Fish Kill of Mullet *Liza klunzingeri* in Kuwait Bay: The Role of *Streptococcus agalactiae* and the Influence of Temperature

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ABSTRACT

A massive fish kill occurred in Kuwait Bay during August and early September 2001. Wild mullet *Liza klunzingeri* represented 99% of the fish kill, whereas the remaining 1% consisted mainly of silvery croaker Otolithes argenteus, giant sea catfish Arius thalassinus and howed erratic swimming, hemorrhages around the mouth, abdomen, pectoral and pelvic fins. They also exhibited internal hemorrhage and exophthalmia. Bacterial isolates from four wild fish species, one cage-cultured species (sea bream *Sparus auratus*) and sewage samples from two beach locations were used to identify the causative bacterium. The biochemical, biophysical and API 20 Strept tests identified the bacterium as β-hemolytic group-B S*treptococcus agalactiae. S. agalactiae* can grow in a range from 18 to 43°C, but not at 5, 12 or 45°C. It can grow in a range between 0.5 to 6.0% NaCl, but not at 6.5%. To investigate the effect of temperature on the susceptibility of fish to infection, healthy mullet kept at two seawater temperatures (25 and 33°C), were intraperitonealy injected with four bacterial doses (101,103,105 and 107 CFU/fish). At 33°C, 100% mortality was produced after 24 h, when fish were injected with 108, whereas the other doses produced the same mortality after three to four days. At 25°C, the mortality varied between 20 and 60% among the different doses. The results clearly implicate the significant role of high temperature in increasing the virulence of *S. agalactiae* causing the massive mullet mortality. The possible relationship of this outbreak to another in August and September 2000 in cultured silver pomfret *Pampus argenteus* is discussed.

INTRODUCTION

During the first week of August 2001, the Environmental Public Authority (EPA/Kuwait) reported a consequential mortality among cage-cultured European seabream *Sparus auratus*

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in Kuwait Bay. The disease outbreak resulted in 100% mortality in two cages. Subsequently, a remarkable mortality started in Kuwait Bay among wild mullet *Liza klunzingeri,* locally known as maid. Other mortalities were also observed among silvery croaker Otolithes argenteus, giant sea catfish *Arius thalassinus* and striped grunt *Rhonciscus stridens.* Since then, the mortality increased till the end of August, decreased in early September and started to cease from mid September. There was an estimated total loss of 2,500 t fish, comprising approximately 99% mullet and about 1% other fish.

There have been several reported outbreaks among wild marine and cultured fish in different parts of the world that were associated with Streptococcus sp. Fish kill was reported in estuarine bays along the Florida and Alabama Gulf coast among eight marine fish species (Plumb *et al.,* 1974). *Streptococcus iniae* was found to be associated with a fish kill that affected thousands of demersal reef fish in several southeastern Caribbean countries from July to September 1999 (Pan American Health Organization, 2000). Other *Streptococcus* sp. have been associated with mortality in mullets *Liza ramada* and *Mugil cephalus* (Hubbert, 1989).

Experimental Streptococcus sp. infection was described in various fish species by different routes of infection. Cook and Lofton (1975) showed that intraperitoneal injection of Streptococcus sp. isolated from moribund menhaden *Brevoortia patronus* kidney causes death in all five species tested. The fish kill in Kuwait Bay has been ascribed ascribed to infection with *S. agalactiae* (Evans *et al.,* 2002, Glibert *et al.,* 2002).

The objective of this manuscript is to provide additional information on the incident in Kuwait. In particular, it will describe the possible sources of *S. agalactiae* which was associated with the fish kill of mullet *L. klunzingeri* and four marine other fish species in Kuwait Bay. In addition, it will document the pathogenicity of *S. agalactiae* in healthy mullet following infection by intraperitoneal injection at two ***seawater temperatures.***

MATERIALS AND METHODS

Fish examination and bacterial isolation

Freshly dead or moribund fish from Kuwait Bay were transported for examination to the Fish Health Laboratory, Mariculture and Fisheries Department (MFD), Kuwait Institute for Scientific Research (KISR). Gross morphological examination and bacterial isolation were carried out on a total of 53 fish samples representing five fish species: mullet (60%), silvery croaker (17%), giant sea catfish (7.5%), striped grunt (7.5%) and cage-cultured gilthead seabream (2%). Samples were taken mostly from the kidney, spleen, brain, eyes and the ascitic fluid. Mashed tissues and fluid were inoculated onto tryptic soy agar (TSA/Difco) and brain heart infusion agar (BHIA/Difco) supplemented with 2.0% NaCl and incubated at 35°C for 24 h.

Environmental parameters

The oceanography team at MFD recorded temperature, salinity, pH and dissolved oxygen during the fish kill duration in Kuwait Bay (6 August to 23 September 2001).

Identification of bacterial isolates

Pure bacterial isolates were characterized following some basic tests: Gram stain, morphology, motility, oxidase and catalase. The Gram-positive, coccoid, oxidase and catalase negative bacterial isolates were further characterized using API 20 Strept (bio-Mérieux, France). Confirmed streptococcal isolates were serologically grouped according to the Lancefield grouping system (1933), using streptococcal grouping kit (Oxoid) for groups A, B, C, D, F, and G.

Mud and sewage samples

For the purpose of detecting possible sources of the causative agent, mud/sediment samples were collected by means of a mud grabber (Ponar Dredge, Wildco), from locations near the Doha fish farm cages, and from other different locations within Kuwait Bay. Sewage samples were also collected from two sewage outfall canals that drain directly into Al-Salam and Beneid Al-Gar beaches. All samples were immediately transported to the laboratory (MFD) in sterilized glass sampling bottles. A 10 ml of each sample was added to 90 mL peptone water for enrichment, and was left for 18 h. Subsequently, the enriched samples were homogenized and serially diluted in peptone water following the conventional tenfold diluting method. Briefly, 1ml of the homogenate was added to 9 ml of peptone water in a test tube to make a total of 10 ml by tenfold dilution. Then, 1 ml of this mixture was added to another 9 ml of peptone water, hence the serial tenfold dilution method. From each diluted test tube, 0.1 ml and 1 ml sample were obtained and transferred aseptically to two dishes containing BHIA and 5% defibrinated sheep blood, using direct spread-plate count technique and pour-plate method, respectively.

Biophysical characterization

Tolerance of the bacterium to various temperatures and NaCl concentrations were determined using BHI broth, following incubation for 24 and 132 h. A representative of 15 bacterial isolates from the brain, kidney, spleen and eyes of the four marine species and sewage samples were used. A single colony from each bacterial isolate was streaked into BHIA and incubated at 35°C for 18-24 h. After incubation, colonies of the bacteria were transferred to 5 ml sterile neutral salt solution (NSS) test tubes. The bacterial density was adjusted to MacFarland # 5. Later, a 0.1 ml of the bacterial suspension was added to 10 ml BHI broth tubes. They were incubated at 5, 12, 18, 25, 37, 40, 43 and 45°C. Tolerance to NaCl for the isolates was determined for 0.5, 1.5, 3.0, 4.5, 5.0, 5.5, 6.0 and 6.5% (w/v) NaCl in BHI broth inoculated as described earlier, and incubated at 35°C.

Antimicrobial susceptibility pattern

Antibiotic sensitivity patterns were determined by the disk diffusion method using Muller-Hinton agar (Oxoid). Eleven antibiotic disks (g/disk) were tested: amoxycillin, flumequin, gentamycin, kanamycin, neomycin, novobiocin, oxolinic acid, oxytetracycline, rifampicin, vancomycin and sulphamethoxazole/trimethoprim.

Fish and tank management

Healthy mullet weighing on average 55 g were used in this study. They were wild-caught and then reared at the hatchery building at MFD. Four treatment doses were used for the fish injection (10^1 ,10^3,10^5, and 10^7 CFU/fish). Five fish per treatment were placed in each of two 100 L seawater tanks, which were maintained at 25 and 33°C. Two control tanks containing five fish each were also used.

Bacterial suspension

Streptococcus agalactiae (Isolate no. 18A) isolated from the kidney of an infected mullet was used. The bacteria that were incubated on BHIA for 18-24 h were suspended in NSS and adjusted the turbidity of MacFarland # 1, which is approximately equivalent to 10^8 CFU/ml in bacterial number. Then, the original suspension was diluted with NSS for each bacterial number required for the experimental infection.

Fish injection

Each 0.1 ml of bacterial suspension, adjusted to 10^1, 10^3, 10^5, and 10^7 CFU/fish was injected intraperitoneally into the fish. Additionally, 0.1 ml NSS was intraperitoneally injected into the 10 fish for the control groups. After the injection, the fish were maintained for 10 days without feeding to observe the survival rates. Half of the rearing seawater was exchanged once a day.

Bacterial isolation

During the experiment, bacterial isolations were obtained from the spleen and brain of the dead fish using BHIA. When the experiment was terminated, bacterial isolations were obtained from the spleen, brain and intestine of all the surviving fish using BHI broth containing 25 ppm oxolinic acid, which can inhibit the growth of most Gram-negative bacteria, but not Streptococcus sp. Inoculates were incubated at 35°C for two days. Bacterial growth was examined using Gram staining to observe any coccal Gram-positive bacteria. When Streptococcus was detected in the broth, a drop of the broth was streaked on BHIA with 5% sheep blood. This was carried out to confirm the streptococcal characteristics based on colony formation, haemolysis, catalase and oxidase reactions. It was finally identified with the API 20 Strept system.

Detection of bacteria in the tissue

Spleen smears were used to detect the bacteria. The smears were fixed with absolute methanol for 3 min and stained with 5% Giemsa solution for 30 min.

RESULTS

Environmental parameters

The average seawater temperature was 32.5°C from 6 August to 23 September, but the highest temperature reached 36.4°C during the first week of August. The average dissolved oxygen (D.O) was 7 mg/l from 6 August to 23 September and the lowest value of D.O during that period reached 3.3 mg/l. The average salinity was 43 ppt and the pH was 8.0 during the same period.

Table1. Characteristics and reactions in API 20 Strept of 64 isolates of *S. agalactiae* isolated from five marine fish and three isolates from the sewage canals in Kuwai Bay (August to September 2001).

Test	Isolate No.																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	17A	18	18A	18B	19	20
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidation/fermentation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hemolysis	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β	β
Growth on:																							
BHIA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
TSA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acetoin production	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of:																							
Hippurate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esculin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Starch	nd	-	nd	nd	-	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pyrrolidonylarylamidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Glucorinase	-	+	+	+	+	-	-	+	+	+	+	+	+	+	-	+	-	-	+	+	+	+	-
β-Galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Alkaline Phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from:																							
Ribose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Arabinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Mannitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	-	-	-	-	-	+	-	+	+	-	-	+	+	+	+	-	-	+	-	-	-	+	+	
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amygdalin	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Identified as *S. agalactiae*	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lancefield grouping test	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B

Isolate Nos. 1-20 were taken from kidney tissues of mulletnd = not determined

Disease symptoms

The 53 fish samples representing the five fish species showed severe reddening around the mouth and haemorrhages at the base of pectoral, ventral and anal fins. Exophthalmus was regularly observed. Internally, haemorrhages, splenomegaly and kidney enlargement was frequently seen. In the silvery croaker, the presence of petechial haemorrhages on the ventral side of the body was distinct with severe internal haemorrhage and excessive production of ascitic fluid.

Identification of bacterial isolates

A total of 64 bacterial isolates (Table 1) from the kidney, spleen, brain, eye and ascitic fluid showed similar physiological characteristics. The serological grouping revealed that all strains reacted with the group-B in the kit, according to Lancefield grouping system. These were Gram-positive cocci, non-motile, negative reactions in oxidase and catalase. A clear transparent zone of haemolysis surrounded the colonies. Therefore, they all revealed the characteristics of group B, β-haemolytic *Streptococcus* sp. These isolates were further characterized using the API 20 Strept system (Table 1) and the results were interpreted using the Analytical Profile Index of the API 20 Strept system. Although the isolates showed some variation in reaction to β-glucuronidase and the acidification patterns in trehalose, all the isolates were identified as *Streptococcus agalactiae.*

Temperature and NaCl tolerance

The selected representative 15 isolates of *S. agalactiae* grew at 18, 25, 37 and 43°C, but not at 5, 12 and 45°C. All the strains of *S. agalactiae* grew in a range from 0.5 to 6% NaCl, but not at 6.5%.

Antimicrobial sensitivity patterns

Antimicrobial sensitivity test of the 15 isolates was conducted on 11 antibiotics. The *S.agalactiae* isolates were sensitive (S) to amoxycillin (25 µg), oxytetracycline (30 µg), rifampicin (30 µg), sulphamethoxazole/(trimethoprim (25 µg), and vancomycin (30 µg) but showed resistance (R) to flumequin 30 µg), gentamycin (10 µg), kanamycin (30 µg), neomycin (30 µg) and oxolinic acid (2 µg). They showed resistance and intermediate reaction to novobiocin (30 µg).

Sediment, mud and sewage

S. agalactiae was only detected from two samples from the two sewage canals (Table 2). It was not detected in any of the sediment/mud samples.

Table 2. Samples from sediment and mud taken near two fish cage, Kuwait Bay and from sewage canals.

Date	Sample No.	Type of sample	Source	*S. agalactiae*
5.9.2001	S1	Sewage	Al-Salam beach	+
5.9.2001	S2	sewage	Al-Salam beach	+
5.9.2001	S4	sewage	Al-Salam beach	-
5.9.2001	S5	sewage	Bneid Al-Gar beach	+
5.9.2001	S6	sediment	Kuwait Bay	-
5.9.2001	S7	sediment	Kuwait Bay	-
5.9.2001	S8	sewage	Bneid Al-Gar beach	-
5.9.2001	S9	sewage	Al-Salam beach	-
19.9.2001	S10	sediment	Kuwait Bay	-
19.9.2001	S11	sediment	Kuwait Bay	-
19.9.2001	S12	sediment	Kuwait Bay	-
19.9.2001	R7	sediment	Cage # 9	-
19.9.2001	R8	sediment	Cage # 22	-
24.9.2001	R1	mud	Kuwait Bay	-
24.9.2001	R2	mud	Kuwait Bay	-
24.9.2001	R3	mud	Kuwait Bay	-
24.9.2001	R4	mud	Kuwait Bay	-
24.9.2001	R5	mud	Kuwait Bay	-
24.9.2001	R6	mud	Kuwait Bay	-

\+ = detected
\- = not detected

Pathogenicity test

Fish injected with 10^7 to 10^3 CFU/fish at 33°C, died within three days and only two fish died at 101 (Table 3). At 25°C, the total numbers of dead fish in each group of five were between one to three. Control fish at both 33 and 25°C did not show any mortality during the experiment. All the dead fish showed haemorrhages around the mouth, pectoral, ventral fins and abdomen, which are similar to the clinical signs of wild infected mullet. Among the surviving fish, *S. agalactiae* was detected in the spleen, brain and intestine. The surviving fish showed some chronic symptoms, such as abscess in the peduncle, whitish nodule on the heart and splenomegaly. Smears from the spleen of the dead fish, which were kept at 33°C, indicated that *S. agalactiae* severely spread in this organ. However, the infection was only observed in the white blood cells of mullet kept at 25°C. Interestingly, *S. agalactiae* was found in the brain, but not in the spleen or intestine, of one fish kept in the control group at 33°C and the fish did not display any signs of the disease.

Table 3. Number of dead fish after experimental infection.

Bacterial number (CFU/mL)	Seawater temp (°C)	Days after injection										Number dead/ total number
		1	2	3	4	5	6	7	8	9	10	
1.9×10^8	33	5	-									5/5
	25	0	0	0	0	2	0	0	0	0	0	2/5
1.9×10^6	33	3	1	1	-							5/5
	25	0	0	1	1	0	0	1	0	0	0	3/5
1.9×10^4	33	3	1	1	-							5/5
	25	0	0	0	1	0	0	1	0	0	0	2/5
1.9×10^2	33	0	2	0	0	0	0	0	0	0	0	2/5
	25	0	0	0	1	0	0	0	0	0	0	1/5
Control	33	0	0	0	0	0	0	0	0	0	0	0/5
	25	0	0	0	0	0	0	0	0	0	0	0/5

DISCUSSION

The biochemical, biophysical tests and API 20 Strept characterization of the 64 isolates support the identity of the isolated bacterium as a beta-hemolytic group-B, *Streptococcus agalactiae*. Although Evans *et al*. (2002) and Glibert *et al.* (2002) reported *S. agalactiae* as a fish pathogen in wild mullet; this is the first report of this bacterium in three other three wild marine fish species in Kuwait. In addition, *S. agalactiae* caused severe mortality among cultured silver pomfret *Pampus argenteus* in 2000 (Al-Marzouk *et al*., 2003) suggesting that the bacterium had been present for at least 12 months.

The phenotypic profile of the isolates from infected mullet in Kuwait corresponds to other fish pathogenic, group-B streptococci, and *S. agalactiae* ATCC mammalian reference strains (Evans *et al*., 2002). Kusuda and Komatsu (1978) reported *S. agalactiae* group-B from American strains isolated from saltwater fish.

Munday *et al*. (1993) and Shoemaker and Klesius (1997) reported that the fish species could play a major role in the severity of streptococcal losses since different species of fish may have different levels of susceptibility to infection. This point of view is in agreement with the species composition of the fish kill in Kuwait Bay, in which mullet comprised 99% of all the dead fish.

The present study showed that S. agalactiae is a euryhaline species, capable of growing in wide range salinity from 0.5 to 6.0% NaCl, but not at 6.5%. It is also a eurythermal, capable of growing in a range from 18 to 43°C but not at 5, 12 or 45°C.

The antimicrobial sensitivities of group-B streptococcal fish isolates to different antibiotics varied. Baya *et al*. (1990) reported that the isolates were sensitive to bacitracin and gentamycin. In the present study, 15 isolates of *S. agalactiae* were sensitive to amoxycillin, oxytetracycline, rifampicin, sulphamethoxazole/tri methoprim and vancomycin but resistance to gentamycin.

The isolated bacterium from sewage samples collected from sewage outfalls near Al-Salam and Benid Al-Gar beaches was identical to the isolates from infected mullet. This has been confirmed at the level of DNA using the random amplified polymorphoic DNA (RAPD) fingerprinting (Jafar Qasem, Biottechnology Department, Kuwait Institute for Scientific Research, personal communication). This confirmation strongly suggests that sewage is a possible source for the contamination of *S. agalactiae*.

The high seawater temperature, salinity and hot summer in Chesapeake Bay were reported to be contributory factors in predisposing blue fish *Pomatomus saltatrix*, striped bass *Morone saxatilis* and sea trout *Cynoscion regalis* to Streptococcus sp. infection (Baya *et al*, 1990). Environmental stresses such as temperature change and poor water quality may influence streptococcal diseases (Shoemaker and Klesius, 1977). High temperature increased the susceptibility of rainbow trout to *Lactococcus garviae* (Munday *et al*., 1993) and rabbitfish to *Streptococcus iniae* (Yuasa *et al*., 1999). The unfavorable stressful environmental conditions can affect the biological efficiency of fish in such a way that it weakens their immune system because stress is known to be immunosuppressive in fish (Schreck, 1996), thus rendering the fish susceptible to bacterial infection. The stressful environmental conditions in Kuwait Bay during August, mainly high water temperature, can explain the apparent enhanced virulence of *S. agalactiae*.

Yuasa *et al*. (1999) reported that *S. iniae* injected intramuscularly caused chronic and acute infections to white-spotted rabbitfish at 25°C and 28-32°C, respectively. In this study, mullet was found to be highly susceptible to *S. agalactiae* infection at 33 °C following intraperitoneal injection. The dead fish showed disease symptoms that resembled the infected wild mullet in Kuwait Bay. Mullet infected at 25°C did not show the severity of infection compared with those kept at 33°C. The bacterium was found only in some white blood cells at 25°C, causing a chronic effect. The mortality of mullet at 33°C supports the hypothesis that high temperature was a stressful factor that increased *S. agalactiae* virulence and caused the epizootic of mullet in Kuwait Bay. Evans *et al*. (2001) found that the tissue distribution of *S. iniae* in the brain might be an important component in relation to behavioral abnormalities and disease initiation. In this study, the isolation of the bacterium from the brain in the control group at 33°C highlighted that the bacterium can be carried asymptomatically in the brain of fish not displaying signs of the disease. This may also indicate that some wild mullet could be carrier fish.

With the appearance of *S. agalactiae* in fish from Kuwait Bay and to some fish culture facilities, there is a serious potential threat to the fish stock and to the future of aquaculture in Kuwait. Therefore, further investigations should be considered to determine other possible sources of the bacterium and different methods of implementation to prevent similar outbreaks in the future.

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