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Short communication

Characterization of beta-hemolytic streptococci isolated from southern sea otters (*Enhydra lutris nereis*) stranded along the California coast

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ABSTRACT

The goal of this study was to characterize the beta-hemolytic streptococci cultured from southern sea otters (*Enhydra lutris nereis*) stranded off the coast of California (USA) and to verify identifications made using the Lancefield system. Lancefield serotyping and biochemical analysis alone was inadequate for isolate characterization. Final identification was based on sequence analysis of a portion of the 16s ribosomal RNA gene from 12 of the 35 total isolates. The majority of isolates (10 of 12; 83.3%) were *Streptococcus phocae* and reacted with Lancefield group G and F antisera or were less frequently untypeable. The remaining isolates belonging to Lancefield group G were identified as *S. dysgalactiae* subsp. *equisimilis* (2 of 12; 16.7%). This is the first report of *S. phocae* in southern sea otters and further evidence of *S. phocae* expressing cell surface antigens compatible with Lancefield group G typing.

1. Introduction

In marine mammals, beta-hemolytic streptococci have been implicated in debilitating disease processes such as pneumonia, septicemia, and opportunistic infections (Howard et al., 1983; Skaar et al., 1994; Swenshon et al., 1998; Thornton et al., 1998; Johnson et al., 2006; Kuiken et al., 2006). These marine mammal isolates of beta-hemolytic streptococci have been identified as *Streptococcus phocae*, *S. dysgalactiae* subsp. *dysgalactiae*, *S. marimammalium*, *S. halichoeri*, *S. iniae*, *S. canis*, and *S. zooepidemicus* (Pier and Madin, 1976; Skaar et al., 1994; Swenshon et al., 1998; Vossen et al., 2004; Lawson et al., 2004; Lawson et al., 2005; Johnson et al., 2006; Kuiken et al., 2006). In particular, *S. phocae* has been isolated from harbor seals (*Phoca vitulina*), grey seals (*Halichoerus grypus*), California sea lions (*Zalophus californianus*), Caspian seals (*Phoca caspica*) and more recently, from Atlantic salmon (*Salmo salar*) (Skaar et al., 1994; Vossen et al., 2004; Gibello et al., 2005; Johnson et al., 2006; Kuiken et al., 2006; Romalde et al., 2008).

Due to similar antimicrobial sensitivity, characterization is not routinely pursued to the species level. Rather, identification of beta-hemolytic streptococcal isolates is typically based on the Lancefield system. Using this system, marine mammal isolates belonging to Lancefield group F, C or those that are untypeable are presumptively identified as *S. phocae*. Marine mammal isolates belonging to group G are sometimes identified as *S. canis* and group L strains are identified as *S. dysgalactiae* subsp. *dysgalactiae* (Skaar et al., 1994; Swenshon et al., 1998; Facklam, 2002). Isolates of *S. phocae* from Atlantic salmon have been typed as group G (Romalde et al., 2008).

This study was designed to further characterize beta-hemolytic streptococcal isolates cultured from southern sea otters (*Enhydra lutris nereis*) that stranded off the coast of California (USA) between June 1998 and June 2002 and to verify the identifications made using the Lancefield

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system. Thirty-five beta-hemolytic streptococcal isolates were characterized by Lancefield grouping, biochemical analysis and 16 s rRNA gene sequencing.

2. Materials and methods

Sterile culturettes (BBL CultureSwab, Becton Dickinson and Co., Sparks, MD) were used for aseptic sample collection and transport to the Microbiology Laboratory, at the Veterinary Medical Teaching Hospital, University of California, Davis. Swabs of orogenital mucosa as well as lesions in live and dead stranded sea otters were collected, streaked for isolation on 5% sheep blood agar plates and incubated at 37 °C at 5% CO_2 for 24 h. Small, beta-hemolytic colonies of catalase negative, Gram-positive cocci were isolated, inoculated into Microbank cryovials (Pro-Lab Diagnostics, Richmond Hill, Canada) and stored at −80 °C.

Lancefield serotyping was performed using a Streptex kit (Wellcome Diagnostics, Dartford, England) into Lancefield group A, B, C, D, F and G by latex bead antibody agglutination (Skaar et al., 1994). Biochemical characterization was performed using an API (analytical profile index) 20 Strep identification system (bioMerieux, Inc., Hazelwood, Missouri), as described (Skaar et al., 1994; Lawson et al., 2005). All isolates were simultaneously incubated with BBL Bacitracin discs (Becton Dickson Microbiology Systems, Cockeysville, MD) and any zone of inhibition was interpreted to indicate susceptibility (Skaar et al., 1994; Swenshon et al., 1998).

Sequence analysis of the 16 s ribosomal RNA gene was performed on a representative sample ($N = 12$) from groups G, F and untypeable. A single colony from each isolate was inoculated onto a 5% sheep blood agar plate and the resulting pure culture was used for DNA extraction. DNA templates were extracted from a crude lysis of bacterial colonies using a QIAamp DNAeasy minikit (QIAGEN, Valencia, CA). Ten microlitres of extracted DNA was added to 90 μL of a master amplification solution that consisted of 50 mM KCL, 10 mM Tris–HCl, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM each of deoxynucleoside triphosphates, 20 pmol each of forward and reverse primers and 2 U of *Taq* DNA polymerase. A forward eubacterial primer, designated 8FPL (5′-CTGCAGAGTTT-GATCCTGGCTCA-3′), and a reverse eubacterial primer, designated 1492RPL (5′-CGGGTTACCTTGTTACGACTT-3′), were used (Foley et al., 1998). Amplification was performed in a thermal cycler (MJ Research, Watertown, MA), as described (Foley et al., 1998). PCR product was separated by electrophoresis on an agar gel, visualized with ethidium bromide and analyzed for appropriate size and quantity, as described (Johnson et al., 2003). PCR products were purified using a Microcon Centrifuge filter device (Millipore Co., Bedford, MA), according to the manufacturer. Sequencing was performed at Davis Sequencing (Davis, CA) using PCR primers. Sequences were manually corrected to exclude low quality product and end-reading errors. The resulting high quality sequences were between 241 and 908 base pairs (bp) in length (mean 658 bp ± 262, correlating with *E. coli* bp 40–698) and were compared with the BLAST database in the National Center for Biotechnology Information.

3. Results

Of the 35 isolates of beta-hemolytic streptococci, 17 (48.6%) isolates were typed as Lancefield group G. Twelve (34.3%) isolates were typed as Lancefield group F, two (5.7%) isolates were typed as Lancefield group C and four isolates (11.4%) were untypeable by the methods used.

Based on bacitracin susceptibility and API Strep 20 profiles, Lancefield group G isolates were identified as either *S. dysgalactiae* subsp. *equisimilis*, *Streptococcus* group L or untypeable. Many of the identifications were considered very good, based on criteria defined by the manufacturer of the API Strep 20 system. Group F, C and untypeable isolates were variably identified as *S. dysgalactiae* subsp. *equisimilis*, *Gemella haemolysans* or untypeable.

Sequence analysis of a portion of the 16 s rRNA gene from 12 selected isolates within Lancefield groups G, F and untypeable resulted in identification of 1/1 (100%) untypeable isolate, 4/4 (100%) group F isolates and 5/7 (71.4%) group G isolates were *S. phocae* (see Table 1). Sequence similarity between these isolates and the reference strain (ATCC# AJ621053.1) was high, between 98 and 100%. The remaining two group G isolates were identified as *S. dysgalactiae* subsp. *equisimilis* and were 99% similar to the reference strain (ATCC# EU075065). All sequences were submitted to GenBank (see below). Biochemical profiles of the select isolates identified by sequence analysis were compared to the previously published profiles of *S. phocae*, *S. canis* and *S. dysgalactiae* subsp. *equisimilis* in Table 2.

4. Discussion

Thirty-five isolates of beta-hemolytic streptococci obtained from southern sea otters of the coast of California between 1998 and 2002 were characterized in this study. Results indicated that the majority of isolates were *S. phocae*, that the *S. phocae* isolates expressed cell surface antigens of Lancefield groups G, F, C or were untypeable, and that acidification of sugars varied slightly from

Table 1
Identification by 16s rRNA gene sequence analysis of 12 sea otter beta-hemolytic streptococcal isolates and percent nucleotide similarity to closest related species.

Streptococcus sp.	ID#	Lancefield group	Similarity (%)[a]	Non-identities[b]
S. phocae	37	–	99	2/240
	67	F	99	2/876
	72	F	99	2/866
	88	F	98	6/452
	91	F	100	0/344
	7	G	99	1/819
	63	G	99	2/879
	64	G	100	0/369
	79	G	100	0/804
	90	G	99	2/435
S. dysgalactiae subsp. *equisimilis*	5	G	99	3/903
	70	G	99	2/888

[a] 16s rRNA gene sequence to ATCC strain AJ621053.1 or EU075065.
[b] Number of dissimilar nucleotides/total number of nucleotides compared to ATCC strain.

Table 2
Biochemical characteristics which differentiate Lancefield group G *Streptococcus phocae* from other streptococcal isolates in marine mammals.

Characteristic	*S. phocae* (*N* = 10)			*S. dysgalactiae* sp. (*N* = 2)	*S. phocae*[a]	*S. canis*[b]	*S. dysgalactiae* subsp. *equisimilis*[c]
Lancefield group	G	F	U[d]	G	F, C, U	G	C, G
Beta-hemolysis	+[e]	+	+	+	+	+	+
Bacitracin susceptibility	v	+	+	−	+	−	−
Production of:							
Alkaline phosphatase	+	+	+	+	+	+	+
Leucine arylamydase	+	+	+	+	ND	+	+
Arginine dihydrolase	v	−	−	+	−	+	+
Alpha-galactosidase	−	−	−	−	ND	±	+
Beta-galactosidase	−	−	−	−	ND	±	+
Beta-glucuronidase	−	−	−	+	ND	±	+
Acid produced from:							
Ribose	v	v	+	+	+	+	+
Lactose	v	v	−	−	−	+	−
Starch	+	v	+	+	−	+	+
Glycogen	+	v	+	+	±	±	+
Hippurate	v	−	−	v	ND	ND	ND
Trehalose	v	v	−	v	ND	±	ND

[a] Skaar et al. (1994).
[b] Devriese et al. (1986), Efstratiou et al. (1994).
[c] Vandamme et al. (1996), Vieira et al. (1998).
[d] (U) Untypeable.
[e] (+) More than 90% positive, (−) more than 90% negative, (v) 11–89% positive, (±) weak reaction, (ND) not done.

previously published reports of *S. phocae*. Fewer isolates were identified as *S. dysgalactiae* subsp. *equisimilis* and were typed as Lancefield group G. This is the first report of *S. phocae* in sea otters and further demonstration of Lancefield group G antigen expression by *S. phocae*.

Standard bacteriological methods were not adequate for identification of the isolates of beta-hemolytic streptococci from the sea otters sampled. Initial identification based on Lancefield grouping was not consistent with the biochemical analysis using the Strep API 20 system. Similar difficulties in identifying beta-hemolytic streptococci from marine mammals and fish have been reported (Gibello et al., 2005; Kuiken et al., 2006; Romalde et al., 2008). Sequencing of the 16 s rRNA gene revealed that a majority of the group G isolates were *S. phocae* and the remainder were *S. dysgalactiae* subsp. *equisimilis*. Sequence analysis of the 16 s rRNA gene is considered a more reliable means for species identification and most consider a minimum of 97–98% sequence similarity as adequate for identification (Kolbert and Persing, 1999; Johnson et al., 2003; Gibello et al., 2005).

Reaction with Lancefield group G antiserum was not observed in the original description of *S. phocae* (Skaar et al., 1994) or in subsequent studies (Henton et al., 1999; Vossen et al., 2004); however, group G isolates of *S. phocae* from California sea lions had been observed by our group (Johnson et al., 2006) and in Atlantic salmon (Romalde et al., 2008). Group C isolates were not sequenced in the present study because of their low prevalence (2/35 isolates) and Lancefield group and biochemical similarity to previous reports of *S. phocae* (Skaar et al., 1994; Vossen et al., 2004; Lawson et al., 2005).

In summary, *S. phocae* groups G and F were the predominate isolates of beta-hemolytic streptococci cultured from stranded southern sea otters. Sequencing of the 16 s rRNA gene was required for identification of isolates as *S. phocae*. Further work is in progress to characterize other bacterial pathogens of sea otters.

5. Nucleotide sequence accession numbers

The nucleotide sequences corresponding to the 16 s rRNA gene of *S. phocae* 37, 67, 72, 88, 91, 7, 63, 64, 79 and 90 have been assigned the respective accession numbers FJ429782–FJ429791 in the GenBank database. Nucleotide sequences corresponding to the 16 s rRNA gene of *S. dysgalactiae* subsp. *equisimilis* 5 and 70 have been accessioned as FJ429792 and FJ429793 in the GenBank database.

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