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Virulence of the fish pathogen *Aeromonas dhakensis*: genes involved, characterization and histopathology of experimentally infected hybrid tilapia

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ABSTRACT: *Aeromonas dhakensis* (*Ad*) CAIM 1873 growth was evaluated at different conditions and antibiotic susceptibility. Mortality and histopathological damages in hybrid tilapia *Oreochromis niloticus* × *O. mossambicus*, and virulence factors caused by *Ad* bacterial cells and extracellular products (ECPs) were evaluated, and the whole genome was obtained. *Ad* grew between 0.0 and 5.5% NaCl at a pH of between 4 and 10 and from 4 to 37°C. The lowest minimum inhibitory concentration was found for enrofloxacin (<5 µg ml^{-1}), and bacteria were resistant to erythromycin, amoxicillin and ampicillin. *Ad* bacterial cells (1.86 × 10^5 cells g^{-1}) and ECPs (0.462 µg protein $fish^{-1}$) were highly virulent to challenged hybrid tilapia and caused over 80% mortality at 24 h. The primary clinical sign caused was haemorrhage, and damage was most marked in the spleen, liver, kidney and brain of fish challenged with bacterial cells. To our knowledge, this is the first report that *Ad* causes pyknotic and karyorrhectic nuclei of erythrocytes in the internal organs of hybrid tilapia, which was the most striking histopathological observation. The virulence of *Ad* to hybrid tilapia may be primarily related to the activity of haemolysins (*hlyA* genes) and cytotoxins (aerolysin *aerA*), along with the production of siderophores and proteases. We also found β-lactamase, tetracycline and multiple antibiotic resistance genes, as well as adherence, iron acquisition, toxins (aerolysin family, haemolysins) and diverse protease genes.

KEY WORDS: *Aeromonas dhakensis* · Virulence · Erythrocyte necrosis · Antibiotic · Virulence genes · *Oreochromis*

INTRODUCTION

Aeromonas species are well known agents of fish disease under stressful conditions. *A. dhakensis* (*Ad* = *A. aquariorum*, *A. hydrophila* subsp. *dhakensis*) (Beaz-Hidalgo et al. 2013) has been misidentified as *A. hydrophila, A. veronii* or *A. caviae*; therefore, the importance that is attributed to this bacterium in fish infections should be re-evaluated due to the changing taxonomy. Globally, clinical strains have been associated with a variety of human diseases (Huys et al. 2002). *Ad* is a ubiquitous Gram-negative rod-shaped bacterium that is commonly isolated from freshwater ponds, aquarium water and ornamental fish (Martinez-Murcia et al. 2008), and it is a normal inhabitant of the gastrointestinal tract of fish widely distributed throughout warm countries. However, *Ad* has also been isolated from chironomid egg masses in Israel (Figueras et al. 2011), diseased fish in Spain (Esteve et al. 2012), eels in South Korea (Yi et al. 2013) and dolphins in Spain (Pérez et al. 2015). In addition, *Ad* has been reported as a fish pathogen in

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aquatic environments throughout the world, including India (Nadiga et al. 2016), Brazil (Carriero et al. 2016), Scotland (Orozova et al. 2009) and Mexico (Soto-Rodriguez et al. 2013).

Scientific reports are scarce regarding fish pathologies caused by virulent *Ad* strains; the mechanisms of pathogenicity have not been established but are considered multifactorial, especially for tropical fish. Among these fish, the main pathological sign in challenged freshwater fish (*Piaractus mesopotamicus*) and in rainbow trout *Oncorhynchus mykiss* is haemorrhagic septicaemia (Orozova et al. 2009, Carriero et al. 2016). Austin & Austin (2012) reported that virulent *Ad* strains caused generalized liquefaction of rainbow trout tissues. Recently, studies with *Ad* have been increasing due to its clinical virulence and recent reclassification of taxonomy. The evidence suggests that clinical *Ad* exhibits greater virulence compared to other *Aeromonas* species and possesses cytotoxic activities against human blood cell lines (Morinaga et al. 2013). In addition, *Ad* strains exhibited high virulence in the *Caenorhabditis elegans* infection model with unusual presentation of rapid lysis of the dead body (Mosser et al. 2015).

Virulence factors observed in clinical samples and from sardine *Ad* genome strains included structural components (type IV pilli), extracellular factors (haemolysin and phospholipase), iron acquisition (siderophore and a ferric uptake regulator), multidrug resistance and a type IV secretion system (Wu et al. 2012, Nadiga et al. 2016). Therefore, given the importance of farmed Nile tilapia worldwide and in Mexico, the present work was conducted to understand the growth tolerances with respect to aquatic environmental parameters (temperature, salinity and pH), susceptibility to commonly used antibiotics, virulence factors and genes of the fish pathogen *Ad* CAIM 1873 isolated from diseased Nile tilapia *Oreochromis niloticus*. The histological changes in hybrid tilapia challenged with both bacterial cells and their extracellular products were also analysed.

MATERIALS AND METHODS

Physical-chemical parameters

For growth tests, 3 assays with 5 replicates for each were conducted. Tubes with 5.0 ml tryptic soy broth (TSB, Bioxon) were evaluated from 1 to 10% NaCl at 0.5% intervals. Tubes for 0.0 NaCl were prepared using the components of the original formula of TSB without NaCl. For the pH tests, tubes with 5.0 ml of TSB were adjusted to pH 4, 5, 6, 7, 8, 9 and 10. All of the tubes were inoculated with 100 µl of the bacterial suspension and incubated at 30°C. In the last assay, the bacterial suspension was inoculated on tryptic soy agar (TSA, Bioxon) and incubated at 4, 20, 37 and 40°C. Bacterial growth was observed for 7 d in all of the assays.

Antibiotic susceptibility

Antibiotic minimum inhibitory concentrations (MICs) against the *Ad* strain were estimated following the method of Hindler (1992). Briefly, a direct colony suspension method was used from a bacterial suspension incubated overnight at 30°C in Mueller-Hinton broth. Nine antibiotics (norfloxacin, enrofloxacin, gentamicin, florfenicol, oxytetracycline, trimethoprim-sulfamethoxazole [TSX], erythromycin, amoxicillin and ampicillin, all from Sigma-Aldrich) were tested in triplicate at 10 concentrations ranging from 0 to 2000 µg ml^{-1} along with a negative control (incubated for 24 h at 30°C). The materials, media, procedures and quality control followed Hindler (1992) and are based on the National Committee for Clinical Laboratory Standards procedure. The quality control strain used was the *Escherichia coli* ATCC 25922 CLSI reference strain.

Bacterial inoculum

The strain *Ad* CAIM 1873 was previously isolated from a diseased *Oreochromis niloticus* and was pathogenic to tilapia (Soto-Rodriguez et al. 2013). The strain was recovered from the cryovials, inoculated in 5 ml of TSB and incubated overnight at 30°C. The colonies were suspended in sterile phosphate-buffered saline (PBS) 0.85% NaCl at pH 7.0, and the cells were centrifuged at 5724 × *g* (10 min at 15°C). The bacterial suspension was adjusted to an optical density of 1.0 at 610 nm, which was equivalent to approximately 10^8 CFU ml^{-1} and serially diluted to achieve densities from 10^5 to 10^7 CFU ml^{-1}. These suspensions were plated onto TSA to determine the real density of the isolates used in the challenges.

Characterization of *Ad* extracellular products (ECPs)

Ad ECPs were obtained by the cellophane plate technique (Zhang & Austin 2000). ECPs were filtered through 0.22 µm pore size membrane filters (Milli-

pore™) and were subjected to a sterility test by inoculation of 100 µl on TSA and incubation overnight at 30°C. The ECP samples were stored at −20°C until use. The protein concentration of the ECPs was determined by the method of Bradford (1976) using bovine serum albumin (BSA, Sigma) as a standard. To evaluate the total proteolytic activity present in the ECP samples, a multiprotein substrate (Azocoll, Sigma-Aldrich) was used following the manufacturer's instructions. An absorbance reading of 1.0 at 520 nm was defined as 1 unit of proteolytic activity (U). The siderophores were quantified following the method of De Meyer & Höfte (1997). The concentration was expressed as µM siderophores (10^8 CFU $ml^{-1})^{-1}$ and corresponding to the 0.5 McFarland scale. Two methods were used to evaluate the haemolytic activity of ECPs on blood agar plates (Dibico®): 5 µl ECPs were inoculated in 2 × 1 cm wells and 100 µl ECPs were spread on agar plates. Plates in triplicate were incubated for 48 h at 37°C, which is the standard temperature for the haemolysis test (Tindall et al. 2007).

Cytotoxic activity of *Ad* ECPs

The 3T3-L1 cell lines (ATCC) were seeded (3×10^4 cells $well^{-1}$) in 24-well plates with 10% foetal bovine serum (FBS) in Dulbecco's modified Eagle medium (DMEM; Gibco BRL). The 3T3-L1 cells were kept at 37°C in a humidified atmosphere with 5% CO_2. After 24 h, the medium was changed to 2% FBS in the same medium, and 48 h after seeding, the cells from 2 wells were counted to determine the number of cells at the time of changing the experimental conditions. Likewise, treatments were added in duplicate in DMEM supplemented with 0.5% BSA at concentrations between 0.332 and 33.2 mg of protein ml^{-1}. After 24 h of incubation, the effects on cell proliferation and cell survival were determined in agreement with García-Gasca et al. (2002). Simple linear regressions between log concentration and cell survival or cell proliferation were used to calculate the median lethal concentration (LC_{50}) and the median inhibitory concentration (IC_{50}), respectively, with at least 3 treatments. Each determination was conducted in duplicate with at least 3 independent experiments.

Challenge with *Ad* cells

Apparently healthy hybrid tilapia were obtained from a local tilapia supplier and acclimated for 1 wk prior to the challenge. Hybrid tilapia averaging 3.3 g body weight in groups of 8 with 3 replicates were randomly placed in 3 l aquaria with aerated freshwater and fed *ad libitum* twice daily with a commercial diet of 33% protein. The organisms were intraperitoneally injected with washed bacterial cells in PBS at doses of 1.86×10^5 and 1.55×10^6 cells g^{-1}. The groups of control fish were inoculated with 100 µl of sterile PBS (pH 7.2). The clinical signs and mortality rate of each group were monitored at regular times over a 5 d period. Internal organs from moribund fish were aseptically dissected, and the spleen, liver, kidney and brain samples were inoculated on TSA, MacConkey and blood agar and incubated at 30°C for 24 h. The organs were immediately preserved in buffered 10% formalin for histological analysis. Dominant colonies were purified, and DNA fingerprinting of the isolates was performed with REP-PCR using the $(GTG)_5$ primer (Gomez-Gil et al. 2004).

Challenge with *Ad* ECPs

Intraperitoneal inoculation of hybrid tilapia (3.2 g body weight) with ECPs was conducted under similar experimental conditions as previously described. Groups of 8 fish were inoculated with 0.178, 0.356 and 0.462 µg protein $fish^{-1}$ in triplicate. Groups of control fish were inoculated with 130 µl of sterile PBS (pH 7.2). The clinical signs and mortality rates of each group were monitored at regular times over a 5 d period. Spleen, liver, kidney and brain samples were preserved in buffered 10% formalin.

Histological analysis

To observe the tissue damage, the internal organs (spleen, liver, kidney and brain) were preserved in buffered 10% formalin from moribund fish during the bacterial cell and ECP challenges. Fixed samples were processed following conventional histological methods (Lee & Luna 1968) and stained with haematoxylin and eosin (H&E) and Price's Giemsa stain and examined under a light microscope.

DNA extraction and sequencing

The DNA of strain *Ad* CAIM 1873 was extracted with a Promega Wizard® Genomic DNA Purification Kit. Library preparation for whole genome sequencing was done with Nextera XT following the manu-

facturer's protocol. The library was sequenced in an Illumina Miniseq platform with a Miniseq Mid Output Kit (2 × 150 cycles). Resulting sequences were de novo assembled with the A5-miseq assembly pipeline (Coil et al. 2015), and the contig annotations were done with the Rapid Annotations using Subsystems Technology (RAST) server (Aziz et al. 2008).

Statistical analyses

The cumulative survival data from the *Ad* challenge were analysed with a Kruskal-Wallis 1-way analysis of variance (ANOVA). The cytotoxicity was compared by ANOVA with Tukey's test for pairwise comparisons and Dunnett's test. Differences were considered to be significant at $p < 0.05$.

Table 1. Antibiotic susceptibility of *Aeromonas dhakensis* CAIM 1873 and *Escherichia coli* ATCC 25922 CLSI reference strain. MIC: minimum inhibitory concentration; TSX: trimethoprim-sulfamethoxazole

Antibiotic	MIC (µg ml^{-1})	
	A. dhakensis CAIM 1873	*E. coli* ATCC 25922
Norfloxacin	10	10
Enrofloxacin	<5	<5
Gentamicyn	500	100
Florfenicol	10	1000
Oxytetracycline	100	50
TSX	2000	>2000
Erythromycin	>2000	>2000
Amoxicillin	>2000	500
Ampicillin	>2000	50

RESULTS

Ad, a motile Gram-negative bacillus (Soto-Rodriguez et al. 2013), grew well at NaCl concentrations ranging from 0.0 to 5.0 g l^{-1} and grew less at 5.5 g l^{-1} 48 h post inoculation (h.p.i.). From 6.0 to 10.0 g l^{-1} NaCl, no bacterial growth was observed (Table S1 in the Supplement at www.int-res.com/articles/suppl/d129p107_supp.pdf). With respect to temperature, *Ad* grew well at 20, 30 and 37°C, but bacteria grew less at 4°C starting at 72 h.p.i. Meanwhile, bacteria grew well at a pH from 5 to 10, but at pH 4, no growth was observed. The lowest MIC was found for enrofloxacin (<5 µg ml^{-1}) and the highest MICs were found for erythromycin, amoxicillin and ampicillin (>2000 µg ml^{-1}; Table 1).

Cytotoxic activity of *Ad* ECPs

The dose-response curves of ECPs on the 3T3-L1 cell line growths showed a concentration-dependent effect. At 5.0 and 10.0 mg ml^{-1}, significant differences in cell mortality (54 and 55%, respectively, $n = 3$, $p < 0.05$) were observed compared with the 0% mortality of the control treatment (Fig. 1a). Meanwhile, 1 mg ml^{-1} only had a cytostatic effect with no significant difference ($n = 3$, $p < 0.05$). Lower ECP concentrations (0.1 and 0.5 mg ml^{-1}) had a cell stimulant effect because all treatments were prepared with BSA, which had cell growth factors. With respect to cellular proliferation, ECP concentrations of 0.1, 0.5 and 1.0 mg ml^{-1} showed significant differences (Tukey test, $n = 3$, $p < 0.05$), and 0.1, 0.5, 1.0, 5.0 and 10.0 mg ml^{-1} ECP concentrations had significantly lower cell proliferation

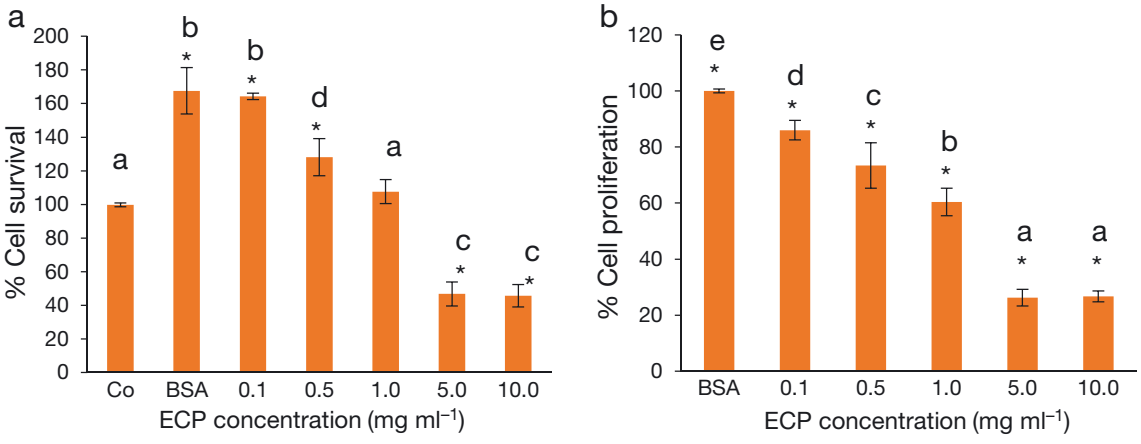

Fig. 1. Effect of *Aeromonas dhakensis* extracellular products (ECP) on (a) 3T3-L1-cell survival and (b) cell proliferation at 24 h incubation. Letters indicate significant differences among treatments (Tukey, $n = 3$, $p \leq 0.05$); asterisks indicate significant differences compared with controls (Dunnett, $n = 3$, $p \leq 0.05$). Co: initial control with foetal bovine serum; BSA: final control with bovine serum albumin

than the control (14, 26, 40, 73 and 74%, respectively, Dunnett's test, n = 3, $p < 0.05$; Fig. 1b). The LC_{50} was 2.21 µg protein ml^{-1}, while the IC_{50} was 1.19 µg protein ml^{-1} (Fig. S1 in the Supplement), indicating that half of the lethal concentration inhibited 50% of the cell proliferation.

The bacterial cells showed haemolytic activity (β-haemolysis), observed as a clear, colourless zone surrounding the colony, with the erythrocytes in the zone completely lysed, and the cells were also susceptible to enrofloxacin (Table 2). The ECPs produced siderophores and had proteolytic, cytotoxic and inhibitory activity on 3T3-L1 cells without haemolytic activity.

Challenges with *Ad* cells and ECPs

Major significant differences were observed depending on the dose; 1.55×10^6 cells g^{-1} caused hybrid tilapia to begin to die after 12 h.p.i., and at 13 h.p.i., the cumulative mortality reached 79.2%. Meanwhile, the lower dose (1.86×10^5 cells g^{-1}) displayed a delay of 36 h (Fig. S2a in the Supplement). The higher ECP dose (0.462 µg protein $fish^{-1}$) caused 100% cumulative mortality at 24 h.p.i.; in contrast, the lower doses (0.356 and 0.178 µg protein $fish^{-1}$) caused only 20% cumulative mortality at the end of the experiment (120 h.p.i.; Fig. S2b). No mortality was observed in the control organisms injected with PBS for either assay.

The clinical signs of the hybrid tilapia challenged with bacterial cells were similar for both doses, but in fish infected with the lower dose, the clinical signs presented at a delayed time. Hybrid tilapia exhibited haemorrhagic zones from the operculum to the pectoral fin starting at 5 h.p.i. (1.55×10^6 cells g^{-1}) and 64 h.p.i. (1.86×10^5 cells g^{-1}; Fig. 2a). The higher (7–13 h.p.i.) and lower dose (96 h.p.i.) caused increased haemorrhagic zones (from the pectoral fin to the anus) and caudal fin erosion (Fig. 2b), and also lethargy and anorexia. The clinical signs of organisms challenged with higher ECP doses (0.462 µg protein $fish^{-1}$) were similar but apparently minor for fish challenged with bacterial cells, except for caudal fin erosion. Lesions in the pectoral fins started at 4 h.p.i., whereas a number of fish showed erratic swimming and abnormal behaviour and were moribund at 6 h.p.i. One hour later, most fish showed slight haemorrhagic lesions in the pectoral fins, a distended abdomen and anal prolapse (Fig. 2c). For fish challenged with lower doses (0.356 and 0.178 µg protein $fish^{-1}$), no clinical signs were observed in dead and surviving organisms. The control group did not show any clinical signs or mortality during the experiment.

Table 2. Characterization of bacterial cells and their extracellular products of *Aeromonas dhakensis* (*Ad*) CAIM 1873. MIC: minimum inhibitory concentration, LC_{50}: median lethal concentration; IC_{50}: median inhibitory concentration; prot: protein

Characteristic	*Ad* CAIM 1873
Bacterial cells	
Haemolytic activity 24 h	+
NaCl tolerance:	
0.0, 1.0, 2.0, 3.0, 4.0, 5.0, 5.5%	+
6.0, 7.0, 8.0, 9.0, 10.0%	–
Temperature tolerance:	
4, 20, 30, 37°C	+
40°C	–
pH tolerance:	
1, 2, 4	–
5, 6, 7, 8. 9, 10	+
Lowest MIC enrofloxacin	<5.0 µg ml^{-1}
Extracellular products	
Total protein	3.32 µg ml^{-1}
Proteolytic activity	0.52 U ml^{-1}
Siderophore production	0.26 µM
Haemolytic activity 24, 48 h	–
Cytotoxic activity (3T3-L1 cells) 24 h	+
Inhibitory activity (3T3-L1) 24 h	+
LC_{50}	2.21 µg prot ml^{-1}
IC_{50}	1.19 µg prot ml^{-1}

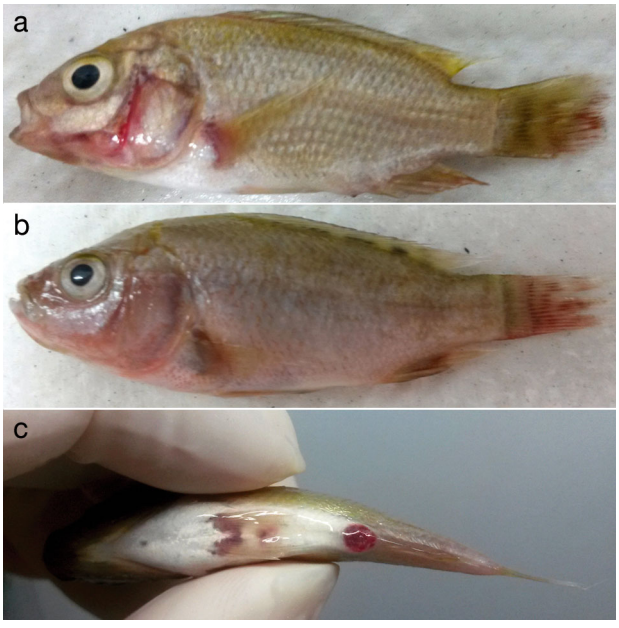

Fig. 2. Hybrid tilapia inoculated with *Aeromonas dhakensis* CAIM 1783 (a,b) cells and (c) extracellular products (ECPs). (a) Haemorrhage in opercula, pectoral fin base and caudal fin erosion, (b) general haemorrhage in ventral area and caudal fin erosion, (c) slight haemorrhage in pectoral-ventral fins and anal prolapse

Histopathology

Tilapia challenged with *Ad* bacterial cells

Within the first 5 h.p.i., *Ad* bacterial cells at 1.86 × 10^5 cells g^{-1} caused lymphocyte infiltration in the liver and pyknotic and karyorrhectic nuclei of erythrocytes in the spleen (Fig. 3a,b). In the next 5 h, inflammation of the kidney and the brain and pyknotic and karyorrhectic nuclei of erythrocytes in the brain were observed (Fig. 3c) with bacilli inside the brain blood capillary (Fig. 3d). Necrosis of erythrocytes in the kidney (Fig. 3e) and bacilli in their intertubular space were also observed (Fig. 3f). The spleen and liver exhibited necrotic erythrocytes (Fig. 3g), bacterial bacilli within the tissue (Fig. 3h) and infiltration of lymphocytes in the liver. After 18 h.p.i., congestion in the liver and an increased severity of necrotic erythrocytes occurred in the spleen and brain. Two fish at 31 h.p.i. exhibited infiltration of focal and multifocal lymphocytes in the liver and kidney, and a granulomatous formation with necrotic tissue was observed in 1 fish. At this time, pyknotic and karyorrhectic nuclei of erythrocytes in the brain continued to be detected.

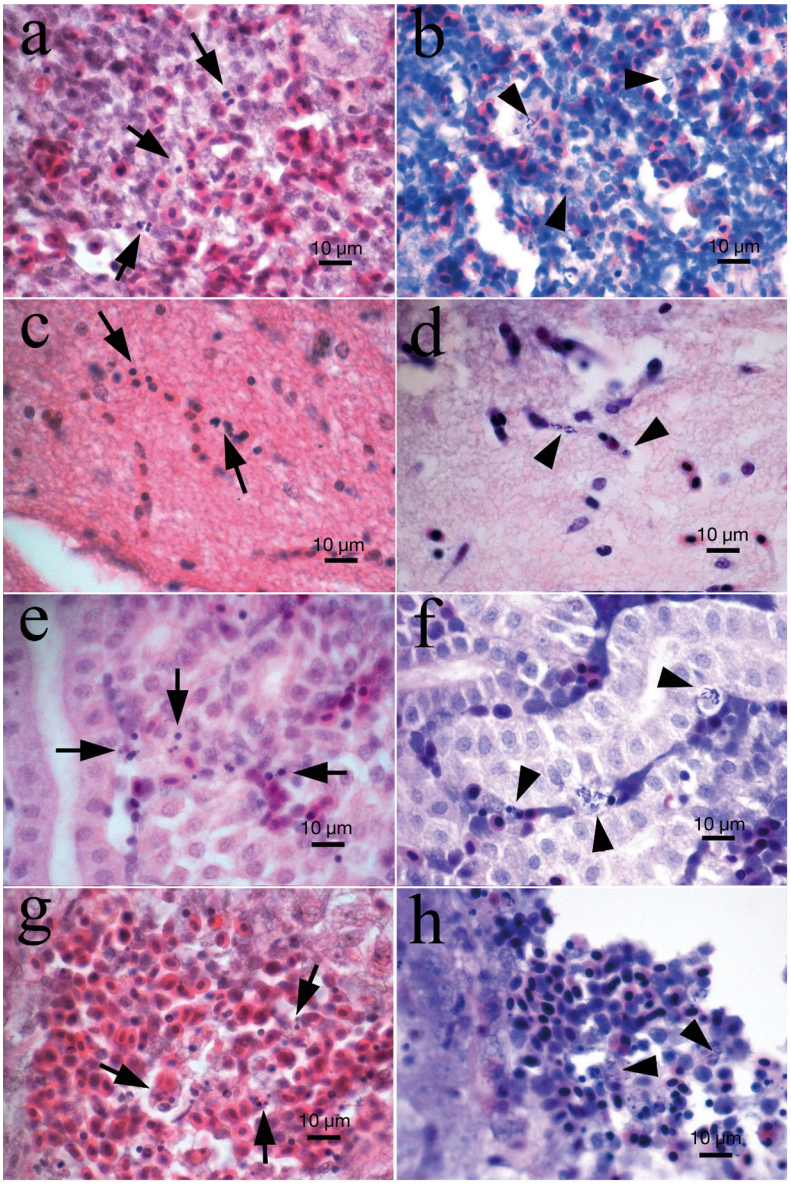

Fig. 3. Histological sections of hybrid tilapia internal organs infected with *Aeromonas dhakensis* CAIM 1783 cells showed pyknotic and karyorrhectic nuclei of erythrocytes (arrows) and presence of bacteria (arrowheads). (a,b) Spleen 3 h post inoculation (h.p.i.), (c,d) brain 7 h.p.i., (e,f), kidney 8 h.p.i., (g,h) liver 8 h.p.i. Haematoxylin & eosin stain (left column), Giemsa stain (right column)

Tilapia challenged with *Ad* ECPs

For the first hours with 0.178 µg protein $fish^{-1}$ (ECPs), the histological damages were similar to organisms challenged with bacterial cells. However, with ECPs, all of the analysed tissues (liver, brain, kidney and spleen) were affected, and the initial stages of focal to multifocal liver congestion were displayed. At 2 h.p.i., focal necrotic erythrocytes and focal lymphocyte infiltration were observed in all tissues (Fig. 4a), and focal to multifocal congestion was also identified. At 4 h.p.i., focal and multifocal lymphocyte infiltration in the liver with necrotic erythrocytes was detected in the most severely affected tissue (Fig. 4b), and no bacteria were observed inside the damaged tissue (Fig. 4c,d). Nevertheless, fewer and smaller lesions were observed at the later time. No abnormal changes were observed in the control groups (not shown) from bacterial cells and ECP challenges.

To examine whether inoculated bacteria were the causal agent of fish mortality and caused internal organ damage, a molecular identification of isolates was conducted. The rep-PCR banding patterns obtained from the pure isolates from 5 to 31 h.p.i were identical to *Ad* CAIM 1873.

Genome

This whole genome shotgun project has been deposited in the DDBJ/ENA/GenBank databases under accession no. PJOL00000000. The version described in this paper is version PJOL01000000. A total of 1 049 856 pair-end reads were obtained, and assembly of these sequenced with a5 produced 193 contigs (N50 64 462, total length 4.928 Mbp, GC% 61.61, 14.6×). Genome annotation produced 4438 coding sequences, 127 RNAs in 532 subsystems. Identification of CAIM 1873 was confirmed by genome taxonomy methods. Average nucleotide identity (ANI; https://github.com/widdowquinn/pyani) between CAIM 1873 and the type strain of *Aeromonas dhakensis* (CECT 7289^T, assembly CDBP01) was 97.3% (ANI MUMmer; http://mummer.sourceforge.net/), and between CAIM 1873 and *A. hydrophila* subsp. *hydrophila* (ATCC 7966^T, assembly CP000462.1), it was 93.3%. The threshold to delimit bacterial species is 97%; a value above this can be considered the same species (Richter & Rosselló-Móra 2009). Furthermore, a digital DNA–DNA hybridization (dDDH) was done between these genomes; CAIM 1873 was compared to the type strains of *A. dhakensis* and *A. hydrophila* subsp. *hydrophila* and had a dDDH of 75.0 and 50.3%, respectively. The value with this methodology to be considered the same species is above 70% (Meier-Kolthoff et al. 2013). Clearly, CAIM 1873 is identified as *A. dhakensis*.

Six β-lactamase genes, 1 tetracycline, 1 multiple antibiotic resistance, and 22 multidrug resistance efflux pump genes were annotated (Table 3), and several genes related to virulence were also found (Table 4): 5 for siderophore (aerobactin) production, 49 for iron acquisition and metabolism, and 9 for regulation of virulence. Ten genes were found related to haemolysins and 38 to proteases. A type I, II and a type III secretion systems were also found along with mannose-sensitive haemagglutinin type IV pili. Three operons related to flagellar production were also annotated.

DISCUSSION

The role of *Aeromonas* as a causative agent of fish disease has been known for decades; however, accurate identification of *Aeromonas* species is necessary to avoid underestimating the prevalence of infections by *Ad* due to erroneous misidentification and potential antimicrobial resistance (Chen et al. 2016). Globally, *Ad* shows a wide range of tolerance to temperatures, salinities and pH levels registered for tilapia culture. Likewise, *Aeromonas* species, similar to *A. hydrophila* isolates from diseased and healthy farmed fish, exhibited growth at 0 but not at 6.0% NaCl (Beaz-Hidalgo & Figueras 2013). This finding indicates that *Ad* can adapt to mild and extreme conditions, such as fresh and saltwater tropical environments, which implies a large risk to farmed aquatic organisms. Fortunately, *Ad* CAIM 1873 showed susceptibility to enrofloxacin, a fluoroquinolone with a broad antibacterial spectrum and high potency that is commonly used to treat bacterial infections affecting aquaculture (Martinez et al. 2006). *Ad* strains isolated from freshwater and wild fish exhibited a higher resistance to multiple antibiotics among *Aeromonas* species (Esteve et al. 2015). Erythromycin resistance shown by *Ad* CAIM 1873 might be caused by the macrolide *MacB* ABC transporter genes (Lin et al. 2009) present in CAIM 1873 in 2 copies. Amoxicillin and ampicillin resistance might be caused by 2 β-lactam resist-

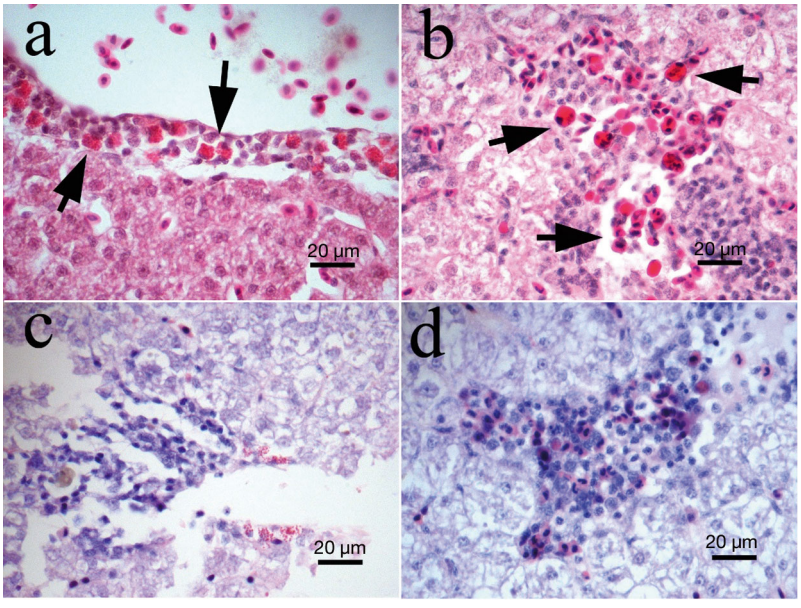

Fig. 4. Microphotographs of hybrid tilapia challenged with *Aeromonas dhakensis* extracellular products (ECPs). (a) Focal inflammation and mastocytes (arrows) in liver 2 h post inoculation (h.p.i.) and (c) liver without bacteria. (b) Moderate inflammation and necrotic erythrocytes (arrow) in liver 4 h.p.i. and (d) liver without bacteria. Haematoxylin & eosin stain (a,b), Giemsa stain (c,d)

ance genes found in the bacteria, which are commonly present in *Aeromonas* species (Aravena-Román et al. 2012). In the genome of the *Ad* strain isolated from Indian oil sardines, 10 genes for the multidrug resistant efflux pump were identified (Nadiga et al. 2016), whereas in our genome, 22 genes were associated with the efflux pump.

This report describes the first pathogenic study in hybrid tilapia challenged with an *Ad* strain, and its virulence was a result of the bacterial cells and their ECPs; both were highly pathogenic to the host fish and caused over 80% mortality at 24 h.p.i. Both treatments were density- and dose-dependent, similar to other *Aeromonas* species in tilapia (Li & Cai 2011, Dong et al. 2017). However, the main external signs caused by *Ad* bacterial cells and ECPs were haemorrhage, caudal fin erosion and anal prolapse, which were different from the clinical signs caused by *A. hydrophila* in tilapia *Oreochromis* sp. (Hamid et al. 2016) and by *Ad* in rainbow trout (Orozova et al. 2009). Parallel experiments by the immersion route conducted under similar experimental conditions and adding *Ad* ECPs and bacterial cells directly, showed that this method did not cause clinical signs or mortality in hybrid tilapia (data not shown). Pridgeon & Klesius (2011) reported that sterilized ECPs failed to kill any channel catfish *Ictalurus punctatus* by bath immersion. Therefore, *Ad* pathology is dependent on the bacterial dose, strain, fish model, the infectious route and the time post infection. Furthermore, our study has described for the first time histological changes in experimentally infected hybrid tilapia caused by *Ad*, and necrotic erythrocytes were the most remarkable histopathological damage that was detected in the spleen, liver, kidney and brain of the challenged hybrid tilapia, as well as lymphocyte infiltration in response to the presence of bacteria in the tissues. In addition, pure bacterial isolates that were identified as *Ad* CAIM 1873 were recovered from all of the internal organs of the moribund fishes. Histopathological examination of tilapia challenged with *Aeromonas* species revealed the most significant findings as severe blood congestion in the brain and liver, haemorrhage in multiple organs, focal necrosis in hepatocytes and pancreas cells, and extensive oedema (Yardimci &

Table 3. Antibiotic resistance genes found in the genome of *Aeromonas dhakensis* CAIM 1873

Subsystem and function of antibiotic resistance	No. of genes
Beta-lactamase	**6**
Beta-lactamase precursors	4
Metallo-beta-lactamase	2
Multidrug resistance efflux pumps	**22**
Acriflavin resistance protein	5
Macrolide export ATP-binding/permease protein *MacB* (EC 3.6.3.-)	2
Macrolide-specific efflux protein *MacA*	2
Membrane fusion protein of RND family multidrug efflux pump	2
Multi antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps	4
RND efflux system, inner membrane transporter *CmeB*	2
RND efflux system, membrane fusion protein *CmeA*	1
RND efflux system, outer membrane lipoprotein *CmeC*	2
Transcription repressor of multidrug efflux pump *acrAB* operon, *TetR* (*AcrR*) family	1
Type I secretion outer membrane protein, *TolC* precursor	1
Multiple antibiotic resistance MAR locus	**1**
Multiple antibiotic resistance protein *MarC*	1
Resistance to tetracycline	**1**
Tetracycline resistance protein, class B *TetA*	1

Table 4. Main pathogenicity mechanisms and virulence factor genes found in the genome of *Aeromonas dhakensis* CAIM 1873

Subsystem and function of virulence	No. of genes
Adherence	**18**
Mannose-sensitive haemagglutinin type IV pili	13
Type IV pili	5
Iron acquisition	**54**
Aerobactin	5
Diverse iron acquisition and metabolism	49
Movement and chemotaxis	**77**
Flagellum	69
Polar flagella	8
Secretion systems	**41**
Type I secretion system	3
Type II secretion system	12
Type III secretion system	26
Toxins	**10**
Aerolysin family beta-barrel pore-forming toxin	1
cya haemolysin	1
Bacillus haemolytic enterotoxin (HBL)	1
hlyA haemolysin	1
hlyA-1 haemolysin	1
Haemolysin-III related	1
Protease	1
RTX toxin	2
Thermostable haemolysin	1
Photox	1
Proteases	**38**
Diverse proteases	34
Metalloproteases	4

Aydin 2011, Dong et al. 2017). The differences observed with *Ad* CAIM 1873 might be due to the bacterial density/dose and time post infection.

The main virulence factors of *Aeromonas* implicated in fish pathology are haemolysins, proteases and lipases (Beaz-Hidalgo & Figueras 2013). In this study, ECPs caused a 17–75% decrease in cell proliferation, and the cytotoxic effect was dose dependent. *Ad* ECPs contained siderophores, proteases and cytotoxins, but no haemolytic activity was observed although bacterial cells displayed strong β-haemolysis; up to 3 haemolysin and diverse protease genes were found in the genome. Clinical *Ad* displayed potent cytotoxic activities against human blood cells and skin fibroblast cell lines (Morinaga et al. 2013, Chen et al. 2014, Hoel et al. 2017), and those authors detected multiple virulence genes, such as the pore-forming toxin gene *aerA* (aerolysin) and the *hlyA*/*aah1* (haemolysin), both also found in CAIM 1873. *Ad* pathogenic to rainbow trout produces the putative virulence factors elastase, haemolysins, lecithinase and lipase (Orozova et al. 2009). The virulence of *Ad* CAIM 1873 to hybrid tilapia could be primarily attributable to haemolytic (haemolysin *hlyA* genes) and cytotoxic activity (aerolysin *aerA*), along with the bacterial siderophores and protease activity, which might produce general organ dysfunction in concert. *Ad* is emerging as one of the most prevalent pathogenic *Aeromonas* species to humans and fish. Future work to understand its mechanisms must include a more complete pathogenesis study.

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