DETECÇÃO DE GENES DO SISTEMA DE SECREÇÃO DO TIPO III EM *Aeromonas hydrophila* E SUA RELAÇÃO COM A VIRULÊNCIA EM TILÁPIA DO NILO

GLEI DOS ANJOS DE CARVALHO CASTRO

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Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Curso de Mestrado em Ciências Veterinárias, para a obtenção do título de "Mestre".

Orientador

Prof. Dr. Henrique César Pereira Figueiredo

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APROVADA em 27 de fevereiro de 2009

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LAVRAS
MINAS GERAIS - BRASIL

À minha mãe Benedita Ribeiro de Carvalho (*in memorian*), de quem só tenho doces lembranças, meu eterno referencial de amor incondicional, perseverança e dignidade.

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Aeromonas hydrophila é uma bactéria patogênica para diversos hospedeiros, dentre eles estão os seres humanos e os peixes. O sistema de secreção do tipo III (T3SS) tem sido relatado como um dos mais importantes fatores de virulência para bactérias Gram negativas, incluindo *A. hydrophila.* Neste estudo protocolos de PCR foram desenvolvidos para detectar os genes do T3SS, *ascV* e *aopB*, e utilizados para avaliação da freqüência em isolados de peixes doentes e de ambientes da aquicultura. Foi avaliada também a relação da presença dos genes com a virulência da bactéria em tilápia do Nilo (*Oreochromis niloticus)*. Os protocolos de PCR desenvolvidos possilitaram a determinação de três perfis diferentes entre os isolados: *ascV*+/*aopB*+, *ascV*+/*aopB*- e *ascV*-/*aop*B-. A freqüência de 62,5% do perfil *ascV*+/*aopB*+ em isolados de peixes doentes foi significativamente diferente ($P < 0,05$) da freqüência de 32,5% em amostras de casos ambientais. Os resultados obtidos nos ensaios *in vivo* demonstraram uma taxa de mortalidade mais alta em grupos de peixes desafiados com bactérias *ascV*+/*aopB*+ que desafiados com *ascV*-/*aopB*- e *ascV*+/*aopB*-. Os resultados obtidos nas análises de freqüência e ensaios *in vivo* indicam que o perfil *ascV*+/*aopB*+ potencializa a virulência bacteriana. Os protocolos de PCR aqui estabelecidos se mostraram eficientes e específicos para detecção de genes do T3SS podendo ser utilizados para tipagem da bactéria *A. hydrophila*.

Palavras- chave: *Aeromonas. hydrophila,* gene *ascV,* gene *aopB,* peixes doentes, ambiente de aquicultura

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ABSTRACT

Carvalho-Castro, Glei dos Anjos de. **Detection of the Type III Secretion System Genes in *Aeromonas hydrophila* and Its Relation with Virulence in Nile tilapia.** 2009. 38p. Dissertation (Master in Veterinary Science) – Federal University of Lavras, Lavras, MG.*

Aeromonas hydrophila is a bacterium which can cause diseases in several hosts, such as humans and fish. The type three secretion system (T3SS) has been reported as one of major virulence factor in Gram negative bacteria, including the fish pathogen *A. hydrophila*. The aims of this study were to develop PCR systems to detect *ascV* and *aopB* genes from the T3SS, to evaluate their frequency in *A. hydrophila* strains isolated from diseased fish and aquaculture environment and the relation between T3SS presence and virulence to Nile tilapia. The PCR developed here was useful to detect the target genes, showing three different profiles among the strains: *ascV*+/*aopB*+, *ascV*+/*aopB*- e *ascV*-/*aop*B-. Higher frequency of *ascV*+/*aopB*+ was verified in isolates from diseased fish than aquaculture environment ($P < 0.05$). Among 64 isolates from diseased fish, *ascV*+/*aopB*+ (62.5%) was the most frequent profile ($P < 0.05$) and promoted more intensive mortality rates. Environmental strains with *ascV*+/*aopB*+ profile were less virulent than isolates from clinical cases. This result suggests that the presence of functional T3SS probably improves the *A. hydrophila* virulence. The PCR assay showed to be a specific and efficient tool for detection of T3SS and this technique can be used for typing *A. hydrophila* isolates.

Keywords: Aeromonas hydrophila, ascV gene, *aopB* gene, diseased fish, environment.

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CAPÍTULO 1

1 Introdução geral

A aquicultura mundial apresentou crescimento médio superior em relação aos demais setores da produção de alimentos de origem animal nos últimos anos. Em todo o mundo, desde 1970, a taxa média anual de crescimento foi de 8,8%, contra 1,4% na pesca extrativista e 2,8% no setor de produção de animais terrestres (Food Agricultural Organization - FAO, 2006). No Brasil também ocorreu aumento na produção de organismos aquáticos, e em 2005, a aquicultura teve participação de 25,6% na produção total de pescados, o que representou cerca de 257.780 toneladas (Instituto Brasileiro de Abastecimento e Meio Ambiente - IBAMA, 2005). Na aquicultura mundial, os peixes são os principais organismos aquáticos cultivados, representando 52% da produção, os moluscos correspondem a 18,6%, os crustáceos a 5% e a produção de plantas e outros 24,4% (Camargo & Pouey, 2005; FAO, 2006). No Brasil, os peixes ocupam o primeiro lugar em produção sendo as espécies mais cultivadas tilápias e carpas. Entretanto, ocorre uma maior produção de crustáceos que moluscos (IBAMA, 2005).

A tilapicultura continua se expandindo no Brasil dentro das regiões Nordeste, Sul e Sudeste. No ano de 2004, o maior produtor de tilápia no país foi o estado do Ceará com 18.000 toneladas (IBAMA, 2005). O resultado dessa expansão foi que, em menos de dez anos, a produção nacional passou de 42.918 toneladas para aproximadamente 70.000 toneladas (IBAMA, 2005). Segundo Lovshin & Cyrino (1998), o Brasil tem potencial para se tornar o maior produtor de pescado cultivado do mundo devido à disponibilidade de águas, ao clima tropical favorável, a maior e mais diversificada fauna de água doce do mundo, além de terras para cultivo de grãos tornando menos dispendiosa a produção de rações.

Entretanto, o consumo “per capita” de pescado no Brasil é considerado baixo (5 a 10 Kg/hab/ano) em relação a outros países do mundo (FAO, 2004), o que pode representar um obstáculo ao desenvolvimento do setor de aquicultura. Contudo, esse consumo apresenta potencial de aumento e estima-se que chegue a 12 Kg/hab/ano. Somente cerca de 10% da população utiliza o pescado em sua alimentação. O hábito de consumo é variável de acordo com a região, com uma taxa de consumo de pescados e derivados de 21% no norte e nordeste e 2% na região sul. Os grandes grupos do setor alimentício detectaram o aumento do consumo de pescados semi-prontos por parte da população, consequentemente iniciando a comercialização desses produtos em diversos centros urbanos do país (Germano & Germano, 2001).

Outros entraves para o aumento da produção na aquicultura são as deficiências na aplicação de medidas preventivas contra doenças e a falta de laboratórios de diagnóstico (Valenti et al., 2000). Dentre os patógenos relacionados às perdas econômicas na aquicultura está *Aeromonas hydrophila*, uma bactéria Gram negativa, ubíqua em ambientes aquáticos. Relatos demonstram que já houve isolamento desse microrganismo de água mineral, de água clorada e de alimentos, principalmente peixes e frutos do mar, o que caracteriza *A. hydrophila* como um sério problema de saúde pública (Kirov et al., 1993; Fernández et al., 2000; World Health Organization - WHO, 2006; Edberg et al., 2007). Diante de tais fatos, essa bactéria foi incluída na primeira e segunda lista dos contaminantes de água da Agência de Proteção Ambiental (Environmental Protection Agency - EPA, 2009) e o monitoramento da sua presença na água de abastecimento dos Estados Unidos da América ocorre desde 2002 (Borchardt et al., 2003).

A. hydrophila é uma bactéria que pode infectar peixes, répteis, anfíbios e mamíferos (Vivas et al., 2004) incluindo seres humanos, sendo transmitida pela água e alimentos de origem vegetal e animal (Subashkumar et al., 2007).

Quando ocorre a transmissão ao homem, o sintoma mais comum é a gastroenterite. Entretanto, o patógeno está relacionado também a quadros de septicemia, síndrome urêmica hemolítica, peritonite, infecção respiratória e feridas cutâneas (Janda & Abbott, 1998). A doença causada pela bactéria em humanos foi inicialmente associada a crianças com idade inferior a dois anos, idosos e indivíduos imunocomprometidos. Entretanto, diversos casos já foram relatados em imunocompetentes (Clark & Chenoweth, 2003).

Em peixes, a doença causada pela *A. hydrophila* é a septicemia hemorrágica bacteriana. Os sinais clínicos variam de lesões superficiais ou profundas na pele a quadros típicos de septicemia. As lesões de pele podem se apresentar inicialmente como áreas de hemorragia e necrose de extensão variada e, em alguns casos, progridem a úlceras acometendo o tecido muscular. Nos quadros clínicos de infecção sistêmica são observados o abdômen distendido, contendo líquido serosanguinolento, a exoftalmia e a presença de petéquias nas vísceras (Noga, 2000). A doença pode estar relacionada ao excesso de matéria orgânica na água, oxigênio dissolvido abaixo das concentrações adequadas e alta densidade animal (Irie et al., 2005).

Estudos anteriores demonstram que a patogênese de *A. hydrophila* ocorre com o envolvimento de diversos fatores de virulência como adesinas, pilli (Quinn et al., 1993; Pepe et al., 1996), Camada S (Dooley & Trust, 1988), enzimas extracelulares (Pemberton et al., 1997; Leung & Stevenson, 1998), exotoxinas (Chakraborty et al., 1984; Chopra et al., 1993) e o Sistema de Secreção do Tipo III (T3SS) (Vilches et al., 2004; Sha et al., 2005; Sierra et al., 2007).

O T3SS foi descrito pela primeira vez por Salmond & Rives (1993) como um mecanismo de exportação de proteínas utilizado por bactérias Gram negativas. Trata-se de uma nanomáquina complexa, constituída por mais de 20 proteínas diferentes, formada por uma estrutura basal ancorada nas membranas

bacterianas que sustenta uma “agulha” externa (Figura 1), sendo também denominado injectiosomo ou “complexo agulha” (Cornelis, 2006; Mueller et al., 2008). O T3SS permite que a bactéria injete proteínas efetoras diretamente no citosol das células hospedeiras causando alterações e possibilitando que o patógeno colonize, se multiplique e, em alguns casos, persista cronicamente no hospedeiro (Coburn et al., 2007).

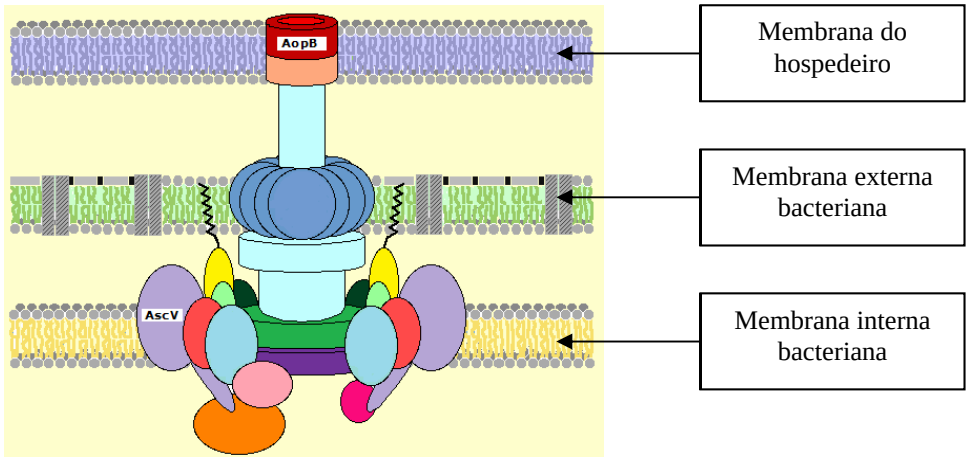

FIGURA 1 – Representação esquemática do injectiosomo, adaptada do T3SS de *Yersinia* sp.*

A análise filogenética de proteínas conservadas do T3SS indicou a evolução de um ancestral comum para sete famílias diferentes de injectiosomos: Chamydiales, Inv-Mxi-Spa, Ssa-Esc, Hrc-Hrp 1, Hrc-Hrp 2, Rhizobiales e Ysc (Troisfontaines & Cornelis, 2005; Cornelis, 2006). Os injectiosomos que ocorrem em patógenos de animais são encontrados em somente três famílias, sendo que o Ysc de Yersinia spp é o arquétipo de uma dessas, em que estão incluídos os Sistemas Asc – dos patógenos de peixes *Aeromonas* spp, Psc - *Pseudomonas aeruginosa*,

* http://www.genome.ad.jp

Vsc – *Vibrio parahaemolyticus*, Lsc – *Photrhabdus luminescens*, Bsc – *Bordetella* spp e Dsc – *Desulfovibrio vulgaris* (Trosfontaines & Cornelis, 2005; Cornelis, 2006). O sistema Asc foi detectado em diferentes espécies de *Aeromonas* e trabalhos anteriores demonstraram que a presença do T3SS está relacionada à virulência da bactéria (Burr et al., 2002; Yu et al., 2004; Fadl et al., 2006).

Dentre os genes codificadores de proteínas do T3SS em *A. hydrophila* estão *ascV* e *aopB*. O primeiro é um gene conservado entre as diferentes espécies que possuem o T3SS e está relacionado à fixação do sistema a membrana interna. Homólogos do gene *aopB* têm como funções a formação do poro na membrana hospedeira e o transporte de proteínas efetoras (Ide et al., 2001; Cornelis, 2002). Essas funções fazem dos genes *ascV* e *aopB* bons marcadores para a detecção do T3SS.

Estudos anteriores compararam a freqüência do T3SS em *Aeromonas* isoladas de diferentes síndromes clínicas em seres humanos e de ambiente utilizando técnicas de hibridização (Chacón et al., 2004; Vilches et al., 2004). Contudo, não há relatos sobre a freqüência do T3SS em *A. hydrophila* isoladas de peixes doentes e de ambiente de aquicultura. O fato de isolar *Aeromonas* spp. de peixes não indica que essas são as causadoras de doenças. Além de essas bactérias serem ubíquas em ambientes aquáticos, elas já foram detectadas na microbiota de peixes saudáveis (Goldschnidt-Clermont et al., 2008). Por isso, estudos de marcadores moleculares são necessários para distinguir amostras avirulentas das virulentas. Consequentemente, esses poderão ser utilizados como ferramentas para diagnóstico, assim como na seleção de amostras para a produção de vacinas.

O presente estudo teve como objetivos o desenvolvimento de sistemas de PCR para detecção dos genes *ascV* e *aopB* na bactéria *A. hydrophila*, a análise da freqüência desses genes em bactérias isoladas de peixes doentes e de

ambiente da aquicultura e avaliação da relação dos genes do T3SS com a virulência em tilápia do Nilo.

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CAPÍTULO 2

Detection of the Type III System Secretion Genes in *Aeromonas hydrophila*, and Its Relation with Virulence to Nile tilapia.

O capítulo 2 foi redigido em formato de artigo e será encaminhado para submissão no Periódico **Veterinary Microbiology**

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ABSTRACT

The aims of this study were to develop PCR systems to detect *ascV* and *aopB* genes from the Type III System Secretion (T3SS), to evaluate their frequency in *Aeromonas hydrophila* strains isolated from diseased fish and aquaculture environment, and the relation between T3SS presence and virulence to Nile tilapia. The PCR assay developed here was useful to detect the target genes, showing three different profiles among the strains: *ascV*+/*aopB*+, *ascV*+/*aopB*- and *ascV*-/*aop*B-. Higher frequency of *ascV*+/*aopB*+ was verified in isolates from diseased fish than aquaculture environment ($P < 0.05$). Among 64 isolates from diseased fish, *ascV*+/*aopB*+ (62.5%) was the most frequent profile ($P < 0.05$) and promoted more intensive mortality rates. Environmental strains with *ascV*+/*aopB*+ profile were less virulent than isolates from clinical cases. These results suggest that the presence of functional T3SS probably improve the *A. hydrophila* virulence. The PCR showed to be a specific and efficient tool for detection of T3SS and this technique can be used for typing *A. hydrophila* isolates.

Keywords: Aeromonas hydrophila, *ascV* gene, *aopB* gene, diseased fish, aquaculture.

1 Introduction

Aeromonas hydrophila is an important pathogen of the family *Aeromonadaceae*, being associated with illness in a broad spectrum of hosts. Ubiquitous in aquatic environments, this Gram negative bacterium have also been isolated from drinking water, chlorinated water and food (mainly fish and seafood), becoming a serious concern to public and animal health (Edberg et al., 2007). Because of this, *A. hydrophila* was included on the First and Second Drinking Contaminant Candidate List by the Environmental and Protection Agency (EPA) and its presence began to be monitored in water supplies since 2002 in the USA (Borchardt et al., 2003).

A. hydrophila has been described as an etiologic agent of diseases in fish, reptiles, amphibians, and mammals (Vivas, et al., 2004). In humans, this bacterium causes septicemia, wound infections, meningitis, peritonitis, hepatobiliary disease, and gastroenteritis, the principal clinical presentation of the illness (Janda & Abbott, 1998). Hemorrhagic septicemia and ulcerative disease are the common manifestations of infection in fish. The main clinical signs are serobloody ascites, exophthalmia, erratic swimming, cutaneous hemorrhage, and skin ulcers surrounded by a hyperemic region (Noga, 2000). Outbreaks caused by *A. hydrophila* in fish farms induce high mortality rates and result in elevated economic losses to the fish farming industry (Fang et al., 2004).

Previous studies have demonstrated that the pathogenesis of diseases caused by *A. hydrophila* is complex, involving several virulence factors such as pili (Pepe et al., 1996), S-layer (Dooley & Trust, 1988), extracellular enzymes (i.e. proteases, amylases, and lipases) (Leung & Stevenson, 1988) and exotoxins (Chopra et al., 1993). Among them, the Type III Secretion System (T3SS) is now considered of great importance (Vilches et al., 2004; Yu et al., 2004; Sha et al., 2005; Sierra et al., 2007). It was first described in 1993 (Salmond & Rives,

1993) as a mechanism used to export proteins by Gram negative bacteria. Also known as needle complex or injectisome, this complex nanomachine is composed by a basal body anchored in the bacterial membranes and an external needle (Cornelis, 2006; Mueller et al., 2008). Detected in many pathogenic bacteria for humans, animals and plants, such as *Salmonella* sp, *Shigella* sp, *Yersinia* sp, enteropathogenic and enterohemorragic *Escherichia coli* (EPEC, EHEC), *Pseudomonas* sp, *Aeromonas* sp as well as other bacteria, especial attention has been given to this system, mainly because its enable the microorganisms to inject protein effectors directly into the citosol of host cells without contact with intercellular space (Burr et al., 2002; Vilches et al., 2004; Yu et al., 2004; Mueller et al., 2008). These effectors change the cell physiology and host immune response, allowing the bacteria to colonize, multiply and in some cases chronically persist in the organism (Coburn et al., 2007). The T3SS was first reported in *A. hydrophila* at the same time in strains isolated from clinical cases of diseases in human and fish and presented a high similarity to T3SS proteins of *A. salmonicida, P. luminescens* and *Yersinia* sp. (Chacón et al., 2004; Vilches et al., 2004; Yu et al., 2004).

Several works have demonstrated the importance of T3SS for *Aeromonas* infection in different hosts. A previous study reported that the deletion of the gene *aopB* in an isolated of a human *A. hydrophila* strain reduced the mortalities caused by bacteria in mice when compared to wild type (Sha et al., 2005). *A. hydrophila* mutants to *aopB* and *aopD* genes showed a significant higher sensitivity to phagocytosis, a decrease in citotoxicity to carp epithelial cells and lower virulence to Blue goumari fish. Likewise, Vilches et al. (2004) verified a reduction in cytotoxic effect on different eukaryotic cell lines and an increase in lethal doses to rainbow trout and mice testing an *ascV* T3SS mutant derived from virulent *A. hydrophila* strain.

Chacón et al. (2004) reported no significant differences between the presence of T3SS genes in *Aeromonas veronii*, *Aeromonas caviae*, and *A. hydrophila* recovered from human cases of intestinal and extraintestinal illness. Nevertheless, to the same mesophilic *Aeromonas* species, Vilches et al. (2004) demonstrated that the frequency of T3SS genes is higher in isolates from human clinical cases when compared with strains isolated from the environment. However, there is no data about the comparative frequency of these genes in isolates from diseaded fish and aquaculture environment.

AscV and *aopB* genes are essential components of *A. hydrophila* T3SS, associated with the fixation of T3SS in the inner bacterial membrane and assembly of the translocon, a channel to deliver effectors into the host cell, respectively. The *ascV* is a highly conserved gene among different bacterial species; it is belonged in yscV injectisome family of *Yersinia* spp. (Cornelis, 2006). This characteristic makes it a valuable target to detect the T3SS. Homologous to genes *YopB* and *YopD* in Yersinia, *EspB* and *EspD* in *E. coli* (Cornelis, 2002; Ide et al., 2001), *aopB* gene is necessary to translocation of effector proteins and to form the pore in the membrane of host cells. Because this essential functions, this gene is a reasonable constituent of T3SS to be screened.

The aims of this study were to develop PCR assays to detect *ascV* and *aopB* genes in *A. hydrophila* strains, to evaluate the frequency of these genes in isolates from diseased fish and aquaculture environment and to access the *in vivo* virulence to Nile tilapia among strains belonged to the different genetic profiles.

2 Materials and methods

2.1 Bacterial strains

A total of 104 *A. hydrophila* strains, obtained from 12 fish farms, located in three different Brazilian states, were evaluated in this study

(Supplementary table 1). From those, 64 were isolated from diseased fish of five species, being from the brain (n=6), spleen (n=5), kidney (n=33), wounds (n=14), liver (n=5) and ascites fluids (n=1). Forty isolates from aquaculture environments were evaluated, being 28 from supply water and 12 from pond water. The reference strain ATCC 7966 (Seshadri et al., 2006) that had the whole genome sequenced and does not have the T3SS, was used as a negative control during PCR development. *A. hydrophila* isolates were biochemically characterized according to Abbott et al. (2003) and bacterial species were confirmed by genotypic identification using PCR-RFLP analysis of the 16s rRNA gene, digested with enzymes *Alu*I and *Mbo*I according to Borrel et al. (1997), with some modifications. The digestion products were resolved in 8% poliacrylamide gel electrophoresis. The isolates were stored at -70°C until use.

2.2 DNA extraction

The strains were thawed, streaked onto Trypticase soy agar (TSA) and incubated at 30°C for 18 h. A single colony from the culture was resuspended in 50µl of sterile ultrapure water, vortexed at high speed for 1 min and incubated at 95° C for 10 min in thermocycler for cell lyses. The extracted DNA was used immediately as template for PCR reactions.

2.3 Oligonucleotide primers

For PCR development, primer sets to detect *ascV* and *aopB* genes from *A. hydrophila* strains were designed. Nucleotide sequences available in NCBI database for *ascV* and *aopB* genes of this bacterium were obtained and aligned for the primer design. The construction and preliminary evaluation of the primers were performed using the softwares DNAMAM (version 4.0 Lynnon Corporation, Canada) and BLAST (http://www.ncbi.nlm.nih.gov/BLAST) for characteristics and specificity analysis, respectively. The primers ascV sense and

antisense were designed based on the highly conserved regions of *ascV* sequences from *A. hydrophila* strains AH1 e AH3 (Genbank accession numbers AY394563 and AY528667) (Vilches et al., 2004; Yu et al., 2004) to amplify a *ascV* fragment of approximately 891 bp. The primers *aopB* sense and antisense were constructed based on *aopB* sequence from *A. hydrophila* strain AH1, aiming to amplify a *aopB* fragment of 951 bp. The four primers were synthesized by Integrated DNA Technologies (USA). The oligonucleotide primers are showed in table 1.

TABLE 1 – Primers for PCR amplification of genes *ascV* and *aopB* from *A. hydrophila* strains.

Primer	*Position in genes of A. hydrophila AH1**	*Sequence (5'-3')*
ascV sense	509-527	AGCAGATGAGTATCGACGG
ascV antisense	1380-1399	AGGCAT TCTCCTGTACCAG
aopB sense	16-33	TACCTGTTGGAATGATTCCG
aopB antisense	947-966	AGTGAACGCCCTCTCTCC

***** Acession number Genbank AY394563

2.4 PCR amplification

PCR reactions were standardized using different concentrations of $MgCl_2$, deoxyribonucleoside triphosphates (dNTPs) mixture solution 100mM (Amresco, USA) primers, enzyme Taq DNA polymerase and annealing temperature for starting amplification. Positive reactions were evaluated according to absence of unspecific products and band intensity for the choice of best reaction. The PCR was performed in a termocycler using a Kit GoTaq flexi DNA polymerase (Promega, USA) which included also 5X Buffer and $MgCl_2$ 25mM. After

standardization, the best PCR mix for *ascV* amplification consisted of 1X Buffer, 0.25µM of each primer, 1.5 mM of $MgCl_{2,}$ 0.1 mM of each dNTP (dATP, dTTP, dGTP and dCTP), 1.25 U of Taq DNA polymerase, and 2 µl DNA template. For the *aopB* gene, the best amplification was achieved using as mix 1X Buffer, 0.25µM of each primer, 3.0 mM $MgCl_{2,}$ 0.2 mM of each of dNTP, 3.0 U of Taq DNA polymerase, and 2 µl of DNA template. All reactions were completed with sterilized ultra pure water for a total volume of 25 µl. *A. hydrophila* ATCC 7966 was included as a negative control in each test since this isolate is T3SS negative (Seshadri et al., 2006). Sterilized ultra pure water was used as a negative control of extraction. The PCR of all isolates was performed twice to confirm reproducibility.

PCR conditions consisted of an initial denaturation step at 95°C for 5 min followed by 35 cycles of amplification in which denaturation, annealing and elongation temperatures were 95°C for 1 min, 58°C for 1min and 30s, and 72°C for 1 min. The final elongation was at 72 ° C for 5 min.

The amplification products were analyzed on 1.5% (w/v) agarose gels electrophoresis with 1X Tris-acetate buffer (0,04 M Tris–acetate, PH 8.4, 1 mM EDTA) and were visualized with a UV transilluminator after staining with ethidium bromide solution (0.5µg mL/ml). A 100 bp ladder DNA molecular marker (New England Biolabs, USA) was used in each electrophoresis.

2.5 DNA sequencing

PCR products for *ascV* and *aopB* genes were purified using a Wizard PCR Preps kit (Promega, USA) and sequenced. Sequencing reactions were performed using a BigDye™ Terminator Cycle sequencing kit (Applied Biosystems, USA) and run on an ABI 3730XL genetic analyzer (Applied Biosystems, USA). Sequences were aligned and then compared with sequences

of *ascV* genes and *aopB* genes available in NCBI database using BLASTn algorithm.

2.6 Experimental infection

To virulence evaluation 22 *A. hydrophila* strains were selected according the results of PCR reactions. From those, nine were *ascV*+/*aopB*+ (positive for the two genes), nine were *ascV*-/*aopB*- (negative for both genes), and four *ascV*+/*aopB*- (positive only to *ascV* gene). The selected strains were inoculated in TSB with calcium depleted conditions, and incubated at 30°C for 6 h under 150 rpm agitation. The bacterial suspension was then adjusted to an optical density of 0.180 ± 0,020 at 600 nm, corresponding approximately to 10^7CFU/mL. Suspensions were serially diluted in 0.5 M phosphate-buffered saline, streaked onto TSA and incubated at 30°C for 18 h for bacterial counting. To prepare the bacterial inoculum, after growth the cells were harvested by centrifugation (3000 x g, 30min) and washed once with phosphate buffered saline (PBS) and resuspended in PBS. Healthy Nile tilapia (*Oreochromis niloticus*) were acquired from a commercial hatchery at an average weight of 25.6 ± 5.3 g for challenge and acclimatized to laboratory conditions by 15 days. Each experimental group comprised eight fish kept in a 57-liter aquarium supplied with flow-through dechlorinated tap water (0.5 L/hour). Fish were maintained on a 12 h: 12 h light/dark period at a water temperature of 26°C and were fed with VITAFISH 32% PB (Matsuda, GO, Brazil) four times a day until satiation. Fish were anesthetized by immersion in a bath containing 100 mg/L benzocaine. All groups were injected intraperitoneally with PBS washed *A. hydrophila* cells, corresponding to 10^6CFU/fish, and the control fish with 0.1 mL of sterile PBS. Challenged fish were monitored for 15 days. Samples of brain, liver and kidney were aseptically collected from all dead fish and inoculated on blood agar to recover the bacteria. After 15 days all fish were sacrificed and

bacteriologic assay was carried out. All experiments *in vivo* were carried out agreed the standards of animal welfare and approved by Ethical Committee from Animal Experiments of Federal University of Lavras, Brazil.

2.7 Statistical analysis

Fisher's Exact Test using SAS® statistical software STAT Version 6.12 (SAS Institute Inc., USA) was applied to determine whether the differences obtained in gene frequencies in isolates from different origins, a *p*- value of < 0. 05 were considered statistically significant.

3 Results

3.1 PCR standardization

Positive PCR reactions were obtained for the genes *ascV* and *aopB* of *A. hydrophila* isolates. Figure 1 presents the fragments of 891 and 951 bp resultants from amplification of respective genes. Negative reactions were verified for *A. hydrophila* ATCC7966 DNA template and ultra pure water, as expected. The sequenced amplicons confirmed the specificity of primers. These results were analyzed by BLASTn algorithm and demonstrated a high degree of similarity between PCR products obtained in this work and *ascV* and *aopB* gene sequences available in NCBI database of *A. hydrophila* (accession number AY52866 and AY394563 respectively) with 98% and 96% of identity.

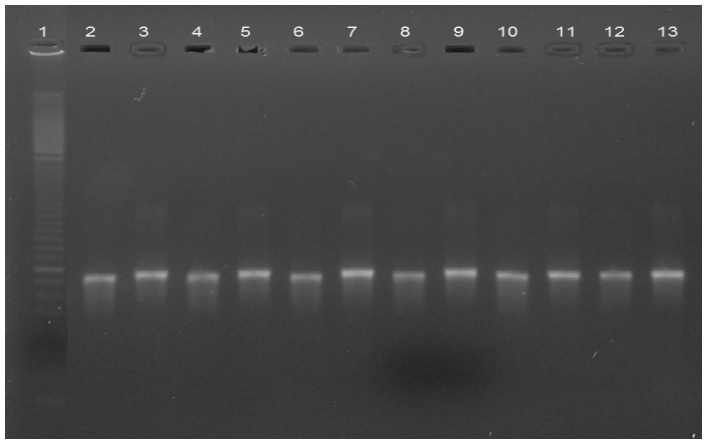

FIGURE 1 – PCR analysis for the genes *ascV* and *aopB* from *A. hydrophila* strains: AE067, AE110, AE 111, AE288, AE403 and AE406, respectively. Lane 1: 100 bp ladder; Lanes 2, 4, 6, 8, 10, 12: *ascV* gene 891 bp fragment amplification; Lanes 3, 5, 7, 9, 11, 13: *aopB* gene 951 bp fragment amplification.

3.2 Frequency of *ascV* and *aopB* genes

The T3SS genes evaluated in this work were detected in *A. hydrophila* isolates from all farms and from all fish species (supplementary table 1). The table 2 shows the distribution of positive and negative strains according to fish farm and source from which they were isolated. Three genetic types occurred in the strains analyzed: *ascV*+/*aopB*+ , *ascV*-/*aopB*- and *ascV*+/*aopB*-. A total of 53 strains were *ascV*+/*aopB*+ being 40 (75.5%) isolated from diseased fish and 13 (24.5%) from environment. Significant difference ($P < 0.05$) was observed among these sources. Four isolates of diseased fish showed profile *ascV*+/*aopB*- (6.25%) (table 2). Among 64 isolates of diseased fish, *ascV*+/*aopB*+ (62.5%) was the highest frequent profile ($p < 0.05$).

TABLE 2 – Frequency of T3SS genes in *A. hydrophila* isolated from diseased fish and aquaculture environment, detected by PCR.

Strain source	Farm												Total (%)
	A	B	C	D	E	F	G	H	I	J	L	M	
Clinical Case													
ascV+/aopB+ [*]		2	16	3			13					6	40 (62.5)
ascV+/aopB-		0	2	2			0					0	4 (6.25)
ascV-/aopB-		0	1	11			6					2	20 (31.25)
Environment													
ascV+/aopB+	2				6	1		2	1	1	1		13 (32.5)
ascV+/aopB-	0				0	0		0	0	0	0		0 (0)
ascV-/aopB-	6				12	3		1	0	2	3		27 (67,5)
Total	8	02	19	16	18	4	19	3	1	3	4	8	104

[*] The frequency of the profile *ascV+/aopB+* in strains from diseased fish was significantly higher than such frequency in strains from aquaculture environments ($P < 0.05$). No significant differences were obtained between other genetic profiles ($P = 0,243$).

3.3 Experimental infection

During the experimental period, none disease signs or mortalities were observed in the control group. In the challenged groups with *ascV+/aopB+* strains, the first signs of disease became four hours post-infection. The major of mortalities occurred until eight hours post-challenge, except to some fish of groups infected with strains AE 110, AE 288 and AE 111, whose mortalities ranged from 48 h to seven days. Bacterial reisolation was carried out from brain, liver and kidney of all dead fish. The main clinical signs observed in those groups were cutaneous hemorrhage (in the base of all fins and in the mouth), ascites with serobloody fluid and, in some fish, exophthalmia and erratic swimming. In contrast, to fish infected with AE 204 (*ascV+/aopB-*) as well as AE 190, AE 220 and AE 266 (all belonging to *ascV-/aopB-*) the mortalities began after 24 h post-inoculation. The diseased fish presented only anorexia, lethargy and skin darkening, followed by death.

Mortality rates varied between 12.5% and 87.5%, in the groups challenged with *A. hydrophila ascV*+/*aopB*+. The strains AE 067, AE 110, AE 111 and AE 406 demonstrated to be highly virulent to Nile tilapia fingerlings, inducing 75%, 75%, 87.5%, and 87.5% mortality rates, respectively (Table3). The strains AE 190, AE 220, and AE 266 belonged to the profile *ascV*-/*aopB*- presented low virulence to Nile tilapia fingerlings, resulting in mortality rates of 37.5%, 12.5%, and 12,5% respectively. Likewise, among the strains belonging to profile *ascV*+/*aopB*- only AE 204 caused disease, with a mortality rate of 12.5 %. Environmental strains with *ascV*+/*aopB*+ profile were less virulent than isolates of clinical cases.

TABLE 3 – Results of experimental infection with *A. hydrophila* stains possessing different genetic profiles for *ascV* and *aopB* genes.

*Strain	Source	Type[b] *ascV/aopB*	Main clinical sign observed[a]	Mortality[c]%
AE 049-02	water supply	+/+	Cutaneous hemorrhage	12.5
AE 067-02	Kidney	+/+	Cutaneous hemorrhage	75
AE 110-02	Kidney	+/+	Cutaneous hemorrhage	75
AE 111-02	Kidney	+/+	Erratic swimming and exophthalmia	87.5
AE 152-02	wound fin	+/+	Cutaneous hemorrhage	25
AE 288-03	Kidney	+/+	Swimming erratic	12.5
AE 344-03	pond water	+/+	Cutaneous hemorrhage	25
AE 403-04	Brain	+/+	Cutaneous hemorrhage	25
AE 406-04	Kidney	+/+	Cutaneous hemorrhage	87.5
AE 080-02	pond water	-/ -	-	0
AE 190-02	Spleen	-/-	Anorexia, lethargy, darkening skin	37.5
AE 220-02	water supply	-/-	Anorexia and lethargy	12.5
AE 221-02	water supply	-/-	-	0
AE 225-02	water supply	-/-	-	0
AE 266-03	water supply	-/-	Anorexia, lethargy, darkening skin	12.5
AE 333-03	pond water	-/-	-	0
AE 410-04	Kidney	-/-	Anorexia, lethargy, darkening skin	12.5
AE 413-04	Kidney	-/-	-	0
AE 178-02	Kidney	+/-	-	0
AE 179-02	Kidney	+/-	-	0
AE 203-02	Liver	+/-	-	0
AE 204-02	Liver	+/-	Anorexia and lethargy	12.5

* Strains randomly chosen according to *ascV*/*aopB* gene profiles for experimental infection.
[a] (+/ +) positive to both genes, (+/ -) positive to *ascV* and negative to *aopB*, (-/ -) Negative to both genes.
[b]- Mortality observed.

4 Discussion

The T3SS is well established as an important virulence factor to many Gram negative bacteria, including pathogenic *A. hydrophila*. In this work we developed PCR methods to detect the genes *ascV* and *aopB*, both responsible to encode essential components of this nanomachine. *ascV* is a conserved gene of T3SS, consequently, a good marker of presence or absence of injectisome in the bacteria. Studies with *yopB* which is homologous to *aopB*, showed that the protein codified by this gene is fundamental for pore formation in the host membrane, being essential to a functional T3SS (Cornelis, 2002). Because of this, *aopB* is an interesting marker of bacterial virulence. Positive results were obtained using PCR technique to identify these genes in *A. hydrophila* strains isolated from diseased fish and aquaculture environment. The PCR products showed high identity with reference sequences used to design the primers. Neither unspecific products nor positive reactions to ATCC 7966 *A. hydrophila* strain were verified with this methodology. Previous studies have been used hybridization procedures to identify T3SS in human and fish isolates of *Aeromonas* sp. (Burr et al., 2002; Stuber et al., 2003; Chacón et al., 2004). These methods are time consuming, labor intensive and expensive. By contrast, the PCR reaction developed here showed to be fast, high specific, with high reproducibility and easy to be implemented in the laboratory routine and, consequently, a reasonable method to proceed virulence typing in *A. hydrophila*..

Previous studies showed that T3SS is related with bacterial pathogenicity and its presence can be used as an indicator of virulence (Stuberr et al., 2003; Chacón et al., 2004). However, there is no data about the comparative frequency of the T3SS in *A. hydrophila* isolates from diseased fish and aquaculture environments. Our results demonstrated that the presence of T3SS is widespread in this bacterial species, since their genes were detected in

all farms independently of the strain source (clinical cases or environment). Higher frequency of *ascV+/aopB+* profile was verified in isolates from diseased fish with a significant difference ($P < 0.05$). It suggests that this system can improve the bacterial infectivity to fish. Similar results of T3SS frequency were obtained by Vilches et al. (2004), evaluating mesophilic *Aeromonas* isolated from environment and clinical cases of human diseases. As the T3SS can be horizontally transferred (Troisfontaines & Cornelis, 2005) and *A. hydrophila* is ubiquitous in aquatic environment, its presence in environmental strains could not be considered uncommon. Further, the bacteria are released by diseased fish, can spread and persist in environment for a long time. During this process, the microorganism can suffer adaptation to survive in water and the low virulence showed by *ascV+/aopB+* strains from this source could due to this. However, the relation between the presence of T3SS in isolates from different sources and *A. hydrophila* virulence to fish is unclear. Recently, some reports helped to establish the concept that T3SS can restrict as well as to promote the colonization of alternative hosts by bacteria and the ecological functions of T3SS in several bacterial species need to be addressed (Preston, 2007).

In this work four *ascV+/aopB-* strains were detected, suggesting that *aopB* gene was absent or these isolates show mutations in the region recognized by the primers, consequently, its could not be detected by the PCR system developed. There are no previous reports evaluating the frequency of this gene as a single marker in *A. hydrophila.* The occurrences of degenerate T3SS, which contains mutations or deletions that become the afunctional virulence factor have been reported in *E. coli* (Tobe et al., 2006). This phenomenon can be a possible explanation for those results.

ascV+/aopB+ strains were more virulent than *ascV+/aopB-* and *ascV-/aopB-* strains in experimental infection, based on the mortality rates observed. Although strains of three genetic profiles caused disease, different clinical signs

were observed in experimental infection performed with their representatives. The isolates *ascV+/aopB+* promoted a sudden disease, with fast evolution and characterized by widespread hemorrhage on body surface and in mucosa. In contrast, fish challenged with *ascV-/aopB-* or *ascV+/aopB-* isolates showed just apathy, anorexia and skin darkening accompanied for low mortality rates. The pathogenesis of *A. hydrophila* is multifactorial and previous studies have been demonstrated the involvement of many virulence factors (Wong et al., 1998; Yu et al., 2005). In spite of that, *ascV+/aopB-* strains seemed not to be so well adapted to promote fish infection like *ascV+/aopB+* strains. Also, these results are in agreement with studies with *A. hydrophila* mutants for T3SS genes that showed a decrease in virulence and lower cytotoxicity (Vilches et al., 2004; Yu et al., 2004; Sha et al., 2005). Low mortality rates were observed in challenge assays performed with *ascV+/aopB+* strains AE 152, AE 288, AE 344 and AE 403. It would be associated with possible variations in the secretion mechanism of the effector toxins by T3SS or different behaviors in global gene expression of these strains in comparison to *ascV+/aopB+* ones that induced high mortality (Francis et al., 2002; Mejia et al., 2008). The overall results obtained in experimental infections suggest that *A. hydrophila*, similar of verified in *Escherichia coli*, can show different subpopulations or pathotypes with variable ability to infect the hosts and induce clinical signs (Kaper et al., 2004).

The present study allows to conclude that the screened genes were more frequent in *A. hydrophila* strains associate with clinical disease in fish, and that *ascV+/aopB+* strains were more virulent to Nile tilapia. The PCR protocols developed for both genes showed to be good assays for virulence genes screening and could be used in routine analyses to *A. hydrophila* virulence typing.

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Competing interest statement

The authors declare no competing financial interests.

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6 CONCLUSÃO

Pode-se concluir com o presente trabalho que a PCR se mostrou específica para os genes alvos do T3SS sendo uma boa ferramenta para tipagem de *A. hydrophila*. Os genes alvos foram mais frequentes em amostras isoladas de peixes doentes que de ambiente de aquicultura. Nos ensaios *in vivo* as amostras que possuiam o perfil gênico *ascV* +/*aopB*+ se mostraram mais virulentas para tilápia do Nilo que as demais, indicando que a presença do T3SS potencializa a patogenicidade da bactéria *A. hydrophila.*

ANEXO

Supplementary TABLE 1 – *Aeromonas hydrophila* strains used in this study

Strain/Year[a]	Fish species	Farm[b]	Brazilian state[c]	Source	Results *ascV/aopB*[d]
AE 067-02	*Piaractus mesopotamicus*	B	MG	Kidney	+/+
AE321-03	*Beta splendens*	B	MG	Ascites fluid	+/+
AE 049-02	-	A	MG	Water supply	+/+
AE 074-02	-	A	MG	Pond water	-/-
AE 080-02	-	A	MG	Pond water	-/-
AE 081-02	-	A	MG	Pond water	-/-
AE 092-02	-	A	MG	Pond water	-/-
AE 103-02	-	A	MG	Water supply	-/-
AE 329-03	-	A	MG	Pond water	+/+
AE 333-03	-	A	MG	Pond water	-/-
AE 110-02	*Oreochromis niloticus*	C	MG	Kidney	+/+
AE 111-02	*Oreochromis niloticus*	C	MG	Kidney	+/+
AE 112-02	*Oreochromis niloticus*	C	MG	Kidney	+/+
AE 113-02	*Oreochromis niloticus*	C	MG	Kidney	+/+
AE 114-02	*Oreochromis niloticus*	C	MG	Kidney	+/+
AE 115-02	*Oreochromis niloticus*	C	MG	Kidney	+/+
AE 116-02	*Oreochromis niloticus*	C	MG	Kidney	+/+
AE 117-02	*Oreochromis niloticus*	C	MG	Kidney	+/+
AE 118-02	*Oreochromis niloticus*	C	MG	Kidney	+/+
AE 178-02	*Oreochromis niloticus*	C	MG	Kidney	+/-
AE 179-02	*Oreochromis niloticus*	C	MG	Kidney	+/-
AE 180-02	*Oreochromis niloticus*	C	MG	Kidney	-/-
AE 119-02	*Oreochromis niloticus*	C	MG	Caudal Wound	+/+
AE 120-02	*Oreochromis niloticus*	C	MG	Caudal Wound	+/+
AE 121-02	*Oreochromis niloticus*	C	MG	Caudal Wound	+/+

(…Continua…)

"Supplementary TABLE 1, Cont."

AE 129-02	*Oreochromis niloticus*	C	MG	Caudal Wound	+/+
AE 130-02	*Oreochromis niloticus*	C	MG	Caudal Wound	+/+
AE 140-02	*Oreochromis niloticus*	C	MG	Face Wound	+/+
AE 152-02	*Oreochromis niloticus*	C	MG	Fin Wound	+/+
AE 190-02	*Rhandia quelen*	D	RS	Spleen	-/-
AE 192-02	*Rhandia quelen*	D	RS	Kidney	-/-
AE 193-02	*Rhandia quelen*	D	RS	Spleen	-/-
AE 194-02	*Rhandia quelen*	D	RS	Liver	-/-
AE 195-02	*Rhandia quelen*	D	RS	Kidney	-/-
AE 196-02	*Rhandia quelen*	D	RS	Spleen	-/-
AE 197-02	*Rhandia quelen*	D	RS	Liver	+/+
AE 198-02	*Rhandia quelen*	D	RS	Kidney	-/-
AE 199-02	*Rhandia quelen*	D	RS	Spleen	+/+
AE 200-02	*Rhandia quelen*	D	RS	Liver	-/-
AE 201-02	*Rhandia quelen*	D	RS	Kidney	+/+
AE 202-02	*Rhandia quelen*	D	RS	Spleen	-/-
AE 203-02	*Rhandia quelen*	D	RS	Liver	+/-
AE 204-02	*Rhandia quelen*	D	RS	Liver	+/-
AE 205-02	*Rhandia quelen*	D	RS	Kidney	-/-
AE 206-02	*Rhandia quelen*	D	RS	Kidney	-/-
AE 208-02	-	E	MG	Water supply	+/+
AE 209-02	-	E	MG	Water supply	+/+
AE 211-02	-	E	MG	Water supply	-/-
AE 212-02	-	E	MG	Water supply	-/-
AE 213-02	-	E	MG	Water supply	-/-
AE 214-02	-	E	MG	Water supply	-/-
AE 215-02	-	E	MG	Water supply	-/-
AE 217-02	-	E	MG	Water supply	-/-
AE 219-02	-	E	MG	Water supply	+/+
AE 220-02	-	E	MG	Water supply	-/-
AE 221-02	-	E	MG	Water supply	-/-
AE 222-02	-	E	MG	Water supply	+/+

(…Continua…)

"Supplementary TABLE 1, Cont."

AE 223-02	-	E	MG	Water supply	+/+
AE 224-02	-	E	MG	Water supply	+/+
AE 225-02	-	E	MG	Water supply	-/-
AE 226-02	-	E	MG	Water supply	-/-
AE 227-02	-	E	MG	Water supply	-/-
AE 255-03	-	F	MG	Pond water	-/-
AE 264-03	-	F	MG	Water supply	-/-
AE 265-03	-	F	MG	Water supply	-/-
AE 266-03	-	F	MG	Water supply	-/-
AE 288-03	*Brycon orbignyanus*	G	MG	Kidney	+/+
AE 295-03	*Brycon orbignyanus*	G	MG	Kidney	+/+
AE 298-03	*Brycon orbignyanus*	G	MG	Kidney	+/+
AE 411-04	*Oreochromis niloticus*	G	MG	Brain	-/-
AE 412-04	*Oreochromis niloticus*	G	MG	Brain	-/-
AE 413-04	*Oreochromis niloticus*	G	MG	Kidney	-/-
AE 414-04	*Oreochromis niloticus*	G	MG	Kidney	-/-
AE 415-04	*Oreochromis niloticus*	G	MG	Kidney	+/+
AE 416-04	*Oreochromis niloticus*	G	MG	Brain	-/-
AE 417-04	*Oreochromis niloticus*	G	MG	Brain	+/+
AE 284-03	*Brycon orbignyanus*	G	MG	Brain	+/+
AE 292-03	*Brycon orbignyanus*	G	MG	Wound	-/-
AE 296-03	*Brycon orbignyanus*	G	MG	Kidney	+/+
AE 297-03	*Brycon orbignyanus*	G	MG	Kidney	+/+
AE 308-03	*Brycon orbignyanus*	G	MG	Tail Wound	+/+
AE 310-03	*Brycon orbignyanus*	G	MG	Tail Wound	+/+
AE 312-03	*Brycon orbignyanus*	G	MG	Tail Wound	+/+
AE 314-03	*Brycon orbignyanus*	G	MG	Tail Wound	+/+
AE 315-03	*Brycon orbignyanus*	G	MG	Lateral Wound	+/+
AE 344-03	-	H	MG	Pond Water	+/+
AE 345-03	-	H	MG	Pond Water	+/+
AE 350-03	-	H	MG	Pond Water	-/-
AE 366-03	-	I	MG	Water supply	+/+

(…Continua…)

"Supplementary TABLE 1, Cont."

AE 372-03	-	J	MG	Water supply	-/-
AE 375-03	-	J	MG	Water supply	+/+
AE 376-03	-	J	MG	Water supply	-/-
AE 389-03	-	L	MG	Water supply	+/+
AE 392-03	-	L	MG	Water supply	-/-
AE 401-03	-	L	MG	Pond water	-/-
AE 402-03	-	L	MG	Pond water	-/-
AE 403-04	*Oreochromis niloticus*	M	RJ	Brain	+/+
AE 404-04	*Oreochromis niloticus*	M	RJ	Brain	-/-
AE 405-04	*Oreochromis niloticus*	M	RJ	Kidney	+/+
AE 406-04	*Oreochromis niloticus*	M	RJ	Kidney	+/+
AE 407-04	*Oreochromis niloticus*	M	RJ	Kidney	+/+
AE 408-04	*Oreochromis niloticus*	M	RJ	Kidney	+/+
AE 409-04	*Oreochromis niloticus*	M	RJ	Kidney	+/+
AE 410-04	*Oreochromis niloticus*	M	RJ	Kidney	-/-

[a] Isolation year

[b] A-M different farms used in this study

[c] MG- Minas Gerais; RS – Rio Grande do Sul; RJ – Rio de Janeiro

[d] (+ /+) positive to both genes, (+/ -) positive to *ascV* and negative to *aopB* (-/ -) Negative to both genes

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