Novel atypical *Aeromonas salmonicida* bath challenge model for juvenile ballan wrasse (*Labrus bergylta,* Ascanius)

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Running title:

Atypical *As* bath challenge model for ballan wrasse

Abstract

Atypical *Aeromonas salmonicida* (a*As*) is currently one of the most routinely recovered bacterial pathogens isolated during disease outbreaks in farmed cleaner fish, ballan wrasse (*Labrus bergylta*, Ascanius). Vibrionaceae family bacteria have also been isolated from ballan wrasse in Scotland. This study determined the infectivity, pathogenicity and virulence of a*As* and Vibrionaceae isolates in juvenile farmed ballan wrasse (n= 50; approx. 2 gr) using a bath challenge and fish were monitored for a period of 16 days. Atypical *As* caused significant mortalities in contrast to Vibrionaceae isolates. Notably, differential virulence was observed between two a*As vapA* type V strains at similar challenge doses. Diseased fish exhibited a

systemic infection where a*As* was detected in all analysed tissues (liver, spleen and kidney) by PCR and qPCR. Macroscopically, moribund and survivor fish exhibited hepatomegaly and splenomegaly. In moribund and surviving fish histopathology showed granulomatous hepatitis with eosinophilic granular cells surrounding bacterial colonies and endocarditis along with splenic histiocytosis. This is the first report of a successful a*As* bath challenge model for juvenile ballan wrasse which provides an important tool for future studies on vaccine efficacy and immunocompetence.

Keywords: atypical *Aeromonas salmonicida*, bath challenge, gross pathology, systemic disease, eosinophilic granular cells (EGCs).

Introduction

Among disease challenges faced by the Atlantic salmon (*Salmo salar*, Linnaeus) farming industry, caligid sea lice infections caused by *Lepeophtheirus salmonis* (Krøyer) and *Caligus elongatus* (Nordmann) had the most significant impacts since the 1960s (Hastein & Bergsjo, 1976; Costello, 2006). Due to large global economic losses to the industry, for example £700 million in 2015 alone (Brooker et al., 2018), there has been considerable investment and innovation on developing sea lice control strategies. The most effective control methods have been bath or in-feed medicated treatments but their efficacy is reduced due to resistance development in lice (Treasurer & Feledi, 2014). Alternative mechanical (hydrolicers, thermolicers, and lasers), physical (snorkel cages and lice skirts) and biological (cleaner fish) treatments have been applied in order to control sea lice (Holan et al., 2017). Biological control through the deployment of wild cleaner fish in salmon cages in Norway and Scotland has been effective and gaining popularity as an environmentally friendly pest management strategy (Treasurer, 2012; Skiftesvik et al., 2013). Commercial scale farming of ballan wrasse (*Labrus bergylta,* Ascanius) and lumpsucker (*Cyclopterus lumpus*, Linnaeus) is currently being used for this purpose. In Scotland, efforts have recently intensified on the farming of ballan wrasse as opposed to using wild caught animals. However, the bacterial pathogen atypical *Aeromonas salmonicida* (a*As*), continues to cause high mortalities during the hatchery and post-deployment stages (Brooker et al., 2018). Depending on the host, typical (salmonids) and atypical (generally non – salmonids) strains of *As* infect a wide range of fresh and marine water fish species. High mortality events have been recorded in farmed species including Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*, L.), Atlantic cod (*Gadus morhua*, L.), Atlantic halibut (*Hippoglossus hippoglossus*, L.) and turbot (*Scophthalmus maximus*, L.) (Austin & Austin, 2016). Atypical *As* strains are very heterogeneous and attempts have been

made to characterise and categorise the different strain types. To date, four main subspecies have been described: *A. salmonicida* subsp. *achromogenes* (Smith, 1963; Schubert, 1967), *masoucida* (Kimura, 1969a, 1969b), *smithia* (Austin et al., 1989) and *pectinolytica* (Pavan et al., 2000). However, some strains cannot be assigned under any of these groups making vaccine development against a*As* difficult. A variety of virulence factors have been characterised for *As* including the A- layer (Munro et al., 1984).The latter is an outer membrane protein of *As* (Udey & Fryer, 1978; Kay & Trust, 1991) which plays an important role in the infection of the host as well as providing protection for the bacterium to the host immune responses (Munn et al., 1982; Kay & Trust, 1991; Daly et al., 1996). Gulla et al. (2016a) demonstrated that *As* strains can be differentiated using the A – layer. Variations can be detected using a partial sequence technique in a region of the virulence array protein gene (*vapA*). In total, 23 A – layer types were differentiated by Gulla et al. (2019) while previously a*As* type V (the most predominant in Scotland) and VI (mainly in Norway) were found to be associated with disease in ballan wrasse (Gulla et al., 2016a). Although there are no commercially available registered licensed vaccines to protect ballan wrasse against a*As*, autogenous vaccines are currently used as an emergency solution in Scotland (Ramirez Paredez et al., 2020). While efficacy testing is not required for commercialising this type of vaccine, data on immune protection elicited by such vaccines is important for optimising treatment efficacy. Challenge models must therefore be developed for ballan wrasse to undertake vaccine testing and assess protective efficacy.

The present study aimed to develop a bacterial bath challenge model for juvenile ballan wrasse using a*As* and Vibrionaceae isolates that had been recovered from disease outbreaks in Scotland but without prior knowledge of their virulence. In addition, the infectivity, pathogenicity and virulence of these bacterial isolates was determined by simulating more natural bacterial portals of entry, as opposed to injection challenge, to provide a vaccination testing platform for the future.

Materials and Methods

Ethics statement

Bacterial infection and vaccination procedures were performed under UK Government Home Office project licences P8E92D8B3 following approval by the Animal Welfare and Ethical Review Body (AWERB) at the Centre for Environment, Fisheries and Aquaculture Science (Cefas) and University of Stirling. Ballan wrasse were treated in accordance with the Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039).

Experimental animals

Juvenile ballan wrasse (n= 1200, 1 ± 0.2 g) were obtained from Otterferry Seafish (Scotland, UK) and transported to Cefas (Weymouth, UK) in January 2018. Fish were kept in four aerated aquaria (50 L) under a 20 h light: 4 h dark photoperiod, with water flows of 1.5 L / min and dissolved oxygen (DO) maintained at 8 ± 0.5 mg / L throughout the experiment. Fish were acclimated to the new environment for three weeks during which temperature was kept constant at 12 ± 1 °C. Temperature was then increased by 0.5 degree / day to reach 15 °C. The fish were fed with Otohime Fish Diet C1 & C2 (Biokyowa, Japan) for 7 h / day using auto feeders during the experiment.

Fish health assessment

The health status of the fish was assessed prior to transportation. Whole larvae (n= 60) were screened for pathogens including common isolated bacteria (a*As* and Vibrionaceae) as well as amoebic gill disease (AGD) by histology, bacteriology and molecular techniques (Yamamoto et al., 2000; Young et al., 2008; Klindworth et al., 2013). In addition, the fish health (n= 60) was reassessed upon arrival to Cefas facilities for bacteriology (swabs from head kidney plated onto sea water agar (SWA)), histopathology (fixed in 10% neutral buffered formalin) and molecular methods to provide further assurances of biosecurity in accordance with the methodological approach reported by Ramirez-Paredes et al. (2020). Whole fish were also fixed in transport media (500 mL G-MEM base, 50 mL foetal bovine serum, 5 mL antibiotic antimycotic stabilised solution, 5 mL glutamax 1, 5 mL penicillin – streptomycin solution, 0.8 mL Tris solution, 2.4 mL sterile 7.5% sodium bicarbonate, OIE) for virology to confirm that fish were not carrying any notifiable viral diseases for further biosecurity assurance as listed in the OIE manual of diagnostic tests for aquatic animals (OIE, 2018). Whole fish samples were fixed in 10% neutral buffered formalin to confirm by histology that fish did not have any existing pathological conditions prior to challenge.

Bacteria preparation

The bacterial strains (n= 8) used were previously recovered from diseased ballan wrasse in hatcheries or cage sites in Scotland or Norway (mentioned where applicable) (Biering et al., 2016; Papadopoulou, 2019). The bacteria used were two strains of a*As vapA* type V (TW 3 / 14 and TW 4 / 14) and members of Vibrionaceae family including two strains of *Vibrio anguillarum* TW 260 / 16 and 12 – 50 2075 F383 – 1 (Norwegian), *Aliivibrio salmonicida* (TW 189 / 16 and TW 322 / 16) and *Photobacterium indicum* (TW 181 / 16 and TW 138 / 16) (Table 1). All bacteria were grown in Tryptone Soya Broth (TSB) + 1.5% NaCl at 22 °C for 24 h with continuous shaking at 150 rpm except atypical *Aeromonas salmonicida* isolates which were grown in TSB at 22 °C for 48 h. The bacteria were centrifuged at 2,000 x g for 10 min, bacterial pellets were washed with phosphate buffered saline (PBS) and then re – suspended in sterile

sea water to an OD_{550} 1. Colony forming unit (CFU) per mL were counted by the 10 – fold dilution method.

Challenge

Ballan wrasse (1.5 ± 0.3 g) were randomly selected from the holding tanks (n= 4) and split into 18 small challenge aquaria (5 L, 50 fish / aquarium). Experimental bacterial infection was performed in duplicate with the exception of an a*As* strain (TW 3 / 14) which was found to be less virulent than TW 4 / 14 at similar doses when previously used for an intraperitoneal (i.p.) injection trial in ballan wrasse (Ramirez Paredez et al., 2020).This strain was prepared in two doses (10^7 and 10^8 CFU/ mL) to assess changes in virulence. The study consisted of 16 challenged (n= 4 bacterial species x 2 strains per species x 2 tanks each) and 2 control groups. The fish were challenged with a*As vapA* type V and Vibrionaceae isolates by bath in a bacterial suspension of an OD_{550} 1.0 while control groups were exposed to sterile sea water (Table 2). All groups were challenged for 4 h at 15 °C with aeration in static conditions. Fish were monitored at least twice per day, with increasing frequency as clinical signs of infection appeared, up to16 days post infection (dpi). Individuals displaying more than two clinical signs of disease (generic and pathogen-specific) were considered as moribund and were humanely killed with an overdose of tricaine methanesulfonate MS-222 (Sigma), followed by destruction of the brain to confirm death. Mortalities were removed from the tanks as soon as they were detected.

Sampling

Head kidney swabs were sampled from a representative number of moribund fish (5 individuals; 10 %) from each tank and plated onto tryptone soya agar (TSA a*As* isolates) or TSA + 1.5 % NaCl (Vibrionaceae) to confirm infectivity and pathogen induced mortality; individuals had only been exposed to one of the four bacteria species. Bacterial colonies were confirmed based on morphology, motility, Gram staining and agglutination test (BIONOR™). Heart, intestine, posterior kidney, liver and spleen samples from moribund fish were preserved in 10 % neutral Buffer Formalin (NBF) for histology. A total of 20 moribund fish (n= 5 x 4 a*As* exposed tanks) were sampled during the trial while 5 survivor fish from each tank were sampled at termination and preserved in 100% ethanol for molecular assessment. In addition, all the survivor fish exposed to TW 3 / 14 (dose 10^7) were sampled (n= 19) at termination as mortalities had not reached a plateau. Samples were screened with molecular assays to determining the presence of a*As* as described below. Mid kidneys, liver and spleen tissue were also sampled from control and all the survivor fish challenged with Vibrionaceae isolates and preserved in 100 % ethanol for molecular analysis.

Histology & Gram Twort stain

A representative number of samples (19 moribund, 12 survivor and 6 control) that had previously been fixed in 10 % NBF were dehydrated (Thermo Shandon Citadel 2000), embedded in paraffin wax, sectioned at 5 µm (Leica RM 2135) and stained with Haematoxylin and Eosin (H&E), while slides not previously stained were dewaxed and stained with Gram Twort. Images were captured using Nikon Eclipse Ni microscope (Nikon, UK) and camera with accompanying software.

DNA extraction and quality check

DNA was extracted from liver, spleen, and mid kidney tissue samples (n= 179, 64 fish x 3 tissues; 13 spleen samples were not included as they were either too small or not sampled) previously preserved in 100% ethanol (Fisher) using a modified salt precipitation method from (Khanam et al., 2016). The extracted DNA was re-suspended in 5 mM Tris. The concentration and purity of the samples was assessed using a NanoDrop® ND-1000 (Thermo Fisher Scientific) and samples were standardised at 50 ng / µL. The DNA integrity was validated by gel electrophoresis and samples were stored at - 20°C until use.

Conventional PCRs – 16S, *vapA* and a*As* type V specific PCR

The presence of bacterial DNA in the fish tissues (liver, spleen and mid kidney) was assessed on the V3-V4 hypervariable region of the *16S* rRNA gene – 16S PCR (Herlemann et al., 2011); if samples were negative no further testing was carried out as bacterial load in the samples was considered below the assay detection limit. Positive samples were then screened with a species specific PCR (*vapA*) for the presence of *As* DNA using a previously published PCR protocol targeting the hypervariable region of *vapA* gene – *vapA* PCR (Gulla et al., 2016a). The presence or absence of a*As vapA* type V was then determined using a previously developed a*As vapA* type V specific PCR (*aAs* type V specific PCR) (Papadopoulou, 2019). The primer pair used for the tissue samples screening with conventional PCRs are listed in Table 3. The relative molecular weight of the amplicons was compared against a 100 bp gene ruler (Thermo Fisher Scientific) on 1 % agarose gel and visualised as previously described.

Quantitative PCR (qPCR)

Aeromonas salmonicida bacterial loads were assessed on tissue samples that were confirmed positive for *As* with the conventional PCRs using a modified qPCR protocol targeting the *vapA* gene (Gulla et al., 2016b). In brief, a 10 µL reaction was set up and the master mix per sample consisted of 0.03 µM of the forward and reverse (Eurofins) primers (Table 3), 0.02 µM of beacon probe (Eurofins), 5.0 µL of Luminaris colour probe qPCR master mix (Thermo Fisher

Scientific), 3.2 µL of Milli-Q water and 1.0 µL of sample or control at 100 ng/µL. Milli-Q water was used as a negative control and the positive control was a*As* type V isolate DNA. The following thermal cycling conditions were run in a LightCycler® 480 Instrument II (Roche Molecular Diagnostics): 1 cycle at 95 °C for 10 min, 45 cycles at 95 °C for 15 sec, 60 °C for 30 sec and 72 °C for 30 sec. The analysis was performed with LightCycler® 480 software (Roche Molecular Diagnostics). The *Aeromonas salmonicida* type strain (NCIMB 1102) was used for the development of the standard curve for the qPCR. The bacteria were grown on TSA at 22 °C. After 48 h an inoculum was transferred into TSB and incubated in a shaker incubator at 22 °C, 150 rpm for 48 h and then bacterial DNA was extracted as described previously. The quality and quantity of the DNA was measured with the methods mentioned and 7 serial dilutions (initial concentration of 40 ng / µL) were conducted using Qubit™ (Thermo Fisher Scientific).

Statistical analyses

All statistical analyses were conducted using the statistical software R (R Core Team, 2020) with the packages survival (Therneau, 2020) and survminer (Kassambara et al., 2020). Survival data were analysed using Kaplan–Meier. Differences between a*As* challenges were analysed by the log-rank test, using the Bonferroni correction for multiple comparisons.

Results

Bath challenge

The bacterial infection dosages are shown in Table 2. Control fish did not show any signs of disease and no mortalities were recorded during the trial. No notable mortalities were recorded for the duplicate groups exposed to *V. anguillarum*, *Aliiv. salmonicida* or *Phot. indicum*, with only 2, 1 and 0 mortalities recorded, respectively. In contrast, fish challenged with the two strains of a*As vapA* type V showed cumulative mortalities of 52 and 60 % (TW 4 / 14 replicates at 2.84 x 10^7 CFU / mL) and 20 % and 62 % (TW 3 / 14 dose at 1.93 x 10^7 CFU /mL and 2.04 x 10^8 CFU /mL, respectively) (Table 2, Figure 1). Survival was higher for individuals challenged with TW 3 / 14 dose at 1.93 x 10^7 CFU /mL than with any other a*As* challenges. The log-rank shows that the challenges statistically differed significantly at the 5% significance level (p=0.00011) and pairwise comparisons indicated that TW 3 / 14 at a dose of 1.93 x 10^7 CFU /mL is statistically significantly different from the other treatments (Table 2). Fish exhibited clinical signs at 5 and 7 days post infection (dpi), for strains TW 4 / 14 and TW 3 / 14, respectively. The first mortalities were recorded at 7 dpi for both strains at the same dose of 10^7 CFU /mL, and 9 dpi for strain TW 3/14 at dose of 10^8 CFU / mL. Mortalities peaked at 13 dpi for the TW 3 / 14 isolate (10^7 CFU /mL; one of the replicates) and 14 dpi for strains TW 3

/ 14 and TW 4 / 14 at 10^7 and 10^8 CFU /mL, respectively (Figure 1). Mortality rate was faster for strain TW 4 / 14 in comparison with fish infected with strain TW 3 / 14 (similar CFU / mL). Pure cultures of a*As* isolated from head kidneys of moribund fish grew on TSA within 48 h at 22 °C.

Gross clinical signs following bath challenge

Moribund fish exhibited external clinical signs of disease including uni or bilateral exophthalmia (Figure 2 A), eroded fins with complete loss of pectoral fins, haemorrhage on dorsal fin and / or tail base (Figure 2 B), skin discoloration, swollen abdomen (ascites), petechiae on the body (Figure 2 B) and haemorrhage between the eyes (Figure 2 A). Exophthalmia and eroded fins were one of the predominant gross pathological changes reported simultaneously with lethargy, loss of buoyancy and imbalance, and loss of appetite. Internally, dark red intestines (Figure 2 C), white nodular lesions in the liver (Figure 2 D), as well as hepatomegaly and splenomegaly were also observed. The liver and spleen were the two main affected organs macroscopically.

Histopathology and Gram Twort staining

In total 6 out of 19 (31.5 %) liver samples from moribund fish analysed exhibited granulomas and increased eosinophilic granular cell activity (Figure 3 A – C). Moribund and survivor fish demonstrated mild scattered single cell necrosis together with mild hepatocellular dissociation, congestion and distended sinusoids (Figure 3 C). In granulomatous livers, small infiltrates of lymphocytes were apparent at the periphery of the granulomas (Figure 3 A - C). The granulomatous formations ranged from large disorganised aggregates of macrophages with eosinophilic granular cells (EGCs) surrounding bacterial colonies (early stage) to well organised, walled off, granulomas (later stage) (Figure 3 A - D). Hepatocellular degeneration was also noted surrounding early lesions (Figure 3 D). Hearts from moribund fish exhibited mild to moderate granulomatous endocarditis and some samples showed endocardial hypertrophy. Intraluminal thrombi containing bacteria were also observed within the atrium (not shown). Over 93.5 % (29 of 31) of the moribund and surviving infected fish that were sampled at the termination of the trial had some degree of peritonitis (Figure 4 C & D), characterised by an increase of EGCs infiltrating the peri-pancreatic fat cells. Control fish had no pancreatic changes (Figure 4 A & B). Spleens of moribund and survivor fish had moderately reactive ellipsoids, mild to moderate histiocytosis and splenic congestion (Figure 5 C & D), which was not evident in control fish spleens (Figure 5 A & B). Spleens from the a*As* infected fish also contained a moderate infiltrate of EGCs. Interestingly, no apparent differences in kidneys from both control and infected fish were observed. EGCs were found in the renal

interstitium of both groups. The intestines from the fish examined microscopically were also very similar with many goblet cells in both control and infected groups. Control fish did not present any pathology in the livers, hearts or spleens.

Gram-Twort stain confirmed the presence of dark pink, Gram-negative rod bacteria forming large colonies within granulomas (Figure 6 C and D) localised in the same area of H&E stained liver sections (Figure 6 A and B).

PCR

16S *PCR*

Initially 179 tissue samples (liver, spleen and kidney) from 64 fish were screened with a broad range 16S rRNA PCR for detection of bacterial DNA (Table 4). Of these samples, 103 tissues (from 59 individuals) had bacterial DNA present, specifically, 39/103 (37.9%) in the liver, 23/103 (22.3%) in the spleen, and 41/103 (39.8%) in the kidney. The majority of these positive samples were subclinical individuals (33/59) that had survived the challenge trial while the rest were moribund individuals (18/59) and control fish (8/59).

***vapA* PCR**

In total, 30 out of 179 (16.8 %) fish tissues (liver, spleen and kidney) screened for *As* species –specific PCR design based on the *vapA* gene of *As* were positive for the bacterium. From the 30 *As* positive organs (from 21 individuals) 13/30 (43.3 %) were detected in the liver, 7/30 (23.3 %) in the spleen, and 10/30 (33.3 %) in the kidney of these fish. Ten out of the 21 fish (48.0 %) had survived the challenge trial (subclinical individuals) and 11 (52.0 %) were moribund (Table 4). All control samples tested (30) were negative. There were no differences in a*As* detection between the individuals challenged with either of the two a*As vapA* type V stains. All the individuals challenged with Vibrionaceae isolates were negative for presence of a*As*.

***aAs* type V-specific PCR**

All control samples tested (30) were negative for a*As* type V. In total, 29 out of 64 fish had the expected PCR product for a*As* type V (254 base pairs) confirming the presence of this type in the tissues tested (43.5% of liver, 19.6% of spleen and 36.9% of kidney samples). From the 29 positive fish, 16 (55.2%) had survived the challenge trial and 13 (44.8 %) were moribund fish (Table 4). There were no discernible differences in the number of positive samples from fish challenged with either of the two a*As vapA* type V stains. All the individuals challenged with Vibrionaceae isolates were negative.

As vapA qPCR

In total, 13 of the 28 fish (or 46.4 %) that were positive by a *As vapA* qPCR were moribund fish and 15 (or 53.6 %) were survivors (subclinical) during the challenge trial. Of the 28 positive fish, 12 (42.8 %) had a high bacterial load with a Crossing point (Cp) value between 18 and 24. The liver (16/41; 39.0 %) and kidney (15/41; 36.6 %) samples had higher bacteria loads that the spleen (10/41; 24.4 %). There were no differences between bacteria load for individuals challenged with either of the two a*As vapA* type V stains. All control samples tested (30) were negative.

Discussion

Bacterial disease outbreaks have impeded advancement of the ballan wrasse industry in Scotland. In the present work one of the two a*As vapA* type V stains was more virulent during the bath challenge experiment developed herein. Infected fish displayed clinical signs, however the disease impacts were more remarkable upon post-mortem examination. Internal lesions in challenged fish were severe including multi focal granulomas in the liver, hepatomegaly and splenomegaly. Eosinophilic granular cells and macrophages were the main leukocytes involved in inflammatory response following histological examination. In contrast, bath challenge of ballan wrasse with *V. anguillarum*, *Alliv. salmonicida* and *Phot. indicum,* did not result in notable mortalities, despite challenging under similar conditions as a*As* isolates.

The significantly higher mortality rates following bath challenge with a*As* compared to all other bacterial species tested in this study, indicates that juvenile ballan wrasse are more susceptible to this bacterium. Both a*As* strains used in the experimental infection were *vapA* type V, which is a predominant *vapA* type (in addition to VI) to which ballan wrasse have been reported susceptible to (Gulla et al., 2016a; Papadopoulou et al., 2020). Fish were exposed to high doses of bacterial broth cultures (2.84×10^7 CFU / mL for strain TW 4 / 14 and 1.93×10^7 CFU / mL and 2.04×10^8 CFU / mL for strain TW 3 / 14), as required for a successful bath challenge model. The cumulative mortalities were higher for strain TW 4 / 14 than TW 3 / 14 at similar doses, which suggested that the former strain was more virulent than the latter even though they belong to the same *vapA* type (V). This is in agreement with our previous findings following an i.p. injection challenge trial (Ramirez Paredez et al., 2020). In addition, in this study, no histological differences, or notable changes in a*As* prevalence, were noted between fish samples collected from fish challenged with the different strains. Furthermore, pure cultures of the bacterium (a*As*) grew on TSA plates from kidney swabs taken from 10 % of moribund fish from each tank and no control fish died throughout the experiment, confirming that a*As* was causing the disease in infected individuals. In a previous study, a*As* (*vapA* type V) induced 75 – 89 % mortalities after i.p. injection in 50 g ballan wrasse and 51 % mortality

with a cohabitation infection challenge (Biering et al., 2016). In the same study a*As* (*vapA* type VI) was notably less virulent by cohabitation challenge (8 %) while i.p. caused 70 – 85 % mortality. This higher mortality rate is, however, not surprising as i.p. administration of the pathogen bypasses the mucosal tissue barriers (*e.g.* skin, gill and gut) and results in higher mortalities in fish (Embregts & Forlenza, 2016; Adams, 2019).

Gross pathology was similar in fish from all tanks challenged with a*As* strains. Exophthalmia and eroded fins were two of the most predominant clinical signs in conjunction with lethargy, loss of equilibrium and inappetence. Externally the response of infected ballan wrasse to a*As* following the bath challenge was less severe in comparison with other fish species like salmonids in which large furuncules on the skin of infected fish are often observed (Austin & Austin, 2016). Similarly, the external clinical disease appears less severe that that reported for other non - salmonids species where the clinical picture includes ulceration in cod (Magnadottir et al., 2002), black rock fish (*Sebastes schlegeli*) (Han et al., 2011), sailfin sandfish (*Artoscopus japonicas*) (Wada et al., 2010) and granulomatous dermatitis in turbot (Farto et al., 2011; Coscelli, Bermúdez, Losada, et al., 2014; Coscelli, Bermúdez, Silva, et al., 2014). Internally, however, the pathological impacts of a*As* are clear whereby the liver, spleen and hearts of infected ballan wrasse were the most affected organs in this study. Hepatomegaly and splenomegaly was noted macroscopically while internally white nodular lesions were present in these organs.

In a recent report of viral haemorrhagic septicaemia (VHS) in the Shetland Islands in Scotland, the presence of EGCs in the pancreas of wrasse species, including ballan wrasse, was described by Munro et al. (2015). Reite and Evensen (2006), in their review on fish inflammatory cells, also reported that the eosinophilic component of leukocytes were dominant in the *Labridae* family. The eosinophilic granular cells observed in the current study were associated with a granulomatous response, surrounding the bacteria in the liver creating the centre of the granulomas. The heart and kidneys were found to be the least affected, despite a*As* being present in a considerable proportion of kidney samples tested by PCR/qPCR, thus perhaps indicating rapid clearance of the bacterium. Specifically, no changes were noted in kidneys of control and infected individuals although EGCs were seen in both groups. Even though the role of EGCs is not completely known in ballan wrasse, their function appears to match mast cells in mammals which release chemical mediators and phagocytose foreign particles (Reite, 1998).

The granulomatous response was also characterised by macrophage infiltration observed in the liver, pancreas, and spleen of fish infected with a*As*. Epithelioid macrophages created the outer layers of the granuloma wall, separating the bacteria from the healthy hepatic cells.

Macrophage infiltration was also observed, replacing the normal splenic tissue, which caused congestion and swelling of the organ. The livers of infected ballan wrasse also showed increased vacuolation similar to the a*As* infection in walking catfish (*Clarias batrachus*) (Menanteau-Ledouble et al., 2016). Necrosis in the kidney, spleen and gut were previously reported in salmonids during a*As* exposure with an increased number of infiltrating macrophages (Menanteau-Ledouble et al., 2016), however such changes were not observed in the kidney or gut in the present study.

Bacterial presence in the tissues of infected and control fish was confirmed with a series of molecular assays. Initially a broad range PCR (16S) was used to determine the presence of bacteria DNA in the samples. *Aeromonas salmonicida* are categorised in 23 known A-layer types and for this work *vapA* type V was detected using a *vapA* type V-specific assay (Papadopoulou, 2019). Bacterial loads were also assessed with the *As* species specific qPCR designed on the A-layer of the virulent array protein gene (Gulla et al., 2016b). In total, 59 out of 64 fish (103 out of 179 tissue samples) had bacterial DNA in their tissues including 8 control fish. However, none of the control fish showed a positive result when screened with the a*As* specific PCRs (*vapA* and type V) and the absence of mortalities in the control replicates during the trial confirmed the lack of potentially confounding bacterial pathogens. In total 21 fish were positive for the presence of *As* DNA (*vapA* PCR) while 26 were positive for *vapA* type V specific PCR. The higher number of positive fish detected with the *vapA* type V specific PCR may suggest a greater sensitivity of the assay for detecting *aAs* (type V) DNA in the samples in comparison with *vapA* PCR. Lastly, the a*As* loads were measured in the same samples by qPCR. From 64 fish samples 28 were positive, making this assay the most sensitive in comparison with the other two PCRs (*vapA* and *vapA* type V specific). This was expected as the qPCR assay is able to detect as little as 7-8 *As* genomes (Gulla et al., 2016b). The varying bacterial loads seen across different organs and samples most likely indicate different stages of infection following the experimental challenge trial. This concurs with (Gulla et al., 2016b) who also showed that qPCR did not always produce positive results when expected due to variable stages of infection or when the DNA concentration of the bacteria is too low.

In this study, the Scottish *V. anguillarum* isolate (TW260/16) caused the second highest cumulative mortality between the different bacteria species tested (4 %). Interestingly, no mortalities were seen from the Norwegian *V. anguillarum* isolate in contrast to a previous trial in Norway where higher mortalities (10 – 15 %) were reported in a bath challenge with two strains of *V. anguillarum* (Biering *et al.*, 2016). Variations in the genome of the bacterium and country of origin of the fish may explain these results (Busschaert et al., 2015; Castillo et al., 2017). The Norwegian isolate included in this study was expected to be more virulent, as bacterial isolates are usually more virulent when coming into contact with hosts from different

geographical locations due to the lack of adaptation to the host species (Anderson & Siwicki, 1994). *Alliv. salmonicida* and *Phot. indicum* were less virulent to the fish resulting in 1 % and 0 % mortalities, respectively. *Alliv. salmonicida* is known to cause cold – water vibriosis in Atlantic salmon and rainbow trout at temperatures between 3 and 10 °C (Egidius et al., 1981) but it does not cause clinical disease at temperatures higher than 14 °C (Colquhoun et al., 2002). This may explain the very low percentage mortality incurred to fish in the present trial despite the fact that these isolates had been isolated from the host at similar 'higher' temperatures (15 °C). It could also indicate that the isolate in these circumstances acted as an opportunistic pathogen to already immunocompromised hosts. However, it should be noted that the lack of mortalities during the infection with *Phot. indicum* could also be related to low numbers of viable bacteria in the suspension used for challenge (2.66×10^5 and 3.12×10^5 CFU / mL) than the target dose 10^8 CFU / mL. This is likely to be due to harvesting of the bacteria towards the end of the exponential phase and perhaps including bacterial cells harvested during the decline (death) phase. We previously noted at least 50 % mortalities in juvenile ballan wrasse when infected by i.p. with *Phot. indicum* (Ramirez Paredez et al., 2020). Growth curve testing and shorter incubation times (< 24 h) may help addressing the problem we encountered in this study to enable reassessment of the pathogenicity of the isolate on juvenile ballan wrasse by bath inoculation.

In conclusion, we successfully developed a bath challenge model for a*As vapA* type V in farmed ballan wrasse for the first time which can now be used as a model for vaccine efficacy testing as it is not invasive and simulates natural bacterial portals of entry in contrast to i.p. injection. The disease caused more severe internal than external pathology in comparison with other species (salmonids and non – salmonids) infected with the same bacterial species. Gross external and internal pathology, alongside histological changes of infected ballan wrasse tissues with the bacterium a*As* were described following experimental a*As* bath challenge conditions. Microscopically, granulomatous hepatitis with EGCs surrounding bacterial colonies and endocarditis along with splenic histiocytosis in moribund and surviving fish were observed. This bath challenge model is now being applied in immunocompetence and vaccine efficacy studies for juvenile ballan wrasse.

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Conflict of interest Statement

The authors declare no conflict of interest

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Adams, A. (2019). Progress, challenges and opportunities in fish vaccine development. *Fish & Shellfish Immunology, 90*, 210-214. doi:https://doi.org/10.1016/j.fsi.2019.04.066

Anderson, D. P., & Siwicki, A. K. (1994). Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. *The Progressive Fish-Culturist, 56*(4), 258-261. doi:https://doi.org/10.1577/1548-8640(1994)056%3C0258:DOPAAS%3E2.3.CO;2

Austin, B., & Austin, D. A. (2016). Aeromonadaceae representative (*Aeromonas salmonicida*). In *Bacterial fish pathogens* 6th ed., pp. 215-321. Springer Cham.

Austin, D., McIntosh, D., & Austin, B. (1989). Taxonomy of fish associated *Aeromonas* spp., with the description of *Aeromonas salmonicida* subsp. *smithia* subsp. nov. *Systematic and applied Microbiology, 11*(3), 277-290. doi:https://doi.org/10.1016/S0723-2020(89)80026-8

Biering, E., Vaagnes, Ø., Krossøy, B., Gulla, S., & Colquhoun, D. (2016). Challenge models for atypical *Aeromonas salmonicida* and *Vibrio anguillarum* in farmed Ballan wrasse (*Labrus bergylta*) and preliminary testing of a trial vaccine against atypical *Aeromonas salmonicida*. *Journal of Fish Diseases, 39*(10), 1257-1261. doi: https://doi.org/10.1111/jfd.12450

Brooker, A. J., Papadopoulou, A., Gutierrez, C., Rey, S., Davie, A., & Migaud, H. (2018). Sustainable production and use of cleaner fish for the biological control of sea lice: recent advances and current challenges. *Veterinary Record, 183*(12), 383. doi:10.1136/vr.104966

Busschaert, P., Frans, I., Crauwels, S., Zhu, B., Willems, K., Bossier, P., Michiels, C., Verstrepen, K., Lievens, B., & Rediers, H. (2015). Comparative genome sequencing to assess the genetic diversity and virulence attributes of 15 *Vibrio anguillarum* isolates. *Journal of Fish Diseases, 38*(9), 795-807. doi:https://doi.org/10.1111/jfd.12290

Castillo, D., Alvise, P. D., Xu, R., Zhang, F., Middelboe, M., & Gram, L. (2017). Comparative genome analyses of *Vibrio anguillarum* strains reveal a link with pathogenicity traits. *Msystems, 2*(1), e00001-00017. doi:10.1128/mSystems.00001-17

Colquhoun, D., Alvheim, K., Dommarsnes, K., Syvertsen, C., & Sørum, H. (2002). Relevance of incubation temperature for *Vibrio salmonicida* vaccine production. *Journal of Applied Microbiology, 92*(6), 1087-1096. doi:https://doi.org/10.1046/j.1365-2672.2002.01643.x

Coscelli, G. A., Bermúdez, R., Losada, A. P., Faílde, L. D., Santos, Y., & Quiroga, M. I. (2014). Acute *Aeromonas salmonicida* infection in turbot (*Scophthalmus maximus* L.). Histopathological and immunohistochemical studies. *Aquaculture, 430*, 79-85. doi:https://doi.org/10.1016/j.aquaculture.2014.04.002

Coscelli, G. A., Bermúdez, R., Silva, A. R. S., de Ocenda, M. V. R., & Quiroga, M. I. (2014). Granulomatous dermatitis in turbot (*Scophthalmus maximus* L.) associated with natural *Aeromonas salmonicida* subsp. *salmonicida* infection. *Aquaculture, 428*, 111-116. doi:10.1016/j.aquaculture.2014.02.038

Costello, M. J. (2006). Ecology of sea lice parasitic on farmed and wild fish. *Trends in Parasitology, 22*(10), 475-483. doi:https://doi.org/10.1016/j.pt.2006.08.006

Daly, J. G., Kew, A. K., Moore, A. R., & Olivier, G. (1996). The cell surface of *Aeromonas salmonicida* determines in vitro survival in cultured brook trout (*Salvelinus fontinalis*) peritoneal macrophages. *Microbial pathogenesis, 21*(6), 447-461. doi:10.1006/mpat.1996.0075

Egidius, E., Andersen, K., Clausen, E., & Raa, J. (1981). Cold-water vibriosis or'Hitra disease'in Norwegian salmonid farming. *Journal of Fish Diseases, 4*(4), 353-354. doi:10.1111/j.1365-2761.1981.tb01143.x

Embregts, C. W. E., & Forlenza, M. (2016). Oral vaccination of fish: Lessons from humans and veterinary species. *Developmental & Comparative Immunology, 64*, 118-137. doi:https://doi.org/10.1016/j.dci.2016.03.024

Farto, R., Milton, D. L., Bermúdez, M., & Nieto, T. (2011). Colonization of turbot tissues by virulent and avirulent *Aeromonas salmonicida* subsp. *salmonicida* strains during infection. *Diseases of Aquatic Organisms, 95*(2), 167-173. doi:10.3354/dao02342

Gulla, S., Bayliss, S., Björnsdóttir, B., Dalsgaard, I., Haenen, O., Jansson, E., McCarthy, U., Scholz, F., Vercauteren, M., & Verner-Jeffreys, D. (2019). Biogeography of the fish pathogen *Aeromonas salmonicida* inferred by *vapA* genotyping. *FEMS microbiology letters, 366*(7), fnz074. doi:10.1093/femsle/fnz074

Gulla, S., Duodu, S., Nilsen, A., Fossen, I., & Colquhoun, D. (2016b). *Aeromonas salmonicida* infection levels in pre-and post-stocked cleaner fish assessed by culture and an amended qPCR assay. *Journal of Fish Diseases, 39*(7), 867-877. doi:10.1111/jfd.12420

Gulla, S., Lund, V., Kristoffersen, A., Sørum, H., & Colquhoun, D. (2016a). *vapA* (A-layer) typing differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously undescribed subtypes. *Journal of Fish Diseases, 39*(3), 329-342. doi:10.1111/jfd.12367

Han, H. J., Kim, D. Y., Kim, W. S., Kim, C. S., Jung, S. J., Oh, M. J., & Kim, D. H. (2011). Atypical *Aeromonas salmonicida* infection in the black rockfish, *Sebastes schlegeli* Hilgendorf, in Korea. *Journal of Fish Diseases, 34*(1), 47-55. doi:10.1111/j.1365-2761.2010.01217.x

Hastein, T., & Bergsjo, T. (1976). The salmon lice *Lepeophtheirus salmonis* as the cause of disease in farmed salmonids. *Rivista Italiana di Piscicoltura e Ittiopatologia (Italy)*.

Herlemann, D. P., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J. J., & Andersson, A. F. (2011). Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *The ISME journal, 5*(10), 1571-1579. doi:10.1038/ismej.2011.41

Holan, A. B., Roth, B., Breiland, M. S. W., Kolarevic, J., Hansen, Ø. J., Iversen, A., Hermansen, Ø., Gjerde, B., Hatlen, B., & Mortensen, A. (2017). Beste praksis for medikamentfrie metoder for lakseluskontroll (MEDFRI)-Faglig sluttrapport. *Nofima rapportserie*.

Kassambara, A., Marcin, K., & Przemyslaw, B. (2020). survminer: Drawing Survival Curves using 'ggplot2'. R package version 0.4.8. . Retrieved from https://CRAN.R-project.org/package=survminer

Kay, W., & Trust, T. (1991). Form and functions of the regular surface array (S-layer) of *Aeromonas salmonicida*. *Experientia, 47*(5), 412-414. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/2044685

Khanam, T., Davie, A., McAndrew, B. J., & Penman, D. J. (2016). DNA sampling from mucus in the Nile tilapia, *Oreochromis niloticus*: minimally invasive sampling for aquaculture-related genetics research. *Aquaculture Research, 47*(12), 4032-4037. doi:10.1111/are.12809

Kimura, T. (1969a). A new subspecies of *Aeromonas salmonicida* as an etiological agent of furunculosis on “Sakuramasu”(*Oncorhynchus masou*) and pink salmon (*O. gorbuscha*) rearing for maturity. Part 1. On the morphological and physiological properties. *Fish Pathology, 3*(2), 34-44.

Kimura, T. (1969b). A new subspecies of *Aeromonas salmonicida* as an etiological agent of furunculosis on “Sakuramasu”(*Oncorhynchus masou*) and pink salmon (*O. gorbuscha*) rearing for maturity. Part 2. On the serological properties. *Fish Pathology, 3*(2), 45-52.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research, 41*(1), e1. doi:10.1093/nar/gks808

Magnadottir, B., Bambir, S., Gudmundsdóttir, B., Pilström, L., & Helgason, S. (2002). Atypical *Aeromonas salmonicida* infection in naturally and experimentally infected cod, *Gadus morhua* L. *Journal of Fish Diseases, 25*(10), 583-597. doi:DOI 10.1046/j.1365-2761.2002.00407.x

Menanteau-Ledouble, S., Kumar, G., Saleh, M., & El-Matbouli, M. (2016). *Aeromonas salmonicida*: updates on an old acquaintance. *Diseases of Aquatic Organisms, 120*(1), 49-68. doi:10.3354/dao03006

Munn, C., Ishiguro, E., & Kay, W. (1982). Role of surface components in serum resistance of virulent *Aeromonas salmonicida*. *Infection and Immunity, 36*(3), 1069-1075. doi:10.1128/IAI.36.3.1069-1075.1982

Munro, A., Ellis, A., McVicar, A., McLay, H. A., & Needham, E. (1984). An exocrine pancreas disease of farmed Atlantic salmon in Scotland. *Helgolander Meeresuntersuchungen, 37*(1-4), 571-586.

Munro, E., McIntosh, R., Weir, S., Noguera, P., Sandilands, J., Matejusova, I., Mayes, A., & Smith, R. (2015). A mortality event in wrasse species (Labridae) associated with the presence of viral haemorrhagic septicaemia virus. *Journal of Fish Diseases, 38*(4), 335-341. doi:10.1111/jfd.12237

OIE. (2018). *Manual of diagnostic tests for aquatic animals, Chapter 2.3*. Retrieved from https://www.oie.int/en/standard-setting/aquatic-manual/access-online/

Papadopoulou, A. (2019). *Health screening and autogenous vaccination strategies for atypical Aeromonas salmonicida in farmed ballan wrasse (Labrus bergylta, Ascanius).* (Doctoral dissertation University of Stirling). Retrieved from http://hdl.handle.net/1893/30672

Papadopoulou, A., Wallis, T., Ramirez-Paredes, J. G., Monaghan, S. J., Davie, A., Migaud, H., & Adams, A. (2020). Atypical *Aeromonas salmonicida vapA* type V and *Vibrio* spp. are predominant bacteria recovered from ballan wrasse (*Labrus bergylta*) in Scotland. *Diseases of Aquatic Organisms, 140*(2), 47-54. doi: https://doi.org/10.3354/dao03432

Pavan, M., Abbott, S., Zorzopulos, J., & Janda, J. (2000). *Aeromonas salmonicida* subsp. *pectinolytica* subsp. nov., a new pectinase-positive subspecies isolated from a heavily polluted river. *International Journal of Systematic and Evolutionary Microbiology, 50*(3), 1119-1124. doi:10.1099/00207713-50-3-1119

Ramirez-Paredes, J. G., Verner-Jeffreys, D. W., Papadopoulou, A., Monaghan, S. J., Smith, L., Haydon, D., Wallis, T. S., Davie, A., Adams, A., & Migaud, H. (2020). A commercial autogenous injection vaccine protects ballan wrasse (Labrus bergylta, Ascanius) against Aeromonas salmonicida vapA type V. *Fish & Shellfish Immunology, 107*, 43-53. doi:https://doi.org/10.1016/j.fsi.2020.09.040

Ramirez Paredez, G., Verner-Jeffreys, D., Papadopoulou, A., Monaghan, S., Smith, L., Haydon, D., Wallis, T., Davie, A., Adams, A., & Migaud, H. (2020). A commercial autogenous injection vaccine protects ballan wrasse (*Labrus bergylta*, Ascanius) against *Aeromonas salmonicida vapA* type V. *bioRxiv*, 2020.2007.2002.183616. doi:10.1101/2020.07.02.183616

Reite, O. B. (1998). Mast cells/eosinophilic granule cells of teleostean fish: a review focusing on staining properties and functional responses. *Fish & Shellfish Immunology, 8*(7), 489-513. doi:10.1006/fsim.1998.0162

Reite, O. B., & Evensen, Ø. (2006). Inflammatory cells of teleostean fish: a review focusing on mast cells/eosinophilic granule cells and rodlet cells. *Fish & Shellfish Immunology, 20*(2), 192-208. doi:10.1016/j.fsi.2005.01.012

Schubert, R. H. (1967). The taxonomy and nomenclature of the genus Aeromonas Kluyver and Van Niel 1936. Part II. Suggestions on the taxonomy and nomenclature of the anaerogenic aeromonads. *International Journal of Systematic Bacteriology, 17*(3), 273-279.

Skiftesvik, A. B., Bjelland, R. M., Durif, C. M., Johansen, I. S., & Browman, H. I. (2013). Delousing of Atlantic salmon (*Salmo salar*) by cultured vs. wild ballan wrasse (*Labrus bergylta*). *Aquaculture, 402*, 113-118. doi:10.1016/j.aquaculture.2013.03.032

Smith, I. W. (1963). The classification of '*Bacterium salmonicida*'. *Microbiology, 33*(2), 263-274.

Therneau, T. (2020). A Package for Survival Analysis in R. R package version 3.1–12. In.

Treasurer, J. (2012). Diseases of north European wrasse (*Labridae*) and possible interactions with cohabited farmed salmon, *Salmo salar* L. *Journal of Fish Diseases, 35*(8), 555-562. doi:10.1111/j.1365-2761.2012.01389.x

Treasurer, J., & Feledi, T. (2014). The physical condition and welfare of five species of wild-caught wrasse stocked under aquaculture conditions and when stocked in Atlantic salmon, *Salmo salar*, production cages. *Journal of the World Aquaculture Society, 45*(2), 213-219. doi:10.1111/jwas.12099

629 Udey, L., & Fryer, J. (1978). Immunization of fish with bacterins of *Aeromonas salmonicida*.
630 *Marine Fisheries Review, 40*(3), 12-17.

631 Wada, S., Ozeki, K., Kurata, O., Hatai, K., Ishida, A., & Ui, K. (2010). Atypical *Aeromonas*
632 *salmonicida* infection in sailfin sandfish *Arctoscopus japonicus*. *Fish Pathology,*
633 *45*(2), 92-95. doi:10.3147/jsfp.45.92

634 Yamamoto, S., Kasai, H., Arnold, D. L., Jackson, R. W., Vivian, A., & Harayama, S. (2000).
635 Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the
636 nucleotide sequences of *gyrB* and *rpoD* genes. *Microbiology, 146*(10), 2385-2394.
637 doi:https://doi.org/10.1099/00221287-146-10-2385

638 Young, N. D., Dyková, I., Nowak, B. F., & Morrison, R. N. (2008). Development of a
639 diagnostic PCR to detect *Neoparamoeba perurans,* agent of amoebic gill disease.
640 *Journal of Fish Diseases, 31*(4), 285-295. doi:10.1111/j.1365-2761.2008.00903.x

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647 Table 1. Bacterial isolates used in this study. All bacterial isolates recovered from diseased
648 ballan wrasse (*Labrus bergylta*) in Scotland with the exception of a single isolate recovered
649 from the species in Norway.
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Bacterial species	**Isolate ID**	**Year of isolation**
Aeromonas salmonicida (*vapA* type V)	TW 3 / 14	2014
Aeromonas salmonicida (*vapA* type V)	TW 4 / 14	2014
Vibrio anguillarum	TW 260 / 16	2016
Vibrio anguillarum	12-50-2075 F383-1*	< 2014
Aliivibrio salmonicida	TW 189 / 16	2016
Aliivibrio salmonicida	TW 322 / 16	2016
Photobacterium indicum	TW 138 / 16	2016
Photobacterium indicum	TW 181 / 16	2016

651 (*) Norwegian isolate

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Table 2. Cumulative mortalities of ballan wrasse (*Labrus bergylta*) infected with atypical *Aeromonas salmonicida*, *Vibrio anguillarum*, *Allivibrio salmonicida* and *Photobacterium indicum.* No mortalities were recorded for control groups.

Bacteria sp.	Bacteria ID	CFU / mL	Cumulative mortalities (%)
Atypical *Aeromonas salmonicida vapA* type V	TW4/14	2.84E+07	52[b]
			60[b]
	TW3/14	1.93E+07*	20[a]
		2.04E+08*	62[b]
V. anguillarum	TW260/16	4.81E+07	4
			0
	12-50-2075 F383-1	2.58+08	0
			0
Aliiv. salmonicida	TW189/16	3.85E+07	2
			0
	TW322/16	4.09E+07	0
			0
Phot. indicum	TW138/16	2.66E+05	0
			0
	TW181/16	3.12E+05	0
			0

(*) No duplicate tanks

(a,b) statistically significant difference at $p < 0.05$.

667 Table 3. Primers used for amplification of broad bacterial DNA, *Aeromonas salmonicida* and
668 atypical *Aeromonas salmonicida vapA* type V specific with conventional PCR and primers for
669 bacterial load with qPCR.

Gene	Oligo sequence	Annealing (°C)	Product size (bp)	Publication
Bacterial *rRNA* 16S	F341: CCTACGGGNGGCWGCAG R805: GACTACHVGGGTATCTAATCC	54.0	485	(Herlemann et al., 2011)
***vapA* partial**	F2: CTGGACTTCTCCACTGCTCA R3: ACGTTGGTAATCGCGAAATC	53.0	625	(Gulla et al., 2016a)
***vapA* partial**	Vspec– F:CAACGGTTTCTGGAGTAATAACTTT Vspec – R:TGCATCAGCAACAGCGGTAGT	57.0	254	(Papadopoulou et al., 2020)
vapA	F: ACTGTCTGTTACCCTGCCA-3´ R: GCTACTTCACCCTGATTGG-3´	60.0		(Gulla et al., 2016b)

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685 Table 4. Ballan wrasse (*Labrus bergylta*) samples (liver, kidney and spleen) from moribund,
686 survivor and control fish screened with a PCR for bacterial presence (16S), atypical
687 *Aeromonas salmonidisa* (a*As*) specific (*vapA* type V) and a*As* type specific PCR (*vapA* type
688 V specific) as well as a qPCR for a*As* loads**.**

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Tissue / Fish status	Total	16S	*vapA*	V specific	qPCR
Liver	63	39	13	20	16
Spleen	53	23	7	9	10
Kidney	63	41	10	17	15
Tissues samples	**179**	**103**	**30**	**46**	**41**
Moribund fish	20	12	11	13	13
Survivors	34*	35	10	16	15
Control fish	10	8	0	0	0
Total number of fish	**64**	**55**	**21**	**29**	**28**

690 (*) includes 19 individuals challenged with TW 3 / 14 (dose 10^7) but mortalities did not reach
691 a plateau at termination.

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Figure 1. Cumulative mortalities of ballan wrasse (*Labrus bergylta*) bath challenged with atypical *Aeromonas salmonicida vapA* type V strain TW 3 / 14 (interrupted line) and TW 4 / 14 (continuous line).

Figure 2. Gross pathology and Internal gross pathology of infected ballan wrasse (*Labrus bergylta*) with atypical *Aeromonas salmonicida* following bath challenge. A) Bilateral exophthalmia and haemorrhage between the eyes, B) eroded pectoral fins and petechiae on the body (arrow head).C) Dark red intestine (circle) D) hepatomegaly and white nodular lesions in the liver (arrow heads).

Figure 3. Ballan wrasse liver infected with atypical *Aeromonas salmonicida*. A) Arrow heads delineating three large granulomas composed of a thick wall of epithelioid macrophages (black arrows) encircling degenerate and non-degenerate eosinophilic granular cells which themselves surround large bacterial colonies B) The region highlighted in a box in A). Detail of wall and centre of granuloma. Degenerate eosinophilic granular cells (E) surrounding atypical *Aeromonas salmonicida* colonies (B), delineated by a dense rim of epithelioid macrophages. Degenerate hepatocytes are present at the periphery (arrow heads) along with small numbers of lymphocytes. C) Multiple poorly organised hepatic granulomas centred on bacterial colonies. Note the moderate distension of hepatic sinusoids throughout the parenchyma. D) Very early site of infection showing bacterial colonies surrounded by a fine rim of karyorrhectic cellular debris (arrow heads), admixed with occasional macrophages, EGCs and lymphocytes. Scale bar 100 µm for A) and C) and 20 µm for B) and D).

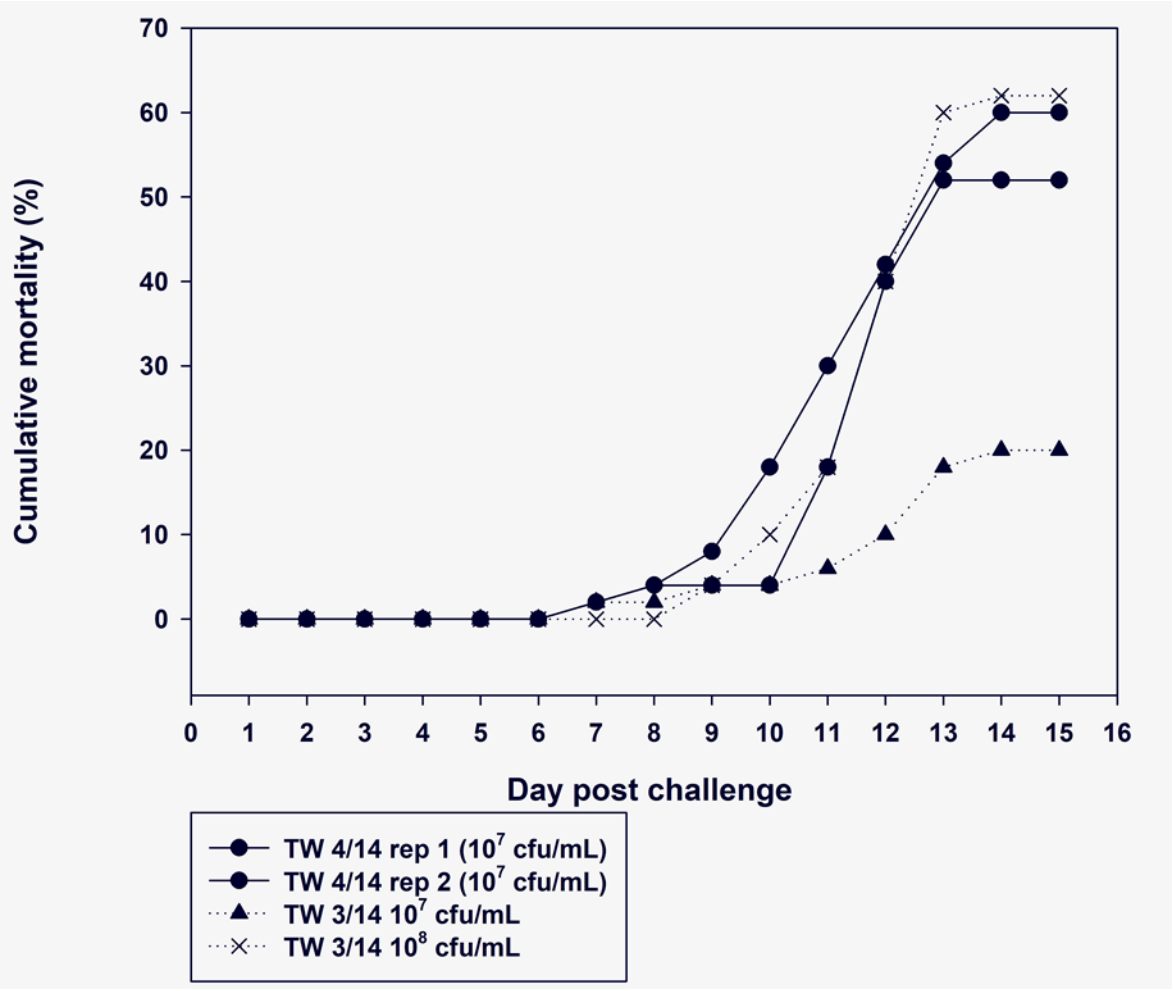
Figure 4. Hematoxylin and Eosin stained slides of ballan wrasse (*Labrus bergylta*) pancreases and peritoneum fixed in 10% neutral buffered formalin from atypical *Aeromonas salmonicida* bath challenge. (A) Healthy pancreas of ballan wrasse (B) Healthy pancreas of ballan wrasse with adjacent fat cells (+), exocrine pancreatic cells, mild infiltrate of eosinophilic granular cells (arrow head). (C) Pancreas of ballan wrasse with peritonitis in a fish infected with atypical

Aeromonas salmonicida. (D) Pancreas with infiltration of phagocytic macrophages and eosinophilic granular cells replacing the peripancreatic adipose cells (arrow head). Scale bar 50 µm for A) and C) and 20 µm for B) and D).

Figure 5. Ballan wrasse (*Labrus bergylta*) control and infected spleens with atypical *Aeromonas salmonicida* stained with H&E. (A) Healthy spleen of a ballan wrasse with normal splenic haematopoietic tissue and ellipsoids (black arrow). (B) Healthy spleen of a ballan wrasse with visible erythrocytes and haematopoietic tissue. (C) Spleen of a ballan wrasse infected with atypical *Aeromonas salmonicida* with infiltration of macrophages replacing the normal splenic tissue. (D) Spleen of a ballan wrasse infected with atypical *Aeromonas salmonicida* with mild congestion and macrophage infiltration (arrow head). Scale bar 20 µm for A) and C) and 10 µm for B) and D).

Figure 6. Ballan wrasse (*Labrus bergylta*) livers infected with atypical *Aeromonas salmonicida.* A and B Gram-negative bacilli-shaped bacteria (arrow head) inside granuloma H&E (scale bar 20 and 10 µm, respectively). B) The region highlighted in a box in A), arrow heads indicate bacilli-shaped bacteria. C and D Gram-negative bacilli-shaped bacteria (arrow head) inside granuloma. D) The region highlighted in a box in C), arrow heads indicate Gram-negative bacilli-shaped bacteria (scale bar 20 and 10 µm, respectively).

739 Figure 1



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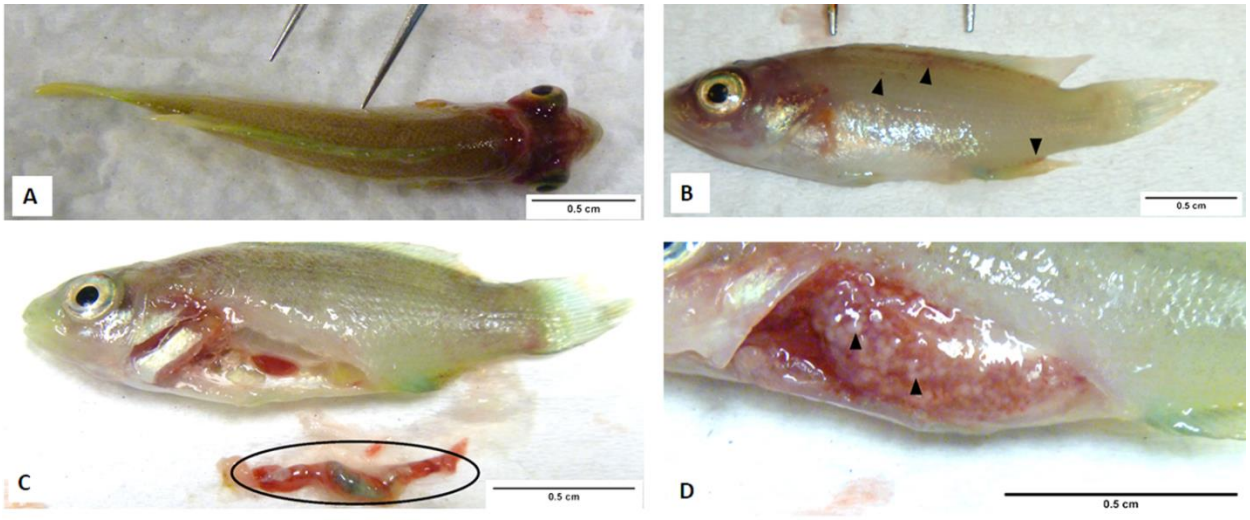
742 Figure 2



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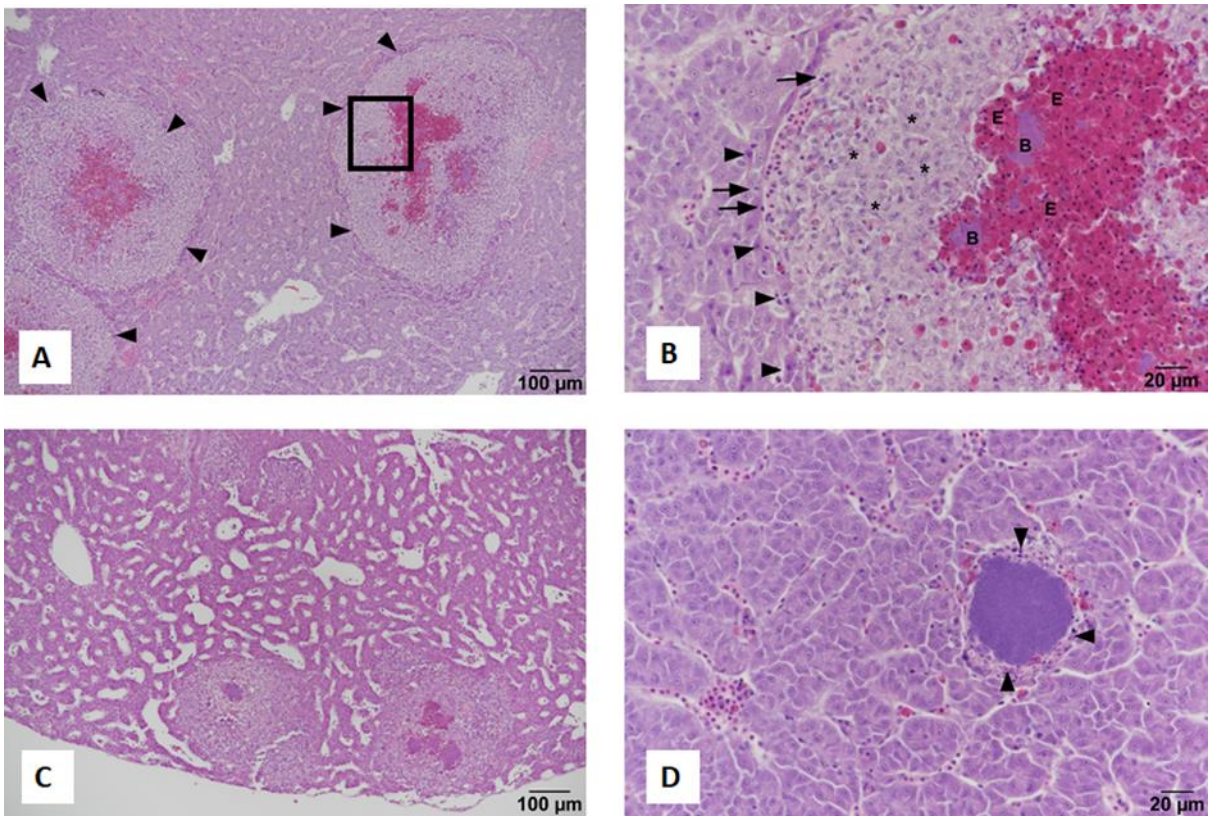
745 Figure 3



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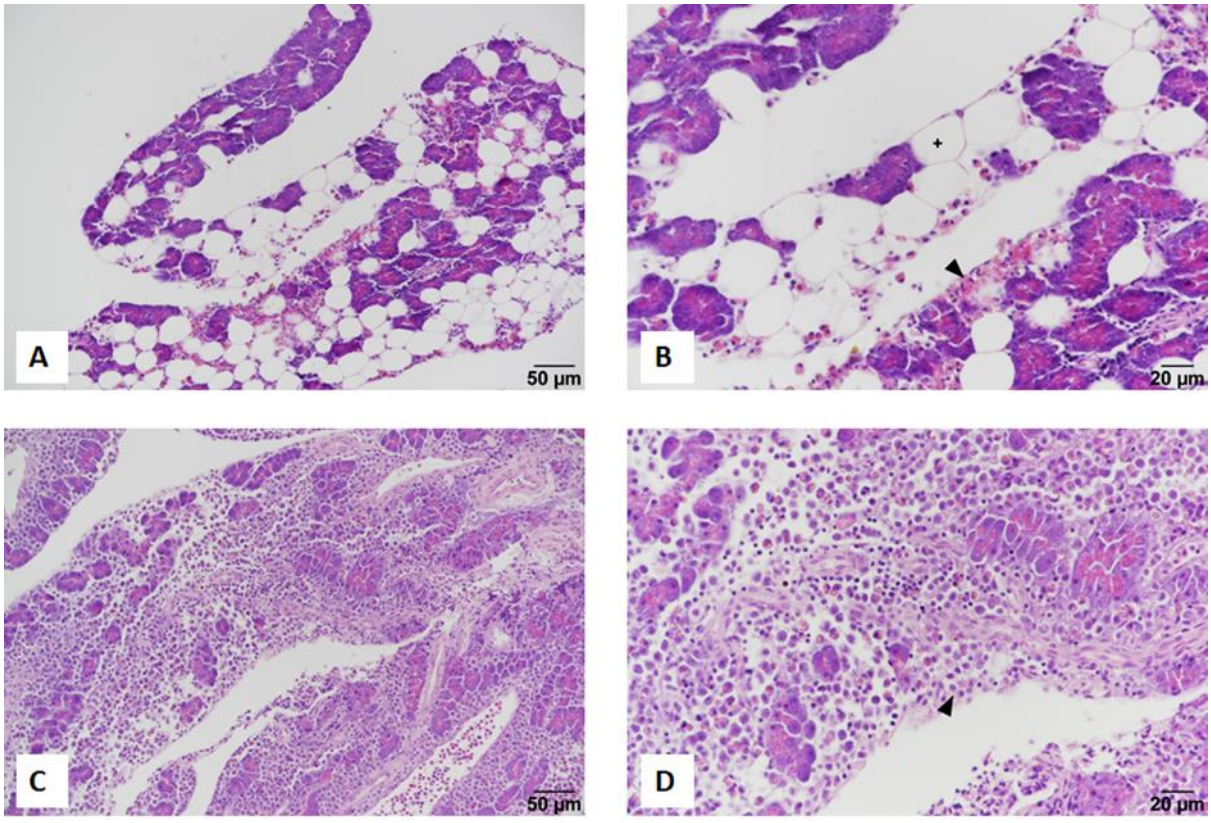
748 Figure 4



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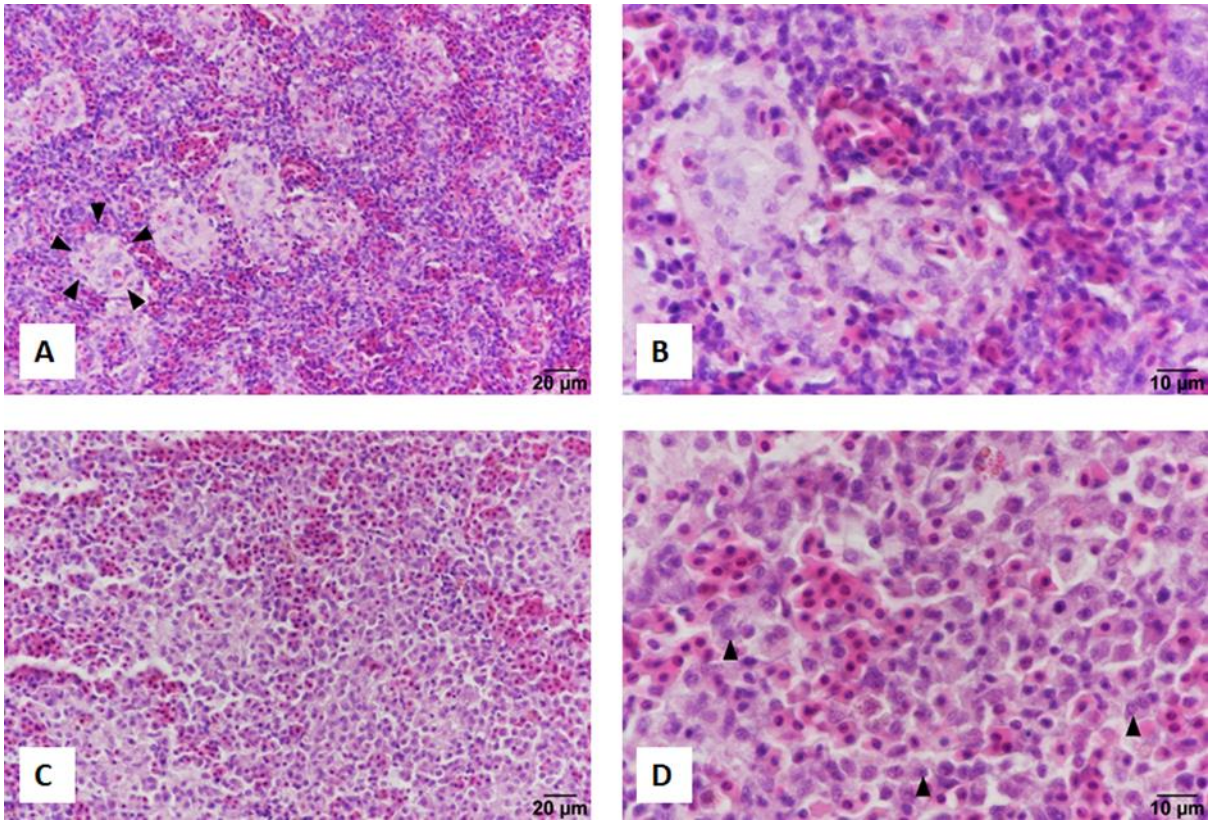
751 Figure 5



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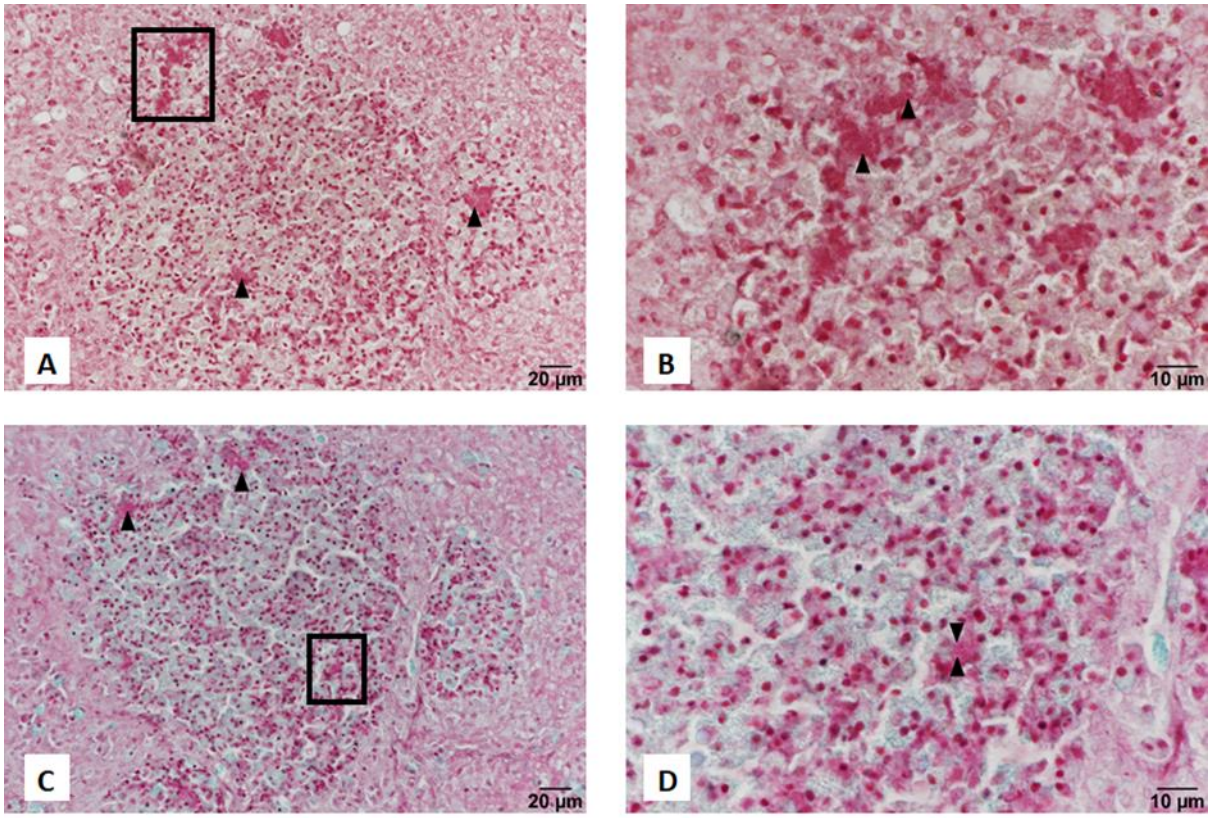
754 Figure 6



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