DOI: 10.7589/2017-07-178

Journal of Wildlife Diseases, 54(4), 2018, pp. 659–670

HYPERMUCOVISCOUS *KLEBSIELLA PNEUMONIAE* ISOLATES FROM STRANDED AND WILD-CAUGHT MARINE MAMMALS OF THE US PACIFIC COAST: PREVALENCE, PHENOTYPE, AND GENOTYPE

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ABSTRACT: Emergent hypermucoviscous (HMV) strains of *Klebsiella pneumoniae* have been reported in multiple marine mammal species; however, there is limited information regarding the epidemiology and pathogenesis of this infection in these species. We determined the prevalence of HMV *K. pneumoniae* in wild-caught and stranded marine mammal populations on the US Pacific Coast. Samples were collected from 270 free-ranging California sea lions (CSLs; *Zalophus californianus*) captured at three discrete sampling sites and from 336 stranded marine mammals of various species. We recovered HMV *K. pneumoniae* only from CSLs, with a prevalence of 1.5% (4 of 275) in stranded animals, compared with 1.1% (3 of 270) in wild-caught animals. We assessed the phenotypic and genotypic variability of recovered HMV *K. pneumoniae* isolates recovered from CSLs (n=11) and of archival HMV and non-HMV isolates from stranded marine mammals (n=19). All but two HMV isolates were of the K2 serotype, whereas none of the non-HMV isolates belonged to this serotype. Of the HMV isolates, 96% (24 of 25) were PCR positive for the HMV-associated gene p-*rmpA*, whereas 92% (23 of 25) were PCR positive for p-*rmpA2*. Genetic fingerprinting by repetitive extragenic palindromic PCR showed four discrete clusters, demonstrating genotypic variability that loosely correlated with phenotype. Antimicrobial susceptibility testing revealed all isolates from stranded CSLs were susceptible to ceftiofur, indicating this antimicrobial agent is an appropriate choice for treatment of HMV *K. pneumoniae* infections in stranded CSLs. Our culture assay could reliably detect HMV *K. pneumoniae* from concentrations as low as 10^2 colony-forming units per milligram of feces. We identified the presence of HMV *K. pneumoniae* in both wild-caught and stranded CSLs from the US Pacific Coast and highlight the need for further studies to evaluate the potential impact of this pathogen on marine mammal health.

Key words: California sea lions, hypermucoviscous, *Klebsiella pneumoniae*, marine mammals, real-time PCR.

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative facultative anaerobic bacterium belonging to the family *Enterobacteriaceae*. Typically found on mammalian mucosal surfaces and in the environment, *K. pneumoniae* has been associated with a wide range of infections in humans and animals (Podschun and Ullmann 1998; Doud et al. 2009). In marine mammals, this bacterium has been isolated from lesions associated with polyarthritis, meningoencephalitis, and peritonitis (Castinel et al. 2007a). Because it can infect multiple hosts and adopt rapidly evolving antimicrobial resistance patterns, this bacterium is recognized as an emerging pathogen in human and veterinary

medicine (Lederman and Crum 2005; Jang et al. 2010).

Hypermucoviscous (HMV) *K. pneumoniae*, considered a hypervirulent form due to its ability to cause life-threatening, community-acquired infection in healthy individuals; its ability to cause metastatic infections; and its greater virulence in animal models (Kong et al. 2012; Shon et al. 2013; Lin et al. 2014), has been associated with pleuritis and suppurative pneumonia in California sea lions (CSLs; *Zalophus californianus*) and with septicemia and meningitis in New Zealand sea lion (NZSL; *Phocarctos hookeri*) pups (Jang et al. 2010; Roe et al. 2015). Little is known about the pathogenesis and epidemiology of this infection in marine hosts, and there is limited information regarding its diagnosis and treatment in both rehabilitated and wild marine mammals.

We aimed to evaluate the prevalence of HMV *K. pneumoniae* in stranded marine mammals and in wild-caught CSLs from the US Pacific Coast. We assessed the phenotypic and genotypic variability of all recovered HMV *K. pneumoniae* isolates as well as of archived isolates collected from marine mammals. In addition, the limit of HMV *K. pneumoniae* detection by culture techniques was evaluated to assess current standards for identification of HMV *K. pneumoniae* in clinical settings.

MATERIALS AND METHODS

Prevalence of HMV *K. pneumoniae* in marine mammals

Between August 2015 and May 2016, 275 stranded CSLs, 53 stranded pacific harbor seals (PHSs; *Phoca vitulina*), three stranded northern elephant seals (NESs; *Mirounga angustirostris*), and five stranded northern fur seals (NFSs; *Callorhinus ursinus*) were sampled after admittance to the Marine Mammal Center (TMMC) in Sausalito, California, for rehabilitation. Stranded animals originated predominantly from the central and northern California coast. Animals of all ages and both sexes were included in this study. Upon admittance, animals were examined by a veterinarian and when possible, mucosal site swabs (oral, rectal, and nasal) were obtained with a sterile Amies bacteriologic swab (Becton, Dickinson and Co. Scientific, Sparks, Maryland, USA). Animals that died during rehabilitation or that were dead upon presentation were necropsied at TMMC, and mucosal sites, lung, liver, kidney, and grossly visible abscesses were swabbed.

Between August 2015 and May 2016, predominantly yearling and juvenile CSLs of both sexes were sampled from Año Nuevo Island, California (n=68) and San Miguel Island, California (n=119), and adult and subadult male CSLs were sampled from Astoria, Oregon (n=83). All wild-caught animals were apparently healthy at the time of capture and sampling, and samples were obtained while animals were under general anesthesia. Swabs of oral, rectal, and nasal sites were obtained similarly those from stranded animals by using the Amies bacteriologic swabs. All wild animal sampling was performed under the National Oceanic Atmospheric Administration National Marine Fisheries Service permits 17115-03 and 16087-01 and with the approval of the University of California–Los Angeles Chancellor's Animal Research Committee in the Office of Animal Research Oversight (ARC 2012-035-11). Hypermucoviscous *K. pneumoniae* isolation prevalence was determined for each of the following groups: stranded animals including CSLs, PHSs, NESs, and NFSs and wild-caught CSLs. Wild-caught CSLs were further categorized by geographic sampling location.

Bacterial isolation

Swabs collected from stranded and wild-caught animals were stored at room temperature and used to inoculate 5% sheep blood agar (SBA) plates (Biological Media Services, University of California, Davis, California, USA) within 7 d of collection. In previous studies, HMV *K. pneumoniae* remained viable for more than 7 d when maintained at these conditions. Inoculated plates were incubated for 48 h at 37 C. Colonies consistent with the appearance of HMV *K. pneumoniae* (grayish, mucoid colonies) were tested for hypermucoviscosity by the string test (Twenhafel et al. 2008). Individual colonies from string test–positive isolates were replated on SBA plates and incubated for 48 h at 37 C, Gram stained, and tested for production of cytochrome oxidase. All Gram-negative, cytochrome oxidase–negative, string test–positive isolates that grew on SBA plates were expanded in Luria broth overnight at 37 C and cryogenically stored (–80 C; 20% [v/v] glycerol) for further characterization.

Archived marine mammal isolates collected from stranded animals at TMMC as well as samples collected from stranded marine mammals in northern California and archived at the University of California–Davis Veterinary Medical Teaching Hospital were evaluated for genotypic

and phenotypic comparisons. Archived isolates were revived on SBA plates as previously described and subjected to the same identification protocols. String test–negative samples identified molecularly as *K. pneumoniae* were categorized as non-HMV *K. pneumoniae* isolates. In total, 30 isolates, including isolates collected during the prevalence survey, in addition to archival HMV *K. pneumoniae* and non-HMV *K. pneumoniae* were included in this study. A *Klebsiella quasipneumoniae* subsp. *similipneumoniae* (formerly *K. pneumoniae* K6; ATCC 700603) was included in the analysis as an outlier for genetic characterization.

Molecular characterization

Genomic DNA was isolated using the DNEasy Blood and Tissue kit (Qiagen, Valencia, California, USA). Aliquots were then stored at –80 C until further use. Isolated DNA was used in endpoint PCR amplification of the *K. pneumoniae* capsular fucose synthesis gene *wcaG* and the K2 capsular type gene wzy_{K2} (Table 1; Turton et al. 2010). Real-time PCR detection of the *Klebsiella* hemolysin gene *khe* and the K1 capsular type mucoviscosity-associated gene *magA* (Hartman et al. 2009) was also performed (Table 1). The K1 and K2 capsular types were targeted, as these are most frequently associated with HMV *K. pneumoniae* in other studies (Fang et al. 2007). The *wcaG* primer was used, as this gene is a putative HMV *K. pneumoniae* virulence factor (Turton et al. 2010) and the *khe* gene was targeted, as it is specific for *K. pneumoniae* (Roe et al. 2015; Table 1). Positive endpoint PCR results were defined as possessing banding patterns similar to positive controls, whereas real-time PCR results were deemed positive when the threshold cycle value was less than <38. Based on previously published evidence indicating that the plasmid-encoded genes *rmpA* and *rmpA2* are highly associated with hypermucoviscosity and virulence (Hsu et al. 2011), real-time PCR assays to detect p-*rmpA* and p-*rmpA2* were developed by the University of California–Davis Real-time PCR Research and Diagnostics Core Facility (Table 1). Both p-*rmpA2* (AB289643) and p-*rampA* (AB289642) sequences from the GenBank were collected using Sequence Analysis and Molecular Biology Data Management software Vector NTI AdvanceTM11 (Thermo Fisher Scientific, Carlsbad, California, USA). A BLASTn (National Center for Biotechnology Information 2017) search of the resulting sequence nonredundant nucleotide database was used to confirm sequence specificity.

Genomic characterization

We designed PCR primers targeting fragments of the β subunit of the bacterial RNA polymerase (*rpoB*) and the B subunit of topoisomerase II (*gyrB*) based on *K. pneumoniae* genomes by using the Primer3 plugin in Geneious® 10.0 (Newark, New Jersey, USA; Table 1). Amplicons were purified using the QIAquick PCR Purification kit (Qiagen), and single Sanger sequencing reads were generated commercially (Eurofins Genomics, Louisville, Kentucky, USA) by using the forward primer for each target. Ambiguous base calls were annotated manually based on corresponding chromatograms and sequences were trimmed to a uniform length in Geneious 10.0. *rpoB* and *gyrB* sequence fragments of *Klebsiella* spp. isolates obtained from aquatic mammals were compared with *K. pneumoniae* genomes identified by a BLASTn search (National Center for Biotechnology Information 2017).

Phylogenetic inferences based on concatenated *rpoB*>*gyrB* gene sequences of *Klebsiella* spp. isolates from marine mammals were made in MEGA 6.0 (Tamura et al. 2013). In brief, all concatenated sequences were aligned with MUSCLE (Edgar 2004), and Bayesian inference criterion identified the Tamura three-parameter model with gamma distribution (five categories +G, parameter=0.2623) as the best-fit nucleotide substitution model for maximum likelihood analysis (Nei and Kumar 2000; Tamura et al. 2013). All positions containing gaps and missing data were eliminated, and the final tree was constructed from 1,000 bootstrap replicates. The concatenated *rpoB*>*gyrB* sequences of *K. quasipneumoniae* ATCC 700603 were included as an outlier.

Repetitive extragenic palindromic PCR (REP-PCR) fingerprinting was performed on case isolates by using the BOX primer (Table 1) following protocols for bacterial genotyping from previous studies (Versalovic et al. 1994; Griffin et al. 2013). Genetic fingerprints generated by the BOX primer were analyzed using Quantity One 4.6.9 software (Bio-Rad Laboratories, Inc., Hercules, California, USA). Band sizes were estimated by comparison with concurrently run standards, and distinct bands were manually annotated to calculate Dice coefficients and generate a dendrogram based on the unweighted pair group method using arithmetic averages. As mentioned, genomic DNA from *K. quasipneumoniae* (ATCC 700603) was included as an outlier.

Antimicrobial susceptibility

The minimal inhibitory concentrations (MICs) of antimicrobial agents to all the *K. pneumoniae* isolates included in this study were tested using the Sensititre AVIANF plate system (Trek Diagnostic System Inc., Cleveland, Ohio, USA). The MIC was determined based on the manufactur-

TABLE 1. Probe/primer sets for each of the PCR targets, including nucleotide sequence and published reference for characterization of hypermucoviscous strains of *Klebsiella pneumoniae* isolated from marine mammals that were stranded or wild caught along the US Pacific Coast in 2015–16.

Target	Primers/probe	Sequence (5′–3′)	PCR method	Reference
p-*rmpA2*	p-*rampA2*-855f	AGGGAGGGCATCCATGAATT	Real time	This study
	p-*rampA2*-1032r	TTATCTAGGTATTTGATGTGCACCATT		
	p-*rampA2*-908pFAM	6FAM-ATCGTGTCACAGATGTA-MGB		
p-*rmpA*	p-*rampA*-395f	GAATTGTAAACCATTATCCACGGC	Real time	This study
	p-*rampA*-572r	ATAAACCTTATGTACCTTTTGCAGCC		
	p-*rampA*-428pVIC	VIC-AGGAACAAGCAGTGCTG-MGB		
Capsular type K2	K2*wzy*-F1	GACCCGATATTCATACTTGACAGAG	Conventional	Turton et al. (2010)
	K2*wzy*-R1	CCTGAAGTAAAATCGTAAATAGATGGC		
Capsular type K1	*magA*-F	CGAAATGTGAACGAATTGATGCT	Real time	Hartman et al. (2009)
	magA-R	GTTTCTGCTGCAGATTCGAAGA		
	magA-Probe	CATCATGCAATAGCCACGT-NFQMGB		
Hemolysin	*khe*-F	GATGAAACGACCTGATTGCATTC	Real time	Hartman et al. (2009)
	khe-R	CCGGGCTGTCGGGATAAG		
	khe-Probe	6FAM-CGCGAACTGGAAGGGCCCG-TAMRA		
wcaG	*wcaG*-F	GGTTGGKTCAGCAATCGTA	Conventional	Turton et al. (2010)
	wcaG-R	ACTATTCCGCCAACTTTTGC		
rpoB	rpoBCM7	AACCAGTTCCGCGTTGGCCTGG	Conventional	Fenollar et al. (2006)
	rpoBCM31b	CCTGAACAACACGCTCGGA		
gyrB	KlebgyrB408F	CGTGGGTACGTACGCGAATA	Conventional	This study
	KlebgyrB1359R	ATGAACGAACTGCTGAGCGA		
BOX	BOX	CTACGGCAAGGCGACGCTGACG	REP-PCR[a]	Versalovic et al. (1994)

[a] REP-PCR = repetitive extragenic palindromic PCR.

er's suggested protocol and the Clinical Laboratory Standards Institute–published protocol (Watts and Clinical and Laboratory Standards Institute 2008). The MIC_{50} was defined as the antimicrobial concentration that inhibited growth of 50% of the isolates.

Detection limit of HMV *K. pneumoniae* by fecal culture assay

A spiking experiment was used to determine the limit of detection of HMV *K. pneumoniae* in feces and in nuclease-free water based on the culture methods we used. Of the three mucosal sampling sites, fecal swabs were selected for this experiment as they were the most likely to be contaminated by organisms that could potentially inhibit growth of HMV *K. pneumoniae*. A fresh stool sample was collected opportunistically from a stranded CSL of unknown *K. pneumoniae* status being rehabilitated at TMMC. The sample was confirmed negative for HMV *K. pneumoniae* by SBA plating for 48 h and summarily performing the string test on any suspect colonies. The sample was then used to create a solution comprised of 10 mg of feces/mL in sterile phosphate-buffered saline. We chose this concentration because it approximated the amount of stool visually present on fecal swabs. Tenfold serial dilutions of isolate HMV *K. pneumoniae* University of California Fish Pathology Lab ID number (UCD) 18 were created and added to 100-µL aliquots of the stool solution to create concentrations ranging from 10^1 to 10^6 colony-forming units (CFU)/mg of feces. Each spiked sample was analyzed in triplicate. To serve as positive controls, 100-µL aliquots of nuclease-free water were similarly spiked in triplicate with the same serial dilutions of HMV *K. pneumoniae* UCD 18. For each group, a triplicate set of nonspiked samples served as the negative control. Using a sterile swab, samples were streaked onto SBA plates and incubated at 37 C for 48 h, after which all plates were examined visually and string tests performed to confirm growth of HMV *K. pneumoniae*.

Statistical methods

The Fisher's exact test was used to identify significant differences between prevalence in wild-caught CSLs vs. stranded CSLs, with an α level set at 0.05. The Wilcoxon rank-sum test was used to evaluate significant differences between MICs for HMV vs. non-HMV isolates and for HMV stranded vs. wild-caught CSLs, with α set at 0.05. All calculations were performed using R statistical software (R Development Core Team 2017).

RESULTS

Prevalence of HMV *K. pneumoniae* in marine mammals

Hypermucoviscous *K. pneumoniae* was recovered from 1.2% (4 of 336) stranded marine mammals sampled at TMMC. All isolates were recovered from CSL. The four animals that were positive either died or were euthanized, two due to likely HMV *K. pneumoniae* infection and two due to other causes. Hypermucoviscous *K. pneumoniae* was recovered from 1.1% (3 of 270) wild-caught CSLs, 1.4% (1 of 68) at Año Nuevo Island, 1.2% (1 of 83) at Astoria, and 0.8% (1 of 119) at San Miguel Island. Wild-caught CSLs were apparently healthy. When just stranded CSLs were considered, isolates were obtained from 1.5% (4 of 275) animals; however, the difference in prevalence between stranded and wild-caught CSLs was not significant (P=1.000).

Bacterial identification

Of the 30 *K. pneumoniae* isolates included in this study, 11 isolates recovered during the prevalence sampling period, seven CSL isolates collected in 2015 before the study period, and seven marine mammal isolates (five CSLs, one harbor porpoise [*Phocoena phocoena*], and one PHS) archived at the University of California–Davis Veterinary Medical Teaching Hospital were HMV. Eighty-four percent (21 of 25) of the HMV isolates were recovered from males. Five archived *K. pneumoniae* isolates (two CSLs and three PHSs) were nonhypermucoviscous with 60% (2 of 3) recovered from males.

Molecular characterization

All 30 recovered isolates and the *K. quasipneumoniae* ATCC 700603 outlier were *khe* positive and *magA* negative. All but two of the HMV isolates were capsular type K2 serotype, whereas all non-HMV isolates did not belong to this serotype. Only one isolate, a non-HMV isolate from a CSL, tested positive for the *wcaG* gene. Of the analyzed HMV isolates, 96% (24 of 25) were positive for

TABLE 2. String test results and molecular characterization for hypermucoviscous isolates of *Klebsiella pneumoniae* isolated from marine mammals that were stranded or wild caught along the US Pacific Coast in 2015–16 evaluated in this study.[a]

Isolate	Species	Sample source	Isolate source	String test	Gene			
					p-*rmpA2*	p-*rmpA*	*K2* (*wzy*)	*wcaG*
Nonhypermucoviscous								
KATTC	Human	NA	NA	−	−	−	−	−
UCD 1	PHS	Ear	Stranded	−	−	−	−	−
UCD 2	PHS	Abscess	Stranded	−	−	−	−	−
UCD 3	CSL	Lung	Stranded	−	−	−	−	+
UCD 4	PHS	Feces	Stranded	−	−	−	−	−
UCD 5	CSL	Pleura	Stranded	−	+	+	−	−
Hypermucoviscous			Stranded				+	
UCD 6a	CSL	Lung	Stranded	+	+	+	+	−
UCD 6b	CSL	Nasal	Stranded	+	+	+	+	−
UCD 7	CSL	Nasal	Stranded	+	+	+	+	−
UCD 8	CSL	Nasal	Stranded	+	+	+	+	−
UCD 9a	CSL	Lung	Stranded	+	+	+	+	−
UCD 9b	CSL	Kidney	Stranded	+	+	+	+	−
UCD 9c	CSL	Diaphragm	Stranded	+	+	+	+	−
UCD 10	CSL	LN abscess	Stranded	+	+	+	+	−
UCD 11	CSL	Kidney	Stranded	+	+	+	+	−
UCD 12	CSL	Thorax	Stranded	+	+	+	+	−
UCD 13	CSL	Thorax	Stranded	+	+	+	+	−
UCD 14	CSL	Pleural fluid	Stranded	+	+	+	+	−
UCD 15	CSL	Thorax	Stranded	+	+	+	+	−
UCD 16	CSL	NA	Stranded	+	+	+	+	−
UCD 17	CSL	Lung	Stranded	+	+	+	+	−
UCD 18	CSL	Pleural fluid	Stranded	+	+	+	+	−
UCD 19	CSL	Thoracic fluid	Stranded	+	+	+	+	−
UCD 20	CSL	CSF	Stranded	+	+	+	+	−
UCD 21	HP	Abdominal fluid	Stranded	+	+	+	+	−
UCD 22	CSL	Pleural fluid	Stranded	+	+	+	+	−
UCD 23	PHS	Lung	Stranded	+	−	+	−	−
UCD 24a	CSL	Oral	Wild caught	+	+	+	+	−
UCD 24b	CSL	Rectal	Wild caught	+	+	+	+	−
UCD 25	CSL	Oral	Wild caught	+	−	−	−	−
UCD 26	CSL	Nasal	Wild caught	+	+	+	+	−

[a] KATTC = outlier strain of *Klebsiella quasipneumoniae* subsp. *similipneumoniae*; NA = not available; UCD = University of California Fish Pathology Lab ID number; PHS = Pacific harbor seal (*Phoca vitulina*); CSL = California sea lion (*Zalophus californianus*); LN = lymph node; CSF = cerebral spinal fluid; HP = harbor porpoise (*Phocoena phocoena*).

HMV associated gene p-*rmpA* and 92% (23 of 25) were positive for p-*rmpA2* (Table 2). One non-HMV isolate tested positive for both p-*rmpA* and p-*rmpA2* (Table 2).

Genomic characterization

All *Klebsiella* spp. isolates recovered from marine mammals were identified by *rpoB* and *gyrB* sequence as *K. pneumoniae*. At *rpoB*, *K. pneumoniae* isolates in this study averaged 99.9% identity (99.8–100%) among them and 99.8–100% similarity to selected *K. pneumoniae* genomes. Similarly, *gyrB* sequences of these isolates averaged 99.8% (99.5–100%) identity among them and 99.5–100.0% identity to *K. pneumoniae* genomes. Comparatively, *K. pneumoniae* spp. isolates recovered

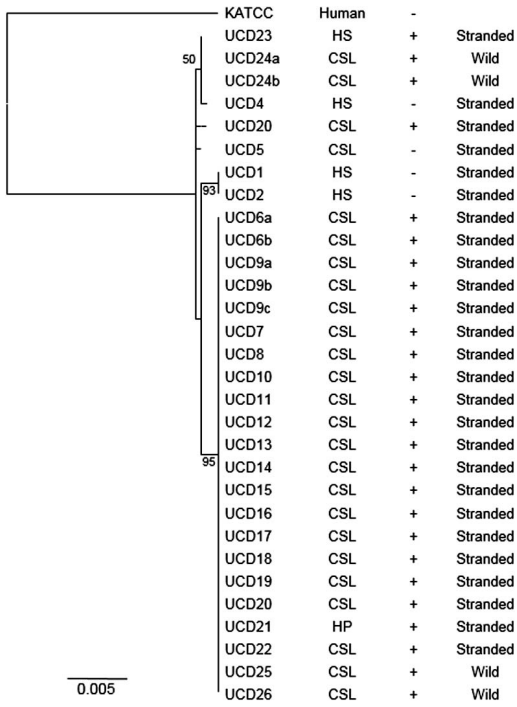

FIGURE 1. Maximum likelihood tree containing *Klebsiella pneumoniae* isolates from marine mammals that were stranded or wild caught in 2015–16 from along the US Pacific Coast. Values at the nodes represent bootstrap confidence values >80, based on 1,000 pseudoreplicates. Scale bar indicates the number of substitutions per site. KATCC=*Klebsiella quasipneumoniae* subsp. *similipneumoniae* (included as an outlier); UCD=University of California Fish Pathology Lab ID number; HS=Pacific harbor seal (*Phoca vitulina*); CSL=California sea lion (*Zalophus californianus*); HP=harbor porpoise (*Phocoena phocoena*); +=hypermucoviscous; −=nonhypermucoviscous; stranded vs. wild=location.

from marine mammals shared 97.3–97.4% similarity at *rpoB* and 94.8–94.9% similarity at *gyrB* to *K. quasipneumoniae* ATCC 700603 genome (GenBank no. CP014696). Phylogenetic analysis of these isolates based on concatenated *rpoB*>*gyrB* sequence was consistent with sequence identity patterns above, suggesting the *K. pneumoniae* isolates from marine mammals described here formed a discrete conspecific group, distinct from *K. quasipneumoniae*. Although variability at these two gene targets was limited, a majority (84.6%, 22 of 26) of the HMV *K. pneumoniae* from marine mammals shared the same *rpoB*>*gyrB* sequence type (Fig. 1).

Genetic fingerprints obtained from REP-PCR formed four discrete patterns (Fig. 2), demonstrating genotypic variability among *K. pneumoniae* from marine mammals from the US Pacific Coast, which loosely correlated with phenotype. Similar to the *rpoB*>*gyrB* concatenated sequences, REP-PCR data suggested the majority of HMV *K. pneumonia* from CSLs (82.6%, 19 of 25) originated from a single clonal phylogroup (Fig. 3). Although a majority (80%, 4 of 5) of non-HMV isolates also clustered together, a greater sample size is necessary to draw more robust conclusions regarding the relative genetic homogeneity of non-HMV isolates from marine mammals.

Antimicrobial susceptibility testing

Range and MIC for each antimicrobial agent were tested using the broth-microdilution method (Table 3). When tested with the Wilcoxon rank-sum test, the MICs for the non-HMV isolates were found to be significantly ($P \leq 0.05$) higher than the MICs for HMV isolates for enrofloxacin ($P=0.036$), ceftiofur ($P<0.001$), oxytetracycline ($P=0.004$), tetracycline ($P=0.003$), amoxicillin ($P=0.021$), spectinomycin ($P=0.009$), trimethoprim/sulfamethoxazole ($P<0.001$), florfenicol ($P<0.001$), and penicillin ($P=0.003$). When HMV isolates from stranded and wild-caught CSLs were compared using the Wilcoxon rank-sum test, the MICs for gentamicin ($P=0.039$), ceftiofur ($P<0.001$), tetracycline ($P=0.028$), amoxicillin ($P=0.018$), florfenicol ($P=0.002$), and penicillin ($P=0.032$) were significantly ($P \leq 0.05$) higher than those in the wild-caught group.

Detection limit of HMV *K. pneumoniae* by fecal culture assay

Swab inocula from 10-mg/mL suspended fecal solutions reliably detected HMV *K. pneumoniae* from concentrations as low as 10^2 CFU/mg feces, with three of three replicate cultures all yielding positive results. Even at concentrations of 10^1 CFU/mg feces, two of three replicates grew string test–

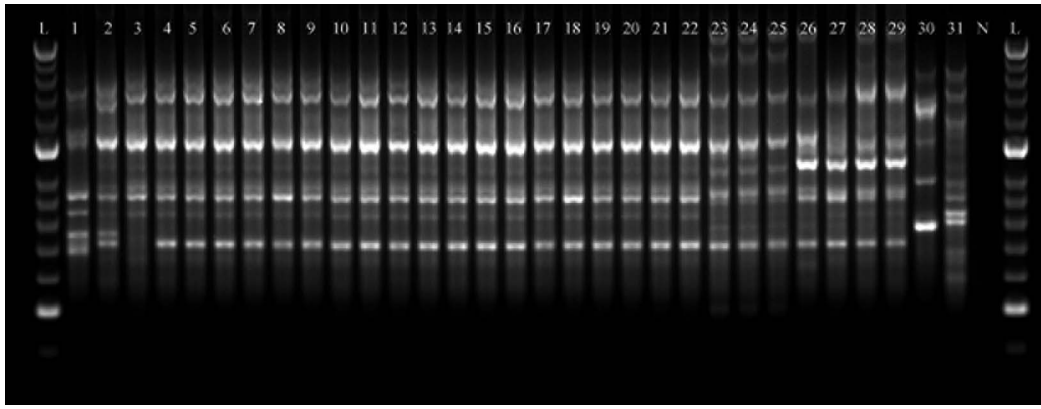

FIGURE 2. Repetitive extragenic palindromic PCR amplification of *Klebsiella pneumoniae* recovered from marine mammals that were stranded or wild caught in 2015–16 from along the US Pacific Coast. Lane 1: University of California Fish Pathology Lab (UCD) 8; Lane 2: UCD 5; Lane 3: UCD 21; Lane 4: UCD 16; Lane 5: UCD 6b; Lane 6: UCD 6a; Lane 7: UCD 14; Lane 8: UCD 7; Lane 9: UCD 17; Lane 10: UCD 18; Lane 11: UCD 10; Lane 12: UCD 15; Lane 13: UCD 13; Lane 14: UCD 22; Lane 15: UCD 12; Lane 16: UCD 9b; Lane 17: UCD 9a; Lane 18: UCD 9c; Lane 19: UCD 19; Lane 20: UCD 11; Lane 21: UCD 20; Lane 22: UCD 25; Lane 23: UCD 24a; Lane 24: UCD 24b; Lane 25: UCD 23; Lane 26: UCD 4; Lane 27: UCD 3; Lane 28: UCD 1; Lane 29: UCD 2; Lane 30: UCD 26; Lane 31: *Klebsiella quasipneumoniae* subsp. *similipneumoniae* outlier. Genetic profiles were generated using the BOX primer. L=hyperladder 50 bp; N=no template control.

positive colonies. All inocula from spiked nuclease-free water samples were culture positive. All unspiked stool solution and nuclease-free water inocula were HMV *K. pneumoniae* culture negative.

DISCUSSION

We investigated the baseline prevalence of HMV *K. pneumoniae* in stranded marine mammals and in apparently healthy wild-caught CSL. The prevalence of HMV *K. penumoniae* was 1.2% among 336 stranded marine mammals tested. No isolates were recovered from PHSs, NESs, or NFSs, likely due to the small number of animals from these species that had stranded during our sampling period. Future studies evaluating larger sample sizes may give a more representative overview of prevalence of HMV *K. pneumoniae* in other marine mammals.

Previous epidemics of *K. pneumoniae* have been identified in NZSLs in New Zealand. The bacterium was found as the etiologic agent of mortality events in this species in 2001–03. Although not evaluated for the presence of HMV *K. pneumoniae* phenotype, these epidemics increased mortality in NZSL pups by three times the mean in nonepidemic years (Castinel et al. 2007b). Evaluation of similar epidemics in NZSLs in 2006–07 and 2009–10 demonstrated HMV *K. pneumoniae* infection as the cause of death in 58% of the pups presented for necropsy (Roe et al. 2015). The baseline prevalence of HMV *K. pneumoniae* in CSLs that we reported was much lower than the reported prevalence of *K. pneumoniae* and HMV *K. pneumoniae* for NZSLs during these epidemics (Castinel et al. 2007a; Roe et al. 2015), which is likely a function of our samples being collected at a time at which there was no active epizootic event, the inclusion of multiple age classes, and sampling of live and dead animals. Larger sample sizes, particularly among species other than CSLs, and extended sampling times over periods of several years targeting at-risk age classes such as young pups, are needed to evaluate the dynamics of HMV *K. pneumoniae* emergence in marine mammals of the US Pacific Coast.

Based on complications HMV *K. pneumoniae* can cause in human nosocomial settings (Podschun and Ullmann 1998; Shon and

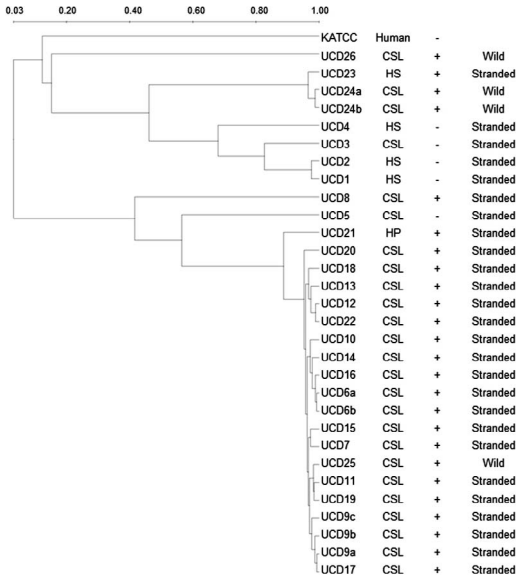

FIGURE 3. Dendrogram generated with Dice coefficient and the unweighted pair group method with arithmetic mean clustering method, showing the genetic similarity among *Klebsiella pneumoniae* isolates by repetitive extragenic palindromic PCR genotyping using the BOX primer (Versalovic et al. 1994). KATCC=*Klebsiella quasipneumoniae* subsp. *similipneumoniae*; UCD=University of California Fish Pathology Lab ID number; CSL=California sea lion (*Zalophus californianus*); HS=Pacific harbor seal (*Phoca vitulina*); HP=harbor porpoise (*Phocoena phocoena*); –=nonhypermucoviscous; +=hypermucoviscous; wild vs. stranded=location.

Russo 2012), the prevalence of this organism in stranded CSLs and in wild-caught CSLs was compared. No significant difference was found between the two. Stranded animals originated from a wild population of CSLs and were sampled only at admission to TMMC. Therefore, it is likely the two populations would have similar prevalence of HMV *K. pneumoniae*. Evaluation of the incidence of this infection and its ability to cause clinical disease in marine mammal hospital populations can help elucidate the importance of HMV *K. pneumoniae* as a nosocomial agent.

Recent studies using isolates recovered from human patients have demonstrated the *rmpA* and *rmpA2* genes, either chromosomally or plasmid encoded, are closely associated with hypermucoviscosity (Hsu et al. 2011). All but one of the HMV isolates recovered from marine mammals possessed the plasmid form of both *rmpA* and *rmpA2*. Interestingly, one of the five non-HMV isolates possessed both p-*rmpA* and p-*rmpA2*. Similar results regarding the hypermucoviscosity phenotype have been found when comparing K1/K2 and non-K1/K2 isolates recovered from liver abscesses in human patients (Yu et al. 2008).

Capsular serotypes K1 and K2 have been most frequently associated with HMV *K. pneumoniae* infection in humans (Fang et al. 2007; Yu et al. 2008). However, of the 77 capsular types identified for *K. pneumoniae*, there are several associated with hypermucoviscosity in addition to types K1 and K2 (Turton et al. 2010). Roe et al. (2015) reported all HMV *K. pneumoniae* isolates recovered from NZSLs belonged to the capsular K2 serotype. Similar findings were reported by Jang et al. (2010) in CSLs. In the current study, most of the HMV isolates belonged to the K2 type. However, two HMV isolates were negative for K1 and K2, suggesting other capsular types may be associated with the HMV phenotype in marine mammals.

In addition to capsular types, the *wcaG* gene is a putative *K. pneumoniae* virulence factor thought to be involved in macrophage evasion (Turton et al. 2010). The use of *wcaG* as a potential marker for HMV *K. pneumoniae* virulence in marine mammals has not been evaluated. All but one of the isolates recovered from marine mammals in our study was negative for *wcaG*, suggesting *wcaG* is not likely a reliable indicator of HMV *K. pneumoniae* type in the species we tested.

There was limited genotypic and phenotypic variability among *K. pneumonia*e isolates based on the markers that we used. Based on *rpoB*>*gyrB* concatenated sequences and REP-PCR, the majority of HMV *K. pneumoniae* from CSLs seem to originate from a single clonal genetic phylogroup, with some geographic exceptions. Similarly, a majority of non-HMV isolates also cluster together. Although promising, more representative isolates are required to make inferences regarding the relative genetic homogeneity of HMV and non-HMV isolates and utility of REP-PCR in virulence screening.

TABLE 3. Range and antimicrobial concentration that inhibited growth of 50% of the isolates (MIC_{50}, μg/mL) of 18 different antimicrobial agents to hypermucoviscous (HMV) and non-HMV *Klebsiella pneumoniae* isolates from marine mammals that were stranded or wild caught along the US Pacific Coast in 2015–16 as determined by broth microdilution.[a]

Antimicrobial agent	Non-HMV		HMV	
	Range	MIC_{50}	Range	MIC_{50}
Enrofloxacin	0.12–2	0.12	NA	0.12
Gentamicin	0.5–1	0.5	0.5–1	0.5
Ceftiofur	1–4	4	0.25–1	0.25
Neomycin	NA	2	NA	2
Erythromycin	NA	4	NA	4
Oxytetracycline	2–4	2	0.25–8	0.5
Tetracycline	2–8	2	0.25–8	0.5
Amoxicillin	2–16	16	0.5–16	1
Spectinomycin	NA	64	8–64	16
Sulfadimethoxine	NA	256	32–256	256
Trimethoprim/sulfamethoxazole	0.5/9.5–2/38	2/38.0	0.5/9.5–1/19	0.5/9.5
Florfenicol	NA	8	1–8	1
Sulfathiazole	NA	256	32–256	256
Penicillin	NA	8	0.06–8	1
Streptomycin	NA	8	8–128	8
Novobiocin	NA	4	0.5–4	4
Tylosin tartrate	NA	20	NA	20
Clindamycin	NA	4	1–4	4

[a] NA = not applicable.

In humans, there has been increasing concern that many of the current strains have developed antimicrobial resistance (Elemam et al. 2009) including recent infections with resistance to all antibiotics tested (Chen et al. 2017). Most of the hypervirulent strains, however, have been susceptible to many common antimicrobial agents, with the exception of ampicillin (Lin et al. 2010). This is consistent with current findings. When the MICs for non-HMV isolates was compared with those of HMV *K. pneumoniae* isolates, non-HMV isolates had higher MICs for many of the antimicrobial agents evaluated. One consideration however is the relatively small sample size of non-HMV isolates available for comparison and the potential for outliers to influence analysis and give the appearance of greater resistance. Sample source must also be taken into consideration, as microenvironments can affect the antimicrobial resistance pattern of many bacteria. Evaluation of more isolates from representative sample sources is needed to determine whether HMV strains in the marine environment are developing resistance profiles similar to previously studied human strains. The presence of this emerging pathogen in marine mammals and the potential acquisition of antimicrobial resistance, however, are causes for concern (Li et al. 2014), given the ability of HMV *K. pneumoniae* to combine hypervirulence with multidrug resistance (Shon and Russo 2012).

In our study, all stranded CSL HMV *K. pneumoniae* isolates exhibited a ceftiofur MIC ($\leq$0.5 μg/mL). This was consistent with doses of ceftiofur typically administered to CSLs at TMMC to treat suspected cases of bacterial pneumonia (Meegan et al. 2013). Therefore, ceftiofur may be an appropriate initial choice for treatment of suspected HMV *K. pneumoniae* infections in CSLs originating from the US Pacific Coast. However, confirmation of clinical effectiveness of this antimicrobial agent needs to be established with the collection of individual case susceptibility

data. Although the sample size was very small, the MICs for HMV wild-caught CSL isolates were significantly higher than those of HMV stranded CSL isolates for several antibiotics including ceftiofur, amoxicillin, tetracycline, gentamicin, florfenicol, and penicillin.

No studies have tested the limit of detection of HMV *K. pneumoniae* in marine mammal stool samples by using the methods used in this study. Cultivation methods can consistently detect 10^1 CFU/g feces of *Salmonella* in CSL feces (Berardi et al. 2014). In our analysis, a 10-mg/mL fecal solution was used for spiking to closely approximate the amount of fecal material that would be obtained on a fecal swab. Based on our results, detection with cultivation techniques is quite sensitive, as it could reliably detect HMV *K. pneumoniae* at concentrations as low as 10^2 CFU/mg. This seems to be a reliable method for detecting the presence of this bacteria in CSL. This is particularly important as most of the HMV *K. pneumoniae* recovered from marine mammals in our laboratory do not grow well on the MacConkey agar typically used to recover *Enterobacteriaceae* from contaminated sites (Jang et al. 2010). Biochemical comparisons between HMV *K. pneumoniae* isolated from different species of marine mammals, including isolates from NZSL (Roe et al. 2015) are warranted to clarify the nutrient requirements of these isolates and to generate better diagnostic methods for this elusive pathogen.

Historically, HMV *K. pneumoniae* has been of minimal concern to the health of aquatic mammals; however, the recent emergence of this pathogen as a cause of clinical infection in marine mammal rehabilitation centers is alarming (Jang et al. 2010). Further detailed phenotypic and genotypic characterization of the isolates examined in this study, and surveys of the prevalence of HMV *K. pneumoniae* in stranded and wild-caught populations, has advanced our understanding of HMV *K. pneumoniae* dynamics in marine mammals of the US Pacific Coast. In addition, these data suggest the *rmpA* gene has potential as a molecular marker for HMV *K. pneumoniae* and molecular diagnostic method for this phenotype. The prevalence of HMV *K. pneumoniae* in other aquatic animals and its persistence in aquatic environments is still unknown.

ACKNOWLEDGMENTS

We thank the members of the University of California–Davis Aquatic Animal Health Laboratory, the Department of Medicine and Epidemiology in the University of California–Davis School of Veterinary Medicine, and the Marine Mammal Center. Funding was provided by the National Science Foundation (OCE-1335657). We thank Irina Udaltsova, Ashley Hill, and Janet Foley (School of Veterinary Medicine, University of California–Davis) and Maris Brenn-White, Ketki Raje, and Yehonatan Berkowic for support.

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Submitted for publication 26 July 2017.
Accepted 13 December 2017.