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Development of a caspase-3 antibody as a tool for detecting apoptosis in cells from rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Background: Apoptosis is an active cell death process mediated by caspases activation, in which different extrinsic or intrinsic signalling pathways result in direct activation of effector caspases. Caspase-3 is considered to be the most important of the executioner caspases, which cause the morphological and biochemical changes detected in apoptotic cells. Different bacterial and virus pathogens have developed different strategies to survive inside the host and overcome natural protections, one of them is inducing apoptotic death in infected cells. We have demonstrated previously that *Piscirickettsia salmonis* activates this process in monocytes/macrophages from salmonid RTS11 cell line both by morphological and caspase detection assays; nevertheless, recognition of caspase activation by western blot was impossible since most of the commercially available antibodies for mammalian caspases are not cross-reacting. **Results:** We have generated a monospecific polyclonal antibody directed to an epitope region of salmonid caspase-3; the selected epitope present high homology with caspase-3 from others teleost species and includes the active site of the enzyme. The peptide was designed using bioinformatics tools and was chemically synthesized using the Fmoc strategy, analysed by RP-HPLC, its molecular weight confirmed by mass spectrometry and its structure analyzed by circular dichroism. The synthetic peptide was immunized and antibodies from ascitic fluid were enriched for immunoglobulins using caprylic acid and then purified by activated affinity columns. The anti-peptide activity of purified antibodies was verified by ELISA, and the ability of the anti-peptide to recognize salmonid caspase-3 activation was demonstrated with the molecule in *P. salmonis* RTS11 infected cells by western blotting, ELISA and immunocytochemistry. **Conclusions:** This is the first antibody available for a fish caspase, specifically for trout caspase-3, whose applications were validated by different immunological assays.

Keywords: antibody, caspase-3, fish apoptosis, *Piscirickettsia salmonis*

INTRODUCTION

Cell death may occur by two opposite modes: programmed or unregulated form; the former process includes apoptosis, autophagy, paraptosis, piroptosis or necroptosis and the latter refers to necrosis (Fink and Cookson, 2005; Elmore, 2007; Vandenabeele et al. 2010). Apoptosis is an evolutionarily conserved and genetically controlled multistep process of cell death in response to a wide variety of stimuli that can be signalled from the external environment or from inside the cell. The mechanism of programmed cell death progresses through a series of morphological changes mediated by the activation of specific cystein proteases named caspases (Fan et al. 2005). Once activated, caspases

selectively cleave many specific target proteins and thereby bring about the characteristic apoptotic morphology. There are two major apoptotic pathways through which these proteases can be activated: the death-receptor and the mitochondrial pathways, which induce initiator caspases 8 and 9, respectively (Elmore, 2007). Signal transduction pathways send the message to the cell death effector caspases, which include caspase-3, the most important terminal caspase essential for execution of apoptosis (Hengartner, 2000; Gupta, 2003; Elmore, 2007).

Caspases are aspartate-specific cystein proteases with a conserved QACXG motif (X can be R, Q or D). They are all expressed as enzymatically inert zymogens (30 to 50 kD) composed of three domains, an N-terminal prodomain, and one large (~20 kD) and one small subunit (~10 kD). In contrast to the strong conservation in the large and small subunits, the size and sequence of the prodomains show little similarity over different species. Activation involves proteolytic processing between domains, followed by association of the large and small subunits to form a heterodimer with two QACXG active sites (Shi, 2002; Fan et al. 2005).

Apoptosis and caspases have been widely investigated in many animal species including *Drosophila*, mammals, and humans; however, information on both the apoptotic process and the enzymes involved are scarce in fish. The caspase-3 gene has been sequenced and characterized in zebrafish (*Danio rerio*) (Yabu et al. 2001; Chakraborty et al. 2006; Eimon and Ashkenazi, 2010), sea bass (*Dicentrarchus labrax L.*) (Reis et al. 2007) and gudgeon (*Gobio gobio*) (Nadzialek et al. 2010), though sequences are available in the databases for other teleostei species, such as trout (*Oncorhynchus mykiss*), salmon (*Salmo salar*), seabream (*Sparus auratus*), Japanese medaka *(Oryzias latipes)*, channel catfish (*Ictalurus punctatus*), mandarin fish (*Siniperca chuatsi*) and tiger puffer (*Takifugu rubripes*).

In a previous study we demonstrated that *Piscirickettsia salmonis*, which is the etiologic agent of a systemic infection named salmonid rickettsial septicaemia (SRS), disease that affects all cultured salmon fish species and salmon production in Chile, induces apoptosis in macrophages and monocyte-like cells from the rainbow trout cell line RTS11, as shown by the morphological and biochemical features of the apoptosis (Rojas et al. 2010). Numerous experiments with a variety of pathogens demonstrate that apoptosis of professional phagocytes is a common event in pathogenesis and plays a pivotal role in the initiation of the infection and the survival of the pathogens, resulting in a decrease in the effectiveness of the immune response and a further spreading of the pathogens to other tissues (Gao and Kwaik, 2000; Navarre and Zychlinsky, 2000; Hasnain et al. 2003; Lai and Sjöstedt, 2003; DeLeo, 2004; Zhang and Bliska, 2005; Carrero and Unanue, 2006; Miyairi and Byrne, 2006). More recently, studies by Soto et al. 2010 demonstrated apoptosis in head kidney macrophages from tilapia (*Oreochromis* spp.) infected with *Francisella asiatica*, which caused substantial mortality in this and other important warm and cold water species.

Compared to other vertebrates, fish caspase-3 share similarities in sequence organization, conserved pentapeptide active-site QACRG and substrate specificity; nevertheless, phylogenetic analysis shows that this corresponds to a divergent evolving group (Yabu et al. 2001; Reis el al. 2007). There are few reports where fish caspase-3 has been detected with commercially available antibodies for mammals; the activation of this enzyme has been described in *Chinook salmon* derived CHSE-214 cells infected with the infectious pancreatic virus (IPNV) using an anti-mouse caspase-3 monoclonal antibody (Amersham, Piscataway, NJ, USA) (Chen et al. 2010). In a transgenic zebrafish cell line incubated with metrodinazole, an anti-activated caspase-3 for human and mouse (BD Biosciences 559565) detected mainly pro-caspase and active-caspase-3 but also other bands with less intensity in a western blotting assay (Chen et al. 2011).

We have previously assayed several commercial antibodies for detection of active caspase-3 in *P. salmonis* infected RTS11 cells by western blot. Our analysis with polyclonal antibodies for human anti-activated caspase-3 and anti-subunits and precursor of caspase-3 (BD Pharmingen 551150, 552785), and also with polyclonal antibody for human caspase-3 (Santa Cruz Biotechnology, Inc sc-7148), resulted in nonspecific recognition of multiple bands of different molecular weights.

In this work we present a monospecific polyclonal antibody designed to recognize an epitope which includes the active site from salmonid caspase-3. The specificity and versatility of the antibody was shown by dot blot, ELISA, western blot and immunocytochemistry and its effectiveness was validated in rainbow trout RTS11 cells induced to apoptosis by *P. salmonis.* This antibody constitutes a valuable

tool to study the molecular mechanisms that regulate cell suicide in fundamental issues such as embryogenesis, tissue homeostasis, disease development and cell-pathogen interactions in salmonids where commercial antibodies to other species usually give very poor results.

MATERIALS AND METHODS

Peptide design

Peptides were designed taking into account the caspase-3 homologous sequence of *Oncorhynchus mykiss* and *Salmo salar* available at the time (GenBank CBY85134 and NM001139921). An alignment with ClustalW (Larkin et al. 2007) was performed with different fish caspase-3 sequences incorporating the conserved motif QACRG included in the active site. To define the best antigenic epitope, the method of Kolaskar and Tongaonkar (Kolaskar and Tongaonkar, 1990) was used in the bioinformatics server from the Immunomedicine group at the Universidad Complutense de Madrid, Spain (http://imed.med.ucm.es/Tools/antigenic.pl). Protscale (Gasteiger et al. 2005) through the Expasy server (Gasteiger et al. 2003) was used to analyze the physicochemical behaviour of the antigenic sequences and identify regions of high hydrophilicity and mean flexibility. The antigenic region in the entire molecule was located using a homology model (pdb 2J30) from the automatic modelling mode in the workspace of the Swissmodel server (Arnold et al. 2006).

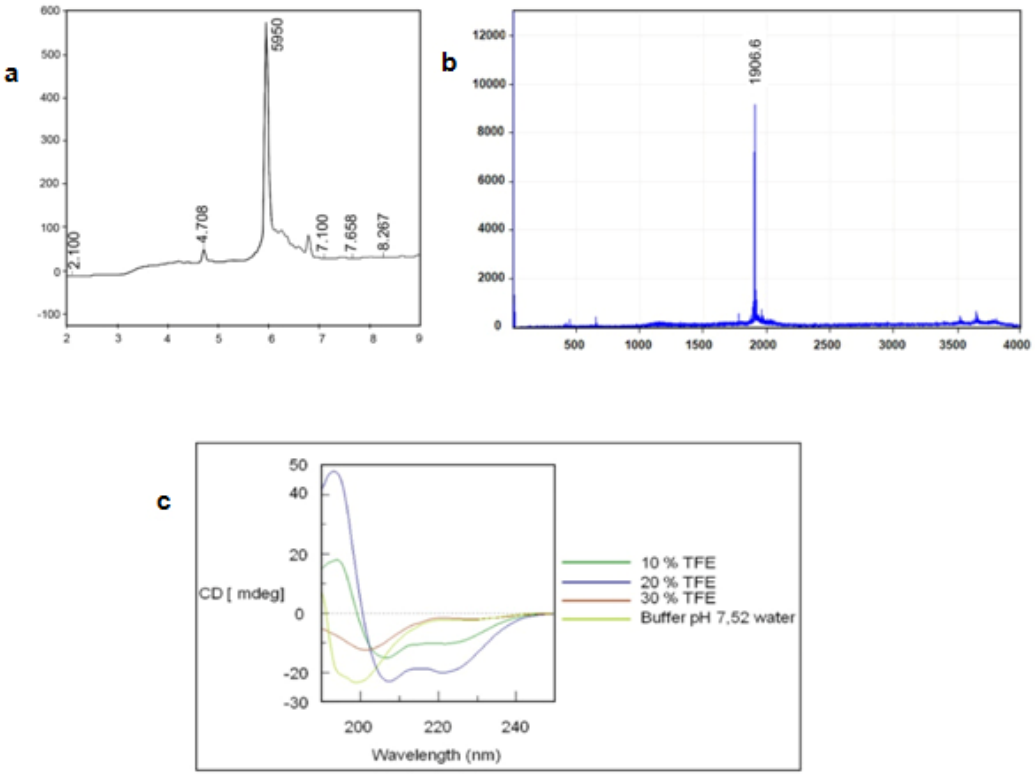

Fig. 1 Peptide characterization. (a) RP-HPLC profile of caspase-3 peptide. (b) Mass spectrometry analysis of caspase-3 peptide. (c) Caspase-3 peptide circular dichroism spectra recorded in the 190-250 nm wavelength interval. Curves show typical α-helix behaviour.

Peptide microanalysis and synthesis

Peptides were chemically synthesized using a solid phase multiple peptide system (Houghten, 1985) with *N*-fluorenylmethoxycarbonyl (Fmoc) protected amino acids (Iris and Rink resin 0.65 meq/g).

Peptides were cleaved by TFA/TIS/H_20 (95%/2.5%/2.5%) (Novabiochem, USA) and purified by reverse-phase HPLC (RP-HPLC) to purity to >95% using a Waters C18 column (0-70% gradient of aqueous acetonitrile); the elution was monitored by UV absorption at 210 nm. Peptides were lyophilized on a Jasco Instrument (Pump PU-2089, Detector UV-2075, Autosampler AS-2055 Jasco, Japan). Mass spectrometry was performed by matrix-assisted laser desorption ionization time of flight on a MALDI-TOF Microflex instrument (Bruker Daltonics INC. MA-USA). Additionally, CD spectra were recorded at room temperature on a Jasco J-810 spectropolarimeter (Jasco, Japan) using a Peltier system PFD-425S. Solutions of the peptide at 0.1 mg/ml in different percentages of 2,2,2-trifluoroethanol (TFE) were used (Roccatano et al. 2002). The recorded spectra are the average of three consecutive scans from 190 to 250 nm using a 1 mm quartz cell. The parameters used were: scan speed 100 nm*min^{-1} data pitch 0.2 nm, 2 sec response, 1 nm band width and 3 scans.

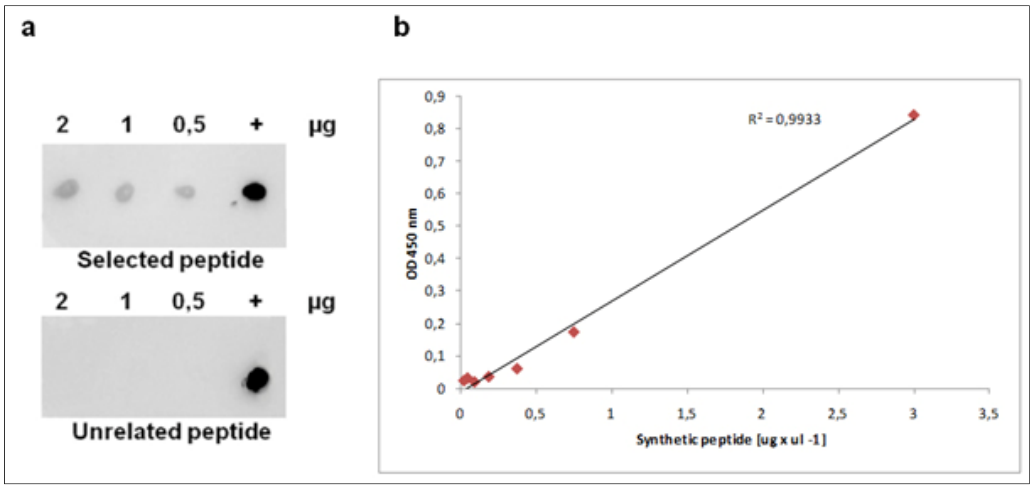

Fig. 2 The selected epitope peptide and its recognition by the developed antibody. (a) Dot Blot shows positive recognition of the selected peptide by ascitic fluid containing the anti-epitope of caspase-3. (b) Linearity of ELISA shows a good correlation between synthetic peptide using IgG purified against the epitope of caspase-3.

Antibody production

To generate the antibodies, CF-1 mice obtained from the Institute of Public Health, Santiago, Chile, were immunized at day 0, 7, 14, 21 by intraperitoneal injection with 60 µg of the epitope peptides and FIS (a T helper cell activator) in 1:1 relation (Prieto et al. 1995) supplemented with 500 µl of Freunds complete adjuvant (Thermo Scientific Inc., USA). On day 6, 0.6 ml of pristane (2,6,10,14-tetramethylpentadecane) (Sigma-Aldrich, MO, USA.) was injected to induce ascitic tumour formation (Narvaez et al. 2010; Bethke et al. 2012). From day 30, mice were anesthetized with a small dose of chloroform and drained with an 18G * 1½" needle (Nipro Corporation, Japan). Ascitic fluid was recovered in the supernatant after centrifugation at 300 g for 10 min and subsequently analyzed by dot blot.

Blot was performed in a 0.45 µm Nitrocellulose membrane (Thermo Scientific Inc., USA) seeding 2, 1 and 0.5 µg of the synthetic peptide and 1 µl of the ascitic fluid containing the anti-epitope of caspase-3 as a positive control. The membranes were blocked with 3% BSA in phosphate-buffered saline (PBSA) for 90 min at 37ºC; washed 5 times with PBS-Tween 0.05% (PBST) and incubated with 0.25 µg/µlof ascitic fluid anti-caspase-3 for 90 min at 37ºC. Following 3 washes the second anti-IgG mouse-HRP antibody (Thermo Scientific Inc., USA) at a ratio of 1:7,000 was added and incubated at 37ºC for 60 min. The membranes were revealed with DAB (3,3 diaminobenzidine, Sigma-Aldrich, MO, USA) and hydrogen peroxide in PBS (10 mg; 10 µl; 10 ml). As a control, we used another synthetic peptide which is not recognized by the anti-epitope of caspase-3.

Anti-caspase ascitic fluids were enriched for IgG by slowly adding 2 vol. of 60 mM sodium acetate (pH 4.0) and 0.4 vol. of caprylic acid. The supernatant was recovered after centrifugation at 10.000 x g for

10 min and IgG were purified by immunoaffinity chromatography using Cyanogen Bromide-Activated Sepharose 4B (Sigma-Aldrich, MO, USA). The IgG fraction was filtered through a 0.22 mm filter (Millipore Corporation, USA) and diluted ten times with phosphate buffered saline (PBS, pH 7.4). Then 0.2 g of gel was prepared with 1 ml of 1 mM HCl. The synthetic epitope (1 mg) was coupled with the prepared gel in a coupling buffer (0.1 M NaHCO3, 0.5 M NaCl pH 8.4). The unreacted active groups were blocked with 0.1 M Tris pH 8. Finally, the prepared mouse ascitic fluid was mixed with the Sepharose-coupled peptide. The mixture was rotated overnight at 4ºC, and afterwards the gel was washed alternating 0.1 M sodium acetate 0.5 M NaCl pH 4 and Tris HCl 0.1 M NaCl 0.5 M pH 8.3. It was then eluted with 100 mM glycine (pH 2.5) and the monospecific antibodies were collected in 1 M Tris (pH 8), labelled as anti-epitope of caspase-3 and stored at -20ºC.

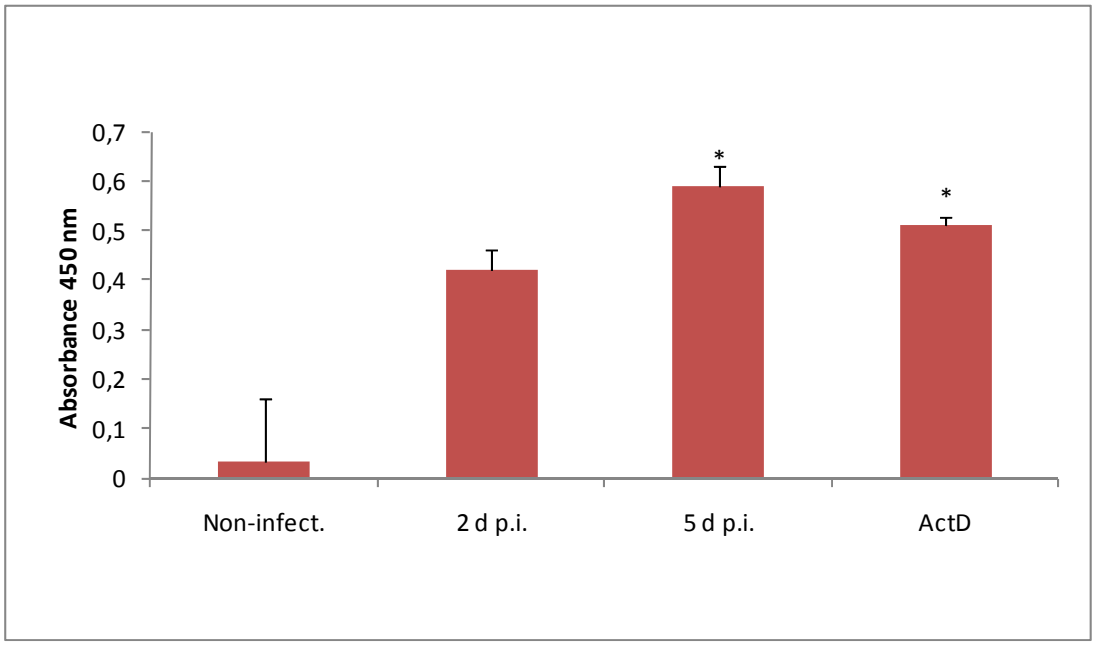

Fig. 3 ELISA analysis of caspase-3 in macrophages-likes RTS 11 cells. Lysate from non-infected, *P. salmonis*-infected from 2 and 5 days post-infection and actinomycin D treated cells were incubated with IgG purified anti-caspase-3. Active caspase-3 in *P. salmonis*-infected cells was detected as the time post-infection increased (* $p < 0.05$).

Antibody quantification and validation

IgG concentration was quantified by ELISA (Corrales et al. 2009). Briefly, multiwell plates were incubated ON at 4ºC with different concentrations of the eluted antibody. After blocking for 2 hrs with 1% BSA in PBS, they were incubated with commercial anti-IgG peroxidase-conjugated antibodies. The plates were washed three times with PBST in JENCONS Millenium 1000 Automatic Microplate Washer, subsequently 100 µl per well 3,3,5-5-tetrametilbenzidine (TMB) single solution (Invitrogen, Paisley, Scotland, UK) was added and incubated for 30 min at RT, the reaction was stopped with 50 µl of sulphuric acid 1N and read at 450 nm with a VERSAmax microplate reader (BIO-TEK Synergy HT). IgG concentration was determined using a commercial mouse IgG standard curve.

Antibody efficiency in peptide recognition was also evaluated by ELISA. Briefly, multiwell plates were incubated ON at 4ºC with serial dilutions of the peptides (from 2 ng/µl). After blocking with 1% BSA in PBS for 2 hrs at 37ºC, they were incubated for 90 min at 37ºC with 40 ng/µl of the anti-epitope of caspase-3 and for 90 min with the second antibody-HRP, at a dilution of 1:7.000. After both incubation plates were washed, they were incubated with TMB and read as described above. As a control, we used another synthetic peptide which is not recognized by the anti-epitope of caspase-3.

P. salmonis infection assays

Monolayers containing adherent macrophage-like RTS11 cells (Ganassin and Bols, 1998) were grown and infected as previously described (Rojas et al. 2009). Briefly, cells were seeded with L-15 medium supplemented with 15% fetal bovine serum (FBS) and then cultivated for 7 days to 80% confluence. Cells were washed with PBS and infected for 1 hr at 20ºC with *P. salmonis* at a multiplicity of infection (MOI) of 50. Cells were rinsed three times with PBS and incubated with fresh medium until 2 or 5 days post-infection.

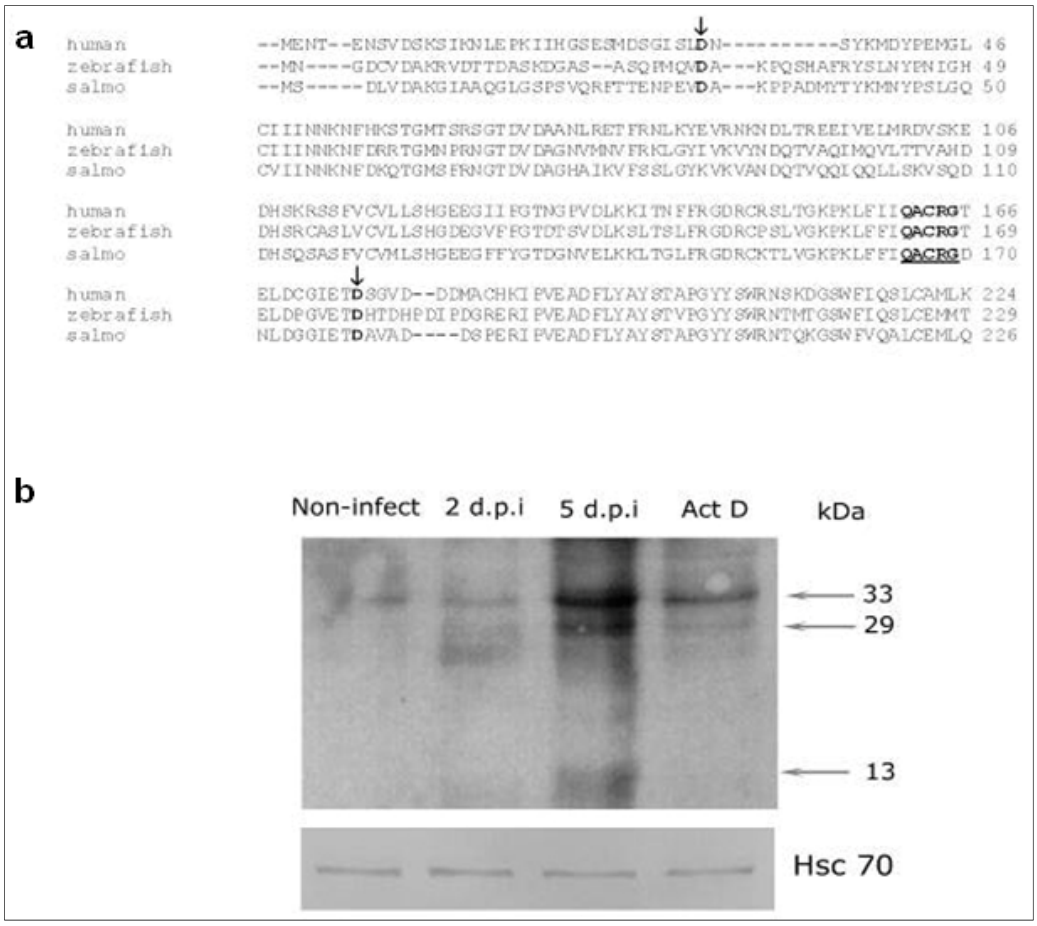

Fig. 4 Western blot analysis of caspase-3 in macrophage-like RTS 11 cells. (a) Alignment of the amino acid sequences of human, zebrafish, salmon and trout caspase-3 (GenBank NP004337, BAB32409, NP001133393 and CBY85134 respectively). Positive cleavage sites are indicated by arrows and the pentapeptide sequence QACRG at the catalytic site is highlighted. (b) Lysate from non-infected cells, *P. salmonis*-infected cells 2 and 5 days post-infection and actinomycin D treated cells were tested with IgG purified anti-caspase-3. Salmonid Hsc 70 was used as housekeeping protein.

ELISA and western blot

Monolayers containing adherent macrophage-like RTS11 cells were grown and infected as described above. Cells were lysed in buffer containing 20 mM Tris-HCl pH 8.0, 1 M NaCl, 0.05%Triton X-100, 3 mM phenylmethylsulphonylfluoride (PMSF), 5 mM EDTA, and 0.2% protease inhibitor cocktail (Sigma-Aldrich, MO, USA) and total proteins were measured by the bicinconinic acid method (BCA protein assay, Pierce, USA).

For ELISA tests, 100 µl samples (15 ng/µl) were incubated overnight at 4ºC and the plates were washed three times with 200 µl of PBST 0.05% in JENCONS Millenium 1000 Automatic Microplate Washer. Samples were blocked with 1% BSA for 4 hrs at 37ºC and incubated with 40 ng/µl of the first antibody for 90 min at 37ºC. After washing, the second antibody, anti-mouse IgG-HRP (Thermo Scientific Inc., USA) at 1:20.000 was incubated for 60 min at 37ºC. 100 µl per well of TMB single solution (Invitrogen, Paisley, Scotland, UK) was added and incubated for 30 min at RT; the reaction was stopped with 50 µl of 1N sulfuric acid and read at 450 nm with a Molecular Devices (VERSAmax) microplate reader.

For western blotting, 30 µg of total proteins from non infected, *P. salmonis* infected and actinomycin induced cells were separated by 15% SDS/PAGE and transferred to a PVDF membrane previously activated in methanol for 5 min and washed in transfer buffer (20% methanol, 0.01% SDS, Tris 25 mM; glycine 192 mM). The gel sandwich was prepared in cassette and placed in the module following the manufacturer instruction's (Mini Trans-Blot BIORAD). The transfer was performed at 100 Volts for 90 min, the PDVF membrane was blocked overnight at 4ºC with 3% in PBSA and incubated with 100 ng/µl of the anti-epitope of caspase-3 for 90 min at 37ºC. After 5 washings, a second antibody at 1:8.000 conjugated to horseradish peroxidase was added for 60 min at 37ºC. The membrane was revealed by ECL western blotting (Pierce, USA) in Bio-Max film (Kodak).

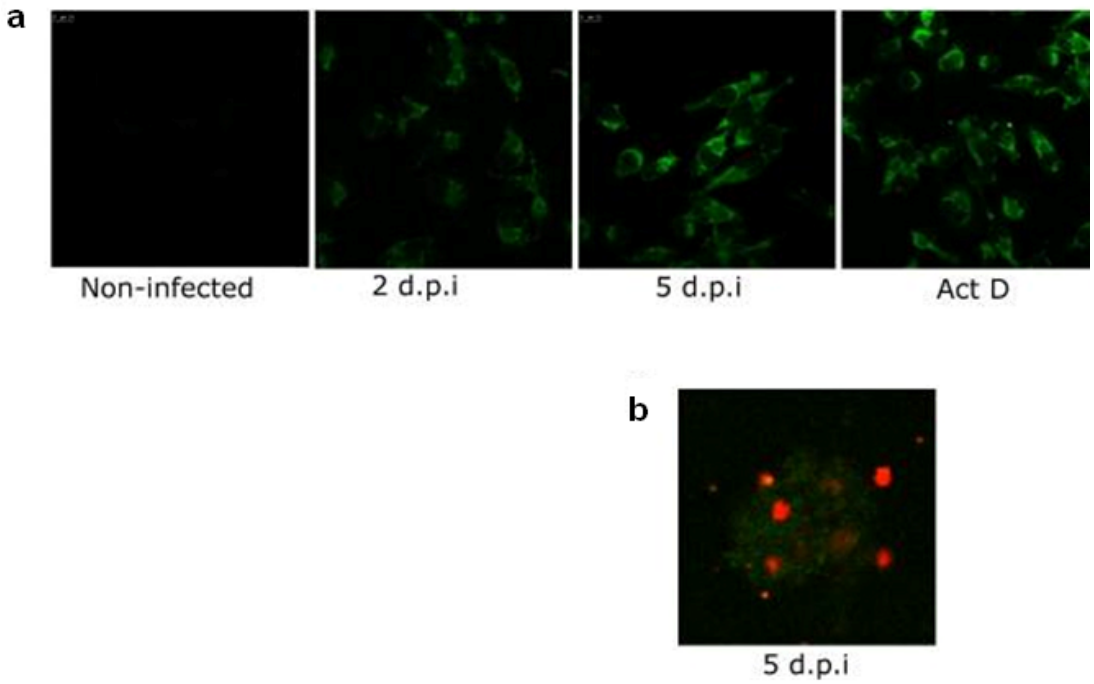

Fig. 5 Caspase-3 induction in macrophage-like RTS 11 cells. Non-infected cells, *P. salmonis*-infected cells and actinomycin D treated cells were incubated with anti-caspase-3. Representative confocal fluorescent image of active caspase-3 detected with *P. salmonis* infected cells. (a) Anti-caspase-3 antibody as the primary antibody and anti-mouse IgG-FITC as the second antibody. (b) Magnification image with those antibodies plus anti-*P. salmonis* and anti-rabbit IgG-Alexa Fluor 532. Scale bar: 25 µm.

Immunocytochemistry

Monolayers containing adherent macrophage-like RTS11 cells were grown onto poly-L-lysine coated coverslips (3.500 to 3.700 cells/coverslip). Infections were performed in triplicate using three independent wells, under conditions described above.

Control non-infected, *P. salmonis* infected and macrophages treated for 30 hrs with 2 mg/ml of the apoptotic inducer actinomycin were evaluated for caspase-3 activation by indirect immunofluorescence (Rojas et al. 2009; Rojas et al. 2010). Cells were fixed and permeabilized with cold methanol-acetic acid at 3:1 (v/v) for 15 min and washed three times in PBS. In order to detect caspase-3 the cells were incubated with 0.25 µg/µlof the caspase-3 anti-epitope and then with 1:100 (v/v) dilution of an anti-mouse secondary antibody conjugated to fluorescein isothiocyanate (FITC) (Sigma-Aldrich, MO, USA). In order to detect *P. salmonis*, cells were incubated with 1:500 (v/v) dilution of an *anti*-ChaPs proteinfrom the bacterium (Marshall et al. 2007) and subsequently labelled with 1:100 (v/v) of an anti-rabbit IgG Alexa Fluor 532-conjugated antibody (Molecular Probes). All incubations were done for 60 min at 20ºC in the dark and afterwards the incubation cells were washed three times with PBS. Cells were mounted with Vectashield mounting medium (Vector Labs., Inc., USA) and examined using a Leica Microsystems Confocal Scanning Laser microscope TCS SP5.

Statistical analyses

Caspase-3 activation in non-infected, infected and induced cells was analyzed using a one-way analysis of variance ANOVA. Differences were considered significant at a level of $p < 0.05$.

RESULTS

Peptide synthesis and identification

The *in silico* analysis of the caspase-3 primary sequences defined the peptide KTLVGKPKLFFIQACRG as the most flexible (average score near 0.5) and the less hydrophobic region (average score near 0). This peptide which included the conserved QACXG motif was chosen as the candidate epitope. The selected epitope was chemically synthesized and its high purity was confirmed by reverse-phase HPLC (RP-HPLC) (Figure 1a). The molecular mass of the peptide was determined as 1906.6 by mass spectrometry Maldi-Tof where the value is consistent with the theoretical mass (Figure 1b). In addition, to identify the secondary structure of the peptide, circular dichroism (CD) spectroscopy was used to reveal a peptide conformation with α-helix tendency (Figure 1c).

Peptide validation

Anti-caspase-3 IgG were enriched by caprylic acid methods and purified by immunoaffinity, maintaining their capacity for peptide recognition. Antibody validation was first shown at the qualitative level by dot blot testing (Figure 2a). Antibody efficiency in peptide recognition was evaluated with serial dilutions of the peptides (ng/ul) by the indirect ELISA methods (Figure 2b). In order to validate antibody specificity and to reject cross reaction, other peptides were tested with the antibody for caspase-3 without positive recognition.

ELISA analysis

Programmed cell death through apoptotic pathways is characterized by caspase activation. The presence of active caspase-3 in macrophage-like RTS11 cells infected with *P. salmonis* was detected by indirect ELISA. A significant increase ($p < 0.05$) of caspase-3 active recognition was detected in *P. salmonis*-infected cells at 5 days post-infection and in actinomycin D treated cells with respect to non-infected control cells (Figure 3).

Western blot analysis of caspase-3 activation

The western blotting analysis shows the activation of caspase-3 in *P. salmonis* infected macrophage-like RTS11 (Figure 4b). In non-infected cells the antibody recognized only the precursor form, which according to the number of amino acid residues described for trout caspase-3 (GenBank CBY85134) has an expected molecular mass of 31 kDa. In infected cells, it recognized both the pro-caspase and the processed forms; accordingly to cleavage sites in trout and salmon sequences, the large caspase-3 subunit has an expected molecular mass of 15 kDa (Figure 4a) (Yabu et al. 2001). The housekeeping protein used was salmonid Hsc 70 (Bethke et al. 2012).

Contrarily, when evaluating commercial anti-caspase-3 antibodies made against both unprocessed (BD Pharmigen 552785 and Santa Cruz Biotechnology, Inc. sc-7148) and active (BD Pharmigen 551150) form of mammal caspase-3, they not recognized the enzyme in RTS11 cells.

In vitro evaluation

We confirmed apoptosis via the presence of active caspase-3 *in situ* in macrophage-like RTS11 cells infected for 2 and 5 days with *P. salmonis* (Figure 5). Using immunocytochemistry, a positive reaction was clearly detected in infected cells but not in non-infected controls cells. Moreover, a significant increase in the number of positive macrophages to active caspase-3 was observed with the increase in days post-infection. Caspase-3 was also detected in macrophages treated with the apoptotic inductor actinomycin D. The localization of active caspase-3, with a punctuate pattern of distribution, was restricted to the cytoplasm and excluded completely from nuclei, as confirmed by confocal microscopy.

DISCUSSION

Apoptosis is a programmed cell death mechanism with the ordered dismantling of the cells showing characteristic morphologic and biochemical changes. This cell death process is evolutionarily conserved and some of its components have stayed through animal evolution, including in fish. In this case, a caspase-3 gene has been identified in zebrafish (Yabu et al. 2001; Chakraborty et al. 2006) and sea bass (Reis et al. 2007), retaining the motifs that are functionally important, such as the active site and the cleavage site at the aspartic residue; nevertheless, phylogenetic analysis shows that fish corresponds to a divergent evolving group, hindering the detection of fish caspase through commercial antibodies developed against mammalian caspases. In order to solve this problem, we have developed an antibody that allows the detection of a salmonid caspase-3.

Apoptosis is part of pathogenesis of several infectious diseases. Different viruses, bacteria and protozoa have developed a survival strategy by activating the apoptotic death of immune cells, mainly macrophages, and so evading defense mechanisms (Gao and Kwaik, 2000; Gaddy and Lyles, 2005; Hong et al. 2005; Santi et al. 2005; Carrero and Unanue, 2006; Miyairi and Byrne, 2006; Rojas et al. 2010). In this work we validated this antibody through different immunological assays using the caspase-3 activation in RTS11 apoptotic cells infected by *P. salmonis* as a model.

ELISA and western blotting analysis show active caspase-3 in *P. salmonis* infected cells. Molecular mass of precursor and subunits of trout caspase-3 are according with the amino acid residues and the cleavage sites described in GenBank. Moreover, molecular weights determined for pro-caspase and active caspase-3 in RTS11 infected cells are according with those predicted in zebrafish and sea bass. In the first species caspase-3 was cloned fused to a His tag sequence at the N-terminus, the western blotting using an anti-His-tag antibody reported the precursor form (31.5 kDa), and an active subunit of 13 kDa (Yabu et al. 2001; Chakraborty et al. 2006). In sea bass, caspase-3 shows an ORF of 281 amino acid residues with a predicted molecular mass of 31.1 kDa; in this case, a human caspase-3 antibody can recognizes only the large subunit of the activated enzyme but not the precursor form (Reis et al. 2007). Moreover, caspase-3 induction was verified by immunocytochemistry assays, there was a time dependent increase in the active form of this enzyme, which co-localized with the bacterium.

Through this antibody we are contributing with an effective tool to better understand the mechanisms of cell death in trout, and to help identifying the involvement of apoptosis in a large number of different events such as embryogenesis, tissue homeostasis, disease development and response to different infective pathogens in this salmonid fish. Moreover, this antibody could be applied for caspase-3 detection in other fish species presenting identical or high homology in the epitope sequence, like salmon or zebrafish respectively; nevertheless, is required to have an established model of apoptosis for this species.

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REFERENCES

ARNOLD, K.; BORDOLI, L.; KOPP, J. and SCHWEDE, T. (2006). The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics,* vol. 22, no. 2, p. 195-201. **[CrossRef]**

BETHKE, J.; ROJAS, V.; BERENDSEN, J.; CÁRDENAS, C.; GUZMÁN, F.; GALLARDO, J.A. and MERCADO, L. (2012). Development of a new antibody for detecting natural killer enhancing factor (NKEF)-like in infected salmonids. *Journal of Fish Diseases,* vol. 35, no. 5, p. 379-388. **[CrossRef]**

CARRERO, J. and UNANUE, E. (2006). Lymphocyte apoptosis as an immune subversion strategy of microbial pathogens. *Trends in Immunology,* vol. 27, no. 11, p. 497-503. **[CrossRef]**

CHAKRABORTY, S.; NANDI, S.; SINHA, S. and GERA, V. (2006). Zebrafish caspase-3: Molecular cloning, characterization, crystallization and phylogenetic analysis. *Protein & Peptide Letters,* vol. 13, no. 6, p. 633-640.

CHEN, P.-C.; WU, J.-L.; HER, G.M.; HER, G.M. and HONG, J.-R. (2010). Aquatic birnavirus induces necrotic cell death via the mitochondrial-mediated caspase pathway. *Fish & Shellfish Immunology,* vol. 28, no. 2, p. 344-353. **[CrossRef]**

CHEN, C.-F.; CHU, C.-Y.; CHEN, T.-H.; LEE, S.-J.; SHEN, C.-N. and HSIAO, C.-D. (2011). Establishment of a transgenic zebrafish line for superficial skin ablation and functional validation of apoptosis modulators *in vivo*. *PLoS ONE,* vol. 6, no. 5. **[CrossRef]**

CORRALES, J.; GORDON, W.L. and NOGA, E.J. (2009). Development of an ELISA for quantification of the antimicrobial peptide piscidin 4 and its application to assess stress in fish. *Fish & Shellfish Immunology,* vol. 27, no. 2, p. 154-163. **[CrossRef]**

DELEO, F.R. (2004). Modulation of phagocyte apoptosis by bacterial pathogens. *Apoptosis,* vol. 9, no. 4, p. 399-413. **[CrossRef]**

EIMON, P.M. and ASHKENAZI, A. (2010). The zebrafish as a model organism for the study of apoptosis. *Apoptosis*, vol. 15, no. 3, p. 331-349. **[CrossRef]**

ELMORE, S. (2007). Apoptosis: A review of programmed cell death. *Toxicologic Pathology,* vol. 35, no. 4, p. 495-516. **[CrossRef]**

FAN, T.J.; HAN, L.H.; CONG, R.S. and LIANG, J. (2005). Caspase family proteases and apoptosis. *Acta Biochimica Byophisica Sinica,* vol. 37, no. 11, p. 719-727. **[CrossRef]**

FINK, S.L. and COOKSON, B.T. (2005). Apoptosis, pyroptosis and necrosis: Mechanistic description of dead and dying eukaryotic cells. *Infection and Immunity,* vol. 73, no. 4, p. 1907-1916. **[CrossRef]**

GADDY, D.F. and LYLES, D. (2005). Vesicular stomatitis viruses expressing wild-type or mutant M proteins activate apoptosis through distinct pathways. *Journal of Virology,* vol. 79, no. 7, p. 4170-4179. **[CrossRef]**

GANASSIN, R.C. and BOLS, N.C. (1998). Development of a monocyte/macrophage-like cell line, RTS11, from rainbow trout spleen. *Fish & Shellfish Immunology*, vol. 8, no. 6, p. 457-476. **[CrossRef]**

GAO, L.Y. and KWAIK, Y.A. (2000). The modulation of host cell apoptosis by intracellular bacterial pathogens. *Trends in Microbiology*, vol. 8, no. 7, p. 306-313. **[CrossRef]**

GASTEIGER, E.; GATTIKER, A.; HOOGLAND, C.; IVANYI, I.; APPEL, R. and BAIROCH, A. (2003). ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Research*, vol. 31, no. 13, p. 3784-3788. **[CrossRef]**

GASTEIGER, E.; HOOGLAND, C.; GATTIKER, A.; DUVAUD, S.; WILKINS, M.; APPEL, R. and BAIROCH, A. (2005). Protein identification and analysis tools on the ExPASy server. In: WALKER, J.M. ed. *The Proteomics Protocols Handbook*, Humana Press. p. 571-607.

GUPTA, S. (2003). Molecular signaling in death receptor and mitochondrial pathways of apoptosis (Review). *International Journal of Oncology*, vol. 22, no. 1, p. 15-20.

HASNAIN, S.; BEGUM, R.; RAMAIAH, K.; SAHDEV, S.; SHAJIL, E.; TANEJA, T.; MOHAN, M.; ATHAR, M.; SAH, N. and KRISHNAVENI, M. (2003). Host-pathogen interactions during apoptosis. *Journal of Biosciences*, vol. 28, no. 3, p. 349-358.

HENGARTNER, M.O. (2000). The biochemistry of apoptosis. *Nature,* vol. 407, no. 6805, p. 770-776. **[CrossRef]**

HONG, J.R.; HUANG, L.J. and WU, J.L. (2005). Aquatic birnavirus induces apoptosis through activated caspase-8 and -3 in a zebrafish cell line. *Journal of Fish Diseases*, vol. 28, no. 3, p. 133-140. **[CrossRef]**

HOUGHTEN, R.A. (1985). General method for the rapid solid-phase synthesis of large numbers of peptides: Specificity of antigen-antibody interaction at the level of individual amino acids. *Proceedings of the National Academic of Sciences of the United States of America*, vol. 82, no. 15, p. 5131-5135.

KOLASKAR, A.S. and TONGAONKAR, P.C. (1990). A semi-empirical method for prediction of antigenic determinants on protein antigens. *FEBS Letters,* vol. 276, no. 1-2, p. 172-174. **[CrossRef]**

LAI, X-H. and SJÖSTEDT, A. (2003). Delineation of the molecular mechanisms of *Francisella tularensis*-induced apoptosis in murine macrophages. *Infection and Immunity*, vol. 71, no. 8, p. 4642-4646. [CrossRef]

LARKIN, M.A.; BLACKSHIELDS, G.; BROWN, N.P.; CHENNA, R.; MCGETTIGAN, P.A.; MCWILLIAM, H.; VALENTIN, F.; WALLACE, I.M.; WILM, A.; LOPEZ, R.; THOMPSON, J.D.; GIBSON, T.J. and HIGGINS, D.G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, vol. 23, no. 21, p. 2947-2948. [CrossRef]

MARSHALL, S.H.; CONEJEROS, P.; ZAHR, M.; OLIVARES, J.; GÓMEZ, F.; CATALDO, P. and HENRÍQUEZ, V. (2007). Immunological characterization of a bacterial protein isolated from salmonid fish naturally infected with *Piscirickettsia salmonis*. *Vaccine,* vol. 25, no. 11, p. 2095-2102. [CrossRef]

MIYAIRI, I. and BYRNE, G.I. (2006). *Chlamydia* and programmed cell death. *Current Opinion in Microbiology*, vol. 9, no. 1, p. 102-108. [CrossRef]

NADZIALEK, S.; PIGNEUR, L-M.; WÉRON, B. and KESTEMONT, P. (2010). Bcl-2 and caspase-3 mRNA levels in the testes of gudgeon, *Gobio gobio*, exposed to ethinylestradiol (EE2). *Aquatic Toxicology*, vol. 98, no. 3, p. 304-310. [CrossRef]

NARVAEZ, E.; BERENDSEN, J.; GUZMÁN, F.; GALLARDO, J.A. and MERCADO, L. (2010). An immunological method for quantifying antibacterial activity in *Salmo salar* (Linnaeus, 1758) skin mucus. *Fish & Shellfish Immunology*, vol. 28, no. 1, p. 235-239. [CrossRef]

NAVARRE, W.W. and ZYCHLINSKY, A. (2000). Pathogen-induced apoptosis of macrophages: A common end for different pathogenic strategies: Microreview. *Cellular Microbiology*, vol. 2, no. 4, p. 265-273. [CrossRef]

PRIETO, I.; HERVÁS-STUBBS, S.; GARCÍA-GRANERO, M.; BERASAIN, C.; RIEZU-BOJ, I.; LASARTE, J.; SAROBE, P.; PRIETO, J. and BORRÁS-CUESTA, F. (1995). Simple strategy to induce antibodies of distinct specificity: Application to the mapping of gp120 and inhibition of HIV-1 infectivity. *European Journal of Immunology*, vol. 25, no. 4, p. 877-883. [CrossRef]

REIS, M.I.R.; NASCIMENTO, D.S.; DO VALE, A.; SILVA, M.T. and DOS SANTOS, N.M.S. (2007). Molecular cloning and characterization of sea bass (*Dicentrarchus labrax* L.) caspase-3 gene. *Molecular Immunology*, vol. 44, no. 5, p. 774-783. [CrossRef]

ROCCATANO, D.; COLOMBO, G.; FIORONI, M. and MARK, A.E. (2002). Mechanism by which 2,2,2-trifluoroethanol/water mixtures stabilize secondary-structure formation in peptides: A molecular dynamics study. *Proceedings of the National Academic of Sciences of the United States of America,* vol. 99, no. 19, p. 12179-12184. [CrossRef]

ROJAS, V.; GALANTI, N.; BOLS, N.C. and MARSHALL, S.H. (2009). Productive infection of *Piscirickettsia salmonis* in macrophages and monocyte-like cells from rainbow trout, a possible survival strategy. *Journal of Cellular Biochemistry*, vol. 108, no. 3, p. 631-637. [CrossRef]

ROJAS, V.; GALANTI, N.; BOLS, N.C.; JIMÉNEZ, V.; PAREDES, R. and MARSHALL, S.H. (2010). *Piscirickettsia salmonis* induces apoptosis in macrophages and monocyte-like cells from rainbow trout. *Journal of Cellular Biochemistry*, vol. 110, no. 2, p. 468-476. [CrossRef]

SANTI, N.; SANDTRØ, A.; SINDRE, H.; SONG, H.; HONG, J.R.; THU, B.; WU, J.L., VAKHARIA, N. and EVENSEN, Ø. (2005). Infectious pancreatic necrosis virus induces apoptosis in vitro and in vivo independent of VP5 expression. *Virology,* vol. 342, no. 1, p. 13-25. [CrossRef]

SHI, Y. (2002). Mechanisms of caspase activation and inhibition during apoptosis. *Molecular Cell*, vol. 9, no. 3, p. 459-470. [CrossRef]

SOTO, E.; FERNANDEZ, D.; THUNE, R. and HAWKE, J.P. (2010). Interaction of *Francisella asiatica* with tilapia (*Oreochromis niloticus*) innate immunity. *Infection and Immunity*, vol. 78, no. 5, p. 2070-2078. [CrossRef]

VANDENABEELE, P.; GALLUZZI, L.; VANDEN BERGHE, T. and KROEMER, G. (2010). Molecular mechanisms of necroptosis: An ordered cellular explosion. *Nature Reviews Molecular Cell Biology*, vol. 11, no. 10, p. 700-714. [CrossRef]

YABU, T.; KISHI, S.; OKAZAKI, T. and YAMASHITA, M. (2001). Characterization of zebrafish caspase-3 and induction of apoptosis trough ceramide generation in fish fathead minnow tailbud cell and zebrafish embryo. *Biochemical Journal*, vol. 360, p. 39-47. [CrossRef]

ZHANG, J. and BLISKA, J. (2005). Role of macrophages apoptosis in the pathogenesis of *Yersinia*. In: GRIFFIN, D. ed. *Role of Apoptosis in Infection, Current Topics in Microbiology and Immunology*, Springer. vol. 289, p. 151-173. [CrossRef]

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