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[Short Communication]

First isolation and identification of *Vibrio vulnificus* (biotype 2) from cultured beluga, *Huso huso* in Iran

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ABSTRACT

By decreasing sturgeon stocks in the Caspian Sea, rearing different sturgeon species especially *Huso huso* was increased in Iran. Under stress conditions the sturgeon can easily be infected by several opportunistic pathogens. In June 2011, mortality happened in 25 - 28°C water temperature, in one of the most important cultured sturgeon farms in Mazandaran province, north of Iran. The mortality rate was 15%. The first clinical signs in moribund fish were lethargy and anorexia. A total of 20 moribund fish was transferred to a central laboratory for more bacteriological examination. Clinical signs including several deep ulcers on body surface, around the head, under operculum, ulcers at the base of the pectoral fins, hemorrhage around the anus, operculum, and pale gills were observed in moribund fish. The main internal signs were hepatomegaly and splenomegaly, liquid accumulation in the intestine and diffuse visceral hemorrhage. The results of morphology and microscopic characterizations and also biochemical tests indicated that *Vibrio vulnificus* (biotype 2) was the etiological agent of mortality in infected fish. This study was the first report of *V. vulnificus* in cultured *H. huso* in Iran.

Key words: *Vibrio vulnificus*, first report, *Huso huso*, Iran

INTRODUCTION

Iran export sturgeon caviar and Persian caviar is the most famous in the world, so that the production of meat and caviar were estimated about 363000 kg and 50192 kg in 2012 respectively (Iranian Fisheries Organization, 2012). By decreasing sturgeon stocks in the Caspian Sea, rearing different sturgeon species especially *Huso huso* was increased in Iran. Between the different sturgeon species, *H. huso* and their hybrids have a good growth performance and survival rates in intensive farming conditions (Bagherzadeh Lakani *et al.*, 2013; Kolman, 2002). Under stress conditions including poor water quality and high density of fish, sturgeon can easily be infected by several opportunistic pathogen bacteria especially *Streptococcus* sp., *Aeromonas* sp., *Yersinia* sp., *Vibrio* sp. and so on (Yanong *et al.*, 2002; Kolman, 2002). *V. vulnificus* is a motile, gram-negative, curved rod- shaped bacterium with a single polar flagellum. This bacterium is a naturally occurring, free-living inhabitant of estuarine and marine environments throughout the world and may be associated with zooplankton and other aquatic biological flora. It is taken up by filter-feeding molluscs such as oysters, clams, mussels and scallops and becomes concentrated in the gut and other tissues. Also, *V. Vulnificus* is commonly found in the intestines of variety of estuarine fish species and these fish may act as a reservoir and transport this bacteria to the other organisms (Storm *et al.*, 2000). Moreover, *V. vulnificus* has been described as an opportunistic human pathogen (Strom *et al.*, 2000; Fouz *et al.*, 2002). Recently, V. *vulnificus* is divided into three distinct biotypes based on phenotypic and host

- range differences. Biotype 1 strains are typically associated with shellfish colonization and human illness. Biotype 2 has been implicated in infections of marine vertebrates, particularly in cultured eels, tilapia and shrimp and is increasingly recognized as a serious pathogen (Storm *et al.,* 2000; Fouz *et al.,* 2002). Biotype 3 has been isolated from wound infections in human in Israel (Buller, 2004). The main objective of this study was the isolation and identification of the mortality agent in cultured beluga, *H. huso*, in the north of Iran.

MATERIALS AND METHODS

Sampling

Sampling was done randomly from cultured beluga,*H. huso* in Mazandarn province, north of Iran. A total of 20 moribund fish was randomly sampled and were individually weighed. Samples were transported to the Caspian Sea Ecology Research Center laboratory for bacteriological examination.

Isolation of bacterium and bacteriological examination

Fish samples (kidney, liver, spleen and body ulcers) were cultured aseptically by streaking a loop onto blood agar (Merck) containing 1.5% NaCl and Tryptic Soy Agar (TSA, Oxoid) supplemented with 2% NaCl and incubated at 28°C for 24 - 48 h. Suspected bacterial colonies were identified using the conventional biochemical media. Specifically, growth at a wide range of Nacl (3%, 5%, 8%) were conducted at 28°C on TSA. In addition, the following tests were also carried out: growth on MacConkey medium, oxides, nitrate reduction, motility, sensitivity to O/129, fermentation of carbohydrate, indole, citrate, bile esculine hydrolysis, urease, gelatin liquefaction, methyl red and Voges-Proskauer (MacFaddin, 2000).

Antimicrobial sensitivity test

Isolated bacterium was tested for antibiotic susceptibility using the disk diffusion method. The most important used antibiotics were sulfamethoxazole/trimetoprim (25 $\mu g.ml^{-1}$), oxytetracycline (20 $\mu g.ml^{-1}$), chlorotetracycline (20 $\mu g.ml^{-1}$), flumequine (30 $\mu g.ml^{-1}$) and oxolinic acid (10 $\mu g.ml^{-1}$).

RESULTS

Mean weight and length of samples was 10 kg ± 500g and 65 cm ± 5 cm respectively. Water temperature, during sampling, was 25 – 28°C. During the outbreak, 15% of *H. huso* died with symptoms such as bleeding ulcers on ventral surface, at base of pectoral fins, under opercula, hemorrhage in vent, necrosis at the base of the pectoral fin (Fig. 1,2,3), paleness of gill (Fig. 4). The main internal signs were hepatomegaly, spleenomegaly, liquid accumulation in the intestine and diffuse visceral petechiae.

The average size of isolated colonies in TSA medium and Blood agar was 2-3 mm in diameter, and was round, raised and opaque morphologically (Fig. 5,6).

The biochemical tests were showed that isolated bacteria were gram negative, motile rods, oxidase and catalase, nitrate reduction and lysine decarboxylase were positive, although indole, ornitine decarboxylase, urease, arabinose, and inositole were negative. The isolate was sensitive to the vibriostatic agent O/129.

Other biochemical tests have been shown in Table 1.

The results of morphology and microscopic characterizations and also biochemical tests indicated that the isolate was *V. vulnificus* biotype 2 which was then confirmed by the central laboratory in the Faculty of Veterinary Medicine, University of Tehran, Iran.

The results of rapidly screening bacterial susceptibility test showed that the isolate was sensitive to sulfamethoxazole/ trimetoprim (25 $\mu g.m^{-1}$) and Oxytetracycline (20 $\mu g.ml^{-1}$) but resistant to chlorotetracycline (20 $\mu g.ml^{-1}$), flumequine (30 $\mu g.ml^{-1}$) and oxolinic acid (10 $\mu g.ml^{-1}$) (Table 2).

Table 1. Biochemical characteristics of isolated *V. vulnificus* from infected *H. huso* (KEY: G: Growth; V: Variable reaction; S: Sensitive; a: Acid production)

Tests	Response	Tests	Response
Catalase	+	Voges-Proskauer (VP)	-
Oxidase	+	O/129	S
MacCankey	G	Glucose	A
No_3 reduction	+	Adonitol	-
Motility	+	Arabinose	-
Indole	-	Inositol	-
Citrate	V	Maltose	A
Esculin hydrolysis	V	Mannitol	V
Arginine dehydrolase	-	Mannose	A
Lysine decarboxylase	+	Raffinose	-
Ornitine decarboxylase	-	Rhamnose	-
Gelatin liquefaction,22 °C	V	Sorbitol	-
Urease	-	Xylose	-
Methyl Red (MR)	+	Trehalose	A

Table 2. Results of rapidly screening bacterial susceptibility test on *V. vulnificus* isolate from infected *H. huso* (ten isolates were tested for each antibiotic)

No	**Antibiotic**	**Result**
1	sulfamethoxazole/trimetoprim (25µg.ml $^{-1}$)	Sensitive
2	Oxytetracycline (20µg.ml $^{-1}$)	Sensitive
3	chlorotetracycline (20µg.ml $^{-1}$)	Resistant
4	flumequine (30µg.ml $^{-1}$)	Resistant
5	oxolinic acid (10µg.ml $^{-1}$)	Resistant

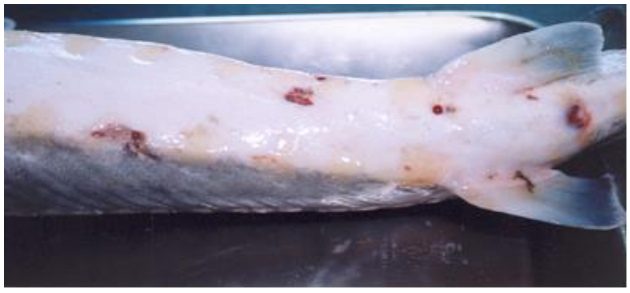

Fig 1. Bleeding ulcers on the ventral surface and at the base of anal fin.

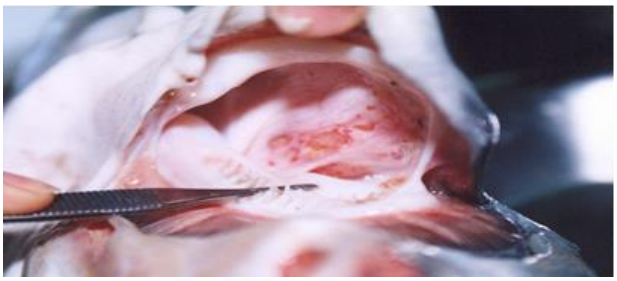

Fig 2. Many ulcers under the opercula.

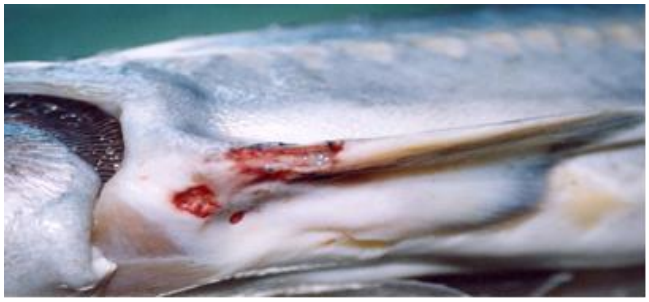

Fig 3. Ulcers at the base of the pectoral fin.

Fig 4. Ulcers around the gill.

Fig 5. Colonies of *V. vulnificus* on TCBS agar.

Fig 6. Colonies of *V. vulnificus* on Blood agar.

DISCUSSION

The results of different studies showed that various bacteria such as: *Streptococcus dysgalactiae., Yersinia ruckeri, Aeromonas hydrophila* and *Flavobacterium hydatis* can cause serious problems in sturgeon fish (Yang & Li, 2009; Vuillaume *et al.,* 1987; Yanong & Floyd 2002; Kolman, 2002).

The present study proved the susceptibility of *H. huso* to vibriosis caused by *V. vulnificus* biotype 2. Biotype 2 has been implicated in infections of marine vertebrates, particularly in cultured eels, tilapia and shrimp and recognized as a serious pathogen (Storm *et al.,* 2000; Fouz *et al.,* 2002; Storm *et al.*, 2000). Moreover, this organism has been described as an opportunistic human pathogen (Strom *et al.*, 2000; Fouz *et al.*, 2002).

V. vulnificus is responsible for important economic losses in intensive culture of European eel (*Anguilla anguilla*) and Nile tilapia (*Oreochromis niloticus*) (Fouz *et al.,* 2002; Fouz *et al.,* 2003). Other fish species, such as sea bass (*Morone saxatilis*), turbot (*Scophthalmus maximus*), sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*) was more resistant than tilapia to *V. vulnificus* (Fouz *et al.*, 2002).

The high temperature is one of the most important factors in the outbreak of vibriosis. The most infections occur during the warmer months of the year (Storm *et al.,* 2000). In this study also, vibriosis was occurred on 26 June when water temperature was 25 - 28ºC.

Several studies have demonstrated that many virulent factors including polysaccharide capsule, Lipopolysaccharide (LPS), hemolysins, toxins, enzymes and iron utilization were correlated with its pathogenesis. *V. vulnificus* produces enzymatic compounds such as hemolysin, elastase, collagenase, lipase, phospholipase, mucinase, chondroitin sulfatase, hyaloronidase, fibrinolysin and a protease with activity against native serum albumin (Strom *et al.,* 2000; Baffon *et al.,* 2001). Three enzymes including elastase, collagenase and chondroitin sulfatase possess elastolytic and collagenolytic activities. They are playing an important role to make lesions in skin and cartilage tissue (Linkous, 1999; Strom *et al.*, 2000; Baffon *et al.,* 2001). External lesions that observed in this study, may be related to mentioned above enzymes activities. The presence of a capsular polysaccharide (CPS) is directly correlated with the virulence of *V. vulnificus* and is essential to its ability to initiate infection. All virulent strains include both encapsulated and non-ecapsulated isolates have an opaque colonial morphology whereas non–encapsulated isolates have translucent colonized and are less virulent or avirulent (Strom *et al.,* 2000). The capsule protects the bacterium by conferring resistance to the bactericidal effects of serum and phagocytosis by macrophages (Linkous, 1999; Baffon *et al.,* 2001). The morphology of our isolate was opaque and confirmed by capsule staining.

CONCLUSION

This study was the first report of *V. vulnificus* in cultured *H. huso* in the north of Iran. *H. huso* could be considered as a susceptible species to *V. vulnificus* biotype 2 in cultured sturgeon farms in the north of Iran. The absence of

reports on the isolation of biotype 2 from diseased fish in Iran could be due to fact that its occurrence is rare in the aquatic environment. More studies would be necessary in farms where there have been previous experience vibriosis outbreaks and the bacterium could be present in water, either associated with particulate material or with fish in a carrier state.

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اولین مورد جداسازی و شناسایی ویبریو ولنیفیکوس (بایوتیپ ۲) از فیل ماهیان پرورشی در ایران

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چکیده

به‌دنبال کاهش ذخایر ماهیان خاویاری در دریای خزر، پرورش گونه‌های مختلف خاویاری از جمله فیل‌ماهی از اهمیت ویژه‌ای برخوردار شده است. در شرایط استرس‌زا ماهیان خاویاری توسط عوامل بیماریزای فرصت طلب مختلف به آسانی درگیر می‌شوند. در تابستان سال ۱۳۹۱، یک مورد همه‌گیری در فیل ماهیان پرورشی یکی از مهمترین مزارع پرورشی استان مازندران در شمال ایران در دمای ۲۸-۲۵ درجه سانتی گراد رخ داد. میزان مرگ و میر ۱۵٪ بود. نخستین نشانه‌های بالینی مشاهده شده در ماهیان در حال مرگ بی‌اشتهایی و بی‌حالی بود. به منظور بررسی‌های باکتریایی بیشتر، تعداد ۲۰ ماهی در حال مرگ به آزمایشگاه مرکزی منتقل شد. در مشاهدات بالینی علایمی از قبیل: زخم‌های عمیق متعدد در سطح بدن، در اطراف سر، زیر سرپوش آبششی، قاعده باله‌های سینه‌ای، خونریزی در اطراف مخرج و آبشش‌ها، نکروز قاعده باله‌های سینه‌ای و کم‌رنگ شدن آبشش ها در این ماهیان مشاهده شد. مهمترین نشانه‌های داخلی مشاهده شده، بزرگ شدن کبد و طحال، تجمع مایع در روده‌ها و خونریزی پتشی در اندام‌های احشایی بود. نتایج آزمایش‌های باکتری‌شناسی و مشاهدات ریخت‌شناسی، ویبریو ولنیفیکوس (بایوتیپ ۲) را به عنوان عامل اصلی بروز تلفات در فیل ماهیان پرورشی نشان داد. این گزارش، اولین مورد شناسایی ویبریو ولنیفیکوس (بایوتیپ ۲) در فیل ماهیان پرورشی در ایران است.

*مولف مسئول