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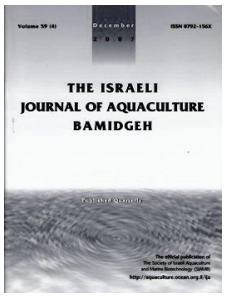

Hafnia alvei: a Pathogen Causing Infectious Intussusception Syndrome (IIS) in Farmed Channel Catfish *Ictalurus punctatus*

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Keywords: Infectious intussusception syndrome; *Ictalurus punctatus*; *Hafnia alvei*; antibiotic susceptibility.

Abstract

Infectious intussusception syndrome (IIS) is a serious problem, causing severe economic losses in farmed channel catfish *Ictalurus punctatus*. Limited information is available on *Hafnia alvei* as a causal agent for this disease. In this study, a virulent strain, temporarily named HN01, was isolated from diseased channel catfish suffering from infectious intussusception syndrome, and identified through phylogenetic analysis and phenotypic characteristics. A phylogenetic tree was constructed to examine isolate HN01 and compare it to other known isolates. Isolate HN01 has also been found to be susceptible to aminoglycosides, quinolones and sulfonamides drugs for veterinary use in aquaculture when screened against a range of common antibiotics. To the best of our knowledge, this is the first report of infectious intussusception syndrome caused by *Hafnia alvei* in farmed channel catfish.

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Introduction

Channel catfish *Ictalurus punctatus* are widely cultivated in Brazil, China, Cuba, Mexico, Russia and the USA (Pool, 2007). With the rapid development of farming techniques, channel catfish has become an important and profitable farmed freshwater fish species in China (Yan et al., 2013). Production increased to over 450,000 tons in 2014 (Yuan & Zhao, 2015). However, bacterial disease has become a major economic problem in catfish aquaculture (Shewmaker et al., 2007). If a sustainable farming industry is to be developed, more attention should be given to bacteriosis.

Infectious intussusception syndrome (IIS) is a serious bacterial disease and causes significant economic losses in channel catfish culture (Liu et al., 2008). Several bacterial pathogens such as *Aeromonas hydrophila, Pseudomonas fluoresces,* and *Stenotrophomonas maltophilia* have been reported to cause IIS in channel catfish (Liu et al., 2008; Geng et al., 2010), however limited information is available on identification of *Hafnia alvei* as a causal agent for IIS in channel catfish.

In the present study, *H. alvei* was isolated from cage-reared channel catfish suffering from IIS in Anhua China in February 2016. The aim of this study is to characterize the phenotype, taxonomic position, and antibiotic sensitivity of this strain. As far as we know, this is the first report of IIS caused by *H. alvei* in farmed channel catfish.

Materials and methods

Channel catfish samples. In Febuary 2016, twenty diseased channel catfish (1271±92 g) suffering from IIS were taken from a catfish farm in Anhua China. The farm had 3,500 acres of cages with catfish stocked at initial rearing density of 40,000 juveniles per acre. Water quality during the disease outbreak was pH 7.21, 0.05 mg/L total ammonia, 0.01 mg/L nitrite and 8.76 mg/L dissolved oxygen. Diseased samples were placed in sterile bags, kept in ice and transported to the laboratory.

Isolation of Bacteria. Each sampled diseased catfish was externally disinfected with 75% alcohol and dissected. 0.1g of affected intestine of each catfish was streaked onto nutrient agar (NA) plates (Sinopharm Chemical Reagent Co., Ltd.). After incubation for 24h at 28^0C, the dominant uniform isolates were purified by streaking and re-streaking onto NA plates. Pure isolates of the dominant colonies were stored at -80^0C supplemented with 15% glycerol. A representative of the dominant isolates, temporarily named HN01, was characterized further in the present study.

Identification of the isolate:

Molecular identification. The extraction of genomic DNA from isolate HN01, as well as PCR amplification and sequencing of its 16S rRNA gene were performed according to our previous study (Cao et al*.,* 2010). The near complete 16S rRNA gene sequence was assembled using MegAlign, Editseq and Seqman software. A search was performed in the National Centre for Biotechnology Information (NCBI) database for sequence homology using the Basic Local Alignment Search Tool (BLAST) program. A phylogenetic tree from the near complete 16S rRNA gene sequence of the isolate and its homologous sequences was constructed using the neighbor-joining method.

Phenotypic identification. Isolate HN01 was identified phenotypically by API 32E system (Qin et al., 2014). The isolate HN01 was grown on NA plates (Sinopharm Chemical Reagent Co., Ltd.) at 28^0C for 24h, and the bacterial suspension was then used to inoculate the Analytical Profile Index (API 32E) test strip (Biomerieux, France) following manufacturer instructions. The test strip was incubated at 37^0C and observed after 24h, and checked against the API identification index and database. Information related to *H. alvei* was obtained from *General manual of systematic and determinative bacteriology* (Dong & Cai, 2001).

Bacterial virulence assay. Bacterial virulence was examined by experimentally infecting healthy cultured channel catfish. One hundred healthy catfish (53.6±2.4 g) were obtained from Baishazhou fishery Co., Ltd. in Wuhan China. Their health status was assessed according to the guidelines in our previous study (Cao et al., 2013). The catfish were acclimated in ten replicate aquaria (ten catfish per aquarium) supplied with 40 L of aerated filtered farming water at 18^0C for 14 days. Prior to the bacterial virulence assay isolate HN01 was inoculated onto NA plate, incubated at 28^0C for 24h, then washed with normal saline into a sterile tube. Cell density was determined by counting colony forming units (CFU) after a ten-fold serial dilution in sterile distilled water. Two replicates of ten healthy fish were injected intramuscularly with 0.1 mL of isolate HN01 at a concentration

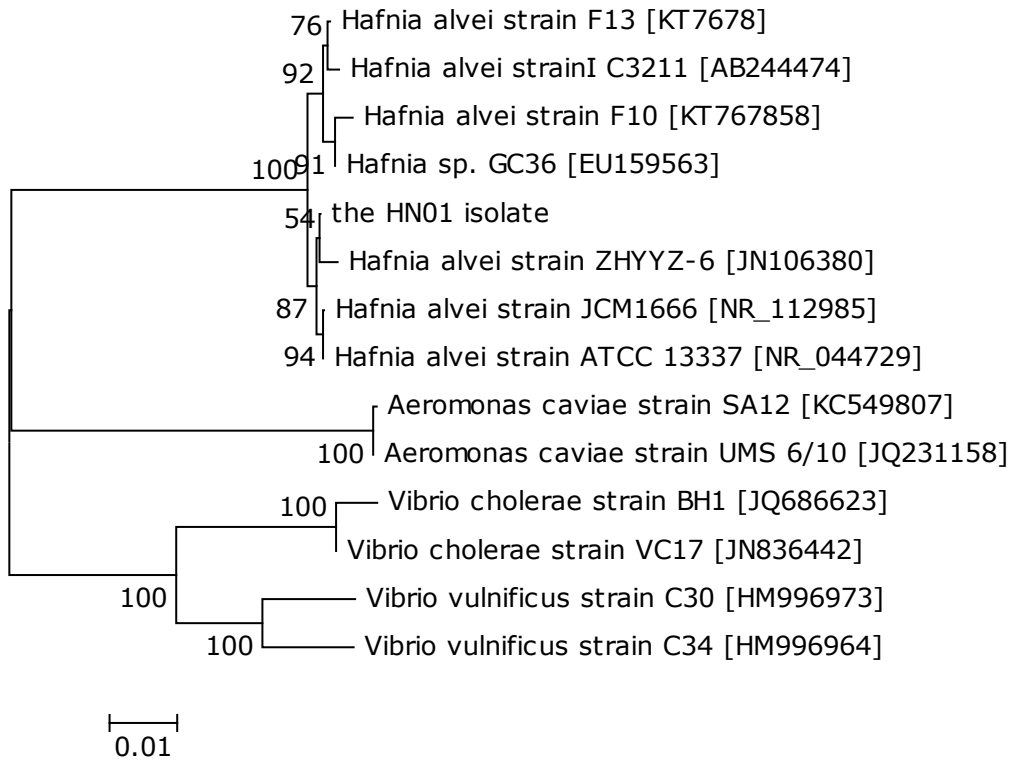
of 6.4 $\times 10^4$ CFU/mL to 6.4 $\times 10^7$ CFU/mL. Another two replicates of ten healthy catfish exposed to the same experimental conditions and injected intramuscularly with 0.1 mL normal saline served as control. The experimental catfish were kept at 18^0C and observed daily for seven days without feeding and water change. Dead catfish were immediately removed and sampled to confirm if mortality was caused specifically by the challenge isolate. The mean lethal dose (LD_{50}) value was calculated using the linear regression method (Won and Park, 2008).

Antibiotic sensitivity assay. Antibiotic sensitivity of isolate HN01 was assayed on NA plates using the Kirby-Bauer disk diffusion method as recommended by Jones et al. (2001). Twenty fishery antibiotic discs were acquired from Hangzhou Tianhe Microorganism Reagent Co., Ltd. Inhibition zones were measured after a 24h incubation period at 28^0C. Antibiotic susceptibility was determined according to manufacturer guidelines.

Results

Identification of the pathogenic isolate. Dominant isolate HN01 isolated from the diseased farmed catfish was identified by molecular and phenotypic methods as *H. alvei.* Its near complete 16S rRNA gene sequence (1400 nucleotides) was submitted to GenBank database (accession no. KX061756). A similarity of 99% was observed in the 16S rRNA gene sequence between the HN01 isolate and other *H. alvei* isolates from the GenBank database. The phylogenetic tree confirms that isolate HN01 is identified with *H. alvei* strain (Figure 1).

Figure 1. A 16S rRNA gene tree of 13 known bacteria and the HN01 isolate constructed using the neighbor-joining method. Bootstrap values (%) are shown besides the clades, accession numbers are indicated beside the names of strains, and scale bars represent distance values.



This is re-confirmed by phenotypic features as *H. alvei* (Table 1) with 100% identity compared to the reference strain.

Table 1. Phenotypic characterization of isolate HN01.

Tests	*Reaction*	
	HN01	*H. alvei*[a]
Arginine dihydrolase	R^-	R^-
Lysine decarboxylase	R^+	R^+
Lipase	R^-	R^-
L-aspartate aminase	R^-	R^-
N-acetyl-β-glucosaminidase	R^-	R^-
α-galactosidase	R^-	R^-
α-glucosaccharase	R^-	R^-
α-maltosidase	R^-	R^-
β-galactosidase	R^-	R^-
β-glucosaccharase	R^+	R^+
β-glucuronidase	R^-	R^-
Urease	R^-	R^-
Orinithine decarboxylase	R^+	R^+
Indole production	R^-	R^-
Malonate utilization	R^-	R^-
Acid production from		
Adonitol	R^-	R^-
Galacturonic acid	R^+	R^+
Inositol	R^-	R^-
L-arabinose	R^+	R^+
L-arabitol	R^-	R^-
L-rhamnose	R^-	R^-
D-arabitol	R^-	R^-
D-cellobiose	R^-	R^-
D-glucose	R^+	R^+
D-maltose	R^+	R^+
D-mannitol	R^+	R^+
D-sorbitol	R^-	R^-
D-sucrose	R^-	R^-
D-trehalose	R^+	R^+
5-ketone-potassium gluconate	R^-	R^-
Palatinose	R^-	R^-
Sodium pyruvate	R^-	R^-

R^+: positive reaction; R^-: negative reaction.[a] The reference strain's data are in accordance with those previously reported (Dong & Cai, 2001).

Isolate HN01 was found to be pathogenic by an experimental injection of the trial organism. Post experimental injection death of catfish increased gradually. Some catfish lost their balance and began to die one day post injection. 10%-95% of the catfish injected with isolate HN01 died (Table 2) at a LD_{50} value of 2.51×10^6 CFU/mL and exhibited the signs of IIS and enteritis as described by Geng et al. (2010). This is similar to that seen in the original diseased catfish (Figure 2). The re-isolated bacteria from experimentally dead catfish were also identified phenotypically and molecularly as isolate HN01. No clinical signs or mortality were noted in the control catfish. This procedure satisfies the conditions of Koch's postulates and proves that *Hafnia alvei* is a casual organism of infectious intussusception syndrome (IIS)

Figure 2. Pathological symptoms of farmed channel catfish suffering from IIS: (a) arrow shows intestinal intussusception; (b) arrow shows enteritis.

Table 2. Cumulative mortality of experimental channel catfish infected by isolate HN01.

Group	*Concentration (CFU/mL)*	*No. of fish*	*Cumulative mortality (%)*	*Average cumulative mortality (%)*
Control	0	10 10	0 0	0
Treated 1	6.4×10^4	10 10	10 10	10
Treated 2	6.4×10^5	10 10	20 20	20
Treated 3	6.4×10^6	10 10	50 60	55
Treated 4	6.4×10^7	10 10	90 100	95

Antibiotic sensitivity. The antibiotic sensitivity of isolate HN01 is shown in Table 3. Data indicate that isolate HN01 is sensitive to amikacin, ciprofloxacin, doxycycline, enrofloxacin, gentamycin, levofloxacin, neomycin, norfloxacin, streptomycin, sulfamethoxazole, tobramycin, and to another nine tested antibiotics. This shows that isolate HN01 has not developed resistance to aminoglycosides, quinolones and sulfonamides antibiotics for use in aquaculture.

Table 3. Susceptibility of isolate HN01 to antibiotics.

Antibiotics	*Content (μg/disc)*	*Inhibition zone diameter (mm)*
Amikacin	30	25.05 ± 0.10^S
Azithromycin	15	0 ± 0^R
Cefradine	30	0 ± 0^R
Chloramphenicol	300	11.96 ± 0.35^R
Ciprofloxacin	5	33.68 ± 0.66^S
Clindamycin	2	0 ± 0^R
Doxycycline*	30	20.19 ± 1.32^S
Enrofloxacin*	5	25.00 ± 0.03^S
Erythrocin	15	6.80 ± 0.45^R
Florfenicol*	75	10.33 ± 0.42^R
Furazolidone	300	0 ± 0^R
Gentamycin*	10	21.44 ± 0.82^S
Levofloxacin	5	30.22 ± 0.57^S
Neomycin*	30	20.00 ± 0.54^S
Norfloxacin	10	31.86 ± 0.28^S
Oxacillin	1	0 ± 0^R
Rifampicin	5	6.54 ± 0.82^R
Streptomycin*	10	18.05 ± 0.61^S
Sulfamethoxazole*	300	25.01 ± 0.28^S
Tobramycin	10	22.96 ± 0.14^S

Data are presented as the mean±standard deviation;
^{S}Sensitive; ^{R}Resistant.*Antibiotics for aquacultural use.

Discussion

H. alvei in aquaculture has been associated with hemorrhagic septicemia in rainbow trout *Oncorhynchus mykiss* (Walbaum)(Gelev et al., 1990), kidney pathology in cherry salmon *Oncorhynchus masou* (Brevoort) (Teshima et al., 1992) and massive mortality in brown trout *Salmo trutta* L. (Rodriguez et al., 1998; Orozova et al., 2014). There is however, limited information on *H. alvei* as a causal agent for IIS in cultured catfish. In this study, we characterized the phenotype, taxonomic position, and antibiotic susceptibility of *H. alvei* HN01. To our knowledge, this is the first report of an *H. alvei* pathogen as a causative agent for IIS in farmed channel catfish.

The pathogenesis of IIS is complex and multi-factorial. Signs of IIS may be induced in channel catfish with bacterial pathogens and their extracellular products, which

contained virulence factors such as enterotoxin and cytotoxin (Wang et al., 2006). This suggests that IIS in channel catfish could probably be caused by bacterial pathogens with virulence factors such as enterotoxin and cytotoxin. *H. alvei* is a well-recognized pathogen with virulence factors including heat stable enterotoxin, cytolytic toxin (Cai et al., 1994; Abbott et al., 2011) as well as fimbriae, siderophore production and resistance to the bactericidal effect of serum (Padilla et al., 2005). IIS caused by *H. alvei* is probably associated with these virulent factors. In the present study, the HN01 isolate was found to cause mortality in healthy channel catfish with a LD_{50} value of 2.51×10^6 CFU/mL. This further demonstrates the potential threat of *H. alvei* to channel catfish farming. Apart from the virulence of the HN01 isolate, there may be other factors such as use of contaminated feed and misuse of feed additives that could induce IIS in channel catfish.

Natural antibiotic susceptibility to aminoglycosides, quinolones, and sulfonamides, has been reported in *H. alvei* (Stock et al., 2005). The HN01 isolate in our study also exhibited similar susceptibility to aminoglycosides, quinolones, and sulfonamide antibiotics as confirmed by Stock et al. (2015), including doxycycline, enrofloxacin, gentamycin, neomycin, streptomycin and sulfamethoxazole used in the fish farming regions. This suggests that this disease may not have resulted from abuse of antibiotics.

In conclusion, the present study reports an *H. alvei* isolate as a causal agent for IIS in cultured channel catfish for the first time. The pathogenicity of the HN01 isolate supports the view that this infection is an emerging threat in the catfish farming.

Acknowledgments

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References

Abbott S.L., Moler S., Green N., Tran R.K., Wainwright K., Janda J.M., 2011. Clinical and laboratory diagnostic characteristics and cytotoxigenic potential of *Hafnia alvei* and *Hafnia paralvei* strains. *J Clin Microbiol.,* 49(9):3122-3126.

Cai J.Y., Yang W.J., Ge S.J., 1994. First isolation of a *Hafnia alvei* pathogen from white granulated sugar. *Food Sci.,* 3: 55-56.

Cao H., He S., Lu L., Hou L., 2010. Characterization and phylogenetic analysis of the bitrichous pathogenic *Aeromonas hydrophila* isolated from diseased Siberian sturgeon. *Isr. J. Aquacult.- Bamidgeh,* 62(3):181-189.

Cao H., Zheng W., He S., Ye X., Xiao G., Yang X., 2013. Identification of a *Vibrio cholerae* isolate as the causal agent of ascites disease in cultured mandarin fish *Siniperca chuatsi* (Basilewsky). *Isr. J. Aquacult.-Bamidgeh,* IJA_65.2013.914.

Dong X.Z., Cai M.Y., 2001. *General manual of systematic and determinative bacteriology.* Science Press, Beijing, 79pp.

Gelev I., Gelev E., Steigerwalt A.G., Carter G.P., Brenner D.J., 1990. Identification of the bacterium associated with haemorrhagic septicaemia in rainbow trout as *Hafnia alvei*. *Res Microbiol,* 141:573-576.

Geng Y., Wang K., Chen D., Huang X., He M., Yin Z., 2010. *Stenotrophomonas maltophilia*, an emerging opportunist pathogen for cultured channel catfish, *Ictalurus punctatus*, in China*. Aquaculture,* 308:132-135.

Jones R.N., Ballow C.H., Biedenbach D.J., 2001. Multi-laboratory assessment of the linezolid spectrum of activity using the Kirby-Bauer disk diffusion method: Report of the Zyvox@ antimicrobial potency study (ZAPS) in the United States. *Diagn Microbiol Infect Dis.,* 40:59-66.

Liu J., Yang W., Li A., He G., 2008. Preliminary study on the etiology of channel catfish intussusception disease. *Acta Hydrobiologica Sinica,* 32(6):824-831.

Orozova P., Sirakov I., Chikova V., Popova R., Al-Harbi A.H., Crumlish M., Austin B., 2014. Recovery of *Hafnia alvei* from diseased brown trout, *Salmo trutta* L., and healthy noble crayfish, *Astacus astacus* (L.), in Bulgaria. *J Fish Dis.,* 37:891-898.

Padilla D., Real F., Gómez V., Sierra E., Acosta B., Déniz S., Acosa F., 2005. Virulence factors and pathogenicity of *Hafnia alvei* for gilthead seabream, *Sparus aurata* L. *J Fish Dis.,* 28:411-417.

Pool T.K., 2007. *Channel catfish review: life-history, distribution, invasion dynamics and current management strategies in the Pacific Northwest.* Washington, USA: University of Washington, 12pp.

Qin L., Xu J., Wang Y., 2014. Edwardsiellosis in farmed turbot, *Scophthalmus maximus* (L.), associated with an unusual variant of *Edwardsiella tarda*: a clinical, aetiological and histopathological study. *J Fish Dis.,* 37:103-111.

Rodriguez L.A., Gallardo C.S., Acosta F., Nieto T.P., Acosta B., Real F., 1998. Hafnia alvei as an opportunistic pathogen causing mortality in brown trout, Salmo trutta L. *J Fish Dis.,* 21:365-369.

Shewmaker P.L., Camus A.C., Bailiff T., Steigerwalt A.G., Morey R.C., Carvalho M.G.S., 2007. *Streptococcus ictaluri* sp. nov., isolated from channel catfish *Ictalurus punctatus* broodstock. *Int J Systematic Evolutionary Microbiol.,* 57:1603-1606.

Stock I., Rahman M., Sherwood K.J., Wiedemann B., 2005. Natural antimicrobial susceptibility patterns and biochemical identification of *Escherichia albertii* and *Hafnia alvei* strains. *Diagn Micr Infec Dis.,* 51:151-163.

Teshima C., Kudo S., Ohtani Y., Saito A., 1992. Kidney pathology from the bacterium *Hafnia alvei*:experimental evidence. *T Am Fish Soc.,* 121:599-607.

Wang K.Y., Geng Y., Huang X.L., Chen D.F., 2006. Infectious intussusception of channel catfish (IICC). *J Modern Fish Inform.,* 21(9): 3-8.

Won K.M., Park S., 2008. Pathogenicity of *Vibrio harveyi* to cultured marine fishes in Korea. *Aquaculture,* 285: 8-13.

Yan C., Xiao Y., Li L., 2013. Current status of the world catfish industry and reconsideration for its market positioning. *China Fisheries,* 6:36-40.

Yuan X.C., Zhao W.W., 2015. *China Fisheries Yearbook*. Beijing: China Agriculture Press, pp31.