Food Microbiology 27 (2010) 327–331

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Detection and characterization of virulence genes and integrons in *Aeromonas veronii* isolated from catfish

Mohamed Nawaz [a,*], Saeed A. Khan [a], Ashraf A. Khan [a], Kidon Sung [a], Quynhtien Tran [a], Khalil Kerdahi [b], Roger Steele [a]

[a] Division of Microbiology, National Center for Toxicological Research, US Food and Drug Administration, Jefferson, AR 72079, USA
[b] Arkansas Regional Laboratory, US Food and Drug Administration, Jefferson, AR 72079, USA

ARTICLE INFO

Article history:
Received 8 May 2009
Received in revised form
29 October 2009
Accepted 2 November 2009
Available online 10 November 2009

Keywords:
Virulence genes
Aeromonas
Catfish
PCR

ABSTRACT

The presence of virulence genes and integrons was determined in 81 strains of *Aeromonas veronii* isolated from farm-raised catfish. Polymerase chain reaction (PCR) protocols were used to determine the presence of genes for cytotoxic enterotoxin (*act*), aerolysin (*aer*A), two cytotonic enterotoxins (*ast*, *alt*), lipase (*lip*), glycerophospholipid:cholesterol acyltransferase (*gcaT*), serine protease (*ser*), DNases (*exu*), elastase (*ahyB*) and the structural gene flagellin (*fla*) in the template DNA. Oligonucleotide primers amplified a 231-bp region of the *act* gene from the template DNA of 97.0% of the isolates. Primers specific for the amplification of the *aer*A gene amplified a 431-bp region of the *aer*A gene from the template DNA of 96.0% of the isolates. None of the isolates contained *ast* or *alt* genes. Oligonucleotide primers specific for the amplification of *lip*, *gcaT*, *ser* and *fla* genes, amplified their respective amplicons from 85.0, 78.0, 82.0 and 80.0% of the isolates. None of the isolates contained *exu* or the elastase genes. Several of the isolates (48.0%) contained class I integrons that confer resistance to multiple antibiotics; various sizes between 0.6 and 3.1 kb were found. None of the isolates contained Class II integrons. Our results indicate that farm-raised catfish may be a source of pathogenic *A. veronii* and that the potential health risks posed by virulent strains of *A. veronii* should not be underestimated.

Published by Elsevier Ltd.

1. Introduction

Aeromonas spp. are inhabitants of a wide range of aquatic ecosystems such as freshwater, estuarine, and coastal waters, and are even found in chlorinated potable water (Janda and Duffy, 1988). They are either mesophilic, motile or psychrophilic, non-motile Gram negative bacteria. These opportunistic bacteria have been associated with several categories of human infections, such as gastroenteritis, peritonitis, endocarditis, meningitis, septicemia, and urinary tract and wound infections (Gosling, 1996). Foods of animal origin, including seafood, and vegetables have been considered important sources of *Aeromonas* spp. infection (Ison and Drake, 2002; Rabaan et al., 2001; Sen and Rodgers, 2004; Soler et al., 2002). Virulent strains of *Aeromonas* spp. have also been isolated from potable water (Alvandi and Anathan, 2003., Granum et al., 1998; Huys et al., 1996; Sen and Rodgers, 2004). The United States Environmental Protection Agency (USEPA) has placed *Aeromonas* spp. on the contaminant candidate list and has actively monitored water supplies for aeromonads since 2002 (USEPA, 2006).

A wide range of putative virulence factors have been detected and studied in several *Aeromonas* spp.(Albert et al., 2000; Gonzalez-Serrano et al., 2002; Kingombe et al., 1999; Sechi et al., 2003); they play a pivotal role in the establishment of infection. Indeed, several studies have reported the detection and characterization of virulence factors in *Aeromonas* spp. isolated from freshwater fish, Tilapia (*Oreochromis niloticus*), humans, meat-producing animals and potable water (Albert et al., 2000; Escarpulli et al., 2003; Granum et al., 1998; Gonzalez-Serrano et al., 2002). However, little is known about the virulent traits of *Aeromonas veronii* isolated from catfish (Nawaz et al., 2006). Pond-raised catfish (*Ictalurus punctatus*) is a popular food fish and the annual production in the US is estimated at $600 million (Aquaculture Outlook, 2001). Earlier, we isolated and identified by molecular techniques 81-strains of *A. veronii* from catfish (Nawaz et al., 2006). Since little is known about the virulence mechanisms involved in *A. veronii*, we decided to investigate whether these isolates contained virulence factors associated with various human diseases.

* Corresponding author. Tel.: +1 870 543 7586.
E-mail address: Mohamed.nawaz@fda.hhs.gov (M. Nawaz).

0740-0020/$ – see front matter Published by Elsevier Ltd.
doi:10.1016/j.fm.2009.11.007

2. Materials and methods

2.1. Growth of bacterial strains and extraction of DNA

Bacteria were isolated from the intestinal contents of farm-raised catfish and identified as per details described earlier (Nawaz et al., 2006). All isolates were stored in Luria-Bertani (LB) broth containing 20% glycerol at −70 °C. Cultures were grown overnight at 37 °C in LB broth or on trypticase soy agar (TSA) plates supplemented with 5% sheep's blood. Genomic DNA was extracted with the QIAamp DNA mini kit (Qiagen, Valencia, CA).

2.2. Detection of virulence and related genes by polymerase chain reaction (PCR)

PCR assays for the amplification of the aerolysin (*aer*A), cytotoxic enterotoxin (*act*), cytotonic enterotoxins (*ast*, *alt*), lipase (lip), glycerophospholipid:cholesterol acyltransferase (*gcat*), serine protease (*ser*), DNase (*exu*), elastase (*ahyB*) and the structural gene, flagellin (*fla*) were performed with the template DNA of the isolates. Primers for the amplification of the virulence and related genes (Table 1) from the template DNA of *A. veronii* were designed by using a primer selection module of the Lasergene program (DNASTAR, Inc., Madison, WI) and synthesized by MWG, Inc. (High Point, NC). The T_m of the primers was calculated by the $2(A + T) + 4(G + C)$ formula. PCR amplification of virulence genes was carried out in a reaction volume of 25 μl by using a PCR Kit (Applied Biosystems, Foster City, CA). Each reaction tube contained 1 μl of bacterial DNA (0.01–0.05 μg), 5 μl of a 10 μM mixture of the primer mix, and 19 μl of PCR mix (200 μl of PCR mix contains: 33.3 μl of 10 × XL buffer II, 27 μl of 25 mM magnesium acetate, 66 μl of 10 mM dNTP mix, 7 μl of *Taq* DNA polymerase and 66.7 μl of water). The thermal cycling conditions consisted of an initial denaturation of 94 °C for 2 min followed by a total of 35 cycles of amplification. Each cycle consisted of 94 °C denaturation for 30 s, annealing for 50 s at 1 °C below the lowest T_m of a given primer pair, and 72 °C extension for 10 min. PCR was started with a initial denaturation at 94 °C for 2 min. The amplified PCR products were maintained at 4 °C. A reagent blank contained all the components of the reaction mixture except template DNA, for which sterile distilled water was substituted. The PCR products were subjected to electrophoresis on 1.2% agarose gels in 1× Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide (1 μg/mL), visualized with UV, and photographed using the Eagle Eye II gel documentation system (Stratagene, La Jolla, CA). A 100-bp DNA ladder (Invitrogen, San Jose, CA) was used as the size standard.

Table 1
Sequence of oligonucleotide primers used in the study[a].

Name of gene	Primer sequence	Product size (bp)	Length (bp)	T_m(°C)
act	F:AGAAGGTGACCACCACCAAGAACA R:AACTGACATCGGCCTTGAACTC	232	24 24	65
ast	F:TCTCCATGCTTCCCTTCCACT R:GTGTAGGGATTGAAGAAGCCG	331	21 21	63
aer	F:CCTATGGCCTGAGCGAGAAG R:CCAGTTCCAGTCCCACCACT	431	20 20	63
alt	F: TGACCCAGTCCTGGCACGGC R: GGTGATCGATCACCACCAGC	442	20 20	64
fla	F:TCCAACCGTYTGACCTC R:GMYTGGTTGCGRATGGT	608	17 17	55
gcaT	F:CTCCTGGAATCCCAAGTATCAG R:GGCAGGTTGAACAGCAGTATCT	237	22 22	65
ser	F:CACCGAAGTATTGGGTCAGG R:GGCTCATGCGTAACTCTGGT	350	20 20	57
ahyB	F:ACACGGTCAAGGAGATCAAC R:CGCTGGTGTTGGCCAGCAGG	513	20 20	59
exu	F:(A/G)GACATGCACAACCTCTTCC R:GATTGGTATTGCC(C/T)TGCAA(C/G)	323	20 20	61
lip	F:CA(C/T)CTGGT(T/G)CCGCTCAAG R:GT(A/G)CCGAACCAGTCGGAGAA	247	18 20	63

[a] The thermal cycling conditions consists of an initial denaturation of 94 °C for 2 min followed by a total of 35 cycles of amplification. Each cycle consisted of 94 °C denaturation for 30 s, annealing for 50 s at 1 °C below the lowest T_m of a given primer pair, and 72 °C extension for 10 min. PCR was started with an initial denaturation at 94 °C for 2 min.

2.3. Confirmation of the PCR amplicon by restriction digestion

Amplified PCR products were purified by the QIAquick PCR purification kit (Qiagen). Unless otherwise stated, the digestion mixture contained 6 μl of the amplicon, 2 μl of sterile distilled water, 1 μl of enzyme and 1 μl of the corresponding buffer. The PCR amplicons were digested for 4 h at 37 °C with appropriate restriction enzymes (Promega Corporation, Madison, WI), respectively. The digested fragments were separated on a 2.0% agarose gel. A 100-bp DNA ladder (Invitrogen, San Jose, CA) was used as the size standard.

2.4. PCR amplification of integrons

Primers used for the amplification of integrons are listed in Table 2. Thermal cycling conditions consisted of an initial denaturation cycle (94 °C for 2 min) followed by 30 cycles of denaturation (95 °C for 45 s), annealing (56 °C for 1 min), and extension (72 °C for 90 s). The final cycle of amplification was carried out at 72 °C for 10 min. The amplified DNA fragments were separated by electrophoresis using 1.2% agarose gels, stained with ethidium bromide (1 μg/mL), visualized with UV, and photographed using the Eagle Eye II gel documentation system (Stratagene, La Jolla, CA).

3. Results and discussion

3.1. PCR amplification of toxin genes (act, aerA, alt, ast)

PCR amplification of the toxin genes from the template DNA of all 81 *A. veronii* isolates from catfish was attempted. The oligonucleotide primers specific for the amplification of *act* amplified the 231-bp region of the gene from 79/81 (97.0%) of the isolates (Fig. 1A, Lane 2). The identity of the 231-bp PCR amplicon was confirmed by restriction digesting with *Alu*I, which produced the predicted 177- and 54-bp DNA fragments (Fig. 1A, lane 3). PCR amplification was also attempted with *aer*-specific primers, amplified a 431-bp portion of the *aer*A gene (Fig. 2, lane 2) from 78/81 (96.0%) of the isolates. The identity of the 431-bp PCR amplicon

Table 2
Oligonucleotide Primers Used for the Amplification of Class I and II Integrons.

Name of the gene	Primer sequence	Size
Class I Integrons	F:GGC ATC CAA GCA GCA AG R:GGC ATC CAA GCA GCA AG	Variable
Class II Integrons	F:CGG GAT CCC CGG CAT GCA CGA TTT GTA R:GAT GCC ATC GCA AGT ACG AG	Variable

Thermal cycling conditions consisted of an initial denaturation cycle (94 °C for 2 min) followed by 30 cycles of denaturation (95 °C for 45 s), annealing (56 °C for 1 min), and extension (72 °C for 90 s). The final cycle of amplification was carried out at 72 °C for 10 min.