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DETECTION OF AEROLYSIN (*aerA*) GENE IN *AEROMONAS HYDROPHILA* STRAINS ISOLATED FROM DISEASED CARP

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Abstract

Bacterial septicemia caused by motile aeromonads is common infection in the intensive fish production. *Aeromonas (A.) hydrophila* is often present in fish populations. Ubiquitous distribution of these bacteria in the aquatic environment, and the stress caused by intensive breeding are predisposing factors for the occurence of the disease. *A. hydrophila* is considered a major cause of septicaemia caused by motile aeromonads. Several *A. hydrophila* extracellular products (ECP) are considered as important factors in pathogenesis, primarily aerolysin (aerA), the extracellular lipase, cytolytic enterotoxin, hemolytic toxin and extracellular proteases. PCR detection of aerolysin (*aerA*) is considered a reliable method of identifying potentially pathogenic *Aeromonas* strains. In spring 2012, after a sudden increase in water temperature, disease occured in common carp population in one fish farm in Serbia. Five specimens of the one-year-old carp with clinical symptoms of motile aeromonas septicaemia were used for isolation of the bacteria. Identification of *A. hydrophila* was done on the basis of morphological, physiological, cultural and biochemical characteristics. PCR amplification of DNA from *A. hydrophila* isolates revealed presence of aerolysin (*aerA*) gene in all examined *A. hydrophila* isolates from carp with motile aeromonas septicaemia.

Key words: *Aeromonas hydrophila*, PCR, aerolysin, carp

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DOKAZIVANJE PRISUSTVA AEROLIZIN (*aerA*) GENA PATOGENIH SOJEVA *AEROMONAS HYDROPHILA* IZOLOVANIH IZ OBOLELIH ŠARANA

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Kratak sadržaj

Bakterijske septikemije izazvane pokretnim predstavnicima roda Aeromonas spadaju u česte infekcije riba u intezivnom gajenju, ali zahvataju i populacije riba otvorenih voda. Široka rasprostranjenost ovih bakterija u vodenoj sredini, i stres uslovljen intenzivnim gajenjem predstavljaju predisponirajuće faktore za nastanak oboljenja. *Aeromonas hydrophila* je primarni ili sekundarni uzročnik bolesti vodenih i kopnenih životinja i ljudi, i njegova patogenost je povezana s faktorima virulencije. *A. hydrophila* se smatra glavnim uzročnikom septikemije izazvane pokretnim aeromonadama. Nekoliko ekstracelularnih proizvoda (ECP) bakterije *A. hydrophila* se smatraju značajnim faktorima u patogenezi, pre svega aerolizin (aerA), ekstracelularne lipaze, citolitički enterotoksin, hemolitički toksin i ekstracelularne proteaze. Detekcija aerA pomoću PCR se smatra pouzdanim načinom identifikacije potencijalno patogenih sojeva *Aeromonas hydrophila.* Kod šarana u prolećnom periodu, prilikom naglog povišenja temperature vode, utvrđena je septikemična forma oboljenja koja je podsećala na prolećnu viremiju šarana. Pet primeraka jednogodišnje mlađi šarana sa kliničkim simptomima bakterijske infekcije su korišćeni za izolaciju bakterija. Identifikacija *A. hydrophila* izvršena je na osnovu morfoloških, fizioloških, kulturelnih i biohemijskih karakteristika. Za identifikaciju gena virulencije odabrane su kolonije *A. hydrophila* izrasle u čistoj kulturi na Rimler-Shotts medijumu. PCR amplifikacijom DNK iz izolata *A. hydrophila* dobijeni su PCR produkti veličine 462bp kod svih ispitivanih uzoraka. U našem istraživanju, pomoću PCR je dokazano prisustvo aerolizin gena kod patogenih sojeva *A. hydrophila* izolovanih iz obolelih šarana.

Ključne reči: *Aeromonas hydrophila*, PCR, aerolizin, šaran

INTRODUCTION

Aeromonas hydrophila, a Gram-negative, motile rod that is a member of the family *Aeromonadaceae* (Joseph & Carnahan 2000; Abbott et al. 2003), has

been widely studied and is regarded as the most important bacterium causing "aeromonosis or haemorrhagic septicaemia or motile aeromonas septicaemia" in fish (Rhaman et al. 2001) and other aquatic animals (Hill et al. 2010; Pearson et al.2000). There have been a number of epidemiological studies indicating Aeromonas species as a cause of diarrheal disease in children, elderly people and immunocompromised patients (Figueras, 2005; von Gravaenitz, 2007).

The widespread of the bacteria in the aquatic environment and the stress caused by intensive breeding are predisposing factors for the disease. Stressful environmental factors, especially high water temperature, high levels of ammonia and nitrite, sudden changes in pH, and low concentrations of oxygen increases the possibility of disease occurence (Jeremic et al. 2005).

Several extracellular products (ECP) of *A. hydrophila* are considered important virulence factors, primarily aerolysin, extracellular lipase, cytolytic enterotoxin, hemolytic toxin and extracellular protease (John et al., 1997, Shome et al., 2005). Detection of virulence genes by PCR is very useful for the identification of pathogenic isolates of aeromonads (Uzbas et al., 2000).

Detection of aerolysin (*aerA*) using PCR and RFLP is considered a reliable for identification of virulent strains of *A. hydrophila* (Kingombe et al., 1999). The aim of this study was to determine the presence of aerA gene in *A. hydrophila* strains isolated from carp with motile aeromonad septicaemia.

MATERIAL AND METHODS

Five samples of the one-year-old carp with clinical symptoms of bacterial infection were used for isolation. From each fish, samples were collected from the kidney, liver, spleen and gills. The samples were streaked on tryptic soy agar (TSA, HiMedia), Mueller-Hinton agar (HiMedia) containing 5% defibrinated sheep blood erythrocytes (BA), selective Rimler-Shotts (RS) media (HiMedia), and incubated at 30°C for 24 to 48^{h}. Following incubation, one typical colony (entire circular, convex, white to greyish, semitranslucent, size 2 to 3 mm, haemolytic) was selected from each plate with a pure culture and subcultivated in order to test the purity of isolates. The isolates were preliminary grouped according to colony morphology, haemolysis, and pigmentation before they were stored at –80°C in 15% glycerol until further characterization. The type strain of Aeromonas hydrophila ATCC 7966 (American Type Culture Collection) was included in the phenotypic characterization.

Isolates were classified as *Aeromonas hydrophila* according to their reactions in the API 20E (Biomerieux) and following conventional tests, based on standard bacterial taxonomic procedures (Holt et al 1994; Austin and Austin, 2007).

For identification of virulence genes, five colonies of *A. hydrophila* were selected and 24 hours old cultures were used for extraction of genomic DNA. DNA extraction was performed using commercial kit (QIAamp DNA Mini Kit, Qiagen) according to manufacturer's protocol. Detection of the aerolysin gene was performed using polymerase chain reaction (PCR) (Chu et al., 2005). Primers were used to detect 462bp aerolysin gene fragment (Aero1: 5'-CTCAGTCCGTGCGACCGACT-3' and Aero2: 5'-GATCTCCAGCCTCAGGCCTT-3'). Amplification was performed by 35 cycles of denaturation at 95° C for 1 min, annealing at 56°C for 2 min and extension at 72°C for 2 min. After amplification, PCR products were characterized by 1.5% agarose gel electrophoresis in Tris–borate–EDTA buffer.

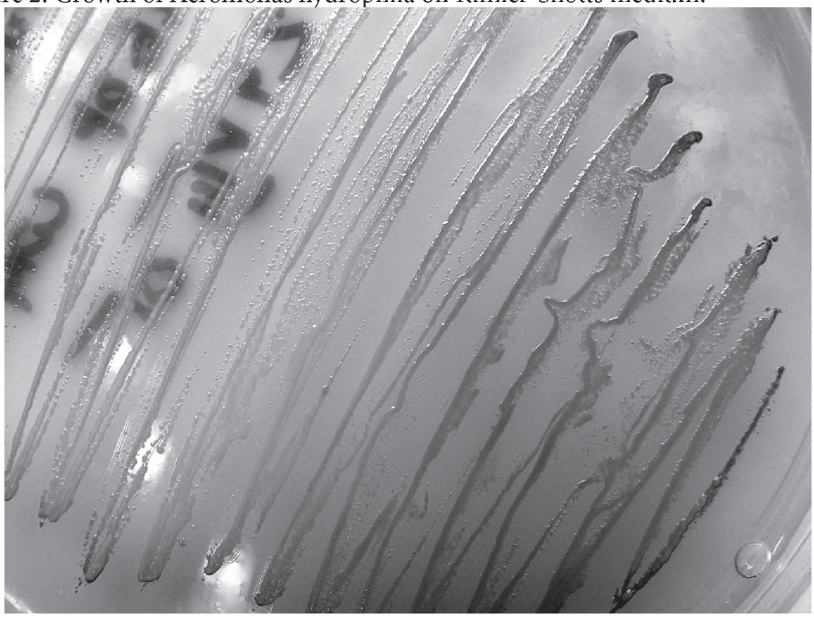
RESULTS

On a carp pond, localized in the northeastern part of the Republic of Serbia, in the spring, during a sudden increase in water temperature, increased mortality of young carp occured. The main signs of the diseased fish were anorexia, exophthalmus, redding due to haemorrhage of the skin and swimming at the surface of the pond, near fresh water supply. In scaled fish, scale pockets become edematous, causing lepidorthosis (Figure 1). Internal organs were edematous with hemorrhage and erythema on liver and kidney.

Figure 1. The pathological symptoms of the common carp suffering from motile aeromonad septicemia.

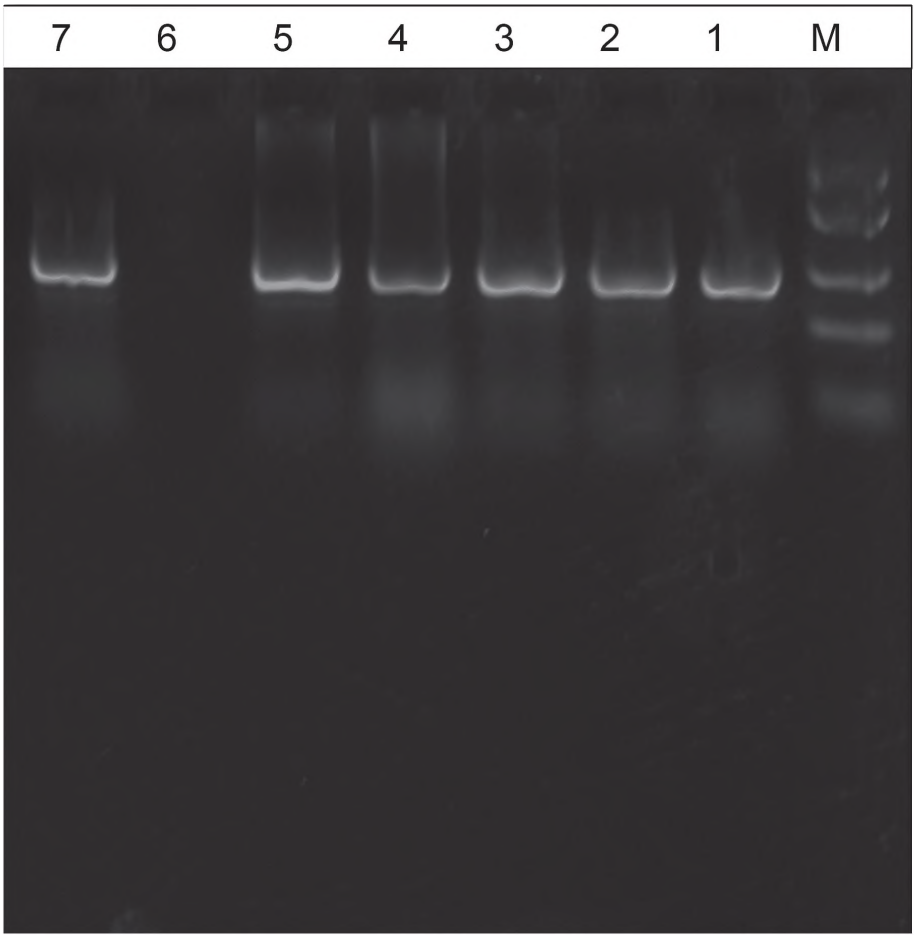
Pure cultures of *Aeromonas hydrophila* were obtained from all samples (Figure 2).

Figure 2. Growth of Aeromonas hydrophila on Rimler-Shotts medium.



A PCR amplification revealed that all *A. hydrophila* isolates were PCR positive for the *aer*A gene.

Figure 3: PCR amplification of *A.hydrophila* isolates for the 426-bp *aer*A gene. Lane M, 100-bp molecular weight marker; lane 1-5, 426-bp *aer*A amplified from the genomic DNA, lane 6-negative control, lane 7-positive control.



DISCUSSION

Motile aeromonads cause different pathologic conditions that include acute, chronic and latent infection. Severity depends on a number of factors including bacterial virulence, type and level of stress, resistance and physiological state of the host. In the acute phase, this condition is characterized by rapid fatal septicemia with little macroscopic evidence of disease. When present, the

most important symptoms are exophthalmos, skin redness and fluid collection in the scale pockets (Faktorovich, 1969).

There is evidence that the motile Aeromonas complex involves secondary and opportunistic pathogens, but ability of *A. hydrophila* to cause disease and death of fish should not be overlooked because occasionally highly virulent strains emerge. Regardless of whether or not the organism serves as a primary or secondary invader of stressed fish, it is often the final insult that leads to death (Plumb and Hanson 2011).

In attempt to explain the pathogenesis of infection caused by *A. hydrophila* several virulence factors were investigated. Toxins with haemolytic, cytotoxic and enterotoxic activities have been described in many *Aeromonas spp.* (Chopra et al., 1990).

Although rare, *A. hydrophila* may cause high mortality among cultured fish without presence of severe external (stressful) influences. This inconsistency may result from the presence of A. hydrophila strains that possess specific virulent or pathogenic characteristics (Plumb and Hanson 2011).

In our study, PCR was performed to detect aerolysin (*aerA*) gene as a genetic marker for the determination of virulence. Role of aerolysin (*aerA*) gene in the pathogenicity of *Aeromonas* genus was previously demonstrated (Kozaki et al., 1989, Shaw, 2003). In present study, PCR amplification of *A.hydrophila* isolates for the 426-bp *aer*A gene, in samples from diseased fish, showed the presence aerolysin gene, which is an essential *A. hydrophila* virulence gene. Similar results were obtained in previous studies with *A. jandaei* (Chacón, 2003). It is well known that the screening of specific cytotoxin and hemolysin genes is the most effective way of detecting and characterizing *Aeromonas* virulence factors (Yousr et al., 2007).

CONCLUSIONS

Despite disagreement among scientists regarding significance of *A. hydrophila* infection, the frequency of its appearance in aquaculture environment, together with a high potential for stress, shows that this problem sholud not be ignored, because it is often what kills the fish. PCR test for the detection of aerolysin gene proved to be a useful tool for the detection of virulent strains of *Aeromonas hydrophila.*

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