Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
Accessible at: www.pvj.com.pk

RESEARCH ARTICLE

Identification and Characterization of a Novel Virulence-Associated Metalloprotease from *Aeromonas hydrophila*

Dai-li Cao, Nan-nan Wang, Cheng-ping Lu and Yong-jie Liu*

College of Veterinary Medicine, Nanjing Agricultural University, Weigang 1, Nanjing 210095, China
*Corresponding author: liuyongjie@njau.edu.cn

ARTICLE HISTORY(14-040)

Received: January 22, 2014
Revised: May 29, 2014
Accepted: July 02, 2014

Key words:
Aeromonas hydrophila
Fish
Histopathology
Metalloprotease
Mice

ABSTRACT

Aeromonas hydrophila is an important fish pathogen causing hemorrhagic septicemia. Proteolytic enzymes may play a critical role in invasive infections of this bacterium. In this paper, a 426-aa protease (designated ahMP) with zinc-binding motif was identified. SDS-PAGE analysis indicated that the recombinant ahMP was 64 kDa in size. The ahMP protease was classified as a zinc-dependent metalloprotease as its activity could be inhibited by zinc specific metal and metal ion chelators. The cells inoculated with ahMP underwent progressively cytopathic changes. To further confirm the role of this protease in *A. hydrophila*, we perform challenge experiments in fish and mice. In the ahMP-injected fish, death occurred within 48 h and the death rate was up to 16.7% (5/30), while most of ahMP-injected mice (8/10) showed clinical signs although there were no deaths. The organs from ahMP-injected mice and fish showed similar histopathological changes, including extensive vascular alterations, exudative lesions and cell necrosis. Together, these results suggest that a novel *A. hydrophila* metalloprotease, ahMP, is identified and is likely an important contributor to pathogenicity of this bacterium in fish.

©2014 PVJ. All rights reserved

To Cite This Article: Cao DL, NN Wang, CP Lu and YJ Liu, 2015. Identification and characterization of a novel virulence-associated metalloprotease from *Aeromonas hydrophila*. Pak Vet J, 35(1): 38-42.

INTRODUCTION

Aeromonas hydrophila, a Gram-negative bacterium, is recognized as the etiological agent of fatal hemorrhagic septicemia and epizootic ulcerative syndrome (Huang *et al*., 2011; Janda and Abbott, 2010; Parker and Shaw, 2011; Cagatay *et al*., 2014; Cheong *et al*., 2014). Also, it causes gastrointestinal and extra intestinal infections in humans. Bacterial pathogenicity depends largely on the expression of virulence factors, which facilitate invasion and ability to bypass or overcome host defense mechanisms (Pridgeon *et al*., 2013). Studies on virulence of bacterial pathogens of fish are essential for the development of new immunoprophylactic and chemotherapeutic reagents to fight bacterial infections (Shayo *et al*., 2012). At present, a number of virulent factors have been investigated and identified in *A. hydrophila*, including pili and adhesins (Bi *et al*., 2007; Guo *et al*., 2013; Khushiramani *et al*., 2012; Wang *et al*., 2013), exotoxins (Li *et al*., 2011), and a number of exoenzymes such as proteases, lipases and amylases (Wu *et al*., 2012; Takahashi *et al*., 2013). The contribution of the proteases to the pathogenesis may be due to their abilities to overcome host defenses and provide nutrients for the bacterial cells, thus promoting invasiveness and establishment of infection (Cascón *et al*., 2000; Zacaria *et al*., 2010). Several major extracellular proteases have been described so far, including a 38-kDa thermostable metalloprotease (Cascón *et al*., 2000), a 19-kDa zinc-proteinase (Loewy *et al*., 1993), and a 22-kDa serine proteinase (Nakasone *et al*., 2004). Although these previous reports showed that the potential virulence factors play role in *A. hydrophila* pathogenicity, a number of unknown virulence factors may be involved because of complex pathogenic mechanism.

Our previous study applied an immunoproteomic technique to survey the extracellular proteins of Chinese vaccine strain *A. hydrophila* J-1 and identified an immunoreactive protein showing close similarity to the putative metalloproteases from *A. hydrophila* ATCC7966 (Ni *et al*., 2010). To investigate its exact properties, in the present study, we expressed and analyzed the protease from *A. hydrophila* J-1. The biochemical properties of the expressed protease showed that it is thermostable and Zn-dependent. And like many of the metalloprotease identified in other pathogens (Hasegawa *et al*., 2008; Sussman *et al*., 2009; Zhang *et al*., 2009), ahMP is a virulence factor of *A. hydrophila* that is involved in tissue damages.

MATERIALS AND METHODS

Bacterial strains and culture: Chinese vaccine strain *A. hydrophila* J-1 (CGMCC 3220) was used in this study. *A. hydrophila* J-1 and *Escherichia coli* were cultured in Luria-Bertani (LB) medium at 28 and 37°C, respectively.

Cloning and expression of ahMP: Oligonucleotides ahMP-F (5'–GCGGAATTCGAAACCATCGACCTGAT-3') and ahMP-R (5'-CCGAAGCTTATCAGGCCGG CTGCGTCT-3') were designed according to sequence information available for *A. hydrophila*ATCC7966 (accession number AHA_0616). The *ahMP* gene from *A. hydrophila* J-1 was cloned into pET28a expression vector and transformed into *E. coli* BL21 competent cells (TaKaRa, China). Expression of the recombinant protein was confirmed on 12% SDS-PAGE. The recombinant protein was refolded using a linear 4.0-0.0 M urea gradient in PBS (pH 7.4) after purified using HisTrap^{TM}HP (GE Healthcare, USA).

Protease activity: Briefly, 10 µl of purified ahMP was added to 100 µl of azocasein (5 mg/ml) in 50 mMTris-HCl buffer (pH 8.0), and then incubated at 37°C for 2 h. Thereafter, 500 µl of 10% trichloroacetic acid was added and kept on ice for 30 min. After centrifugation at 12,000 rpm for 10 min, 500 µl of the supernatants were added to 500 µl of 1 MNaOH. Then the absorbance of mixture was measured at 442 nm. One unit of activity was defined as the amount of enzyme required to cause one unit increase in absorbance under the experimental conditions.

Characterization of the protease: The optimum temperature for protease activity was determined at a range of 4-100ºC. The effect of metal ions was determined using phenylmethylsulfonyl fluoride (PMSF), EDTA, 1,10-phenanthroline (OPA) and SDS. The purified protease was preincubated at 37ºC for 30 min with each chemical, followed by residual protease activity assay. The influence of various metal ions (Ca^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+} and Fe^{2+}) was investigated by adding them to the reaction mixture at a final concentration of 10 mM.

Cytotoxic effect of ahMP: Briefly, the epithelial papilloma of carp (EPC) cells was cultured to confluence in 24-well plates at 28°C. Purified ahMP was added to EPC monolayers at a concentration of 2 mg/ml. After incubation for 12 h, 24 h and 36 h, respectively, the cells were used either for the determination of viability using CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, China) or for morphological observation under an inverted microscope (Nikon).

Challenge assay in mice: ICR mice (6 weeks) were purchased from the Animal Experiment Center of Yangzhou University. They were randomly divided into two groups (n= 10 per group). In challenge group, individual mice were injected intraperitoneally (i.p.) with 200 µl of purified metalloprotease (2 mg/ml). Controls were injected with sterile PBS. Mortalities were recorded daily for 1 week. At 48 h after injection, blood samples were obtained for routine hematology, while the tissues including lung, heart, spleen and kidney were collected from mice euthanized humanely for histopathological examination.

Challenge assay in fish: Chinese breams weighing approximately 25 g were obtained from the Freshwater Fisheries Research Center and acclimated in a concrete pool for 15 days at 20°C. Then they were randomly divided into two groups (n= 30 per group). In challenge group, fish were injected i.p. with 200 µl of purified metalloprotease (1 mg/ml). Controls were injected with sterile PBS. Mortalities were recorded daily for 1 week.

Statistical analysis: All statistical analyses were performed with statistical package (SAS 9.0, USA). Repeated measures ANOVA models were used to test the statistical significance between two groups.

RESULTS

Cloning and sequencing of *A. hydrophila* ahMP gene: The putative metalloprotease gene was amplified from *A. hydrophila*J-1 genome by PCR. Then its sequence was submitted to GenBank with the accession number KF147981. The ahMP protein, consisting of 426 amino acids with 41 basic amino acids, 51 acidic amino acids, 129 hydrophobic amino acids and 134 hydrophobic amino acids, is encoded by an ORF of 1266 nucleotides. Further analysis showed that this domain contains a metallo-proteases signature motif (HEXXHXUGUXHU, where X is any amino acid, coordinates zinc and contains catalytic Glu).

Expression and purification of ahMP protein: The *ahMP* gene was cloned into the pET-32a and transformed into *E.coli* BL21. After induced by IPTG, most metalloprotease were in inclusion body form (Fig. 1). The dissolved inclusion body was further purified by Ni-NTA column. The molecular weight of the recombinant enzyme was approximately 64 kDa as estimated by SDS-PAGE, which agreed well with the calculated one.

Characterization of the protease: The enzyme retained its activity over a wide temperature range between 4ºC and 70ºC, with the optimum temperature of 50ºC (Fig. 2). The effects of various chemicals were shown in Table 1. SDS (5%) strongly inhibited enzyme activity whilst zinc specific metal chelator 1,10-phenanthroline and EDTA reduced the enzyme activity more than half of the activity. The activity of ahMP was uninhibited by serine protease inhibitor PMSF. The addition of Mg^{2+}, Ca^{2+}, Cu^{2+} and Fe^{2+} did not significantly affect enzyme activity. These results suggest that ahMP is a zinc dependent metallo-protease.

Cytotoxic effect on EPC: The cells inoculated with ahMP underwent progressive morphological changes. At 12 h after inoculation, the cells became rounded and about 75% of the cells remained attached (Fig. 3A). At 24 h, approximately 25% of the rounded EPC cells remained (Fig. 3B). By 36 h post infection, all the rounded EPC cells detached from each other and also from the well (Fig. 3C), while EPC cells inoculated with PBS showed no marked changes and adhered tightly to their neighbors (Fig. 3D).

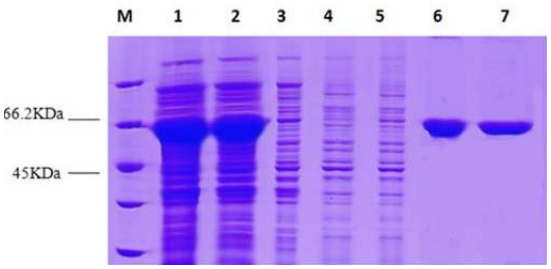

Fig. 1: SDS-PAGE analyses of ahMP expression. Lane M, molecular weight marker; lane 1 and lane 2, pET32a-ahMP in IPTG-induced *E. coli* BL21; lane 3, pET32a in IPTG-induced *E. coli* BL21; lane 4 and lane 5, purified supernatant proteins; lane 6 and lane 7, purified precipitate proteins of ahMP.

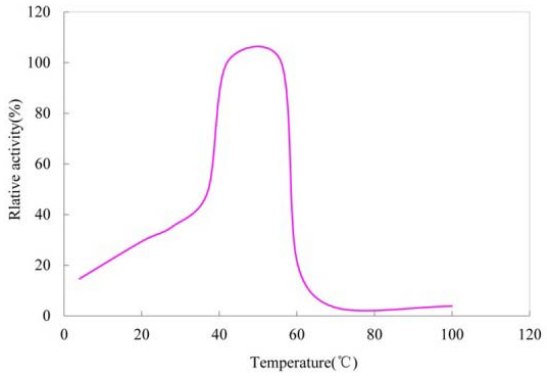

Fig. 2: Effect of temperatures on ahMP activity. Enzyme reaction was performed in 100 ml of 50 mMTris–HCl (pH 8.0) containing 5 mg/ml of azocasein at varying temperatures. The enzyme activity at 50°C was defined as 100%.

In order to determine the extent to which ahMP could be cytotoxic to EPC, we used the lactate dehydrogenase (LDH) release test to measure cytotoxicity levels. The ahMP protein was found to be cytotoxic to EPC in a time-dependent manner. At 12 h, ahMP showed lower toxicity to the cells with the level of 5.78%. However, the cytotoxicity levels (25.46%) at 24 h were significantly higher ($P<0.01$). In particular, the level of cytotoxicity was up to 65.71% at 36 h.

Challenge assay in mice: At 24 hours after injection with the purified ahMP, the mice (8/10) manifested lethargy and less activity, and the clinical signs lasted for 24 hours. Then the mice showed tolerance and no mice were dead.

The hematological analysis showed a significant difference in the number of white blood cells (WBCs) between ahMP-injected mice and controls, whereas no significant differences were found in the other parameter values (Table 2).

All tissues collected from the injected mice with ahMP showed significant pathological changes. In the heart, the fiber of cardiac muscle underwent atrophy with spatium intermusculare thickened. Also, significant hemorrhage was found in cardiac muscles (Fig. 4A). In the lung, alveolar septa became thickened or ruptured. Alveolar capillaries became distended and were filled with red blood cells (Fig. 4B). Some adjacent alveoli fused to form a large lumen. In bronchioles and adjacent alveoli, epithelial cell regeneration was accompanied by fibrovascular tissue proliferation and inflammatory cell

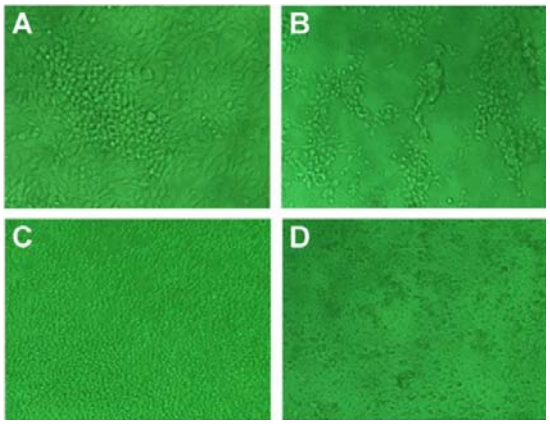

Fig. 3: Micrographs of EPC cells. (A) EPC cells incubated with purified ahMP for 12 h. (B) EPC cells incubated with purified ahMP for 24 h. (C) EPC cells incubated with purified ahMP for 36 h. (D) EPC cells incubated with PBS for 36 h.

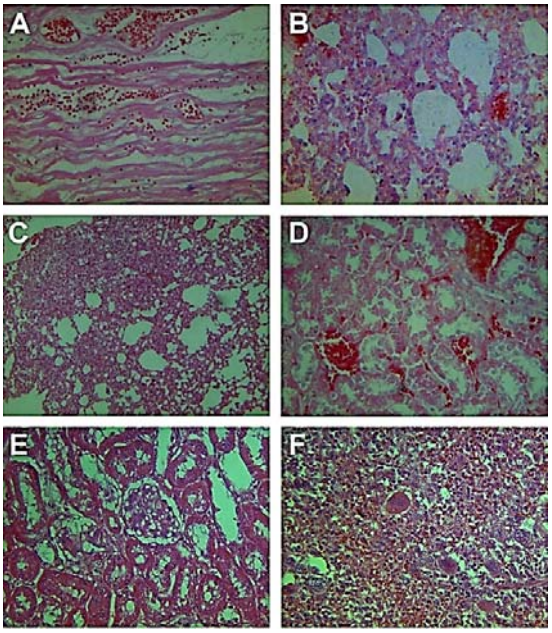

Fig. 4: Histopathologic appearances of H&E-stained heart, lung, kidney and spleen of mice injected with purified metalloprotease (400 ×). (A) Heart, showing hemorrhage in cardiac muscles and atrophy of muscle cells. (B) Lung, showing alveolar capillary filled with erythrocytes and thickened alveolar septum. (C) Lung, showing epithelial cell regeneration, fibrovascular tissue proliferation, and inflammatory cell infiltration. (D) Kidney, showing a large number of erythrocytes accumulated between renal tubules. (E) Kidney, showing many vacuoles in the cytoplasm of convoluted tubular epithelia. (F) Polynuclear macrophage in the red pulp of the spleen.

infiltration (Fig. 4C). Configurations of the kidney tubules and collecting ducts almost disappeared. Epithelia of renal tubules showed degeneration and necrosis. A large number of erythrocytes are accumulated between renal tubules (Fig. 4D). Besides, the cytoplasmas of convoluted tubular epithelia were filled with many vacuoles of varying sizes (Fig. 4E). A lot of polynuclear macrophages were seen in the red pulp of the spleen (Fig. 4F).

Challenge assay in fish: The breams inoculated with ahMP showed a higher death rate (5/30) within 48 h, but there were no death records after 72 h. Most dying fish

Table 1: Effect of metal ions and inhibitors on ahMP activity

Chemicals	Concentration	Residual activity (%)
control	–	100
EDTA	10 mM	43.93
SDS	5% (v/v)	31.75
PMSF	10 mM	113.3
1,10-phenanthroline	10 mM	47.62
Zn^{2+}	10 mM	46.07
Mg^{2+}	10 mM	84.20
Cu^{2+}	10 mM	85.52
Fe^{2+}	10 mM	93.44
Ca^{2+}	10 mM	95.08

Table 2: Changes of hematological parameters in the mice after injected with the purified ahMP protein

Hematological parameters	ahMP-injected mice	PBS-injected mice
Red blood cells (10^6/μl)	10.42±0.62	10.03±0.78
Hematocrit (%)	52.42±3.00	50.73±4.27
MCV (fl)	50.33±0.52	50.67±0.82
Hemoglobin (g/dl)	29.18±0.43	29.37±0.31
RDW (%)	15.85±0.34	15.90±0.54
White blood cells (10^3/μl)	6.07±1.89**	16.15±9.05
Platelet number (10^3/μl)	345.00±55.00	325.2±83.89

**Represent a significant difference between the ahMP-injected mice and control (P<0.01). MCV: mean corpuscular volume; RDW: red blood cell volume distribution width.

presented the clinical sign of hemorrhage. Further, we performed the histopathological examination for the breams which have survived from ahMP inoculation. Our result showed that the percentages of breams presenting the histological alterations were 28% (7/25) and 0 (0/30) after injected with ahMP and PBS buffer, respectively. Major histological changes were found in head kidney, heart and liver, including hemorrhage and exudative lesions (data not shown).

DISCUSSION

In the present work, we describe the cloning, expression and characterization of ahMP, a novel metalloprotease from *A. hydrophila* strain J-1. The inhibition study showed that the proteolytic activity of recombinant ahMP was inhibited by metal ion chelators, such as EDTA and 1,10-phenanthroline, also by SDS. In contrast, the serine protease inhibitor PMSF did not affect the proteolytic activity of ahMP. Besides, the present study also showed that ahMP was most likely a zinc-dependent metalloprotease as it was still inhibited by zinc specific metal. It has been reported that most metallo-proteases were zinc-dependent proteins (Häse and Finkelstein, 1993). The primary sequence motif HEXXH was found in many zinc-containing proteases, and was suggested to be indicative of membership in the family (Jongeneel *et al*., 1989). The zinc-binding motif, HEXXH is present in the deduced amino acid sequence of ahMP, which further demonstrates that ahMP is a zinc metallo-protease.

Extracellular metalloproteases are widely distributed in the bacterial world. In pathogenic bacteria, these enzymes are involved in colonization and invasion during host-pathogen interaction, apart from providing nutrients for the microorganism. Some metalloproteases have been considered to play a key role in pathogenesis. Bjornsdottir *et al*. (2009) suggested that the metallopeptidase MvP1 of *Moritella viscosa* could cause extensive tissue necrosis and hemorrhages at the site of injection, and thus aid in the invasion and dissemination of this bacterium in the host by causing tissue destruction. Yang *et al*. (2007) reported that an extracellular zinc metalloprotease, EmpA, is a putative virulence factor of *Listonella anguillarum* (formerly *Vibrio anguillarum*). However, the metallo-protease Vvp of *Vibrio vulnificus* is not an essential lesion factor, since a Vvp-defective mutant exhibited similar lesions to those caused by the wild type strain in eels (Valiente *et al*., 2008). Another study about *Vibrio* (Labreuche *et al*., 2010) showed the zinc metalloprotease impairs the host cellular immune defense, which suggested that the metalloprotease indeed related to the pathogenicity.

To determine the pathological actions of the ahMP protein, we perform animal experiment in two host models, mice and fish. Most of ahMP-injected mice showed clinical signs although there were no deaths. The ahMP protein could have a more harmful effect on fish and even be lethal to the host. In the ahMP-injected fish, death occurred within 48 h and the death rate was up to 16.7% (5/30). The organs from ahMP-injected mice and fish showed similar histopathological changes, including extensive vascular alterations, exudative lesions and cell necrosis. The histopathologic presentations were in agreement with previous reports in *A. hydrophila*-infected animals (Angka, 1990; Rey *et al*., 2009). The organ most severely affected in fish was the head kidney. This finding is in accordance with that from the earlier investigation in the eels injected with *V. vulnificus* extracellular products (ECPs) (Valiente *et al*., 2008). The head kidney serves as a principal immune organ in fish, which is responsible for phagocytosis, antigen processing, formation of IgM and immune memory (Tort *et al*., 2003). This organ showed severe necrosis after ahMP injection, which could explain why the ahMP-injected fish died so quickly.

The hematological analysis in the ahMP-injected mice showed that the metalloprotease ahMP did not cause apparent changes in the blood parameters indicative of anemia: hemoglobin levels, hematocrit and number of red blood cells. However, it should be noted that there was a significant decrease in number of WBCs. The obvious alternation caused by ahMP may be a novel mechanism contributing to *A. hydrophila* pathogenicity. Further studies are required to evaluate this hypothesis.

Conclusion: This study has focused on identifying a novel metalloprotease. Based on sequence and characteristic analyses, the protease is classified into a family of zinc metalloproteases. Moreover, it has been clearly shown that the metalloprotease (ahMP) acts as one of the critical virulence factors in the pathogenicity of *A. hydrophila.* Hopefully, this study will lead to a deeper understanding of the virulence of this bacterium.

Authors' contributions: Liu YJ, Cao DL and Lu CP conceived and designed the review. Cao DL and Wang NN executed the experiment and analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

Acknowledgment: This work was supported by National Nature Science Foundation (31072151, 31372454) and

Aquatic Three New Projects in Jiangsu Province (D2013-5-4).

REFERENCES

Angka SL, 1990. The pathology of the walking catfish, *Clarias batrachus* (L.), infected intraperitoneally with *Aeromonas hydrophila*. Asian Fish Sci, 3: 343-351.

Bi ZX, YJ Liu and CP Lu, 2007. Contribution of AhyR to virulence of *Aeromonas hydrophila* J-1. Res Vet Sci, 83: 150-156.

Bjornsdottir B, OH Fridjonsson, S Magnusdottir, V Andresdottir, GO Hreggvidsson and BK Gudmundsdottir, 2009. Characterisation of an extracellular vibriolysin of the fish pathogen *Moritella viscosa*. Vet Microbiol, 136: 326-334.

Cascón A, J Yugueros, A Temprano, M Sánchez, C Hemanz, JM Lunego and G Naharro, 2000. A major secreted elastase is essential for pathogenicity of *Aeromonas hydrophila*. Infect Immun, 68: 3233-3241.

Cagatay, T Ifakat, Sen and E Beyhan, 2014. Detection of pathogenic aeromonas hydrophila from rainbow trout (*Oncorhynchus mykiss*) farms in Turkey. Int J Agric Biol, 16: 435-438.

Cheong JY, AAN Azwady, G Rusea, UA Noormasshela, AGN Shaziera, AA Azleen and M Muskhazli, 2014. The availability of astaxanthin from shrimp shell wastes through microbial fermentations, *Aeromonas hydrophila* and cell disruptions. Int J Agric Biol, 16: 277-284.

Guo SL, Y Wang, RZ Guan, JJ Feng, QH Yang, PP Lu, LL Hu and JP Zhao, 2013. Immune effects of a bivalent expressed outer membrane protein to American eels (*Anguilla rostrota*). Fish Shellfish Immun, 35: 213-220.

Häse CC and RA Finkelstein, 1993. Bacterial extracellular zinc-containing metalloproteases. Microbiol Mol Biol Rev, 57: 823-837.

Hasegawa H, EJ Lind, MA Boin and CC Häse, 2008. The extracellular metalloprotease of *Vibrio tubiashii* is a major virulence factor for Pacific oyster (*Crassostrea gigas*) larvae. Appl Environ Microbiol, 74: 4101-4110.

Huang KF, MH Hung, YS Lin, CL Lu, C Liu, CC Chen, YH Lee, 2011. Independent predictors of mortality for necrotizing fasciitis: a retrospective analysis in a single institution. J Trauma, 71: 467-473.

Janda JM and SL Abbott, 2010. The genus *Aeromonas*: taxonomy, pathogenicity and infection. Clin Microbiol Rev, 23: 35-73.

Jongeneel CV, J Bouvier and A Bairoch, 1989. A unique signature identifies a family of zinc-dependent metallopeptidases. FEBS Lett, 242: 211-214.

Khushiramani RM, B Maiti, M Shekar, SK Girisha, N Akash, A Deepanjali, I Karunasagar and I Karunasagar, 2012. Recombinant *Aeromonas hydrophila* outer membrane protein 48 (Omp48) induces a protective immune response against *Aeromonas hydrophila* and *Edwardsiella tarda*. Res Microbiol, 163: 286-291.

Labreuche Y, F Le Roux, J Henry, C Zatylny, A Huvet, C Lambert, P Soudant, D Mazel and JL Nicolas, 2010. *Vibrio aestuarianus* zinc metalloprotease causes lethality in the Pacific oyster *Crassostrea gigas* and impairs the host cellular immune defenses. Fish Shellfish Immun, 29: 753-758.

Li J, XD Ni, YJ Liu and CP Lu, 2011. Detection of three virulence genes *alt*, *ahp* and *aerA* in *Aeromonas hydrophila* and their relationship with actual virulence to zebrafish. J Appl Microbiol, 110: 823-830.

Loewy AG, UV Santer, M Wieczorek, JK Blodgett, SW Jones and JC Cheronis, 1993. Purification and characterization of a novel zinc-proteinase of *Aeromonas hydrophila*. J Biol Chem, 268: 9071-9078.

Nakasone N, C Toma, T Song and M Iwanaga, 2004. Purification and characterization of a novel metalloprotease isolated from *Aeromonascaviae*. FEMS Microbiol Lett, 237: 127-132.

Ni XD, N Wang, YJ Liu and CP Lu, 2010. Immunoproteomics of extracellular proteins of *Aeromonas hydrophila* China vaccine strain J-1 reveal a highly immunoreactive outer membrane protein. FEMS Immunol Med Microbiol, 58: 363-373.

Parker JL and JG Shaw, 2011. *Aeromonas* spp. clinical microbiology and disease. J Infect, 62: 109-118.

Pridgeon JW, PH Klesius, L Song, D Zhang, K Kojima and JA Mobley, 2013. Identification, virulence and mass spectrometry of toxic ECP fractions of West Alabama isolates of *Aeromonas hydrophila* obtained from a 2010 disease outbreak. Vet Microbiol, 164: 336-343.

Rey A, N Verján, HW Ferguson and C Iregui, 2009. Pathogenesis of *Aeromonas hydrophila* strain KJ99 infection and its extracellular products in two species of fish. Vet Rec, 164: 493-499.

Shayo SD, CJ Mwita and KM Hosea, 2012. Virulence of *Pseudomonas* and *Aeromonas* bacteria recovered from *Oreochromis niloticus* (Perege) from Mtera hydropower Dam; Tanzania. Ann Bio Res, 3: 5157-5161.

Sussman M, JC Mieog, J Doyle, S Victor, BL Willis and DG Bourne, 2009. *Vibrio* zinc metalloprotease causes photoin activation of coral endosymbionts and coral tissue lesions. PloS One, 4: e4511.

Takahashi E, H Kobayashi , H Yamanaka, M Nakanishi, A Tateishi, T Abe, S Arimoto, T Negishi and K Okamoto, 2013. Analysis of carboxy terminal domain of metalloprotease of elastolytic *Aeromonas hydrophila*. Biol Pharm Bull, 36: 1174-1182.

Tort L, JC Balasch and Mackenzie S, 2003. Fish immune system. A crossroads between innate and adaptive responses. Immunología, 22: 277-286.

Valiente E, F Padrós, J Lamas, A Llorens and C Amaro, 2008. Microbial and histopathological study of the vibriosis caused by *Vibrio vulnificus* serovar E in eels: the metalloprotease Vvp is not an essential lesional factor. Microb Pathog, 45: 386-393.

Wang N, Z Yang, MF Zang, YJ Liu and CP Lu, 2013. Identification of Omp38 by immunoproteomic analysis and evaluation as a potential vaccine antigen against *Aeromonas hydrophila* in Chinese breams. Fish Shellfish Immun, 34: 74-81.

Wu L, YN Jiang, Q Tang, HX Lin, CP Lu and HC Yao, 2012. Development of an *Aeromonas hydrophila* recombinant extracellular protease vaccine. Microb Pathog, 53: 183-188.

Yang H, J Chen, G Yang, XH Zhang and Y Li, 2007. Mutational analysis of the zinc metalloprotease EmpA of *Vibrio anguillarum*. FEMS Microbiol Lett, 267: 56-63.

Zacaria J, AP Delamare, SO Costa and S Echeverrigaray, 2010. Diversity of extracellular proteases among *Aeromonas* determined by zymogram analysis. J Appl Microbiol, 109:212-219.

Zhang WW, YH Hu, HL Wang and L Sun, 2009. Identification and characterization of a virulence-associated protease from a pathogenic *Pseudomonas fluorescens* strain. Vet Microbiol, 139: 183-188.