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Authors: Evans, Joyce J., Pasnik, David J., Klesius, Phillip H., and Al-Ablani, Salam

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FIRST REPORT OF *STREPTOCOCCUS AGALACTIAE* AND *LACTOCOCCUS GARVIEAE* FROM A WILD BOTTLENOSE DOLPHIN (*TURSIOPS TRUNCATUS*)

Joyce J. Evans,[1] David J. Pasnik,[1,4] Phillip H. Klesius,[2] and Salam Al-Ablani[3]

[1] Aquatic Animal Health Research Laboratory, United States Department of Agriculture, Agricultural Research Service, Chestertown, Maryland 21620, USA
[2] Aquatic Animal Health Research Laboratory, United States Department of Agriculture, Agricultural Research Service, Auburn, Alabama 38756, USA
[3] Mariculture and Fisheries Department, Kuwait Institute for Scientific Research (KISR), Safat 13109, Kuwait
[4] Corresponding author (email:dpasnik@msa-stoneville.ars.usda.gov)

ABSTRACT: The isolation and characterization of two bacterial species, *Streptococcus agalactiae* and *Lactococcus garvieae*, previously unreported in wild marine mammals are described from a freshly dead bottlenose dolphin, *Tursiops truncatus*, from Kuwait Bay, Kuwait, in September 2001. Conventional and rapid identification systems were used to determine that isolates from muscle and kidney were *S. agalactiae* and *L. garvieae*, respectively. The isolates were gram-positive, catalase-negative, oxidase-negative, nonhemolytic cocci. The *S. agalactiae* was serotyped to group antigen B, whereas the *L. garvieae* could not be assigned to any serogroup. These Kuwait isolates displayed considerable homogeneity with corresponding American Type Culture Collection (ATCC) type isolates. Although the dolphin *S. agalactiae* isolate was nonhemolytic, it was biochemically similar to *S. agalactiae* isolated from mullet sampled in the concurrent Kuwait Bay fish kill. Some biochemical heterogeneity was observed between the dolphin isolates and corresponding mammalian ATCC type isolates, especially with Voges Proskauer, alanine-phenylanaline-proline arylamidase, and alpha-galactosidase tests. Nile tilapia, *Oreochromis niloticus*, experimentally infected with the dolphin *S. agalactiae* and *L. garvieae* isolates experienced 90% and 0% mortalities, respectively. This is the first isolation of *S. agalactiae* and *L. garvieae* from a wild marine mammal, and the microbial characteristics established here provide pertinent information for the future isolation of these bacteria.

Key words: Bacteriology, bottlenose dolphin, *Lactococcus* spp., *Streptococcus* spp., *Tursiops* spp.

INTRODUCTION

Streptococcal infections have been reported to cause significant morbidity and mortality among marine mammals. Multiple streptococcal species have been isolated, including *Streptococcus iniae* from captive Amazon river dolphins (*Inia geoffrensis*)(Pier and Madin, 1976; Bonar and Wagner, 2003); *Streptococcus equi* in North Atlantic pilot whales (*Globicephala melaena*) (Higgins et al., 1980); *Streptococcus mitis* from beluga whales (Buck et al., 1989); *Streptococcus phocae* from harbor seals (*Phoca vitulina*), grey seals (*Haliochoerus grypus*) (Skaar et al., 1994), and fur seals (*Aretocephalus pusillus pusillus*) (Henton et al., 1999); *Streptococcus dysgalactiae* subsp. *dysgalactiae* from harbor porpoises (*Phocoena phocoena*) (Swenshon et al., 1998); *Streptococcus zooepidemicus* and several unspeciated β-hemolytic *Streptococcus* sp. (Dunn et al., 2001) in *Tursiops* sp.; *Streptococcus halichoeri* from grey seals (Lawson et al., 2004); and *S. marimammalium* from gray and harbor seals (Lawson et al., 2005). These isolates were sampled from various organs and represented multiple disease processes, such as skin abscessation, bronchopneumonia, pyelonephritis, myocarditis, and osteomyelitis (Higgins et al., 1980; Buck et al., 1989; Skaar et al., 1994; Swenshon et al., 1998; Henton et al., 1999; Bonar and Wagner, 2003), though the bacteria can often be considered normal flora in some marine mammal species (Olsen et al., 1994; Sugita et al., 1996).

The group B streptococcal (GBS) organism, *Streptococcus agalactiae*, and *Lactococcus garvieae* have not been iso-

lated from wild marine mammals but are significant pathogens and of clinical significance for terrestrial mammals and for fish. *Streptococcus agalactiae* causes neonatal meningitis in humans and mastitis in cows (Elliott et al., 1990; Bohnsack et al., 2004; Lindahl et al., 2005). *Lactococcus garvieae* has been isolated from clinical specimens of human skin, blood, and urine and is also a known etiological agent of mastitis in cows (Collins et al., 1983; Elliott et al., 1991; Vela et al., 2000). Furthermore, GBS organisms have been shown to cause significant mortalities among numerous wild and cultured fish species, including menhaden (*Brevoortia patronus*) (Plumb et al., 1974), bullminnows (*Fundulus grandis*) (Rasheed and Plumb, 1984), striped bass (*Morone saxatilis*) (Baya et al., 1990), mullet (*Liza klunzingeri*), and tilapia (*Oreochromis niloticus*) (Evans et al., 2002a). Likewise, *L. garvieae* is an established pathogen of fish, causing a variety of clinical signs and significant mortalities among fish worldwide. Affected fish species include rainbow trout (*Oncorhynchus mykiss*) (Ravelo et al., 2001; Chang et al., 2002), yellowtail (*Seriola quinqueradiata*) (Zlotkin et al., 1998a), and grey mullet (*Mugil cephalus* L.) (Chen et al., 2002). Streptococcal organisms are also of concern to public aquaria, because they affect aquarium species such as danios, *Brachydanio rerio* and *Brachydanio albolineatus*, and minnows (*Tanichthys albonubes*) (Ferguson et al., 1994). Given their propensity to infect both fish and terrestrial mammals, *S. agalactiae* and *L. garvieae* presumably could affect marine mammals in the wild or in captivity.

In this paper, we characterize *S. agalactiae* and *L. garvieae* sampled from a freshly dead bottlenose dolphin (*Tursiops truncatus*) from Kuwait Bay. This is the first isolation of these bacteria from wild marine mammals, and *S. agalactiae* and *L. garvieae* should therefore be considered among the list of potential marine mammal pathogens.

MATERIALS AND METHODS

Clinical history

In September 2001, a single freshly dead bottlenose dolphin was found in Kuwait Bay near the Al-Haishan beach between Subiya and Kazimah, Kuwait. The dolphin was sampled ancillary to a fish epizootic involving several fish species in Kuwait Bay (Evans et al., 2002a; Glibert et al., 2002). The dolphin was placed in a freezer after recovery and examined and necropsied 48 hr later. No samples for histopathologic examination were taken because the dolphin had been frozen and therefore likely to display histologic artifacts.

Bacteriology

Swab samples were taken aseptically for microbiologic examination from the epaxial muscle, kidney cortex, spleen, and heart of the partially frozen dolphin, and samples were processed according to the methods of Evans et al. (2002a, b). Samples were taken using sterile Remel Bacti-Swab NPG collection and transport systems (Remel, Lexena, Kansas, USA), and they were sent in transport tubes with unbroken media ampoules to the laboratory in Auburn, Alabama, USA within 4 days. Evans et al. (2002b) indicated that unbroken media ampoules optimized the viability of gram-positive bacteria, including *S. iniae* and *S. agalactiae*. Isolates remained viable for 10 days under these dry conditions, though the most prolific growth occurred after 4 days. Further, the authors indicated that *S. agalactiae* could not be recovered from fish organ swab samples with broken media ampoules.

The transported swabs were used to inoculate 5 ml of tryptic soy broth (TSB; Difco, Detroit, Michigan, USA) enriched with defibrinated sheep blood. The inoculated TSB was incubated for 3 to 4 hr at 27 C, a temperature routinely used for culture of fish pathogens and used for the culture of *S. agalactiae* from the Kuwait Bay fish epizootic (Evans et al., 2002a). Broth cultures were used to commence and enhance growth of bacteria from transport tubes before agar plate culture (Evans et al., 2002a, b). Broth samples were then streaked on 5% sheep blood agar (SBA; Remel) and incubated for 18–24 hr at 27 C. Pure colonies were then sampled and subjected to morphologic and biochemical tests for phenotypic characterization.

Isolate characterization

Colony morphology and hemolysis were observed on the SBA plates; cell morphology

was assessed with Gram-stained smears. Although culture elicited mostly pure isolates, small numbers of gram-negative organisms and gram-positive, catalase-positive organisms were isolated from the spleen and heart samples but were not characterized. Only gram-positive, catalase-negative isolates were studied because of their predominance in fish sampled during concurrent mass mortalities in Kuwait Bay (Evans et al., 2002a; Glibert et al., 2002). Presumptive identification tests of gram-positive, catalase-negative isolates from the muscle and kidney were based on conventional bacterial biochemical characteristics and were quality controlled and validated using the following American Type Culture Collection (ATCC) type isolates: *S. agalactiae* ATCC 13813 (nonhemolytic) and 27956 (β-hemolytic) and *L. garvieae* ATCC 43921 (American Type Culture Collection, Rockville, Maryland, USA). The dolphin and ATCC type isolates were further characterized using the API Rapid ID 32 Strep system (bioMérieux, Hazelwood, Missouri, USA) and the MicroLog3™ system (Biolog Inc., Hayward, California, USA), which are well-established systems for streptococcal species identification when combined with conventional tests (Freney et al., 1992; Wünsche and Babel, 1996). These two systems compare results to mammalian, veterinary, and clinical databases, and the results presented here were also manually compared with results from piscine bacterial isolates. The API Rapid ID 32 Strep system was used according to manufacturer instructions; results were compared with the manufacturer database, and the T index was interpreted accordingly as good identification ($T>0.25$); very good identification ($T>0.50$); or excellent identification ($T>0.75$). For the MicroLog3™ system, BIOLOG gram-positive microplates were inoculated according to manufacturer instructions and incubated for 24 hr at 35 C. BIOLOG results were compared with the Microlog and User databases, and a similarity index of >0.500 was considered an excellent identification. According to the methods of Lancefield (1933) and Evans et al. (2002a), the isolates were serogrouped with a Slidex strepto kit for grouping of streptococci groups A, B, C, D, F, and G (bioMerieux Industry, Marcy l'Etoile, France).

Infectivity trials

Experimental infectivity trials were undertaken to determine the pathogenicity of the *S. agalactiae* and *L. garvieae* isolates on fish. Nile tilapia were used as the test model because they have been involved in natural streptococcal disease outbreaks and were previously used in *S. agalactiae* infectivity experiments (Evans et al., 2002a). Ten tilapia (mean weight=40 g) were each injected intraperitoneally (IP) with 1×10^7 colony-forming units (CFU) *S. agalactiae*, and another 10 tilapia were each injected IP with 9.5×10^6 CFU *L. garvieae*. Ten control fish were inoculated with sterile TSB. Fish were maintained at 25 C in 57-l aquaria and monitored for 7 days. All moribund or dead fish were removed and brain and head kidney sampled for recovery of the injected bacteria. No samples were taken for histopathologic examination.

RESULTS

Clinical findings

The adult female dolphin measured 2.35 m long. After removal from Kuwait Bay, the carcass of the freshly dead dolphin was stored in a freezer until necropsy. The dolphin remained in good postmortem condition without bloating or eye opacity, but with intact, well-defined viscera and firm muscles (Geraci and Lounsbury, 1998). The dolphin also appeared to be in good nutritional state and its stomach contained a freshly dead mullet (*Liza klunzingeri*). Moderate, multifocal skin ulcerations and subcutaneous bruising were observed at sites across the body, and these lesions were exclusive of signs of moderate bird predation noted on the dorsal aspect of the dolphin. No gross external lesions suggesting human interaction were noted, and no significant lesions were noted internally.

Isolate characterization

Using conventional phenotypic characteristics, API Rapid ID 32 Strep system tests, and BIOLOG system tests, the pure cultures grown from the partially frozen dolphin muscle and kidney were identified as *S. agalactiae* and *L. garvieae*, respectively. Phenotypic and biochemical characteristics were compared to corresponding ATCC isolates subjected to the same identification tests (Table 1). Examination of all isolates revealed gram-positive, oxidase-negative, catalase-negative cocci.

TABLE 1. Results of phenotypic characterization of *Streptococcus* and *Lactococcus* isolates from a bottlenose dolphin (*Tursiops truncatus*) and American Type Culture Collection (ATCC) strains using conventional (con) and API Rapid ID 32 Strep system (api) tests.[a]

	Phenotypic characteristics										
	Streptococcus agalactiae						*Lactococcus garvieae*				
	ATCC 13813/ 27956		Dolphin muscle		Evans et al. 2002a[b]		ATCC 43921		Dolphin kidney		Ravelo et al. 2001[c]
Reaction	con	api	con	api	con	api	con	api	con	api	con/api
Gram's stain	+		+		+		+		+		+
Cell morphology	c		c		c		c		c		c
Growth at 10 C	−		−		−		+		+		
Hemolysis	N/β		N		β		α		N		α
Oxidase	−		−		−		−		−		−
Catalase	−		−		−		−		−		−
CAMP[d]	+/+		+								
Pyrrolidonyl arylamidase	−	−	−	−	−	−	−	+	+	+	+
Voges Proskauer	−	+	−	+	−	+	+	+	−	+	+
Hippurate hydrolysis	+	+	+	+	+	+/−	+	−	−	−	+
β-glucosidase		−		−		−		+		+	+
α-galactosidase		+		−		+/−		−		−	−
β-galactosidase		−		−		−		−		−	+/−
β-glucuronidase		−		−		+		−		−	−
Alkaline phosphatase		+		−		+/−		−		−	−
N-acetyl-β-glucosaminidase		−		−		−		−		−	+/−
Alanine-phenylanaline-proline arylamidase		+		−		+		+		+	+
β-mannosidase		−		−		−		−		−	+/−
Urease		−		+		−		−		−	−
Bile-esculin tolerance	−		−		−		+		−		
Acid from:											
Ribose		+		+		+		−		+	
Mannitol		−		−		−		+		−	+
Lactose		+		+		−		+/−		−	+
Raffinose		−		+		−		−		−	−
Sucrose		+		+		+		+/−		−	+
Glycogen		−		−		+/−		−		−	−
Cyclodextrin		−		−		−		+/−		−	+
Maltose		+		+		+		+		−	+
Pullulan		−		+		+/−		−		−	+/−
Melezitose		−		−		+/−		−		−	+/−
Methyl-β-glucopyranoside		+		+		+		+		+	
Tagatose		+		+		+/−		+/−		−	+
Group antigen	B		B		B		NG		NG		NG

[a] Symbols denote the following: + = positive; − = negative; N = nonhemolytic; blank space = not tested or test not included; c = cocci; NG = no group. All isolates were negative for the following reactions: starch hydrolysis, glycyl-tryptophane arylamidase, sorbitol, L-arabinose, D-arabitol, and melibiose. All isolates were positive for the following reactions: leucine aminopeptidase, arginine deamination, and trehalose.

[b] Sample from mullet, *Liza klunzingeri*, in Evans et al., 2002a.

[c] Sample from rainbow trout, *Oncorhynchus mykiss*, in Ravelo et al., 2001. No distinctions were made between results of conventional tests and API Rapid ID 32 Strep system tests.

[d] The lytic phenomenon after the original authors: Christie, Akins, and Munch-Petersen (Christie et al., 1944).

All isolates were negative for the following reactions: β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, β-mannosidase, glycyl-tryptophane arylamidase, sorbitol, L-arabinose, D-arabitol, glycogen, melezitose, melibiose, and starch hydrolysis. All isolates were positive for the following reactions: leucine aminopep-

tidase, arginine deamination, and trehalose.

The *S. agalactiae* muscle isolate was nonhemolytic on SBA. The *S. agalactiae* ATCC type isolates showed hemolytic variability as no hemolysis (13813) and β-hemolysis (27956). All of the *S. agalactiae* isolates were bile-esculin– and pyrrolidonyl arylamidase–negative and leucine aminopeptidase–positive in conventional or API Rapid ID 32 tests. The Voges Proskauer test for the muscle and *S. agalactiae* ATCC type isolates were negative by conventional methods but positive when determined by the API Rapid ID 32 kit. The CAMP (the lytic phenomenon after the original authors: Christie, Atkins, and Munch-Petersen [Christie et al., 1944], test was positive for the dolphin muscle and the *S. agalactiae* ATCC type isolates.

The kidney and *Lactococcus garvieae* ATCC type isolates were nonhemolytic and alpha-hemolytic, respectively. The pyrrolidonyl arylamidase test was positive, except for the conventional test of the *L. garvieae* ATCC type isolate. The Voges Proskauer tests were negative for the kidney and *L. garvieae* ATCC type isolates, except for the conventional test of the *L. garvieae* ATCC type isolates.

Some variation was observed when comparing the hydrolysis and acid-production results between the dolphin isolates and their corresponding ATCC isolates. The dolphin muscle sample was positive for urease, raffinose, and pullulan, and negative for alpha-galactosidase, β-galactosidase, alkaline phosphatase, and alanine-phenylalanine, whereas the *S. agalactiae* ATCC type isolates produced the opposite results for these tests. The dolphin kidney isolate was positive for ribose, and negative for mannitol, lactose, sucrose, cyclodextrin, maltose, and tagatose, whereas the *L. garvieae* ATCC type isolate produced the opposite results for these tests. The dolphin muscle isolate and *S. agalactiae* ATCC type isolates were classified into Lancefield's serologic group B, but no group antigen could be established for the other isolates.

Despite the variations in API Rapid ID 32 Strep tests for *S. agalactiae* and *L. garvieae* isolates, the dolphin isolates displayed API Rapid ID 32 Strep system profiles characteristic of *S. agalactiae* (99.9% probability, 0.94 T) and *L. garvieae* (99.9% probability, 0.85 T). The ATCC type isolates provided expected results corresponding to *S. agalactiae* (99.0%, 0.40 T) and *L. garvieae* (96.7%, 0.86 T). After 16–24 hr incubation, BIOLOG GP-All microplate analysis using the manufacturer database identified the dolphin muscle isolate as *S. agalactiae* (100% probability, 0.812 similarity) and the kidney isolate as *L. garvieae* (100% probability, 0.705 similarity).

Infectivity trials

Experimental infection of tilapia with the dolphin *S. agalactiae* isolate caused 90% mortalities within 6 days postinoculation. Behavioral changes were noted among the *S. agalactiae*–challenged fish before mortality: the affected tilapia stayed stationary on the bottom of the tank, were unresponsive to feed, and exhibited serpentine swimming, "C"-shaped body curvature, changes in body coloration, and rapid opercular movement. *Streptococcus agalactiae* was isolated from all the cultured fish. There were no mortalities among tilapia injected with *L. garvieae*, although at 5 days postinfection, 60% of these fish appeared stationary, unresponsive to feed, and confined to the bottom of the tank. However, on the sixth day and through the end of the experiment, all of the fish appeared to behave normally. No mortalities and no behavioral changes were observed among the control fish.

DISCUSSION

The phenotypic and biochemical data from conventional and multitest systems (API Rapid ID 32 Strep and BIOLOG

systems) indicated that the dolphin muscle isolate was *S. agalactiae* and the dolphin kidney isolate was *L. garvieae*; this is the first report of *S. agalactiae* and of *L. garvieae* from wild marine mammals. Conventional tests showed that the dolphin isolates' phenotypic characteristics closely matched those of their corresponding ATCC type isolates and previously studied piscine isolates (Ravelo et al., 2001; Evans et al., 2002a). Further, the nonhemolytic dolphin *S. agalactiae* isolate can be considered among other atypical nonhemolytic GBS reported from human, bovine, and piscine origin (Wilkinson et al., 1973; Vandamme et al., 1997). Conventional test result inconsistencies among isolates can be attributed to variability because of different clinical sources (Eldar et al., 1999). The *S. agalactiae* and *L. garvieae* ATCC type isolates were isolated from terrestrial mammals (McDonald and McDonald, 1975; Collins et al., 1983), and differences in environmental, geographic, and mammalian sources may factor into the phenotypic or biochemical characteristics of the bacteria. However, the characteristics presented in this paper should facilitate future identification of *S. agalactiae* and *L. garvieae* according to phenotypic and biochemical properties.

No definitive link between the bacterial isolations and the death of the dolphin was established here. As in other dolphin pathogen studies (Pier and Madin, 1976; Bonar and Wagner, 2003), Koch's postulates were not fulfilled here because of practical, legal, and ethical issues regarding marine mammal research. However, the dolphin *S. agalactiae* isolate was significantly pathogenic to fish, causing 90% mortalities in tilapia within 6 days in experimental infectivity trials. The behavioral changes among these challenged fish were similar to fish studied in the Kuwait Bay epizootic. *Streptococcus agalactiae* is a known pathogen of fish and can cause clinical and histopathologic changes, including exophthalmia, meningoencephalitis, hepatocyte vacuolization and necrosis, and splenic necrosis and congestion (Rasheed et al., 1985; Eldar et al., 1994; Evans et al., 2002a). Further, *S. agalactiae* is also a well-established pathogen of mammals, including humans and cows (Collins et al., 1983; Elliott et al., 1991; Bohnsack et al., 2004; Lindahl et al., 2005). Streptococcal bacteria have been associated with marine mammal morbidity and mortality, often following the development of cutaneous lesions, severe bronchopneumonia, metritis, and septicemia (Higgins et al., 1980; Howard et al., 1983; Dunn et al., 2001).

Streptococcus agalactiae and *L. garvieae* are cosmopolitan in their distribution, existing and adapting to a broad range of environmental conditions. The isolation of *S. agalactiae* and *L. garvieae* from a wild marine mammal may have multiple implications for the epidemiology of these organisms in wild and captive animals and their transmission between species. Streptococcal species have been cultured from a number of marine mammal organs, such as the skin, blowhole, trachea, lungs, pharynx, and uterus; these organs may provide clues into the routes of entry (Higgins et al., 1980; Buck et al., 1989; Henton et al., 1999; Bonar and Wagner, 2003). Infections of streptococci are known to occur after ingestion of materials containing streptococcal organisms (Minami, 1979; Bromage and Owens, 2002). Bonar and Wagner (2003) suggested that a captive Amazon River dolphin suffering from abscesses was exposed to *S. iniae* through food fish. Therefore, the Kuwait Bay dolphin may have been exposed to *S. agalactiae* after eating the mullet found in its stomach. The dolphin *S. agalactiae* isolate exhibited similar physical and biochemical characteristics as mullet sampled from the Kuwait Bay fish epizootic (Evans et al., 2002a). Thus, outbreaks of disease in fish are significant, because they have the potential to affect other cohabitating aquatic species (Zlotkin et al., 1998b; Bromage and Owens, 2002). This may be intensified in predatory populations that

come in contact with and ingest infected prey. Moreover, an infected marine mammal could directly transmit the bacteria to other marine mammals or fish through shedding (Swenshon et al., 1998). Although this may not be a serious problem in wild marine mammals, it would be accentuated in captive populations housed in confined areas.

The capture of wild marine mammals and fish for public display may present the opportunity for streptoccocal outbreaks in aquaria. Evans et al. (2002a) cultured *S. agalactiae* from the brain and eye of a captive mullet previously captured from the wild prior to the epizootic and maintained in a Kuwait public aquarium (Scientific Centre). Although mortality of captive mullet and other fish species did not occur in the aquaria, a carrier state was determined. The health of captive marine mammals and fish should be routinely screened through nonlethal hematologic sampling and bacteriologic culture to detect carrier state and help inhibit the potential spread of infections to other marine mammals and captive fish. Furthermore, feed items fed to captive marine mammals should be screened for potential pathogens or treated by gamma-irradiation, and vaccination of marine mammals may also be considered as a preventative measure (Dunn et al., 2001).

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