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Streptococcus iniae, a bacterial infection in barramundi *Lates calcarifer*

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ABSTRACT: The cause of ongoing mortality in barramundi *Lates calcarifer* (Bloch) in seawater culture was identified as *Streptococcus iniae* by biochemical and physiological tests. This is the first published record of this bacterial species in Australia and the first confirmed report of *S. iniae* causing mortality in barramundi. The bacterium was highly pathogenic for barramundi when challenged by bath exposure. The pathogen was found to have a LD_{50} of 2.5×10^5 and 3.2×10^4 colony-forming units at 48 h and 10 d respectively. Experimental challenge of barramundi resulted in high levels of mortality (>40%) within a 48 h period. Ten days after the challenge, *S. iniae* could not be isolated from kidney, spleen, liver or eye of surviving fish. However, the organism was easily isolated from the brain of both moribund and healthy fish, indicating that barramundi can carry the bacterium asymptomatically.

KEY WORDS: *Streptococcus iniae* · *Lates calcarifer* · Disease studies

INTRODUCTION

It is with increasing regularity that streptococcosis is being identified as the cause of major losses in the aquaculture industry. The syndrome streptococcosis has been used to describe fish disease caused by lactic acid bacteria, including streptococci, enterococci and lactococci (Hawkesford 1997). Recent reviews have indicated that there has been at least 40 separate outbreaks of streptococcosis worldwide (Schmidtke 1995, Bromage 1997, Hawkesford 1997). Streptococcosis appears to have very few limitations in regard to geographic boundaries or host range, with outbreaks occurring in aquaculture facilities worldwide and in many different cultured species. The vast majority of outbreaks due to members of the genus *Streptococcus* have occurred in cultured fishes including rainbow trout *Oncorhynchus mykiss* (Hoshina et al. 1958, Bragg & Broere 1986, Ceschia et al. 1992), yellowtail *Seriola quinqueradiata* (Minami et al. 1979), tilapia *Tilapia* spp. (Eldar et al. 1994, Perera et al. 1994), and rabbitfish *Siganus canaliculatus* (Foo et al. 1985), and more recently an *Enterococcus*-like bacterium was isolated from moribund freshwater prawns (Cheng et al. 1998).

There have been several reported outbreaks of streptococcosis in Australia, occurring primarily in cultured rainbow trout (e.g. Carson & Munday 1990, Carson et al. 1993). The first report of streptococcosis in the tropics of Australia was from an investigation by Queensland Department of Primary Industries Oonoonba Veterinary Laboratory (QDPI OVL) and reported in Moody (1992), who briefly described the involvement of a streptococcal pathogen in mortalities in sea-farmed barramundi.

Since 1992, mortality due to streptococcosis in sea-cultured barramundi has increased and the pathogen is now considered as the most important bacterial species affecting the successful sea-culture of barramundi in northern Queensland (I. Patch pers. comm. 1997). The disease usually persists as a low-level, chronic infection with mortalities occurring daily, mainly in juvenile fish. The clinical signs of disease are very similar to those described by other authors (Kusuda et al. 1976, Perera et al. 1994), including bilateral exopthalmia, darkened body pigmentation and ascites. However, acute episodic outbreaks occur throughout the warmer months with mortalities reaching 70% overnight and clinical signs being limited to mild corneal opacity.

This paper reports on the identity of the bacterium responsible for the on-going mortality of sea-cultured

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barramundi, and establishes, via Koch's postulates, that this bacteria is the cause of these outbreaks.

METHODS

Bacterial isolation. Moribund barramundi displaying clinical signs of the disease were obtained from affected farms and transported live to the laboratory. Bacterial isolation was performed from the brain, spleen, kidney, liver and eye. Swabs from these sites were streaked onto blood agar (BA Oxoid, Basingstoke, UK) supplemented with 5% sterile sheep's blood (laboratory animal). Bacterial cultures were incubated at 27°C for 48 h.

Bacterial characterisation. Bacterial isolates were identified using the classical plate and test tube tests (Smibert & Krieg 1981, Hardie 1984, Facklam & Washington 1991, Coleman et al. 1992) and the API 20 STREP identification system (BioMerieux, Marcy l'Etoile, France). Lancefield groupings A–F were performed using the Streptex system (Murex Diagnostics, USA). Isolates of *Enterococcus faecalis* and *Streptococcus pyogenes* were run in parallel to the streptococcal-like isolates to serve as controls. The API 20 STREP was conducted as per the manufacturers instructions except that the incubation temperature was reduced to 27°C to represent the ambient environmental temperature. Test tube tests were performed by suspending the bacterial culture obtained from a BA plate into 5 ml of sterile phosphate buffer saline (PBS), using a sterile swab, until a cell density equal to 4 MacFarlane units was obtained. Bacterial suspension (100 µl) was then dispensed into each of the tubes containing the varying substrates and incubated for 24 h at 27°C. Reactions were scored positive (+) or negative (–) with regard to colour change by comparison to an uninoculated solution. Two known bacterial controls, *E. faecalis* and *S. pyogenes*, were characterised simultaneously using both systems.

Fish husbandry under experimental conditions. Barramundi fingerlings of an average total length 100 mm were obtained from NQ Barramundi (Townsville, Australia). A sample of 15 fish were subject to bacterial isolation as described above to determine the health status of experimental animals.

The experimental infection trials were performed in glass aquaria maintained at a salinity of 35 ppt and 27°C.

Determination of the lethal dose 50%. Seven groups (6 experimental and 1 control) of 10 barramundi were placed into individual glass aquaria and were allowed to acclimatise over a period of 2 wk before the experiments began. A single bacterial isolate (no. 28-97-L-B) was passaged 3 times through barramundi without culture on hard media following the methods of Eldar et al. (1995a). Following the final passage, a bacterial swab was taken from the brain of the first moribund barramundi, streaked onto BA and incubated for 24 h at 27°C. The culture was then re-suspended in PBS until a cell density of 10^8 colony-forming units (cfu) per ml was achieved. The range of 10^2 to 10^7 was used to provide a broad range of challenge densities. Each group of fish were placed into a 10 l container of seawater containing the appropriate dilution of the bacteria or neat seawater for the control. After an exposure period of 1 min, the fish were removed and placed into their respective aquaria.

Determination of the lethal dose for 50% of the animals (LD_{50}) at 48 h and 10 d was conducted using a probit analysis and the mortality curves were compared using survival analysis in the statistical package SPSS (SPSS Inc.).

Experimental challenge of *Lates calcarifer* with isolates of *Streptococcus iniae*. Three isolates of *S. iniae* (nos. 48-97-L-K, 28-97-L-B, 21-97-W), were randomly chosen from the isolates obtained from moribund farmed barramundi. Ten fish per isolate were then challenged by bath exposure to *S. iniae* equal to the LD_{50} at Day 10. All mortalities were recorded, and dead and moribund fish were subject to bacterial isolation. All fish surviving at Day 10 were euthanised and bacterial isolation performed.

RESULTS

Morphology and characterisation

Bacterial isolation from 31 moribund barramundi received from the farm revealed that *Streptococcus*-like isolates could be obtained, in almost pure culture, from the brain of all infected fish (Table 1). The bacterium could also be isolated to varying degrees from all the other organs sampled.

The bacterial isolates were characterised as follows: Gram-positive, catalase negative, oxidase negative cocci, up to 1 µm in diameter. The cocci were most often seen occurring as long chains in broth culture. Growth on BA media resulted in small white umbonate colonies that were surrounded by a small area of beta

Table 1 *Streptococcus iniae* infecting *Lates calcarifer*. Percentage of recovered isolates from the various organs (5) of 50 naturally infected barramundi

	Brain	Kidney	Spleen	Liver	Eye
Isolation (%)	100	92	50	12	28

Table 2. *Streptococcus iniae* infecting *Lates calcarifer*. Comparison of the biochemical and physiological characteristics of the present isolates and the results obtained by other authors. +: positive, –: negative, ND: not done

Test	Percentage of present isolates (n = 31)	Pier & Madin (1976)	Kusuda et al. (1976)	Stoffregen et al. (1996)
Haemolysis (β)	100	+	ND	+
Gram stain (+)	100	+	+	+
Oxidase	0	–	–	–
Catalase	0	–	–	–
Motility	0	–	–	–
Growth in/at: 10°C	6	+	+	ND
45°C	0	–	+	–
pH 9.6	0	–	+	ND
6.5% NaCl	0	–	+	+
0% bile	0	–	+	ND
Methylene blue milk 0.1%	100	+	+	ND
Hydrolysis of: Starch	100	+	–	ND
Hippurate	0	–	–	–
Esculin	100	+	+	+
Voges-Proskauer	0	ND	+	–
Pyrrolidonylarylamidase	100	ND	ND	+
α-Galactosidase	0	ND	ND	ND
β-Glucuronidase	100	ND	ND	ND
Acid from: Arabinose	0	–	–	–
Fructose	100	+	ND	+
Glucose	100	+	+	+
Inulin	0	–	ND	–
Lactose	0	–	–	–
Mannitol	100	+	+	+
Mannose	100	+	+	ND
Raffinose	0	–	–	–
Ribose	100	ND	ND	ND
Salicin	100	+	+	+
Sucrose	100	+	–	–
Sorbitol	0	–	+	–
Trehalose	100	+	–	+
Xylose	0	ND	–	–
β-Galactosidase	0	ND	ND	ND
Alkaline phosphatase	100	ND	ND	ND
Leucine arylamidase	100	ND	ND	ND
Arginine dihydrolase	100	ND	ND	ND

haemolysis and a larger area of alpha haemolysis. In stab culture in BA tubes, all isolates showed complete beta haemolysis.

Isolates obtained from barramundi all displayed identical biochemical characteristics suggesting that they were all the same or a closely related species. Using the commercial API20 STREP system resulted in a profile number of 4 5 6 2 1 1 7, which corresponded to an unacceptable match to *Streptococcus equisimilis* (APILAB PLUS, BioMuriex). The API 20 STREP system successfully identified the *Enterococcus faecalis* and *Streptococcus pyogenes* control isolates.

No variation was observed in regard to the biochemical profile using the commercial and typical testing methods. However, the additional tests performed in the conventional tests allowed identification of the isolates as *Streptococcus iniae* from comparison with the results from the literature (Pier & Madin 1976) (Table 2). The present isolates only differed in their inability to grow at 10°C after 48 h, with only 2 of the 31 isolates (6%) being able to grow at this temperature, whereas the type isolate was reported to have good growth. No reaction was observed from the present isolates in the agglutination test to Lancefield A–F antisera.

Challenge of *Lates calcarifer* with *Streptococcus iniae*

No bacterial pathogens were isolated from any of the fish screened prior to the challenge experiments. The LD_{50} of the isolate (no. 28-97-L-B) when the fish were challenged via bath exposure was determined to be 2.5×10^5 cfu and 3.2×10^4 cfu at 48 h and 10 d respectively. When the fish were subject to the 10 d LD_{50}, the time to the first death was usually between 16 to 24 h, and in all cases, 40% of the experimentally challenged fish were dead within 48 h (Fig. 1). Statistical comparison of the mortality curves revealed that the isolates displayed similar mortality schedules.

Clinical observations

Fish that died within 48 h displayed darkened, but patchy body colouration, mild corneal opacity, and an increased ventilation rate. Fish were frequently seen swimming with part of the head out of the water. Before death, fish sank to the bottom, lost the ability to orientate themselves in the water, and appeared to have difficulty ventilating. As the experiment continued beyond 48 h, the typical signs of streptococcosis became increasingly evident. Exopthalmia and corneal

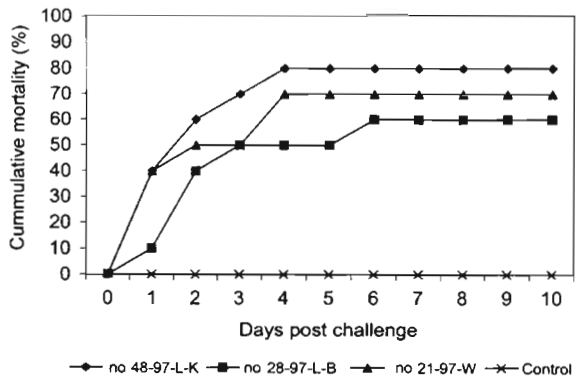

Fig. 1. *Streptococcus iniae* infecting *Lates calcarifer*. Comparison of mortality curves of the barramundi challenged with isolates of *S. iniae*

opacity were always present; often the eye was completely necrotic, resulting in complete blindness. The body of the fish was darkened, and in extreme cases the fish swam in a spiralling motion. Fish were able to respond to physical stimuli, but had little control over movement. Internally, ascites was present, the spleen and kidney were both enlarged when compared to the control animals.

Bacterial isolation from dead and moribund fish resulted in pure cultures of *Streptococcus iniae* from the brain, kidney and spleen. We were unable to isolate *S. iniae* from the spleen or kidney of any surviving fish on Day 10. However, pure isolates were infrequently isolated from the brain of the survivors. No mortality occurred in any of the control fish and the bacterium was not recovered from any organ.

DISCUSSION

The inability of the present isolates to grow at/in 45°C, 6.5% NaCl, pH 9.6 or 40% bile, excludes the isolates from the genus *Enterococcus* (Facklam & Washington 1991). Taxonomic status of the isolate was determined by the comparison of the results with the original report of the type isolate of *Streptococcus iniae* (Pier & Madin 1976). The results obtained suggest that the present isolates are biochemically and physiologically similar to the isolate obtained by Pier & Madin (1976) from freshwater Amazon dolphins. This is the first time that this particular *Streptococcus* has been reported in Australia, and the first confirmed report of this pathogen causing disease in barramundi. It must be noted that, as *S. iniae* is currently regarded as an exotic pathogen to Australia, there were restrictions against the importation of the ATCC type strain into Australia, and therefore comparitive characterisation could not be performed between the type strain and local isolates.

One previous report of streptococcosis in barramundi occurred in China in 1990 (Huang et al. 1990), where the disease caused mortality ranging between 16.7 and 32.6%. The causative agent of disease was reported as a *Streptococcus* sp., but variations in the isolates obtained from moribund fish with regards to biochemical tests and serology indicate that more than one species was involved in the outbreak. The isolates obtained from the Chinese barramundi displayed vague similarity in biochemical responses to that of the 2nd isolate of *S. iniae* that was described by Pier et al. (1978) from another captive Amazon freshwater dolphin. Whether or not this isolate is indeed *S. iniae* is uncertain.

Foo et al. (1985) reported a streptococcal infection in cultured rabbit fish *Siganus canaliculatus* from a farm in Singapore. Barramundi and grouper *Epinephelus tauvina* were also being cultured at the same location, but did not succumb to the disease. This finding is important as the aetiological agent was later reported by Stoffregen et al. (1996) as a subspecies of *S. iniae*.

The results from the experimental challenge of barramundi with *Streptococcus iniae* indicate that it can cause morbidity and mortality via a natural route of infection. The subsequent re-isolation of the pathogen from these fish fulfills Koch's postulates and demonstrates that *S. iniae* is indeed the aetiological agent of the disease observed at the farms.

The isolation of the bacterium from the brain of all naturally infected fish, and only from brain of fish surviving experimental challenge highlights that the bacterium can be carried asymptomatically in the brain of fish not displaying signs of the disease. It is well known that antibodies cannot penetrate the blood-brain barrier (Gudkovs 1988). The dectection of an anti-streptococcal humoral immune response has been shown to confer protection against true members of the genus *Streptococcus* in both tilapia and rainbow trout (Eldar et al. 1995b, 1997). Therefore, if protection against streptococcal infections in barramundi occurs via the same mechanism, this would help explain why the brain remains infected while the other organs test negative for the bacterium. This has serious implications for the culture of barramundi, because even the most diligent farmers removing infected stock will be unable to remove all infected specimens from the farm. These fish will serve as reservoirs of *S. iniae* in an otherwise healthy population.

The isolation of *Streptococcus iniae* is extremely significant to the future of aquaculture in tropical Australia. Streptococcosis has already shown itself to be a major limiting agent in the successful culture of a variety of temperate-water fish species in many countries including Japan (Kusada et al. 1976), Australia (Carson & Munday 1990) and South Africa (Boomker et al. 1979). Now, with the appearance of a streptococcal pathogen in the tropics of Australia, there is a significant emerging threat to profitable aquaculture in that region.

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