Pathogenesis of *Aeromonas hydrophila* strain KJ99 infection and its extracellular products in two species of fish

A. Rey, N. Verján, H. W. Ferguson, C. Iregui

The distribution of antigen and pathological changes induced by an experimental infection with *Aeromonas hydrophila* strain KJ99, and its extracellular products, were studied in two species of fish. The microorganism was disseminated systemically and the haemodynamic and tissue changes were similar to those observed in septicaemia of mammals. Intussusception, degeneration and necrosis of the nervous plexus and muscular layers of the gastrointestinal tract were common findings.

Aeromonas hydrophila is a Gram-negative aerobic and facultative anaerobic, oxidase-positive motile bacterium that inhabits aquatic environments and the gastrointestinal tract of healthy fish. It also commonly occurs in foods like fish, milk, red meats and poultry (Roberts 1993, Handfield and others 1996, Aoki 1999). It causes disease and mortality mainly in freshwater fish but sometimes in marine fish (Aoki 1999). The bacteria also infect human beings and cause lesions ranging from gastroenteritis to septicaemia (Sha and others 2002). The organism is therefore considered a threat to the health of fish and people.

A hydrophila possesses many factors related to its virulence, such as extracellular products including aerolysins, α- and β-haemolysins (Howard and others 1987, Hirono and Aoki 1991), enterotoxins, proteases, haemagglutinins and adhesins (Chopra and Houston 1999, Sha and others 2002). However, the pathogenesis of the disease has not been fully elucidated in fish. Several studies have assessed the virulence (LD50) of different strains of *A hydrophila* (Asao and others 1984, Santos and others 1988, Khalil and Mansour 1997), but the environmental stressors involved in the onset of disease, the possible route of entry of the bacteria (Roberts 1993), their distribution within the fish and the evolution of the pathological process (Grizzle and Kiryu 1993), have rarely been assessed. On the other hand, the lethal effects of the extracellular products of *A hydrophila* have been reported in several species of fish without the description of any histological lesions (Khalil and Mansour 1997).

This paper provides a detailed description of the tissue distribution and morphological changes induced by *A hydrophila* or its extracellular products after the experimental infection of two commercial species of fish, white cachama (*Piaractus brachypomus*) and tilapia hybrids (*Oreochromis* species), and proposes a possible pathogenesis for the disease.

Materials and methods

Fish and experimental conditions

Clinically healthy white cachama (mean [sd] bodyweight 55 [5] g) and tilapia hybrids (bodyweight 35 [5] g) provided by a commercial supplier were acclimatised in 70 litre glass tanks (five fish per tank) for two weeks. They were fed a commercial diet, which was withheld for 24 hours immediately before they were inoculated. The water temperature was maintained at 26 ± 1°C, the pH was 6·8 ± 0·2, and the oxygen concentration was in the range of 5 to 8 mg/l.

Bacterial strain

The *A hydrophila* strain KJ99 used was isolated from a natural outbreak of disease in Nile tilapia (*Oreochromis aureus*) farmed in Kyushu island, Japan. It was initially identified by its basic biochemical properties and its haemolytic activity in brain heart infusion medium with ovine blood.

TABLE 1: Primers used for the PCR amplification of *Aeromonas hydrophila* KJ99 toxin-related genes

Gene	Primer sequences (5´ to 3´)	PCR amplicon
ahh1	GCCGAGCGCCCAGAAGGTGAGTT GAGCGGCTGGATGCGGTTGT	130 base pairs (bp)
aerA	CAAGAACAAGTTCAAGTGGCCA ACGAAGGTGTGGTTCCAGT	309 bp
alt	ATGACCCAGTCCTGGCACG ACGCGCCATCGCCAACGAT	1104 bp
ast	ATGCACGCACGTTACCGCCAT CTTTTTCACCGCAGCGGGTT	1905 bp

Veterinary Record (2009) **164,** 493-499

A. Rey, BVM, MSc,
C. Iregui, BVM, DVM,
Laboratory of Pathology, Faculty of Veterinary Medicine, National University of Colombia, Bogotá, Colombia
N. Verján, BVM&Z, MSc, PhD,
Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Konan, 4-5-7 Minato, Tokyo 108-8477, Japan

H. W. Ferguson, BVM&S, PhD, DipACVP, FRCPath, MRCVS,
Institute of Aquaculture, University of Stirling, Stirling FK9 4LA

E-mail for correspondence:
alreyc@unal.edu.co

TABLE 2: Biochemical properties of Aeromonas hydrophila strain KJ99

Characteristic	Result of biochemical test
Oxidase	+
Catalase	+
Urea	–
Citrate	–
Diffusable pigment	–
Motility	+
Ornithine decarboxylase	–
Mannitol fermentation	+
Esculin hydrolysis	+
Gas production from glucose	+

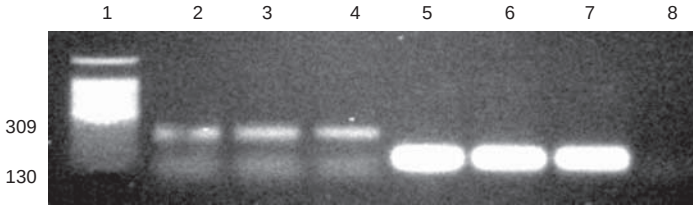

FIG 1: Detection and identification of *Aeromonas hydrophila* haemolysin and aerolysin genes by amplification of fragments in the PCR assay. Lane 1 100 base pair (bp) ladder (Invitrogen), Lanes 2, 3 and 4 *A hydrophila* showing the *aerA* gene (309 bp fragments), Lanes 5, 6, 7 *A hydrophila* showing the *ahh1* gene (130 bp fragments), Lane 8 PCR-negative control

Its identity was confirmed by the amplification of the extracellular haemolysin gene (*ahh*1) (Hirono and Aoki 1991), the aerolysin gene (*aer*A) (GenBank accession number M16495), the *Aeromonas* labile toxin gene (*alt*) (Chopra and others 1996) and the stable toxin gene (*ast*) (Sha and others 2002) using the primers described in Table 1. Its virulence was confirmed by the results of three consecutive infections of tilapia hybrids carried out before this study.

Production of extracellular products

A hydrophila strain KJ99 was inoculated on to tryptic soy agar (TSA) plates and incubated at 28°C for 24 hours. The bacterial cells were collected in 5 ml phosphate buffered saline (PBS) and 1 ml of the culture was inoculated into 500 ml of tryptic soy broth, incubated at 28°C with continuous shaking for 48 hours, and then centrifuged at 6000 *g* at 4°C for 60 minutes. The supernatant was filtered through a 0·22 µm nitrocellulose membrane (Millipore) and concentrated 26-fold by dehydration in a 250-7U dialysis membrane (Sigma-Aldrich) at 4°C for seven hours (Santos and others 1988). The concentrated extracellular products were kept at 4°C until use.

Haemolytic activity The extracellular products (1·5 ml) were diluted from 1:2 to 1:1024 in 0·01M Tris-HCl buffer, pH 7·2, containing 0·9 per cent sodium chloride. Each dilution was mixed with the same volume of a 1 per cent suspension of rabbit erythrocytes and incubated at 37°C for 90 minutes, then centrifuged at 1000 *g* for five minutes and the absorbance measured at 550 nm in a spectrophotometer (Spectroquant Nova 60; Merck) (Asao and others 1984). A standard curve of haemolysis was constructed by using the absorbances measured in suspensions of rabbit erythrocytes lysed with distilled water. A haemolytic unit (HU) was defined as the reciprocal of the highest dilution of a sample that lysed 50 per cent of the erythrocytes (Asao and others 1984).

Proteolytic activity and protein concentration To determine the proteolytic activity, 500 µl of the extracellular products were mixed with 500 µl casein solution in 0·1M glycine-sodium hydroxide buffer, pH 9·6. The solution was incubated at 28°C for 10 minutes, and then 50 µl 1M trichloroacetic acid was added to stop the reaction. The solution was centrifuged at 6000 *g* at 4°C for 10 minutes and the absorbance of the supernatant was measured at 280 nm wavelength in the spectrophotometer. Casein mixed with trichloroacetic acid was used as a control. A proteolytic unit (PU) was defined as the quantity that produced a 0·01 increase in absorbance (Khalil and Mansour 1997). The protein concentration was determined by the method described by Bradford (1976).

Infectious dose

Serial dilutions (from 10^{-1} to 10^{-10}) of the bacteria were made, starting from a concentration of 100 mg of microorganisms/ml of PBS and 0·1 ml of each dilution was injected intraperitoneally into 10 tilapia hybrids (Santos and others 1988, Khalil and Mansour 1997). The fish injected with the original bacterial solution and with the 10^{-1} dilution died, but fish injected with the other

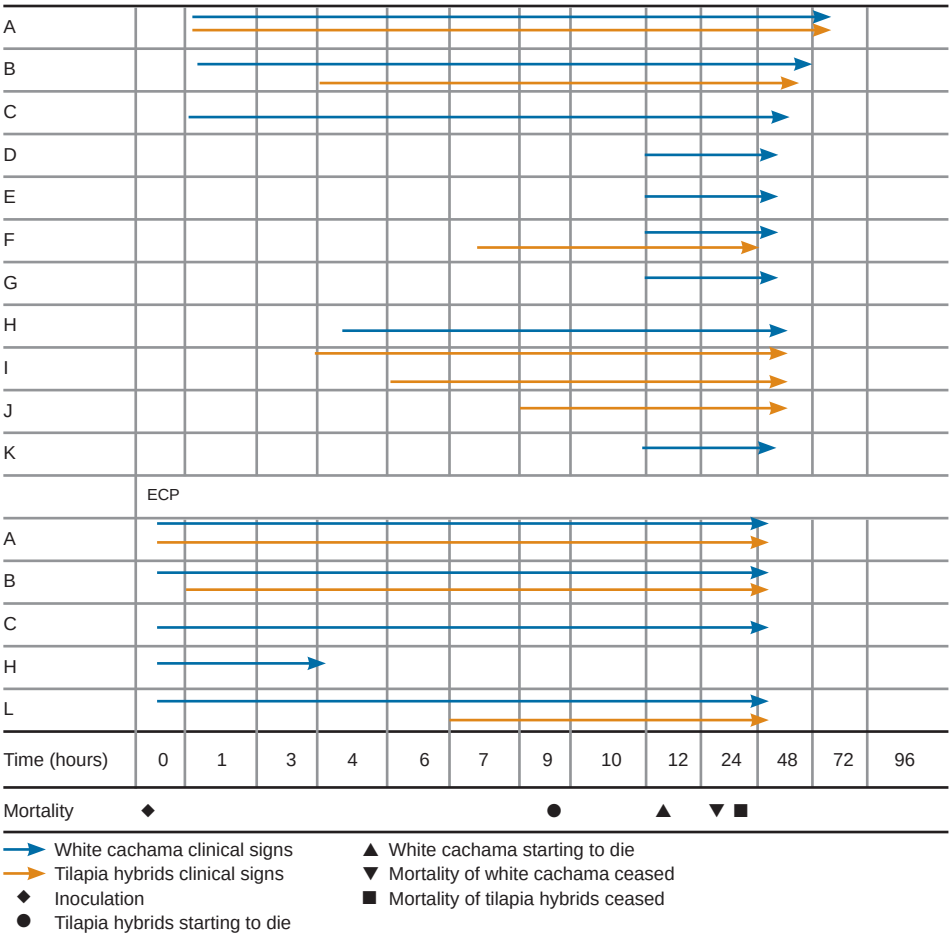

FIG 2: Clinical signs and gross changes in white cachama (*Piaractus brachypomus*) and tilapia hybrids (*Oreochromis* species) exposed to *Aeromonas hydrophila* strain KJ99 and its extracellular products (ECP). A Anorexia and lethargy, B Increased respiratory frequency, C Increased mucus production, D Expulsion of blood-stained fluid through the anus, E Surface haemorrhages, F Excitement and disorientation, G Bumping of aquarium walls, H Ascites or abdominal cavity haemorrhages, I Folding or wrinkling of intestinal walls, J Severe intussusceptions, K Foul smelling and 'rotten' appearance of the abdominal viscera, L Fish lying on bottom of aquarium

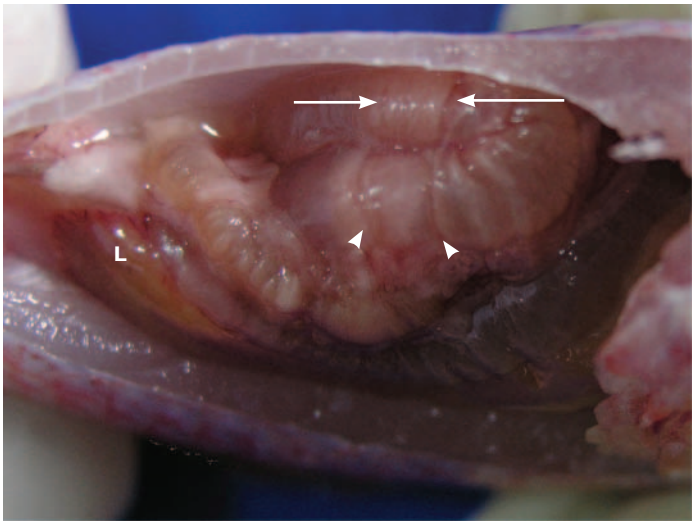

FIG 3: Gastrointestinal tract of a tilapia hybrid (*Oreochromis* species) infected with *Aeromonas hydrophila* strain KJ99, showing severe intussusception (arrowheads) and folding and wrinkling on the intestinal walls (arrows). L Congested liver

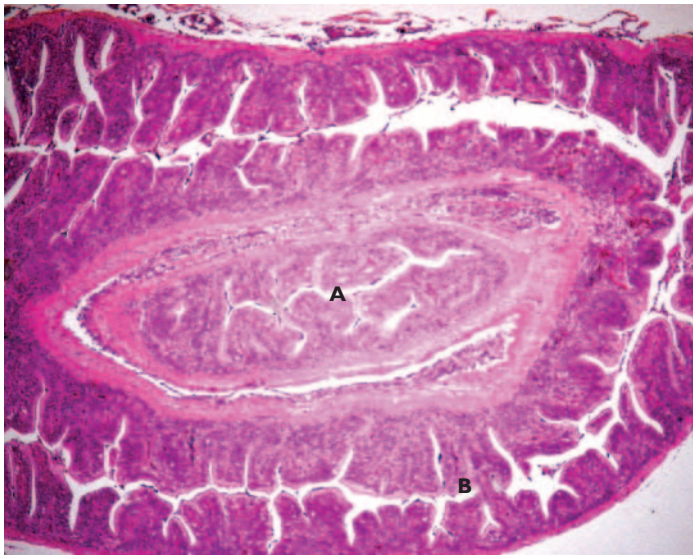

FIG 4: Microscopic appearance of the gastrointestinal tract of a tilapia hybrid (*Oreochromis* species) infected with *Aeromonas hydrophila*, showing intussusception. A Intussuceptum, B Intussuscipiens. Haemotoxylin and eosin. x 40

dilutions survived. Using a second range of dilutions between 10^{-1} and 10^{-2} a dilution of 1:60 (equivalent to $5{\cdot}8 \times 10^{7}$ colony-forming units (cfu)/0·1 ml) was identified as the dose at which clinical signs of the disease occurred, with a minimum survival period of 24 hours. The extract of extracellular products was diluted from 1:2 to 1:1024, and 0·1 ml of each dilution was inoculated intraperitoneally into two fish weighing 10 (1) g. Finally, a 1:2 dilution that induced the desired clinical signs of disease was used for the challenge experiment.

Experimental infection with *A hydrophila* strain KJ99 and inoculation of extracellular products

Twenty-eight white cachama were inoculated intraperitoneally with the infectious dose of *A hydrophila* strain KJ99. Two infected fish were killed every hour and they and any fish that died were examined. Subsequently, moribund fish were examined at each time until the mortality ceased. The surviving fish were killed 96 hours after the fish had been infected. Seven fish were injected with 0·1 ml PBS as controls, and two of them were killed immediately and the others after 96 hours at the end of the experiment. The same experimental design was used with the tilapia hybrids.

A dose of 0·1 ml of the extracellular products was inoculated into 22 white cachama weighing 10 (1) g and into 22 tilapia hybrids weighing 22 (1) g. Two fish of each species were killed five, 15 and 30 minutes, and one, two, six, 12, 24, 48, 72 and 96 hours later. Seven fish were inoculated with 0·1 ml PBS as controls and two of them were killed immediately and the other after 96 hours at the end of the experiment.

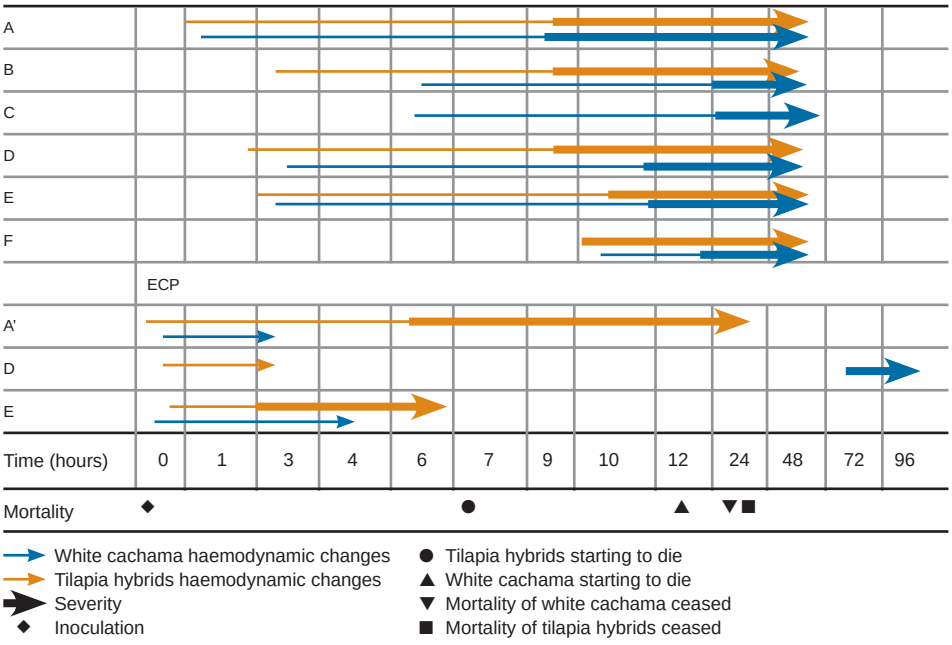

FIG 5: Haemodynamic changes in white cachama (*Piaractus brachypomus*) and tilapia hybrids (*Oreochromis* species) induced by *Aeromonas hydrophila* strain KJ99 and its extracellular products (ECP). A Systemic haemodynamic changes (congestion, haemorrhages, vascular permeability, ascites), A' Local haemodynamic changes in the abdominal wall, peritoneum and gastrointestinal tract, B Bacteria in blood vessels, C Thrombus and embolus, D Macrophages with yellow-green pigment in spleen vasculature, E Oedema in gastrointestinal submucosa, mucosal detachment and haemorrhages, F Myocardial haemorrhages

Clinical signs and postmortem examination

Clinical signs were recorded and the fish were anaesthetised with 100 mg/l of tricaine methane sulfonate (MS222; Argent-Lab). The fish were killed by transecting the spinal cord behind the skull for postmortem examination and the gross lesions were recorded.

Histopathology

Samples of liver and kidney from the moribund animals were inoculated on to TSA plates to verify the infection. The fish were fixed in 10 per cent buffered formalin. Samples of the tissues were processed to paraffin wax blocks and sections were stained with haematoxylin and eosin. The isolates of the organism were examined morphologically and by biochemical tests.

Immunohistochemisty

Paraffin wax-embedded sections were used to detect the antigen of *A hydrophila* strain KJ99 by the indirect immunoperoxidase (IIP) technique (Mayer and Walker 1987). Specific polyclonal antiserum (anti-*A hydrophila* strain KJ99) was produced in rabbits and used as the primary antibody. The secondary antibody was recombinant G-protein conjugated with peroxidase

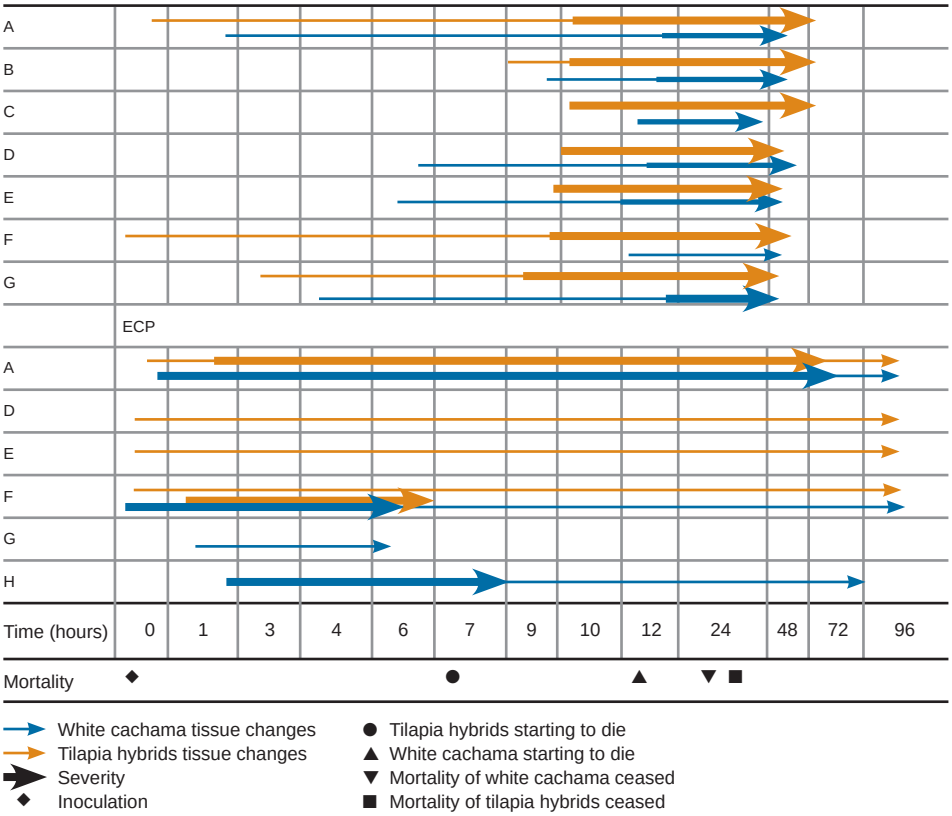

FIG 6: Tissue changes induced in white cachama (*Piaractus brachypomus*) and tilapia hybrids (*Oreochromis* species) by *Aeromonas hydrophila* strain KJ99 and its extracellular products (ECP). A Necrosis of abdominal viscera, B Systemic bacterial proliferation, C Liver degeneration and necrosis, D Hyaline degeneration and vacuolation of gastrointestinal muscle layers, E Stomach muscular layer 'rings' and neuronal cells (nervous plexus) degeneration, F Erythrocytes degeneration (hyaline structures) in spleen and kidney, H Hyaline degeneration of renal tubular cells

(Sigma-Aldrich). Optimal dilutions were established by the indirect immunodot method. Tissues of fish injected with *A hydrophila* KJ99 served as positive and negative controls, using anti-*A hydrophila* KJ99 or pre-immune serum respectively.

Results

Bacterial strain identification

A biochemical test provided a preliminary identification of the *A hydrophila* strain KJ99 (Table 2); the bacterium was β-haemolytic in brain heart infusion medium with ovine blood. Its identity was confirmed by the amplification of the extracellular haemolysin gene (*ahh*1) and the aerolysin gene (*aer*A) (Fig 1). The presence of these two genes helped to group the strain in genotype 4, as described by Wang and others (2003). The amplification and sequencing of the enterotoxin genes (*alt* and *ast*) provided 97 per cent and 98 per cent identical deduced amino acid sequences to *A hydrophila* ALT and AST enterotoxins, respectively.

Biological activities of the extracellular products of *A hydrophila* strain KJ99

The extracellular products contained 2·39 mg protein/ml, 32 HU/ml and 40 PU/ml. The infectious dose was 11·95 µg/g fish and contained 1·6 HU and 2 PU per fish.

Clinical signs and gross changes

Fig 2 summarises the main clinical signs and gross changes shown by white cachama and tilapia hybrids exposed to *A hydrophila* strain KJ99 or its extracellular products. Briefly, the fish became lethargic and anorectic, with increased respiratory frequency and mucus production. They became excited and disorientated, bumping into the aquarium walls. There was ascites or haemorrhaging in the abdominal cavity. The white cachama expelled blood-stained fluid through the anus and developed superficial haemorrhages. In the tilapia hybrids, folding or wrinkling of the intestinal wall and severe intussusception were commonly observed (Figs 3, 4).

Microbiology and histopathology

The microbiological isolates were morphologically and biochemically compatible with *A hydrophila* strain KJ99 (Table 2). A diagrammatic representation of the main haemodynamic changes observed in both species of fish inoculated with *A hydrophila* strain KJ99 or its extracellular products is shown in Fig 5. The systemic changes induced included congestion, leakage of protein from blood vessels, and haemorrhage and detachment of the mucosa in the gastrointestinal tract. Bacteria appeared in the liver vasculature around four hours after inoculation in the tilapia hybrids and after about six hours in the white cachama. Aggregates of leucocytes, thrombocytes and erythrocytes were detected after four hours on the vascular endothelia of both species. Macrophages containing yellow-green pigment appeared in the blood vessels and spleen from two hours onwards. The haemodynamic changes induced in both species of fish by the organism's extracellular products were mild and multifocal.

A diagrammatic representation of the tissue changes during the infection process is shown in Fig 6. The main lesions induced included systemic inflammation and necrosis of the abdominal viscera, especially in organs such as the spleen and the gut. In the latter the nervous ganglia in the serosa were notably involved; the neuronal cells were vacuolated (Fig 7) and had cytoplasmic hyaline inclusions (Fig 8). Frequently observed lesions included hyaline degeneration and vacuolation of the gastrointestinal muscle layers (Fig 9), and muscular layer 'rings' in the stomach, suggesting that the muscle cells were in a hypercontractive stage. The extracellular products induced mainly degeneration of haematopoietic cells, including erythrocytes, giving them a rounded, hyaline appearance, particularly in the kidney and spleen, and degeneration and necrosis of other tissues such as the pancreas, and the muscular layers of the gut. The changes induced were mild one hour after inoculation, but the severity and extent of the lesions increased with time and were severe after nine hours in the tilapia hybrids and after 12 hours in the white cachama. Bacterial proliferation was observed in the mesenteries, pancreas, muscle layers of the gut, liver, spleen and kidney, and within leucocytes.

Immunohistochemistry

The optimal dilutions of the primary and secondary antibody were 1:800 and 1:1500, respectively. A distinct staining pattern due to the bacteria was observed from one hour after inoculation (Fig 10). The intensity of staining increased as the infection progressed, and it was severe after about 10 hours. In the white cachama, the endocardium, spongy myocardium and coronary blood vessels were stained after three hours and became densely stained from 19 hours onwards; the vascular endothelium of their gills became stained after five hours and it was severe after about 12 hours. Bacterial staining was first observed on the serosa of the gut after six to seven hours, and staining was also detected in the muscular layers, becoming severe after seven to nine hours. The staining was associated with detachment and exfoliation of the mucosa after about 12 hours in the white cachama and after about 24 hours in the tilapia hybrids. No staining was observed in the control fish.

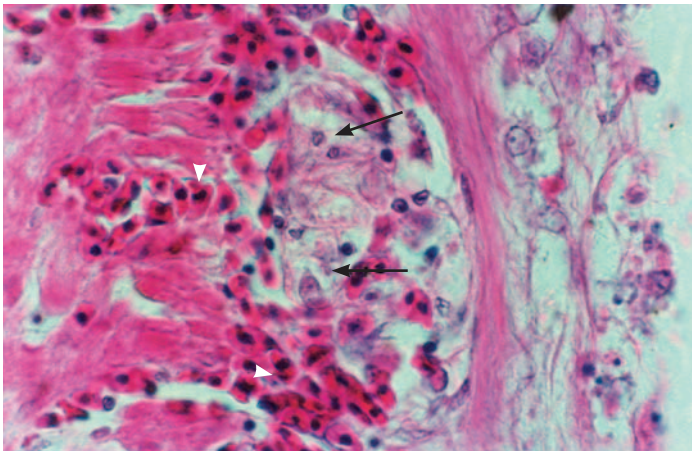

FIG 7: Nervous ganglion on the external muscular layer of the stomach of a tilapia hybrid (*Oreochromis* species) infected with *Aeromonas hydrophila* strain KJ99 (10 hours after inoculation), showing vacuolated neuronal bodies (arrows) with a degenerate appearance and haemorrhage (arrowheads). Haematoxylin and eosin. x 1000

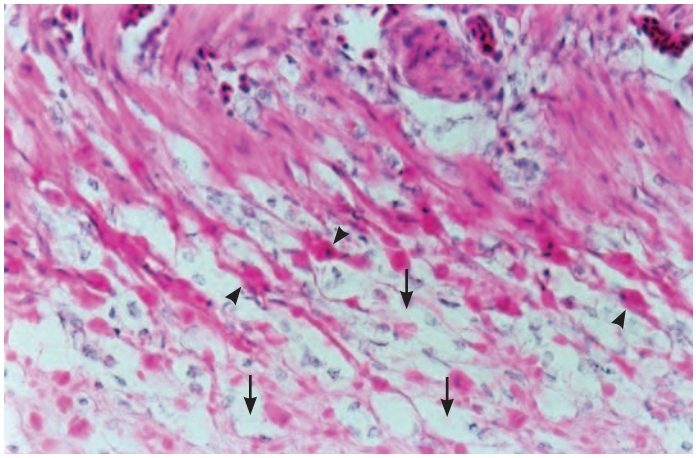

FIG 9: Muscular layer of the intestine of a white cachama (*Piaractus brachypomus*) infected with *Aeromonas hydrophila* strain KJ99 (21 hours after inoculation), showing hyalinisation (arrowheads) and translucent vacuolation of the muscle cells (arrows). Haematoxylin and eosin. x 400

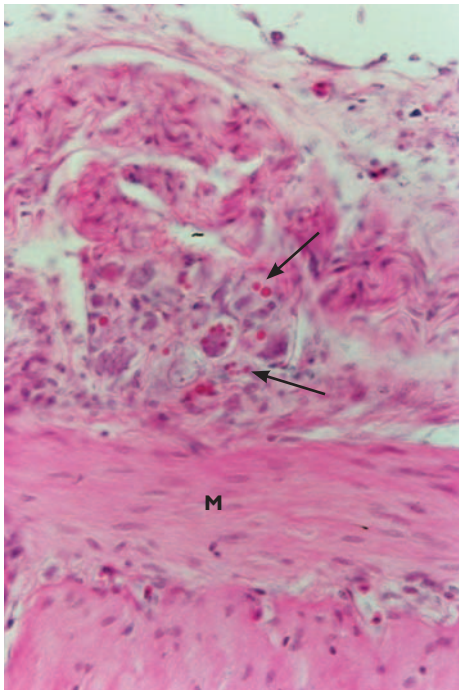

FIG 8: Nervous ganglion on the external layer of the intestinal musculature (M) of a white cachama (*Piaractus brachypomus*) infected with *Aeromonas hydrophila* strain KJ99 (23 hours after inoculation), showing hyaline drops in the cytoplasm and surrounding the neuronal bodies (arrows). Haematoxylin and eosin. x 400

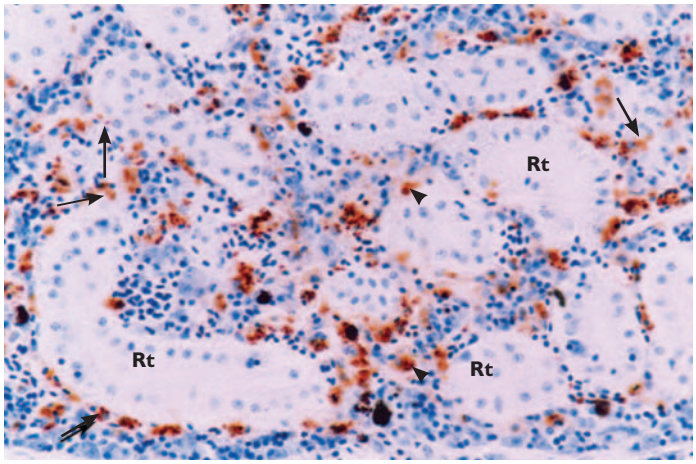

FIG 10: Staining of the bacterium outside (arrows) and inside the renal portal macrophages (arrowheads) associated with the peritubular capillaries of a white cachama (*Piaractus brachypomus*) infected with *Aeromonas hydrophila* strain KJ99 (27 hours after inoculation). Rt Renal tubules. Indirect immunoperoxidase. x 400

Discussion

Taken as a whole, the results suggest that the experimental infection targets the gastrointestinal tract, particularly its muscle layers and nervous plexus, and this target may underlie the pathogenesis of the naturally occurring disease.

Identifying an infectious dose that could induce clinical signs and lesions made it possible to begin to understand the pathogenesis of the disease, rather than merely the final effects of an LD50 dose used for virulence studies. The dose of extracellular products was also lower than the lethal dose reported by Santos and others (1988). A lethal dose does not reveal the continued damage and nature of the disease during the host-pathogen interaction (Casadevall and Pirofski 2001).

The bacteria and their extracellular products induced the same systemic haemodynamic (microcirculatory) changes in both species of fish, changes that were similar to those described in mammalian septicaemia. Inflammation was more severe with the bacterium, but degeneration and necrosis of the muscle layers of the gut were more severe with the extracellular products. Most of the mediators of the immune-inflammatory response induced by *A hydrophila* and/or its toxins through its interaction with mammalian leucocytes have been described in fish (Qin and others 2001). It is therefore not unexpected that the fish developed a response during the septicaemic process similar to that described in mammals.

The clinical signs and gross changes were similar to those described in naturally infected rainbow trout (Aydin and others 1997), and also in catfish infected experimentally with *A hydrophila* (Gaines 1972). There was minor skin haemorrhaging at the injection site and around the anus, the latter a common location for lesions (Gaines 1972). There was more severe haemorrhaging when blood-stained fluid flowed through the anus of the white cachama five or 10 minutes before the fish collapsed and died. In contrast, the tilapia hybrids often had a severely contorted intestine wall, due to varying degrees of intussusception (Figs 3, 4).

Similar haemorrhages and blood-stained ascites have been observed in several fish species infected with *A hydrophila* (Gaines 1972, Roberts 1993, Aydin and others 1997). In the present study, the gut was severely affected in both species and it appeared that there was a gradual increase in its vascular permeability, culminating in haemorrhages into the intestinal lumen and detachment of the mucosa. The muscle layers of the gut showed a pattern of hyaline rings, suggesting a hypercontractive process, with the possibility that the hyperactivity of the muscle cells (culminating in intussusception) was the result of increased activity of the local nervous system.

In the advanced stages of the infectious process these neurons were degenerate or necrotic (Fig 7). Lesions in the enteric nervous system and intestinal muscularis similar to those described have been reported in septicaemic and endotoxaemic processes in other species, including horses (Oikawa and others 2007) and rabbits (Hahn and others 2005). The hyaline inclusions in the cytoplasm of neurons of the myenteric and submucosal plexuses suggest the accumulation of neuronal mediators (Fig 8). Cytoplasmic vacuolation (Fig 9) and/or nuclear pyknosis have been reported in grass sickness in horses (Obel 1955, Whitwell 1997).

The IIP technique revealed a consistently close relationship between the bacteria and the neurons and muscle cells. Myoelectric patterns of intestinal motility induced by enteric bacteria or their toxins have been described in mammals (Husebye and others 2001, Tanabe and others 2004). These motility patterns are responsible for fluid propulsion in the intestine of animals infected by *Escherichia coli*, *Vibrio cholerae*, *Salmonella* Typhimurium or their toxins (Navarre and Roussel 1996). The involvement of the local nervous system and the hyperactivity of the muscle layers may be a defence mechanism aimed to eliminate the invading bacteria or their toxic products, a process culminating in the bloody fluid oozing through the anus of the white cachama. Investigations of the patterns of myoelectric activity in the gastrointestinal musculature of fish may help to explain the hypermotility stage proposed in this study.

The extracellular products induced milder haemodynamic changes but more severe tissue changes than the bacteria. Similar results were obtained with the extracellular products of *V anguillarum* in rainbow trout (Lamas and others 1994). In contrast, Fouz and others (1995) observed more severe clinical signs and histological lesions with the extracellular products of *V damsela* than with the live bacteria in *Scophthalmus maximus*. The severity of the tissue lesions induced in this study may have been due mainly to protease activity. Wang and others (2003) suggested that the cause of the cytotoxicity of *Aeromonas* species and their extracellular products may be multifactorial and that the products (α- and β-haemolysins, aerolysin, ACT, ALT and AST enterotoxins, proteases and RNases) may be acting either alone or in concert. Nevertheless, the composition of the extracellular products of different strains of *A hydrophila* could be different, and the culture medium and temperature conditions could also affect their composition (Khalil and Mansour 1997).

The extracellular products induced larger accumulations of hyaline structures in lymphoid organs than were induced by the bacteria alone. These hyaline structures were accompanied by chromatin fragments and resembled necrotic erythrocytes. Haemolysis of erythrocytes and iron inside splenic macrophages, liver and cranial kidney have been reported during infections with *A hydrophila* (Grizzle and Kiryu 1993). However, in this study there was no evidence of complete haemolysis, because the specific staining for free iron was negative. A wide range of biological functions related to the β-haemolysin (aerolysin) of *A hydrophila* has been described, including haemolytic and proteolytic activities lethal to fish (Khalil and Mansour 1997, Nelson and others 1997). The severe hyaline changes in the erythrocytes suggest the involvement of a similar toxin to cause such an apoptotic-like death.

The intraperitoneal inoculation of *A hydrophila* strain KJ99 into tilapia hybrids and white cachama produced haemodynamic changes similar to those observed in the septicaemic process in mammals. The bacterium itself seems to play a significant role in initiating the pathological process, particularly in inducing inflammation. However, some of the extracellular products released during the infection could also play an important role in the pathogenesis, probably inducing degeneration and necrosis (Khalil and Mansour 1997). The IIP showed that the microorganism colonises and moves forward through the blood vessel walls until it reaches the blood stream to establish a systemic infection. Gross and histopathological changes were found in the gastrointestinal musculature and the local nervous plexus, including intussuceptions not previously reported. The gastrointestinal tract appears to be critically important in the pathogenesis of *A hydrophila* septicaemia, and may also play a part in other Gram-negative bacterial infections in fish.

Acknowledgements

This study was supported by grants from COLCIENCIAS-Instituto Colombiano para el Desarrollo de al Ciencia y la Technolgia 'Francisco José de Caldas'. *A hydrophila* strain KJ99 was kindly provided by H. Kawatzu, Miyazaki, Japan.

References

AOKI, T. (1999) Motile Aeromonads (*Aeromonas hydrophila*). In Fish Diseases and Disorders. Vol 3: Viral, Bacterial and Fungal Infections. Eds P. Woo, D. Bruno. Wallingford, CABI Publishing. pp 427-453

ASAO, T., KINOSHITA, Y., KOZAKI, S., UEMURA, T. & SAKAGUCHI, G. (1984) Purification and some properties of *Aeromonas hydrophila* hemolysin. *Infection and Immunity* **46,** 122-127

AYDIN, S., CILTAS, A. & AKYURT, I. (1997) Investigations in systemic *Aeromonas hydrophila* infections appeared in Rainbow trout (*Oncorhynchus mykiss* Walbaum) in 1991-1995. Proceedings of the Mediterranean Fisheries Congress. Izmir, April 9 to 11, 1997. pp 359-369

BRADFORD, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248-254

CASADEVALL, A. & PIROSKI, L. (2001) Host-pathogen interactions: the attributes of virulence. *Journal of Infectious Diseases* **184,** 337-344

CHOPRA, A. K. & HOUSTON, C. W. (1999) Enterotoxins in *Aeromonas* – associated gastroenteritis. *Microbes and Infection* **1,** 1129-1137

CHOPRA, A. K., PETERSON, J. W., XU, X., COPPENHAVER, D. H. & HOUSTON, C. W. (1996) Molecular and biochemical characterization of a heat-labile cytotonic enterotoxin from *Aeromonas hydrophila*. *Microbial Pathogenesis* **21,** 357-377

FOUZ, B., NOVOA, B., TORANZO, A. E. & FIGUERAS, A. (1995) Histopathological lesions caused by *Vibrio damsela* in cultured turbot *Scophthalmus maximus* (L.): inoculations with live cells and extracellular products. *Journal of Fish Diseases* **18,** 357-364

GAINES, J. L. (1972) Pathology of experimental infection with *Aeromonas hydrophila* (Chester) Stanier, (Bacteria: Pseudomonadales) in the channel catfish, *Ictalurus punctatus* (Rafinesque). PhD thesis, Auburn University

GRIZZLE, J. M. & KIRYU, Y. (1993) Histopathology of gill, liver and pancreas, and serum enzyme levels of Channel catfish infected with *Aeromonas hydrophila* complex. *Journal of Aquatic Animal Health* **5,** 36-50

HAHN, C. N., WHITWELL, K. E. & MAYHEW, I. G. (2005) Neuropathological lesions resembling equine grass sickness in rabbits. *Veterinary Record* **156,** 778-779

HANDFIELD, M., SIMARD, P., COUILLARD, M. & LETARTE, R. (1996) *Aeromonas hydrophila* isolated from food and drinking water: hemagglutination, hemolysis, and cytotoxicity for a human intestinal cell line (HT-29). *Applied Environmental Microbiology* **62,** 3459-3461

HIRONO, I. & AOKI, T. (1991) Nucleotide sequence and expression of an extracellular hemolysin gene of *Aeromonas hydrophila*. *Microbial Pathogenesis* **11,** 189-197

HOWARD, S. S. P., GARLAND, W. J., GREEN, M. J. & BUCKLEY, J. T. (1987) Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. *Journal of Bacteriology* **169,** 2869-2871

HUSEBYE, E., HELLSTRÖM, P., SUNDLER, F., CHEN, J. & MIDTVEDT, T. (2001) Influence of microbial species on small intestinal myoelectric activity and transit in germ-free rats. *American Journal of Physiology. Gastrointestinal and Liver Physiology* **280,** G368-G380

KHALIL, A. H. & MANSOUR, E. H. (1997) Toxicity of crude extracellular products of *Aeromonas hydrophila* in tilapia, *Tilapia nilótica*. *Letters in Applied Microbiology* **25,** 269-272

LAMAS, J., SANTOS, Y., BRUNO, D., TORANZO, A. E. & ANADON, R. (1994) A comparison of pathological changes caused by *Vibrio anguillarum* and its extracellular products in rainbow trout (*Oncorhynchus mykiss*). *Fish Pathology* **29,** 79-89

MAYER, R. J. & WALKER, J. H. (1987) Uses of polyclonal antiserum. In Immunochemical Methods in Cell and Molecular Biology. Academic Press. pp 79-95

NAVARRE, C. B. & ROUSSEL, A. J. (1996) Gastrointestinal motility and disease in large animals. *Journal of Veterinary Internal Medicine* **10,** 51-59

NELSON, K. L., RAJA, S. M. & BUCKLEY, J. T. (1997) The glycosylphosphatidylinositol-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin Aerolysin. *Journal of Biological Chemistry* **272,** 12170-12174

OBEL, A. L. (1955) Studies on grass disease. The morphological picture with special reference to the vegetative nervous system. *Journal of Comparative Pathology* **65,** 334-346

OIKAWA, M., OHNAMI, Y., KOIKE, M., PARK, C-H. & OYAMADA, T. (2007) Endotoxin-induced injury of the central, autonomic and enteric nervous systems and intestinal muscularis in thoroughbred horses. *Journal of Comparative Pathology* **136,** 127-132

QIN, Q. W., OTOTAKE, M., NOGUCHI, K., SOMA, G., YOKOMIZO, Y. & NAKANISHI, T. (2001) Tumor necrosis factor alpha (TNF-α)-like factor, produced by macrophages in rainbow trout, *Oncorhynchus mykiss*. *Fish and Shellfish Immunology* **11,** 245-256

ROBERTS, R. J. (1993) Motile aeromonad septicaemia. In Bacteriology of Fish Disease. Eds V. Inglis, R. Roberts, N. Bromage. Blackwell Scientific Publications. pp 143-155

SANTOS, Y., TORANZO, A. E., BARJA, J. L., NIETO, T. P. & VILLA, T. G. (1988) Virulence properties and enterotoxin production of *Aeromonas* strains isolated from fish. *Infection and Immunity* **56,** 3285-3293

SHA, J., KOSLOVA, E. & CHOPRA, A. (2002) Role of various enterotoxins in *Aeromonas hydrophila*-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and

evaluation of their enterotoxic activity. *Infection and Immunity* **70,** 1924-1935
TANABE, Y., CALLAND, J. & SCHIRMER, B. (2004) Effects of peritoneal injury and endotoxin on myoelectric activity and transit. *Journal of Surgical Research* **116,** 330-336
WANG, G., CLARK, C., LIU, C., PUCKNELL, C., MUNRO, C., KRUK, T., CALDEIRA, R., WOODWARD, D. & RODGERS, F. (2003) Detection and characterization of the hemolysin genes in *Aeromonas hydrophila* and *Aeromonas sobria* by multiplex PCR. *Journal of Clinical Microbiology* **41,** 1048-1054
WHITWELL, K. (1997) Histopathology of grass sickness: comparative aspects of dysautonomia in various species (equine, feline, canine, leporids). Proceedings of the 1st International Workshop on Grass Sickness. EMND and related disorders. Eds C. Hahn, V. Gerber, C. Herholtz, I. Mayhew. Bern, October 26 to 27, 1995. pp 18-20