × 7 days). Mortality started again a week later	20% daily at 20 days old; low grade at 51 days old
Batch C	35–60; 43	At 18 days old, abnormal body darkening and emaciation reported, treated with oxytetracycline bath.	Increasing mortality
Batch D	10; 20	Darkened body, emaciation and abdominal distension. Abnormal swimming behaviour	Increasing mortality

chamber at room temperature. Slides were then washed again in TBS and goat anti-rabbit-IgG horseradish peroxidase (Sigma) was added to the slides at 1/50 dilution for 30 min at room temperature. After washing with TBS, the reaction was visualized by incubating the slides for 10 min with 3′,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma) in the presence of H_2O_2. The reaction was stopped by immersing slides in tap water after which they were counterstained with haematoxylin for 3 min before being dehydrated and mounted. Controls included normal rabbit serum (1/1000 in TBS) in place of the primary antibody (negative), *Pangasius* experimentally inoculated with *E. ictaluri* (positive) and seabass fry with no lesions (negative).

A summary of the history and clinical manifestations is found in Table 1. These seabass fry were reared in fibreglass tanks with flow-through, sand-filtered sea water, and fed rotifers until 15 days old, then rotifers and artemia until 21 days old before being weaned onto a manufactured diet. This disease affected the fry from 3 weeks of age, often resulting in a cumulative mortality of 80–100%. Affected fish were abnormally dark, emaciated (skinny), had abdominal distension (called 'pot-belly' by farmers) and a red swollen anal region with trailing faecal casts. Ectoparasites were not observed in wet-mount microscopic examination of skin scrapings, gill biopsies or squashed whole fry, nor were any seen on histopathology.

Histopathological lesions in mildly affected fish included focal or extensive areas of multi-focal, relatively mild, mucosal inflammation associated with the presence of bacterial colonies within mucosal epithelium. In such cases the lamina propria was infiltrated with a mixture of inflammatory cells, but they were primarily mononuclear. Fish with these mild changes had no significant lesions elsewhere. More severely affected fish had lesions that involved the full thickness of the intestinal or gastric wall, with dilation and often perforation, and with granulomatous and necrotizing lesions, often with caseonecrotic cores, present in the gut wall and on the serosal surface (Fig. 1). Such lesions were accompanied by peritonitis, with activation of mesothelium and the presence of numerous peritoneal macrophages (Fig. 2). Large numbers of bacteria were usually present within these lesions. The bacteria were relatively large, varying in shape from coccoid to bacillary, and were often present as small tightly grouped colonies suggesting an intracellular location. Special stains showed them to be Gram-negative and non-acid-fast. Many fish had these bacteria widely disseminated within most organs, including kidney and spleen, and in many cases accompanied by a necrotizing and granulomatous inflammatory response. When the peritonitis was advanced, the musculature of the body wall was also involved.

The appearance of the bacteria and the associated lesions were both reminiscent of the disease recently reported from Vietnam in *Pangasius* (Ferguson, Turnbull, Shinn, Thompson, Dung & Crumlish 2001) and shown to be closely associated with

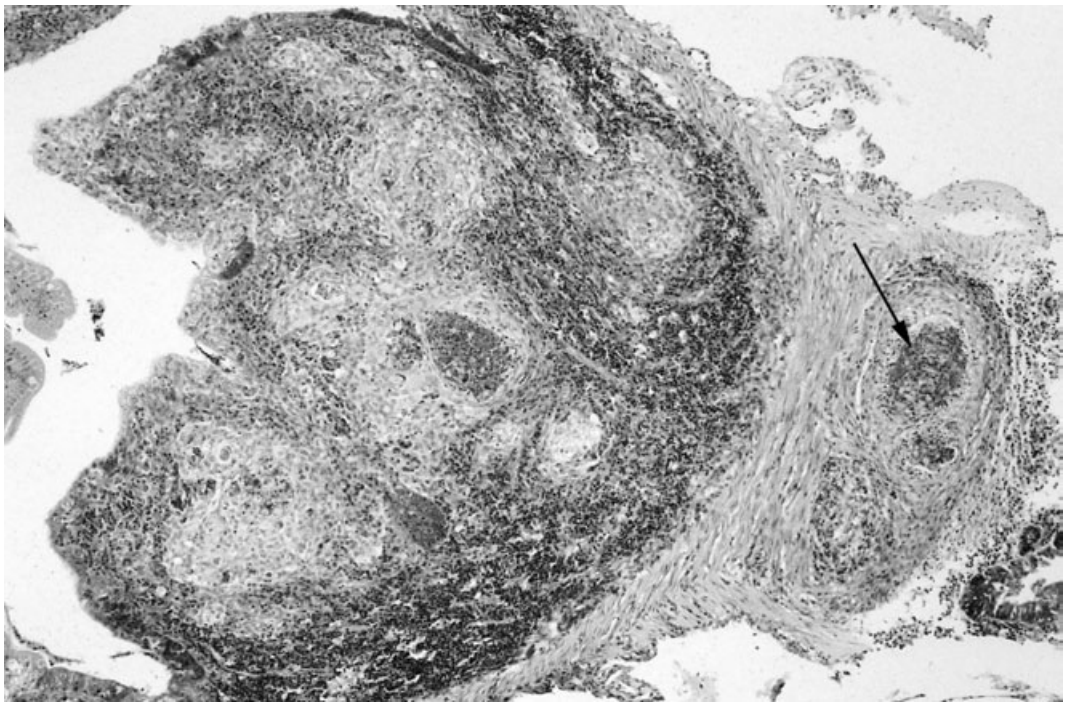

Figure 1 Asian seabass with chronic granulomatous enteritis involving serosal surface (arrow) (H&E, ×185).

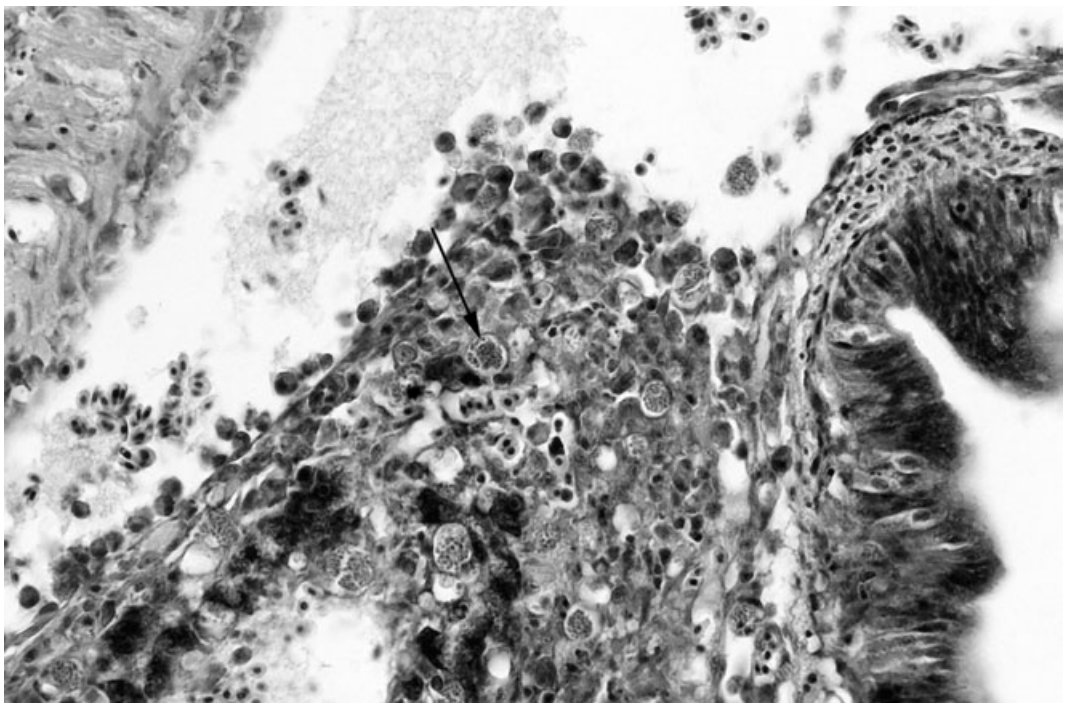

Figure 2 Asian seabass showing pancreatitis and peritonitis. Bacteria can be seen within the inflamed pancreas (arrow) (H&E, ×630).

E. ictaluri (Crumlish, Dung, Turnbull, Ngoc & Ferguson 2002). Accordingly, immunohistochemistry was performed on selected sections in order to confirm or eliminate this possibility. The *E. ictaluri* antiserum showed the presence of large numbers of positive bacteria, even at 1/1000 dilution,

corresponding to the tightly grouped colonies as seen in H & E. A few colonies of smaller bacteria were negative, however. All of the control sections were appropriately positive (experimental inoculation with *E. ictaluri*) or negative, including the *E. tarda* antiserum at both 1/100 and 1/1000 dilutions.

The peritonitis seen in many fish helps to explain the clinical observation of 'pot-belly', while the trailing faecal casts suggesting protein loss are typical of fish with enteritis. The presence of intestinal lesions that varied in severity from mild multi-focal to ulcerative, necrotizing, granulomatous, and subsequently to perforation, peritonitis and bacterial dissemination, suggests that the disease may have started as a mucosal infection of the gastrointestine. Overall, the pathological findings strongly suggest that this disease had a bacterial aetiology.

Bacterial enteritis is a disease that is less well recognized in fish than in mammals or birds, but it does occur. Reported examples include enteric septicaemia of catfish associated with *E. ictaluri* (Newton, Wolfe, Grizzle & Plumb 1989) and summer enteritis syndrome of salmonids (Michel, Bernardet, Daniel, Chilmonczyk, Urdaci & de Kinkelin 2002), although in neither of these two diseases is perforation a feature. The pathological picture in the seabass was similar in some respects to that seen in experimental infection of channel catfish with *E. ictaluri* (enteric septicaemia of catfish, ESC) in which the earliest lesions were seen in the intestinal mucosa and olfactory tracts 2 days post-infection (Newton *et al.* 1989). One major difference from ESC was the absence of lesions in the brain, even in those seabass with widely disseminated lesions. The necrotizing and granulomatous changes in the seabass were also similar to those recently described in *Pangasius* from Vietnam (Ferguson *et al.* 2001; Crumlish *et al.* 2002), although intestinal lesions as seen in the seabass were not so obvious. In both the *Pangasius* and seabass, therefore, there is an association with *E. ictaluri.*

In combination with appropriate histopathological changes, the positive IHC reaction to *E. ictaluri* antiserum suggests the possibility that this organism was causally involved in this disease, although whether this is true for all such outbreaks of clinically similar disease remains to be determined. The presence of some colonies of bacteria that did not stain positively in IHC suggests either that the polyclonal antiserum was reacting differently to possibly younger, smaller actively dividing bacteria, or that another species of bacterium was present. Given the fact that many fish had perforation of the intestinal wall, the involvement of more than one bacterial species would not be unexpected.

Alongside further in-depth histopathology of clinically similar outbreaks to build up a more complete picture of the full range of lesions that can be anticipated, these preliminary results also point towards the pressing need for some thorough bacteriological investigations to try and establish the presence of *E. ictaluri* and its role in the development and progression of these outbreaks. Fish in the early stages of disease would be preferable for these investigations, prior to perforation of the intestine and the large number of contaminants that inevitably accompany such an event.

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