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Experimental *Streptococcus iniae* infection in barramundi (*Lates calcarifer*) cultured in Vietnam

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Abstract: *Streptococcus iniae* has become one of the most important fish pathogens. This work describes first isolation of this bacterial species from cultured barramundi in Khanh Hoa, Vietnam. Three *Streptococcus iniae* strains (Aum, C4 and CR3) were tested for virulence in barramundi model using intraperitoneal injection at concentrations of 10^2-10^7cell per ml. The results showed that the LD50 of Aum, C4 and CR3 when the barramundi were challenged via intraperitoneal were determined to be $10^{4.8}$, $10^{5.6}$ and $10^{5.8}$ CFU respectively. Moribund barramundi diplayed similar clinical symtoms, i.e., erratic swimming, haemorrhage at base of fins, tail rot. Fifteen days after challenge, *S. iniae* could not be isolated from kidney, spleen, liver or brain of surviving fish.

Key Words: *Streptococcus iniae,* barramundi, experiment infection

Introduction

Streptococcal infections are becoming an increasing problem in aquaculture and have been reported worldwide in a variety of fish species. Streptococcosis of cultured fish causes main economic losses in the aquaculture industries of many countries such as Israel (Eldar *et al.*, 1995), Japan (Kitao, 1993) and Korea (Baeck *et al.*, 2006). The first of *Streptococcus iniae* was described from an Amazonian freshwater dolphin (*Inia geoffrensis*) with "golf ball disease" in San Francisco (Pier and Madin, 1976). The bacterium was later found in another fresh water dolphins in an aquarium in New York (Pier *et al.*, 1978). Several outbreaks of *S. iniae* occurred in fish during 1970's and 1980's in Japan (Kitao *et al.*, 1981; Nguyen *et al.*, 2002), Singapore (Foo *et al.*, 1985), Israel and Taiwan (Eldar *et al.*, 1995), but most of these infections were initially misdiagnosed as other bacteria, and

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were only later recognized to be caused by *S. iniae*. Subsequently, *S. iniae* was discovered in various cultured fish stocks, especially hybrid tilapia (AL-Harbi, 1994), barramundi (*Lates calcarifer*) (Bromage *et al.*, 1999), and red drum (*Sciaenops ocellatus*) (Eldar and Ghittino, 1999). Recently, *S. iniae* has been isolated from diseased humans suffering from cellulitis, meningitis, and bacteremia, indicating a threat to public health (Weinstein *et al.*, 1997).

Since Nov. 2008 to Nov. 2009, mortalities in barramundi cultured in Khanh Hoa, Vietnam were observed. This work describes first isolation of bacteria isolated from moribund barramundi, identified as *Streptococcus iniae*.

Materials and methods

Isolation of bacteria

Barramundi were collected from farms in Khanh Hoa and transported live to center for aquatic animal health and breeding studies (CAAHBS), Nha Trang University. Bacterial samples were isolated from the brain, liver and kidney. Isolated bacteria were cultured on trypticase soy agar (TSA), (Merk, Germany) KF streptococcus agar (supplemented with 10ml/l of 1% 2,3,5-triphenyl tetraolium chloride) (Merk, Germany) and blood agar base (Merk, Germany) supplemented with 5% sterile defibrinated blood at 28^{o}C for 24-48h. Isolated bacteria were stored frozen at -80^{o}C in TSA broth supplemented 20% glycerol.

Bacterial characterization

All bacterial isolates were identified using the biochemical tests described in Bergey's Manual of Determinative bacteriology (Brenner *et al*. 2005) and the API 20 STREP test kit (Bio Mérieus ®, France) to compare the biochemical and physiological characteristics of the present isolates with reference strain (ATCC-29178) and the results of other authors.

Fish and challenge experiments

Groups of 20 barramundi juveniles (9-11cm) produced by CAAHBS were kept in 500L fiberglass tanks containing seawater (31‰) where aeration was supplied through an air stone. The fish were fed daily to satiation with NRD (feed for barramundi, INVE, Thailand). The temperatures of tanks were 29 ± 2^{o}C throughout the experimental period. Pure isolates of *S. iniae* (Aum, CR3 and C4) were cultured on TSB broth supplemented 1.5% NaCl as 0.2L volumes in 1L flasks on shaking incubator at 27^{o}C for 24h and centrifuged at 1000 g for 15 minute then washed twice and resuspend in PBS at various concentrations, ranging from 10^2 to 10^7 CFU.mL^{-1}. Each group of fish was injected 0.2 ml of bacterial suspension into intraperitoneally to fish. The control group were injected 0.2ml phosphate buffer saline (PBS). Lethal dose for 50% of the animals (LD50) at 15 day after injection was calculated by probit analysis in the statistical package SPSS.

Tab. 1: Information on fish, pathological signs of diseases sea bass and isolated bacteria (L: Liver; K: Kidney and B: Brain)

Date	Source	Fish No.	Size (cm)	Pathological signs	Growth medium									Mortality rate	Abbreviation	LD50 tested
					TSA			TCBS			KF					
					L	K	B	L	K	B	L	K	B			
11/2008	Ninh Hoa	3	8	haemorrhage in gills, anus	+	+								>50%	C1	
11/2008	Ninh Hoa	2	11	haemorrhage under skin, anus	+	+								>50%	C4	X
02/2009	Vung Ngan	2	26	eruption in mouth and wound in kidney	++	++		+	+	+				>30%	C6 (VN1)	
06/2009	Vung Ngan	6	15	eruption and wound in kidney	++	++		+	+	+				>40%	VN2 (VN)	
07/2009	Cam Ranh	3	8	haemorrhage in gills, skin	++	+++	-	+	++	-				10 fishes per day	CR3	X
10/2009	Cam Ranh	1	30	normal	++	++	+	-	+	-	+	+	+	Non	CL1	
11/2009	Cam Ranh	1	35	normal	+	++	-	-	-	-	-	+	-	Non	CL2	
12/2009	Van Ninh	2	11	ragged tail	+	+	-	-	-	-	-	+	-		Auc	
12/2009	Van Ninh	1	12	haemorrhage liver, pop eye	-	+	-	-	-	-	-	-	-		Aum	X
	Ria I														Ria 1	

Antibiotic sensitivity

Antibiotic susceptibility test was conducted according to disc diffusion method using Mueller-Hinton agar (Merck, Germany) (Bauer *et al*. 1966). The commercially disc including Norfloxacine (10µg), Ciprofloxacin (5 µg), Sulphamethoxazol/trimethoprim (23.75/1.25 µg), Ampicillin (10 µg), Doxycycline (30 µ g), Erythromycin (15 µg), Amoxicyclin (25 µg), Nalidicid acid (30 µg), Oxytetracycline (30 µg), Gentamycin (10 µg), Cephalexin (30 µg) and Streptomycin (10 µg) were placed on the surfaces of the Muller-Hinton agar places by a sterile forceps and gently pressed to make even contact then incubated for 24h at 30°C. The results were recorded as resistant or susceptible based on zone diameters of inhibition, including the diameter of the disc.

Results

Fish pathology

Naturally infected barramundi used for isolation of bacteria showed typical clinical signs of streptococcal infection, including loss of equilibrium, exophthalmia and opacity of the eye, loss of appetite, lethargy and irregular movement. Some fish displayed darkening of the skin, emaciation and proximal margins of the pectoral fins, accumulation of fluid in the peritoneal cavity hemorrhaging of the internal organs, pale livers and enlarged spleens, were also found. Figures 1A and 1B showed the clinical signs from experimental infected fish, the same as in natural outbreaks from farming in Khanh Hoa province, Viet Nam.

The barramundi at these farms had mortalities ranging from 30% to 50%. The information on fish and isolated bacterial were shown in Table 1.

Phenotypic characteristics

The result of identification the phenotype profile of 8 strains showed that on the blood agar incubated at 28°C for 48h, their colonies approach 1mm, opaque and β-haemolytic (Fig. 1C), oxydase negative, the cocci were most often seen occurring as long chains in broth culture (Fig. 1D).

All isolates were similar in phenotypic and biochemical characteristics. In the API 20 strep test, the isolates were positive with esculine (ESC), Pyrrolidonyl acrylamidase (PYRA), Leucine aminopeptidase (LAP), D-ribose (RIB), D-manitol (MAN) and D-trehalose (TRE) (Table 2). API20 STREP analyses resulted in profile numbers 4113115 and 4142114 corresponding to an unacceptable and acceptable match.

Challenge of barramundi with *S. iniae*

Mortality began on day 2, increased on day 5 and ceased at day 10. Gross signs from experimental fish were similar to natural infected barramundi. Moriburn fish diplayed darken body colour, exophthalmia and irregular movement. Hemorrhaging of the internal organs or the skin, pale livers and swollen

Tab. 2: Characteristics of *S. iniae* isolates from infected barramundi and reference strain

Test	Present isolates					*S. iniae* ATCC	*S. iniae* (*)
	Aum/CL1	C1/C4	Auc/CL2	CR3	Ria1		
Fish	Barramundi cultured in Vietnam					Dolphin	
Gram staining reaction	+	+	+	+	+	+	+
Cell morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Catalase production	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-
Voges Proskauer	-	-	-	-	-	-	NT
Haemolysis (5% sheep RBC)	α/β	β	α/β	β	β	β	β
Grow on/in:							
Brain heart infusion	+	+	+	+	+	+	+
Tryptic soy agar	+	+	+	+	+	+	+
Tryptic soy broth	+	+	+	+	+	+	+
Blood agar	+	+	+	+	+	+	+
Temp. 10°C	-	-	-	-	-	-	-
Temp. 27°C	+	+	+	+	+	+	+
Temp. 35°C	+	+	+	+	+	+	NT
NaCl 6.5%	-	-	-	-	-	-	NT
Hippurate (HIP)	-	-	-	-	-	-	NT
Esculin (ESC)	+	+	+	+	+	+	
Pyrrolidonyl acrylamidase	+	+	+	+	+	+	NT
α-Galactosidase (α-GAL)	-	-	-	-	-	-	+
β-Glucuronidase (β-GUR)	-	-	-	-	-	-	NT
β-Galactosidase (β-GAL)	-	-	-	-	-	-	NT
Alkalin Phosphatase (PAL)	-	-	-	-	-	-	
Leucine Aminopeptidase (LAP)	+	+	+	+	+	+	+
L-arginie (ADH)	-	+	-	+	+	+	-
D-ribose (RIB)	+	+	+	+	+	+	-
L-arabinose (ARA)	-	-	-	-	-	-	+
D-manitol (MAN)	+	+	+	+	+	+	-
D-sorbitol (SOR)	-	-	-	-	-	-	-
D-lactose	-	-	-	-	-	-	NT
D-trehalose	+	-	-	+	-	+	NT
Inulin	-	-	-	-	-	-	NT
D-raffinose	-	-	-	-	-	-	NT
Starch (AMD)	+	-	+	-	-	+	NT
Glycogen (GLYG)	-	-	-	-	-	-	NT

NT: not test
(*) Data from Bergey's manual of systematic bacteriology (Brenner et al., 2005)

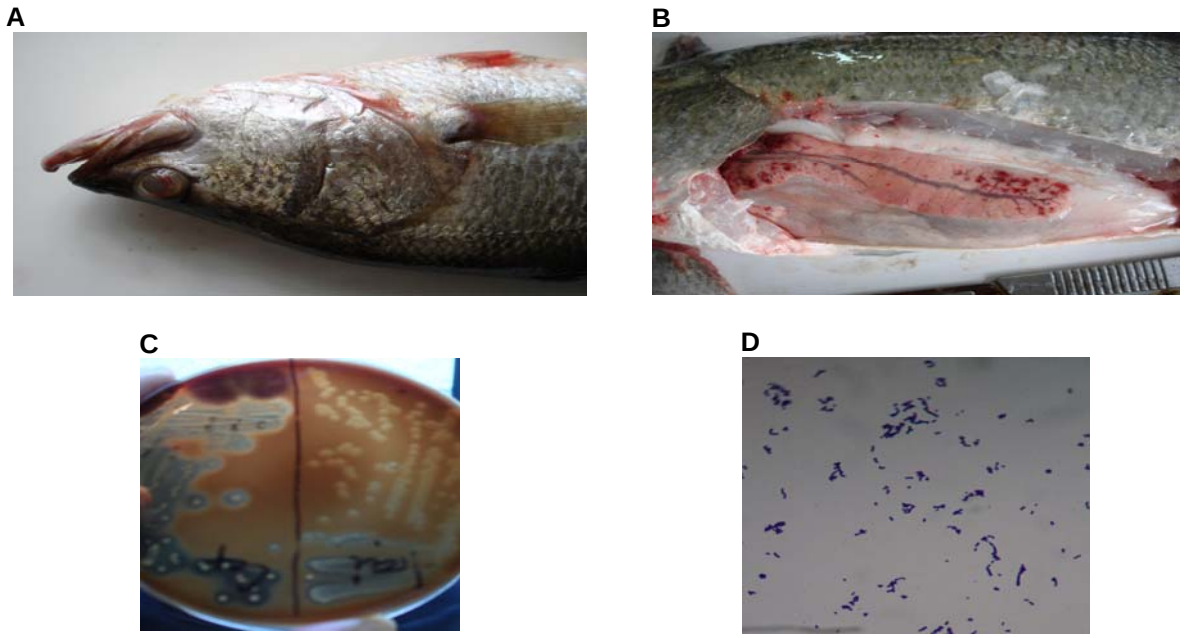

Fig. 1: *Streptococcus iniae* isolated from barramundi. A: Diseased fish used for isolation of bacteria. B: Dead fish from challenged experiment. C: β-haemolysis (left), α-haemolysis (right). D: Gram stained bacteria.

kidneys were also found. No mortality occurred in the control groups. Bacterial isolation from dead and moribund fish resulted in pure cultures of *streptococcus iniae* from the brain, kidney and spleen. The LD50 of Aum, C4 and CR3 when the barramundi when challenged by intraperitoneal injection were determined to be $10^{4.8}$, $10^{5.6}$ and $10^{5.8}$ CFU, respectively (Fig. 2).

Antibiotic susceptibility

The isolates were sensitive to seven antibiotics including Norfloxacine, Ciproffloxacin, Sulpham-ethoxazol/trimethoprim, Ampicillin, Erythrom-ycin, Doxycycline and Amoxicyclin but were resistant to Nalidicid acid, Oxytetracycline, Gentamycin, Cephalexin and Streptomycin (Table 3).

Discussion

Streptococcus iniae was isolated from diseased barramundi in the warm season. *S. iniae* is known to have a temperature dependent pathogenicity and disease outbreaks are known to occur during the warm season (Bromage *et al.*, 1999; Kusuda and Salati, 1999). Bacteria were isolated from several organs of infected fish and thus there were a systemic infection with high numbers of

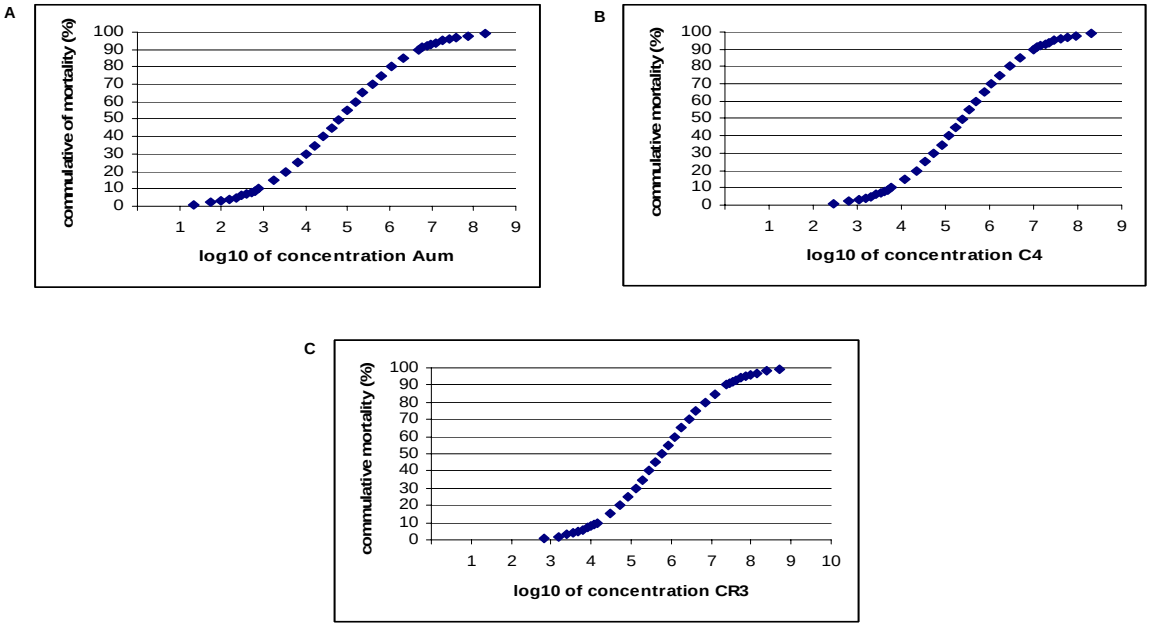

Fig. 2: Mortality curves of barramundi inoculated *Streptococcus iniae* into intraperitoneal.

Tab. 3: Antibacterial susceptibility and resistance of *S. iniae* isolates to eleven antibiotics

Antibiotics	Conc. (µg/disc)	Mean of inhibition zone ± SE (mm)
Norfloxacine	10	26 ± 3.2
Ciprofloxacin	5	24 ± 2.6
Sulphamethoxazol/trimethoprim	23.75/1.25	16 ± 0.9
Ampicillin	10	21 ± 2.7
Doxycycline	30	28 ± 4.6
Erythromycin	15	22 ± 4,1
Amoxicyclin	25	23 ± 1,4
Nalidicid acid	30	0
Oxytetracycline	30	10 ± 3.4
Gentamycin	10	8 ± 1.6
Cephalexin	30	0
Streptomycin	10	0

bacteria. Pure isolates of bacteria obtained by growth on agar. On blood agar small colonies up to 1mm diameter, with opaque center and translucent border were obtained, colonies surrounded by a small to moderate area of β-hemolysis passing to α-hemolysis (Brenner *et al.*, 2005). This is in accordance with Bergey's manual, *Streptococcus iniae*, saying the *S. iniae* are spherical cells, encapsulated, up to 1.5μm diameter, in broth culture cocci arranged in long chains.

β-hemolytic streptococci seem to occur widely throughout the animal worlds as pathogenic agents in fish. Taxonomic status of the isolates were determined by the comparison of the results with the reference strain and the original report of the type isolate of *Streptococcus iniae* (Pier and Madin, 1976). The results obtained in this study suggest that the isolates are biochemically and physiologically similar to *S. iniae* that has been isolated in previous studies (Bromage *et al.*, 1999; Colorni *et al.*, 2002; Perera *et al.*, 1994) except for L-arginine, D-trehalose and starch biochemically. The isolates Aum, Auc, CL1 and CL2 were positive for starch and negative for ADH and glycogen acidification. ADH negative isolates are considered to belong to serotype II of *S. iniae* (Bachrach et al., 2001b, Barnes et al., 2003, Nho et al., 2009, Shoemaker et al., 2010) while the isolates C1, C4, CR3, Ria1 and reference strain were ADH-positive isolates and belong to serotype I (Bachrach *et al.*, 2001a, b; Barnes *et al.*, 2003). The rapid API 20 STREP system failed to identify the isolates from barramundi. This lack of identification has previously been reported by many researchers using Api 20 Strep or Rapid ID 32 Strep (Facklam *et al.*, 2005; Klesius *et al.*, 2006; Lau *et al.*, 2003; Poyart *et al.*, 1998; Weinstein *et al.*, 1997) because there are not *S. iniae* in database of the rapid API 20 STREP system (Agnew and Barnes, 2007).

The results from the experimental challenge of barramundi with *Streptococcus iniae* showed that *S. iniae* caused mortalities in injected fish causing symptoms in accordance with those seen in naural outbreaks. This showed that it was very likely that the etiological agent of the disease observed at the farms were caused by the isolates found to be *S. iniae*. *S. iniae* infections in barramundi were systemic infections of the brain, liver and kidney. In our experimental infections, The LD50 of Aum, C4 and CR3 when the barramundi were challenged by intraperitoneally was determined to be $10^{4.8}$, $10^{5.6}$ and $10^{5.8}$ CFU, respectively. The virulence of barramundi isolates in this study seem to be lower than the *S. iniae* from barramundi cultured in Australia and Thailand which had LD50 were 3.2×10^4 CFU and 1.08×10^4 CFU per fish respectively (Bromage *et al.*, 1999; Suanyuk *et al.*, 2010). However, It is difficult to compare the virulence of *S. iniae* isolates because disease progression in fish is dependent on the route of infection, fish age

and other environmental and water quality factors (Agnew and Barnes, 2007).

The isolates of *S. iniae* were sensitive to 7/12 of antibiotics used in this study. Similar results have been reported by various authors Aamri *et al.* 2010). However, they were resistant to 5/12 of antibiotics. A previous study (Aamri *et al.*, 2010) showed that *S. iniae* were resistant 4/15 of antibiotics. They also were resistant only 2/12 of antibiotics in a study in Thailand (Suanyuk *et al.*, 2010) and they were susceptible to all of the antibiotics and concentrations tests in Park's study (Park *et al.*, 2009). By comparison, our results indicated that *S. iniae* used in this study were more resistant to antibiotics than other ones in previous reports.

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