



Genetics and pathology associated with *Klebsiella pneumoniae* and *Klebsiella* spp. isolates from North American Pacific coastal marine mammals

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ARTICLE INFO

Keywords:

Hypermucoviscous
Marine mammal
serum resistance
Blood killing
Klebsiella pneumoniae
Peritonitis
Sea otter
Septicemia

ABSTRACT

Southern sea otters (SSO: *Enhydra lutris nereis*) are a federally-listed threatened subspecies found almost exclusively in California, USA. Despite their zoonotic potential and lack of host specificity, *K. pneumoniae* and *Klebsiella* spp. have largely unknown epizootiology in SSOs. *Klebsiella pneumoniae* is occasionally isolated at necropsy, but not from live SSOs. Hypermucoviscous (HMV) *K. pneumoniae* strains are confirmed pathogens of Pacific Basin pinnipeds, but have not been previously isolated from SSOs. We characterized the virulence profiles of *K. pneumoniae* isolates from necropsied SSOs, evaluated killing of marine mammal *K. pneumoniae* following *in vitro* exposure to California sea lion (CSL: *Zalophanus californianus*) whole blood and serum, and characterized lesion patterns associated with *Klebsiella* spp. infection in SSOs. Four of 15 SSO *K. pneumoniae* isolates were HMV and all were recovered from SSOs that stranded during 2005. Many *K. pneumoniae* infections were associated with moderate to severe pathology as a cause of death or sequela. All HMV infections were assessed as a primary cause of death or as a direct result of the primary cause of death. *Klebsiella*-infected SSOs exhibited bronchopneumonia, tracheobronchitis and/or pleuritis, enteritis, *Proflicollis* sp. acanthocephalan peritonitis, septic peritonitis, and septicemia. All SSO HMV isolates were capsular type K2, the serotype most associated with HMV infections in CSLs. Multiplex PCR revealed two distinct virulence gene profiles within HMV isolates and two within non-HMV isolates. *In vitro* experiments investigating CSL whole blood and serum killing of *K. pneumoniae* suggest that HMV isolates are more resistant to serum killing than non-HMV isolates.

1. Introduction

Klebsiella pneumoniae is an encapsulated, gram-negative, non-motile bacillus in the order Enterobacterales that is commonly found in the environment and on mucosal surfaces (Bagley, 1985). It is a common nosocomial pathogen in human and veterinary medicine (Tsay et al., 2002; Brisse and Duijkeren, 2005). This bacterium has a broad host range and can rapidly develop antimicrobial resistance (Holt et al.,

2015). *Klebsiella* spp. have been isolated from diverse terrestrial and marine mammals, but few reports exist for sea otters (*Enhydra lutris*) (Castinel et al., 2007; Whitaker et al., 2018; Miller et al., 2020).

Hypermucoviscous (HMV) *K. pneumoniae* are highly invasive, hypervirulent strains of *K. pneumoniae* that can cause severe localized or systemic, community-acquired infections in healthy hosts. HMV strains are more virulent than non-HMV *K. pneumoniae* isolates in animal models (Twenhafel et al., 2008; Shon et al., 2013). When grown *in vitro*

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<https://doi.org/10.1016/j.vetmic.2021.109307>

Received 6 May 2021; Received in revised form 30 November 2021; Accepted 11 December 2021

Available online 13 December 2021

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on agar plates, HMV *K. pneumoniae* strains produce copious capsular mucopolysaccharides, as indicated by a mucoid string >5 mm long when stretched from the plate with an inoculating loop (Shon et al., 2013). HMV *K. pneumoniae* isolates commonly express multiple virulence genes, including *rmpA* (a regulator of the mucoid phenotype), *magA* (capsular serotype K1), *K2wzy* (capsular serotype K2), and siderophore genes among others, that allow the pathogen to infect host cells while avoiding host innate and adaptive immune responses (Shon et al., 2013; Holt et al., 2015; Struve et al., 2015; Soto et al., 2016). This emerging pathogen has been considered the next “superbug” (Shon and Russo, 2012).

HMV *K. pneumoniae* strains have been isolated from California sea lions (CSL: *Zalophus californianus*) with suppurative peritonitis, arthritis, pneumonia, and pleuritis; New Zealand sea lion pups (*Phocarcos hookeri*) with meningitis, subdural hemorrhage, and septic arthritis; Pacific harbor seals (*Phoca vitulina richardii*; PHS); and a harbor porpoise (*Phocoena phocoena*) (Spraker et al., 2007; Jang et al., 2010; Roe et al., 2015; Seguel et al., 2017; Whitaker et al., 2018).

Our composite microbiological data from assessment of >500 necropsied southern sea otters (SSO: *Enhydra lutris nereis*) to date suggest that *Klebsiella* spp. are not common commensals for these animals; all *Klebsiella* spp.-positive, necropsied SSOs from 1997 through 2020 are included in the current study. In addition, assessment of >120 apparently healthy, wild-caught SSOs were sampled, including swabs from the skin, upper respiratory tract, oropharynx, rectum, prepuce, or vagina were inoculated onto non-selective media (Blood and MacConkey agar) yielded no *Klebsiella* spp. isolates (M. Miller, unpub. data).

The objectives of this study were to evaluate the pathogenic profiles of *K. pneumoniae* isolates from SSOs, CSLs, and PHSs using a novel multiplex-PCR for known *Klebsiella* virulence genes. Stranding and necropsy records were reviewed to assess potential gross and microscopic pathology associations for *Klebsiella* spp.-positive SSOs. Phenotypic and genotypic variations of *K. pneumoniae* isolates from eight SSOs that stranded between 1998–2007, eight CSLs examined between 1994–2016, and four PHSs examined between 2012–2016 were also compared. Finally, we challenged non-HMV and HMV *K. pneumoniae* isolates from SSOs, CSLs, and PHSs with whole blood and serum from healthy CSLs to assess bacterial persistence *in vitro*.

2. Materials and methods

2.1. Bacteria

Freshly dead or euthanized SSOs that stranded throughout central California, USA (Fig. 1) or died in captivity were necropsied at the Marine Wildlife Veterinary Care and Research Center (MWVRC; Santa Cruz, CA) between 1997 and 2020 using a standardized protocol (Miller et al., 2020). Sterile swabs inoculated from tissue or body fluids during necropsy were shipped overnight to the Microbiology Laboratory of the Veterinary Medical Teaching Hospital at the University of California-Davis for bacterial isolation and identification. Subsamples of all major tissues and body fluids were also cryopreserved at -80°C at MWVRC.

Bacteriological swabs were inoculated onto tryptic soy agar supplemented with 5% sheep blood (SBA; Hardy Diagnostics, Santa Maria, CA) and incubated at 35°C in 5% CO_2 for 24–72 h. Isolates were identified using conventional microbiological methods including spot testing (cytochrome oxidase, indole), triple sugar iron agar (Biological Media Services, University of California Davis, CA; BMS), Christensen’s urea agar (BMS), sulfur-indole-motility agar (BMS), citrate agar (BMS), and microbial identification using Analytical Profile Index 20E (API 20E Biomerieux, Durham, NC). Purified bacterial isolates were stored on ProLab Microbank Beads (Thermo Fisher Scientific, Waltham, MA) at -80°C for further study. Cryopreserved isolates ($n = 8$) were revived by streaking onto SBA and incubated at 35°C in 5% CO_2 overnight. The identity of all cryopreserved isolates was confirmed via matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Biotyper, Bruker, Fremont, CA) following manufacturer protocols.

Necropsied SSOs were enrolled in the study only if *Klebsiella* spp. were detected via bacterial culture. Detailed information for each SSO is available in Supplementary Material, Table 1. For any necropsied SSO with a previous *Klebsiella* spp. isolate that had not been cryopreserved, necropsy tissues cryoarchived at -80°C were thawed and fresh bacterial swabs were collected and submitted for isolation. The five most common sources of *Klebsiella* spp. isolation from necropsied SSOs (lung, kidney, lymph node, liver, and/or heart; see Supplementary Material, Table 1) were prioritized for bacterial isolation; inoculated swabs were streaked on SBA (BMS) and incubated at 37°C in ambient atmosphere for 72 h. During incubation, bacterial colonies were evaluated for morphology consistent with *K. pneumoniae* and a string test was performed to identify



Fig. 1. Map of California showing the stranding locations of southern sea otters (*Enhydra lutris nereis*) that were culture-positive for *Klebsiella* spp. Sea otter 3057-98 was not included as it came from a captive setting following stranding. Cases are indicated by red dots; parenthetical numbers represent multiple otters recovered in the same general area. Asterisks (*) represent the stranding locations of southern sea otters infected with biochemically confirmed hypermucoviscous *Klebsiella pneumoniae* (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Table 1

Multiplex PCR primers used for genetic characterization of *Klebsiella pneumoniae* isolates recovered from southern sea otters (*Enhydra lutris nereis*), Pacific harbor seal (*Phoca vitulina richardsi*), and California sea lion (*Zalophus californianus*).

Target Gene	Virulence Factor	Virulence Factor Function	Sequence (5'-3')	Size (bp)	References
<i>magA</i>	Capsular serotype K1	K1 capsular polysaccharides	F: GAATATTCCGACTCCCTCTCC R: TAGGACCGTTAATTTGCTTTGT	797 bp	Bojer et al., 2010
<i>K2wzy</i>	Capsular serotype K2	K2 capsular polysaccharides	F: GACCCGATATTCATACTTGACAGAG R: CCTGAAGTAAAATCGTAAATAGATGGC	641 bp	Turton et al., 2010
<i>K5wzy</i>	Capsular serotype K5	K5 capsular polysaccharides	F: TGGTAGTGATGCTCGCGA R: CCTGAACCCACCCCAATC	280 bp	Turton et al., 2010
<i>iroD</i>	Salmochelins	Iron acquisition	F: GCATAGGCGGATACGAACAT R: CACAGGGCAATTGCTTACCT	556 bp	This study
<i>iutA</i>	Aerobactin	Iron acquisition	F: ACCTGGGTTATCGAAAACGC R: GATGTCATAGCCTGATTGC	1115 bp	Tang et al., 2010
<i>fyuA</i>	Yersiniabactin	Iron acquisition	F: GTAACAATCTTCCGCTCGGCAT R: TGACGATTAACGAACCCGAAGGGA	845 bp	Spurbeck et al., 2012
<i>allS</i>	Allantoin metabolism island	Carbon and nitrogen source	F: CCGAAACATTACGCACCTTT R: ATCACGAAGAGCCAGGTAC	508 bp	Yu et al., 2008
<i>rmpA/rmpA2</i>	RmpA/RmpA2	Regulates mucoid phenotype	F: GCGAATTAACCTGGACTACCTCTG R: GTTTACAATTCGGCTAACATTTTCTTTAAG	321 bp	Fang et al., 2007
<i>clbB</i>	Colibactin	Genotoxic molecule	F: CAAAAATCAGCCATCCA R: ACACAAAACTACCGACA	665 bp	This study
<i>mrkB</i>	Type 3 fimbriae	Promotes biofilm formation	F: TGCCACACCGGATACCATTACCAC R: AACGGCCACTTCACTGCGACATC	514 bp	This study
<i>fimH</i>	Type 1 fimbriae	Mediates adhesion to host epithelial cells	F: GCGCGGTGATTATGATT R: AGGCGGTATTGGTGAAGA	369 bp	This study
<i>Kpn</i>	<i>Klebsiella pneumoniae</i> rRNA spacer	Unique to <i>K. pneumoniae</i>	F: ATTTGAAGAGGTTGCAAACGAT R: TTCACTCTGAAGTTTCTTGTTCT	130 bp	Turton et al., 2010

the HMV phenotype (Fang et al., 2004). Cryopreserved *K. pneumoniae* strains obtained from stranded or moribund CSLs and PHSs examined at The Marine Mammal Center (TMMC, Sausalito, CA) from 1994 to 2016 were also revived following the same procedures mentioned above to compare with strains from SSOs.

2.2. Molecular characterization

Two multiplex PCR assays were designed to detect capsule serotypes and factors associated with hypervirulence. The capsule assays included detection of K1, K2, and K5 serotypes. Genes known to be associated with increased virulence in humans (Chen et al., 2004; Wu et al., 2009; Struve et al., 2015) were assessed, including the regulator of mucoid phenotype, the mucoviscosity-associated gene, the gene associated with

allantoin metabolism, the genotoxin colibactin, three siderophores (aerobactin, salmochelins, and yersiniabactin), and two fimbrial adhesins (type 1 and type 3 fimbriae). Primers specific for the 16S–23S internal transcribed spacer in *K. pneumoniae* were also included for species verification (Turton et al., 2010). The two multiplex PCRs, including primer sequences and the specific genes targeted for each capsule serotype or virulence factor, are summarized in Table 1. Reference strains expressing capsule serotypes K1, K2 and K5, and selected *K. pneumoniae* strains with available whole genome sequences were used to validate the multiplex PCR assays (Struve et al., 2015).

Genomic DNA was extracted from bacterial isolates using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer protocols. All DNA samples were stored at –20 °C until used. PCR assays were completed using a Multiplex PCR Kit (Qiagen) in a 50

Table 2

Lesion prevalence (defined by lesion presence/absence independent of the primary or contributing causes of death) in necropsied southern sea otters (*Enhydra lutris nereis*) with culture confirmed *Klebsiella* spp. and hypermucoviscous (HMV) and non-HMV *K. pneumoniae* infections. Total number of animals with each lesion is indicated; percentage of animals positive for the lesion noted in parentheses.

Observed Lesion	Non-HMV- positive <i>K. pneumoniae</i> (n = 11)	HMV-positive <i>K. pneumoniae</i> (n = 4)	Other <i>Klebsiella</i> spp. (n = 3)	Total (All <i>Klebsiella</i> spp., n = 18)
Septicemia	10 (91) ⁺	4 (100)	2 (67)	16 (89)
Acanthocephalan peritonitis (<i>Profilicollis</i> sp.)	9 (82)	4 (100)	0	13 (72)
Lymphadenomegaly	7 (64)	2 (50)	3 (100)	12 (67)
Suppurative lymphadenitis	6 (55)	1 (25)	2 (67)	9 (50)
Suppurative pneumonia, tracheobronchitis, and/or pleuritis	3 (27)	3 (75)	3 (all early or mild) (100)	9 (50)
Septic peritonitis (secondary to acanthocephalans or intestinal perforations)	6 (55)	2 (50)	0	8 (44)
Abscess	3 (27)	1 (25)	3 (100)	7 (39)
Infected wounds*	2 (18)	1 (putative) (25)	3 (100)	6 (33)
Current or healed intestinal perforations	5 (45)	0	0	5 (28)
Gastroenteritis, enterocolitis, or intestinal displacements	3 (27)	1 (25)	0	4 (22)
Suppurative meningitis and/or encephalitis	2 (mild) (18)	1 (25)	0	3 (17)
Suppurative nephritis, cystitis, or vulvovaginitis	2 (18)	0	0	2 (11)
Suppurative cholecystitis	0	1 (25)	0	1 (6)
Suppurative periostitis	1 (9)	0	0	1 (6)
Suppurative polyarthritis	0	0	1 (33)	1 (6)

* Trauma due to mating (nose wounds), fighting, shark bite, boat strike or fishing line or hook entanglement.

⁺ Percentage of southern sea otters within each group where the defined lesion was present at necropsy, independent of whether it was considered a cause of death or sequela. Please see the manuscript text for additional details.

µL reaction that included 2x Qiagen Multiplex PCR master mix, 0.2 µM forward and reverse primers, 5 µL DNA at concentration of ~50 ng/µL, and RNase-free water to volume using a SimpleAmp Thermal Cycler Applied-Biosystem (Thermo Fisher Scientific). PCR conditions included a 15 min denaturation at 95 °C, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 90 s, extension at 72 °C for 90 s, and a final extension at 72 °C for 10 min. PCR products were visualized using a MyECL Imager (Thermo Fisher Scientific) after electrophoresis of the amplified DNA in a 2% agarose gel containing 1% SYBR safe DNA gel stain (Thermo Fisher Scientific).

2.3. Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MIC) for 18 different antimicrobials were identified using Sensititre AVIAN1F Vet AST plates (Thermo Fisher Scientific) in cation-adjusted Mueller Hinton broth (Thermo Fisher Scientific) following Clinical and Laboratory Standards Institute (CLSI) protocols (CLSI, 2020). *Escherichia coli* strain ATCC 25922 was used as a quality control. Inoculated blood agar plates were incubated at 37 °C for 24 h, bacteria inoculated on the Sensititre plates according to manufacturer protocols, and bacterial growth was checked visually 24 and 48 h post-inoculation. The MIC was defined as the lowest concentration where no visible growth was exhibited. Breakpoints were interpreted following those for Enterobacterales in dogs (CLSI, 2018, 2020).

2.4. Potential lesion associations

Case records from all necropsied SSOs that were culture-positive for *Klebsiella* spp. in one or more tissues from 1997 through 2020 were assessed for possible bacterial-associated pathology. Associations were assessed in two ways: 1.) *Klebsiella* spp. detection relative to all primary or contributing causes of death and sequelae at necropsy, and 2.) Presence/absence of defined health conditions for all enrolled SSOs, regardless of whether each condition was considered a cause of death or sequela at necropsy (Table 2). Assessment #1 was performed to examine whether *Klebsiella* spp. detection was associated with the primary or contributing causes of death or sequelae, based on all findings from necropsy and histopathology. Assessment #2 was performed to identify health conditions that could indicate potential routes of *Klebsiella* spp. bacterial invasion, although these patterns would need to be verified or ruled out through subsequent research.

SSOs commonly exhibit multiple moderate to severe health conditions at necropsy (Miller et al., 2020), and careful assessment of the most common causes of death is important for prioritizing conservation

efforts (Tinker et al., 2021), so this level of detail is important. Distinguishing core causes of death and sequelae also ensures that risk assessments are performed on animal groups with the highest possible similarity.

Klebsiella spp. infection was considered a potential contributor to animal morbidity and death (as a cause of death or sequela) by a veterinary pathologist based on review of all findings from gross necropsy, microbiology, and histopathology. Factors that were considered in this assessment included bacterial isolation from observed lesions and visualization of morphologically compatible bacteria (short, stout encapsulated or non-encapsulated bacterial rods) within lesions on histopathology (Fig. 2).

For each enrolled SSO, the primary cause of death was the most severe process that likely caused stranding. Secondary, tertiary and quaternary (cumulatively “contributing”) causes of death represent independent processes that were also present at necropsy, were deemed moderate or severe, and likely contributed to stranding (Miller et al., 2020). Necropsy findings were defined as sequelae if they occurred as a direct consequence of a primary or contributing cause of death and contributed to the risk of death but were not independent processes. An example would be a white shark (*Carcharodon carcharias*) bite as a primary cause of death, with post-traumatic bacterial infection as a sequela (Miller et al., 2020). Miller et al. (2020) confirmed the importance of assessment of both causes of death and sequelae. For example, that study showed that most SSOs survive the acute traumatic effects of shark bite, but later succumb to bacterial infections that would not have occurred if the traumatic event had not happened, providing a portal for bacterial entry (Miller et al., 2020).

Although case detail varied, all available clinical, gross necropsy, histopathology, and diagnostic data were reviewed to identify *Klebsiella*-associated lesion patterns. This included detection of morphologically compatible bacteria (short, stout, Gram-negative, encapsulated, or non-encapsulated encapsulated rods) on histopathology for *Klebsiella*-positive SSOs (see Figs. 2–4 for examples). Assessed lesions and conditions that could be potential indicators for routes of *Klebsiella* spp. bacterial invasion (Table 2) included presence/absence of septicemia, *Proflicollis* sp.-associated peritonitis, septic peritonitis, current or healed intestinal perforations, lymphadenomegaly, suppurative lymphadenitis, infected wounds or abscesses, pneumonia, potential bacterial gastroenteritis, enterocolitis, intestinal displacements, suppurative meningitis, and other suppurative conditions that could be attributed to bacterial infections. These conditions are part of standardized lesion documentation for SSOs and were assessed during necropsy and retrospectively via review of archival photographs and case records.

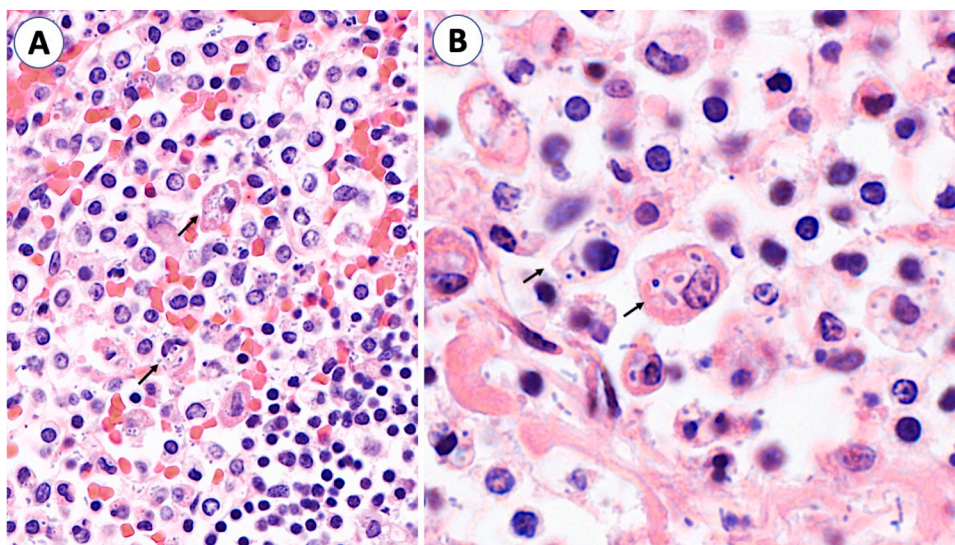


Fig. 2. A.) Case 4449-05: Microscopic views of retropharyngeal lymph node draining a large (20 cm diameter) fascial abscess on the neck of a southern sea otter (*Enhydra lutris nereis*). Hypermucoviscous *Klebsiella pneumoniae* was isolated in pure culture from the fascial abscess, lymph node, brain and heart blood. At low magnification (A.), short, stout bacterial rods were visible throughout the section, including within the cytoplasm of macrophages (arrows). At higher magnification (B), thick, pale outer capsules were apparent around the bacterial rods (arrows). Hematoxylin and eosin stain. Bar = 12 µm (A), and Bar = 10 µm (B).

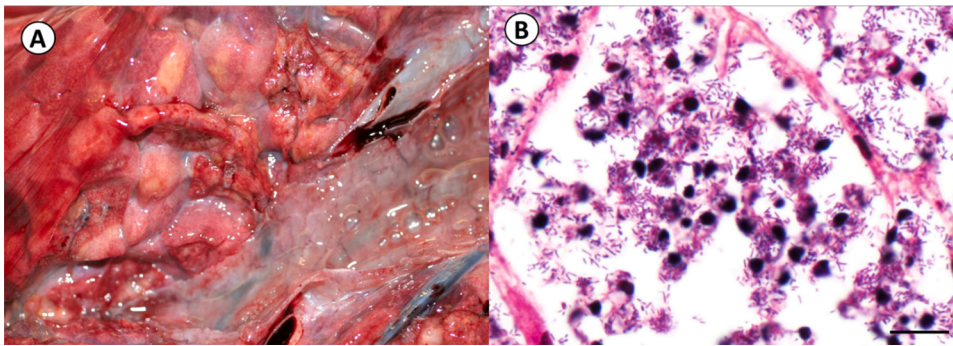


Fig. 3. Southern sea otter (*Enhydra lutris nereis*) 4486-05: A.) Gross view of bronchus and perihilar lung tissue; there is multifocal distension and yellow discoloration of perihilar alveoli (upper left), and moderate mucopurulent bronchial exudate (Lower right). Hyper-mucoviscous *K. pneumoniae* was isolated in pure culture from lung, hilar lymph node, and heart blood. B.) Gram stain of lung from the same sea otter; alveoli are filled with macrophages, neutrophils and myriad free and intracytoplasmic Gram-negative bacterial rods. Brown-Brenn stain. Bar = 25 μ m (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).



Fig. 4. Microscopic views of small intestinal wall from two southern sea otters (*Enhydra lutris nereis*) with concurrent *Profilicollis* sp. acanthocephalan parasite and suspected or confirmed *Klebsiella pneumoniae*-associated septic peritonitis. A.) Case 4164-05: A *Profilicollis* sp. acanthocephalan parasite is migrating through the intestinal wall. Most of the body of the parasite is still within the intestinal lumen (lower left), while the swollen proboscis has perforated through the intestinal serosa (upper right). The arrow indicates a dense accumulation of bacterial rods along the external surface of the parasite. B.) Higher magnification view of the area indicated by the arrow in A, showing the dense population of bacterial rods along the external surface of the transmigrating parasite. Hyper-mucoviscous (HMV) *K. pneumoniae* and coagulase negative staphylococci were isolated from heart blood at necropsy. C.) High magnification view of the external surface of another intramural *Profilicollis* sp. parasite from the same otter, showing the dense population of bacterial rods along the surface of the transmigrating parasite. D.) Case 4127-04: Serosal surface of small intestine at a point of *Profilicollis* sp. acanthocephalan parasite perforation through the intestinal wall. The parasite has passed into the peritoneal cavity, but dense clusters of bacterial rods are still present along the acanthocephalan's migratory pathway through the intestinal wall and at the serosal surface, where they are associated with focal omental adhesion, abscessation and necrosis. Mixed non-HMV *K. pneumoniae* and hemolytic *E. coli* were isolated from peritoneal fluid, heart blood, and brain tissue at necropsy. All sections hematoxylin and eosin stain. Bar = 250 μ m (A), 125 μ m (B), and 15 μ m (C and D).

Animals were considered positive for the above conditions if documentation was found in the case record or identified via gross photographs and/or slide review by a veterinary pathologist. Negative cases were those where the condition of interest was reported as absent in the case record and/or could be excluded through pathologist review of the case record, photos, histopathology, or other means. Cases were noted as unknown if the record, available photographs, and histology lacked sufficient detail to confirm or exclude a defined condition (Miller et al., 2020).

Presence/absence of septicemia was diagnosed based on all findings from bacterial culture, gross necropsy, and histopathology. In addition to direct visualization of bacteria in tissues, lymphatics, and blood

vessels, septic SSOs often exhibited increased neutrophil density on histopathology (e.g., in brain, lung, lymph nodes, spleen, kidney and liver), antemortem leukocyte toxicity, and intracytoplasmic bacteria within leukocytes.

Because one or more bacterial species can contribute to observed pathology, areas of mixed bacterial growth, and isolation of bacteria other than *Klebsiella* spp. were summarized in Supplementary Material, Table 1. Lesions were reported as *Klebsiella* spp.-associated when *Klebsiella* spp. was successfully isolated from the affected tissue and/or morphologically compatible bacteria (short, stout encapsulated or non-encapsulated bacterial rods) were observed in bacterial-mediated lesions on histopathology. *Klebsiella* spp. were considered a cause (or

potentially an additive cause in combination with other bacteria) of a lesion if *Klebsiella* spp. were isolated from tissues with lesions or adjacent tissues (such as a draining lymph node) and morphologically compatible bacteria were observed on histopathology. Given our preliminary state of knowledge and small sample size, additional studies will be needed to further refine and confirm the pathophysiology of *Klebsiella* spp. infections in SSOs.

2.5. Whole blood killing assay

Whole blood bacterial killing assays were performed following protocols by Locke et al. (2007) with modifications. Briefly, whole blood from healthy, rehabilitated CSLs with no known history of *K. pneumoniae* infection was collected into EDTA vacutainer tubes (Greiner Bio-One, Kremsmünster, Austria) during routine release examination. Blood was used within 4 h in *in vitro* assays. Bacterial isolates (Table 3) were grown overnight on SBA at 37 °C. Bacterial concentration (colony forming unit; CFU/mL) was adjusted in phosphate-buffered saline (PBS) to $\sim 1.5 \times 10^5$ CFU/mL and mixed in a 1:1 vol/vol ratio with EDTA whole blood. Inoculated blood was incubated at 37 °C for 1.5 h and then spot plated on SBA by serial dilution to determine counts of viable *K. pneumoniae*. Bacterial survival in whole blood was compared to a control group of 1.5×10^5 CFU/mL *K. pneumoniae* mixed in a 1:1 vol/vol ratio with sterile PBS. Whole blood experiments were performed in six replicates to confirm consistency of results and results were averaged among HMV and non-HMV isolates.

Table 3

Klebsiella spp. isolates from southern sea otters (*Enhydra lutris nereis*), California sea lions (*Zalophus californianus*), and Pacific harbor seals (*Phoca vitulina richardsi*) used in this study.

Isolate Label	Stranding Date	Sex	Age Class [†]	<i>Klebsiella</i> Species Recovered	Recovery Location	HMV	Virulence Analysis?
Southern Sea Otters							
3057–98	08/1998	F	5	<i>Klebsiella pneumoniae</i>	Left inguinal LN, mediastinal LN, soft palate, bladder	–	+
3384–00	07/2000	F	1	<i>K. pneumoniae</i>	Colon	–	–*
3814–02	12/2002	F	4–5	<i>K. pneumoniae</i>	Lung	–	–*
3908–03	04/2003	M	4	<i>K. pneumoniae</i>	Heart blood	–	+
3938–03	06/2003	F	4	<i>K. pneumoniae</i>	Abdominal fluid	–	–*
4125–04	02/2004	M	3	<i>K. pneumoniae</i>	Toe injury	–	–*
4127–04	02/2004	M	4	<i>K. pneumoniae</i>	Heart blood, abdominal fluid, brain	–	+
4379–05	01/2005	M	4	<i>K. pneumoniae</i>	Heart blood	–	–*
4449–05	04/2005	F	3	<i>K. pneumoniae</i>	Brain, neck abscess, left retropharyngeal LN, lung, heart blood	+	+
4451–05	04/2005	M	3	<i>K. pneumoniae</i>	Heart blood	+	+
4486–05	05/2005	F	4	<i>K. pneumoniae</i>	Lung, hilar LN, heart, brain	+	+
4614–05	11/2005	M	2	<i>K. pneumoniae</i>	Heart blood	+	+
4869–06	10/2006	M	4	<i>K. ozaenae</i> -like	Heart blood	–	–*
4922–07	02/2007	M	3	<i>K. pneumoniae</i>	Peritoneal fluid, kidney abscess	–	+
5006–07	05/2007	F	4	<i>K. pneumoniae</i>	Heart, peritoneal fluid	–	–*
5552–09	07/2009	F	3	<i>K. pneumoniae</i>	Left axillary LN	–	–*
6130–11	06/2011	F	5	<i>K. ornithium</i>	Nasal pus	–	–*
2789–97	01/2012	F	5	<i>K. oxytoca</i>	Right sublumbar LN	–	–*
Pacific Harbor Seals							
1	04/2012	F	1	<i>K. pneumoniae</i>	Ear	–	+
5	05/2015	F	1	<i>K. pneumoniae</i>	Abdominal fluid	–	+
6	04/2016	M	1	<i>K. pneumoniae</i>	Feces	–	+
California Sea Lions							
2	1994	F	2	<i>K. pneumoniae</i>	Lung	–	+
4	1995	M	2	<i>K. pneumoniae</i>	Pleura	–	+
3	04/2012	M	1	<i>K. pneumoniae</i>	Abscess	–	+
10	01/2015	M	2	<i>K. pneumoniae</i>	Lymph node abscess	+	+
11	02/2015	F	4	<i>K. pneumoniae</i>	Kidney	+	+
12	05/2015	M	2	<i>K. pneumoniae</i>	Thorax	+	+
7	08/2015	M	3	<i>K. pneumoniae</i>	Nasal	+	+
8	08/2015	M	2	<i>K. pneumoniae</i>	Nasal	+	+
9	08/2015	M	3	<i>K. pneumoniae</i>	Lung	+	+

* Not cryopreserved, only isolated to assess for hypermucoviscous phenotype. Sex: F = female, M = male.

[†] Age: 1 = pup, 2 = immature adult/juvenile/yearling, 3 = subadult, 4 = adult, 5 = aged adult. LN = lymph node.

2.6. Serum killing assays

Complement-based killing (serum killing) of *K. pneumoniae* strains from SSOs, CSLs, and PHSs (Table 3) was assessed as described by Soto et al. (2010). Briefly, pooled serum from healthy, rehabilitated CSLs with no known history of *K. pneumoniae* infection was collected into serum separator tubes during routine release examination. Tubes were centrifuged at 3000 xg for 10 min to achieve adequate serum separation and sera from multiple animals and tubes were pooled. Bacterial isolates (incubated overnight on blood agar and suspended in PBS to 1.5×10^4 CFU/mL as above) were mixed in a 1:4 vol/vol ratio with serum that was passed through a 0.2 µm pore size filter and incubated at 37 °C for 3 or 24 h. Pooled heat inactivated serum (heated at 56 °C for 45 min to inactivate complement, but leaving other potential humoral components such as antibodies intact) inoculated with same amount of bacteria as the activated serum was used as a control. Colony counts were determined using a 6 × 6 drop plate method (Chen et al., 2003). Serum experiments were performed in five replicates for the 3 h incubation, and three replicates for the 24 h incubation period. Results were averaged among HMV and non-HMV isolates.

2.7. Statistical analysis

Bacterial persistence (culturable bacteria) in the different treatments compared in whole-blood and serum killing experiments were subjected to statistical analysis. Statistical significance was determined using two-way ANOVA tests with Tukey's multiple comparisons test (significance

if $p < 0.05$) in GraphPad Prism (version 8.3.0, GraphPad Software, La Jolla, CA).

3. Results

3.1. Identification of HMV *K.pneumoniae*

Klebsiella spp. isolates were obtained from 18 necropsied SSOs from 1997 through 2020, including 15 *K. pneumoniae* strains and three *Klebsiella* spp. strains (Table 3). Of the 15 *K. pneumoniae* isolates, 4/15 were HMV-positive, and all four HMV strains were from SSOs that died during 2005 (Supplementary Material, Table 1). Only one SSO case from 2005 was infected with a non-HMV *K. pneumoniae* strain. The four SSOs with HMV *K. pneumoniae* infections were of variable age: one pup, two subadults, and an adult (Supplementary Material, Table 1). Three of the HMV *K. pneumoniae*-infected SSOs stranded within 57 km of each other over an eight-week period (Fig. 1). The fourth SSO stranded approximately 203 km north of the other animals and six months later. The remaining 11 *K. pneumoniae* isolates were non-HMV strains. The three *Klebsiella* spp. isolates included one each of *K. oxytoca*, *K. ozaenae*-like and *K. ornithium* (Supplementary Material, Table 1).

HMV *K. pneumoniae* was the sole pathogen isolated in one case; all other HMV *K. pneumoniae*-positive cases yielded mixed bacterial growth, sometimes including other known or suspected bacterial SSO pathogens, including *Streptococcus phocae*, *Vibrio parahemolyticus*, *Salmonella enterica*, *Proteus* spp., *Pseudomonas* spp., and hemolytic *E. coli* (Miller et al., 2020).

Klebsiella spp. were commonly isolated from lymph nodes, heart blood, and peritoneal fluid (Table 3 and Supplementary Material, Table 1). Six SSOs had *K. pneumoniae* recovered from more than one location. Among *Klebsiella* spp.-positive SSOs, three or more anatomical sites were culture-positive at necropsy for 2/4 HMV *K. pneumoniae* strains, 2/11 non-HMV *K. pneumoniae* strains, and 0/3 isolates of other *Klebsiella* species. For SSO 4449–05, HMV *K. pneumoniae* was isolated in pure culture from five different anatomic sites, and myriad short, stout, thickly encapsulated bacterial rods consistent with *K. pneumoniae* were identified within a *K. pneumoniae*-positive abscess and an adjacent lymph node on histopathology (Fig. 2). Six of the eight CSL *K. pneumoniae* isolates were HMV and two were non-HMV. All four PHS isolates were non-HMV *K. pneumoniae*.

Table 4

Multiplex PCR results for 12 virulence genes associated with hypermucoviscous (HMV) *Klebsiella pneumoniae* infections using both HMV and non-HMV *K. pneumoniae* isolates from southern sea otters (SSO: *Enhydra lutris nereis*), Pacific harbor seals (PHS: *Phoca vitulina richardsi*), and California sea lions (CSL: *Zalophus californianus*) (blank boxes indicate a negative result).

Isolate	Species	HMV	<i>Kpn</i>	<i>magA</i>	<i>K2wzy</i>	<i>K5wzy</i>	<i>iroD</i>	<i>iutA</i>	<i>fyuA</i>	<i>allS</i>	<i>rmpA</i>	<i>clbB</i>	<i>mrkB</i>	<i>fimH</i>
4486–05	SSO	Yes	+		+		+	+	+		+		+	+
4451–05	SSO	Yes	+		+		+	+	+		+		+	+
4449–05	SSO	Yes	+		+		+	+	+		+		+	+
4614–05	SSO	Yes	+		+			+	+		+		+	+
3908–03	SSO	No	+										+	+
3057–98	SSO	No	+										+	+
4922–07	SSO	No	+					+	+				+	+
4127–04	SSO	No	+					+	+				+	+
1	PHS	No	+										+	+
5	PHS	No	+										+	+
6	PHS	No	+										+	+
2	CSL	No	+										+	+
3	CSL	No	+										+	+
4	CSL	No	+				+	+			+		+	+
7	CSL	Yes	+		+			+			+		+	+
8	CSL	Yes	+		+			+			+		+	+
9	CSL	Yes	+		+			+			+		+	+
10	CSL	Yes	+		+			+	+		+		+	+
11	CSL	Yes	+		+			+	+		+		+	+
12	CSL	Yes	+		+			+	+		+		+	+

3.2. Molecular characterization

Phenotypical and molecular characterization of *K. pneumoniae* isolates are presented in Tables 3 and 4. All isolates used for multiplex PCR were confirmed as *K. pneumoniae* based on amplification and sequencing of the 16S–23S internal transcribed spacer (Table 1) (Turton et al., 2010). All isolates were negative for *magA* (capsular serotype K1), *K5wzy* (capsular serotype K5), *clbB* (colibactin), and *allS* (allantoin metabolism island). Within each host species, there were multiple isolates that had identical virulence gene profiles, including three of the four SSO HMV isolates. The fourth SSO HMV isolate (4614–05) only varied from the other three by absence of *iroD*, and was recovered from an animal that was spatially and temporally separated from the other three animals. HMV *K. pneumoniae* isolates obtained from SSOs and CSLs had similarities between virulence gene profiles, but none were identical. However, there were identical profiles for several non-HMV SSO and CSL isolates. Two of the non-HMV SSO isolates (4922–07 and 4127–04) were positive for *iutA* and *fyuA* but were otherwise identical to the other two non-HMV isolates (3908–03 and 3057–98).

3.3. Antimicrobial susceptibility

HMV and non-HMV isolates from SSOs were all susceptible to enrofloxacin and gentamicin and all resistant to amoxicillin (Table 5, CLSI, 2018, 2020). There was variable resistance to ceftiofur, tetracycline, trimethoprim/sulfamethoxazole, and florfenicol (Table 5). Other antimicrobials did not have established breakpoints for Enterobacterales (CLSI, 2018, 2020), but all isolates had the same MIC for erythromycin, spectinomycin, sulphathiazole, novobiocin, and tylosin tartrate (Table 5). SSO 4486–05, the SSO that grew pure *K. pneumoniae* on culture, had a streptomycin MIC of 256, while all other SSOs had an MIC of <8 for this antimicrobial.

3.4. Lesion/Condition prevalence

Patterns of *Klebsiella* spp. detection in relation to all primary or contributing causes of death and sequelae are summarized in the Supplementary Material, Table 1. *Klebsiella pneumoniae* infection was considered a primary cause of death or a sequela to a primary cause of death for 100 % of HMV positive *K. pneumoniae* cases. Non-HMV *K. pneumoniae* was considered a primary cause of death or a sequela to a primary cause of death for 64 % (7/11) of non-HMV *K. pneumoniae*

Table 5

Minimum inhibitory concentrations ($\mu\text{g}/\text{mL}$) of antimicrobial drugs for *Klebsiella pneumoniae* isolates from southern sea otters (*Enhydra lutris nereis*). Susceptibility interpretation following Clinical Laboratory Standards Institute breakpoints of Enterobacterales for dogs (2018, 2020) (S = susceptible, I = intermediate, R = resistant, no interpretation = breakpoints not available in CLSI reference for Enterobacterales). Control *E. coli* ATCC 25,922 within CLSI reference ranges.

Antimicrobial Drug	4127–04	3057–98	3908–03	4127–04	4451–05 (HMV)	4486–05 (HMV)	4449–05 (HMV)	4614–05 (HMV)
Enrofloxacin	< 0.12 (S)	0.12 (S)	< 0.12 (S)	< 0.12 (S)	< 0.12 (S)	< 0.12 (S)	< 0.12 (S)	< 0.12 (S)
Gentamicin	< 0.5 (S)	< 0.5 (S)	< 0.5 (S)	< 0.5 (S)	< 0.5 (S)	< 0.5 (S)	< 0.5 (S)	< 0.5 (S)
Ceftiofur	1 (S)	> 4 (I/R)	2 (S)	1 (S)	1 (S)	2 (S)	2 (S)	< 0.25 (S)
Neomycin	< 2	< 2	< 2	4	< 2	< 2	< 2	< 2
Erythromycin	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
Oxytetracycline	> 8	> 8	1	> 8	0.5	0.5	0.5	2
Tetracycline	> 8 (I/R)	> 8 (I/R)	0.5 (S)	> 8 (I/R)	0.5 (S)	0.5(S)	0.5 (S)	0.5 (S)
Amoxicillin	> 16 (R)	> 16 (R)	> 16 (R)	> 16 (R)	> 16 (R)	> 16 (R)	> 16 (R)	1 (R)
Spectinomycin	< 8	< 8	< 8	< 8	< 8	< 8	< 8	< 8
Sulphadimethoxine	> 256	> 256	> 256	> 256	256	> 256	> 256	> 256
Trimethoprim/ sulfamethoxazole	< 0.5/9.5 (S)	> 2/38 (I)	< 0.5/9.5 (S)	> 2/38 (I)	< 0.5/9.5 (S)	< 0.5/9.5 (S)	< 0.5/9.5 (S)	< 0.5/9.5 (S)
Florfenicol	2 (S)	4 (S)	4 (S)	2 (S)	> 8 (I/R)	4 (S)	4 (S)	< 1 (S)
Sulphathiazole	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 256
Penicillin	> 8	> 8	> 8	> 8	> 8	> 8	> 8	0.5
Streptomycin	< 8	< 8	< 8	< 8	< 8	256	< 8	< 8
Novobiocin	> 4	> 4	> 4	> 4	> 4	> 4	> 4	> 4
Tylosin tartrate	> 20	> 20	> 20	> 20	> 20	> 20	> 20	> 20
Clindamycin	> 4	> 4	> 4	> 4	> 4	> 4	> 4	4

cases. Non-HMV *K. pneumoniae* was considered a sequela to a secondary or tertiary cause of death for 27 % (3/11) non-HMV *K. pneumoniae* cases, and in one case non-HMV *K. pneumoniae* infection was considered incidental. For 100 % of SSOs infected with other *Klebsiella* spp., infection was considered a sequela to a primary or secondary cause of death (Supplementary Material, Table 1). Examples of HMV and Non-HMV *K. pneumoniae*-associated gross and microscopic pathology are illustrated in Figs. 2–5; cases depicted in these image plates are indicated by an asterisk under column 2 of the Supplementary Material, Table 1.

All HMV *K. pneumoniae*-positive SSOs exhibited perimortem septicemia, and 75 % of HMV cases exhibited suppurative pneumonia, tracheobronchitis, and/or pleuritis. When *Klebsiella* spp. positive SSOs were assessed for health condition presence/absence at necropsy (regardless of whether each condition was considered a primary or contributing cause of death) (Table 2), the most common gross and microscopic findings were septicemia (16/18), acanthocephalan peritonitis (13/18) (Fig. 4A–D), lymphadenomegaly (12/18), suppurative lymphadenitis (9/18) (Fig. 2A, B), suppurative pneumonia,

tracheobronchitis and/or pleuritis (9/18) (Figs. 3A, B, 5 C), and septic peritonitis (8/18) (Fig. 4D) (Table 2). Other common lesions included abscesses (7/18) (Fig. 5A, B) or infected wounds (6/18), and current or healed intestinal perforations (5/18) (Figs. 4A–D and 5 A, B). Less common conditions in *Klebsiella* spp.-infected SSOs included gastroenteritis, enterocolitis, or intestinal displacements (4/18), suppurative meningitis and/or encephalitis (2/18), suppurative nephritis, cystitis, or vulvovaginitis (2/18), and one case each of suppurative polyarthrititis, periostitis, and cholecystitis (Fig. 5C, D).

Although our sample size is too small at present for definitive conclusions, the lesion distribution and sources of bacterial detection varied somewhat between SSOs infected with *K. pneumoniae* and other *Klebsiella* spp. All non-*K. pneumoniae* detections ($n = 3$) were associated with SSOs that had infected wounds, and early or mild suppurative pneumonia, tracheobronchitis, and/or pleuritis, suggesting potential portals for bacterial entry; none of these animals were diagnosed with acanthocephalan peritonitis at the time of necropsy. In contrast, 100 % of HMV *K. pneumoniae* isolates ($n = 4$), and 82 % of non-HMV

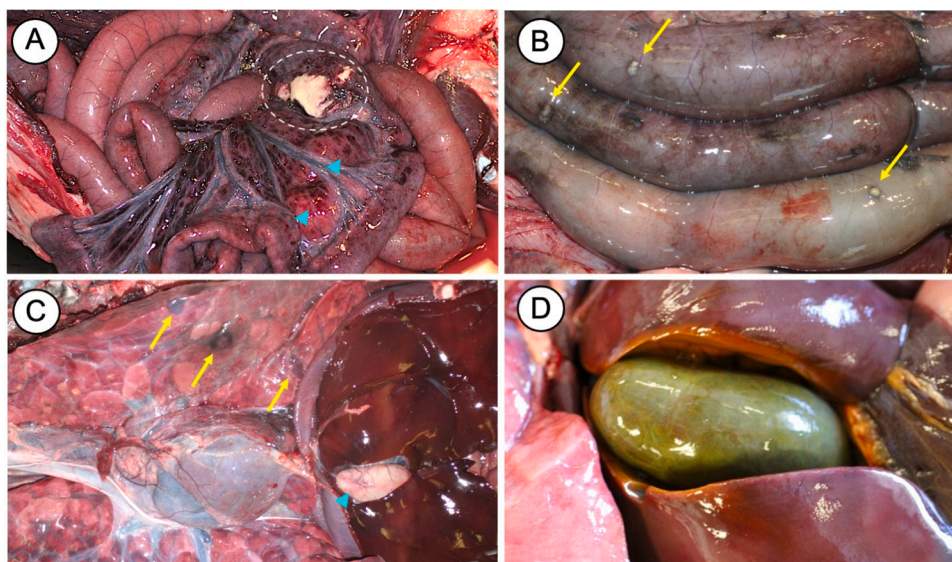


Fig. 5. Gross pathology photos from southern sea otters (*Enhydra lutris nereis*) infected with *Klebsiella pneumoniae*. (A) Case 3908-03: Focally severe intestinal perforation and mural necrosis near the mesenteric border resulting in a large serosal and mesenteric abscess with secondary septic peritonitis and purulent effusion. The abscess was peeled away to show its location (white oval). Heart blood was culture-positive for mixed *K. pneumoniae* and *Escherichia coli*. Mesenteric ecchymoses are indicative of bacterial peritonitis (arrowheads). (B) Another view of intestine from case 3938-03: Serosal abscesses at multiple sites of *Profilicollis* sp. transmigrating through the intestinal wall (arrows). (C) Case 4486-05: Abnormal gallbladder with diffusely thickened, tan-white opaque wall (arrowhead). The gallbladder lumen was filled with fibrinopurulent exudate admixed with bile. Hypermucoviscous positive *K. pneumoniae* was isolated from the brain, heart blood, lung, and hilar lymph node. The yellow arrows indicate multiple foci of fibrinosuppurative and necrotizing bronchopneumonia. (D) Normal sea otter gallbladder for comparison (the rich green color is slightly exaggerated due to postmortem

decomposition) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

K. pneumoniae strains (n = 11) were isolated from SSOs with acanthocephalan peritonitis. These results suggest, but do not confirm a possible enteric route for HMV and non-HMV *K. pneumoniae* exposure in SSOs (Table 2). In addition, perimortem septicemia was suspected for 100 % of HMV *K. pneumoniae* isolates, and 91 % of non-HMV *K. pneumoniae* strains, compared with 66 % for other *Klebsiella* spp. Our results, although preliminary, suggest that routes of bacterial exposure differ for *K. pneumoniae* and other, potentially less pathogenic *Klebsiella* spp. Potential portals for *Klebsiella* spp. invasion include traumatic, mating, or fight wounds, enteric disease, acanthocephalan-associated or other types of intestinal perforations, aspiration of material into the respiratory tract, and ascending urinary tract infection (Table 2).

3.5. Susceptibility of *Klebsiella pneumoniae* to serum and whole blood

All *K. pneumoniae* tested were resistant to CSL whole blood killing under the conditions investigated. There were no significant differences in survival of HMV compared to non-HMV isolates in whole blood from CSLs ($p > 0.05$) (Fig. 6).

HMV *K. pneumoniae*-strains exhibited significantly greater survival (98.0 %, $p < 0.0001$) after 3 h of incubation in active serum than non-HMV isolates (80.6 %, Fig. 7A). Non-HMV isolates exhibited significantly higher survival ($p < 0.0001$) in heat-inactivated serum (101.1 %) compared to active serum (80.6 %). No significant differences were observed for survival of HMV isolates in inactivated compared to active serum.

Following 24 h of incubation, HMV isolates demonstrated significantly greater survival and growth in both heat-inactivated (243.5 %) and active serum (229.6 %) compared to non-HMV isolates (143.7 % heat-inactivated, 92.4 % activated, $p < 0.0001$ for both; Fig. 7B). As with the 3 h incubation period, non-HMV isolates exhibited significantly higher survival in inactivated serum (143.7 %) compared to active serum (92.4 %, $p < 0.0001$), while no significant difference was noted for HMV isolates.

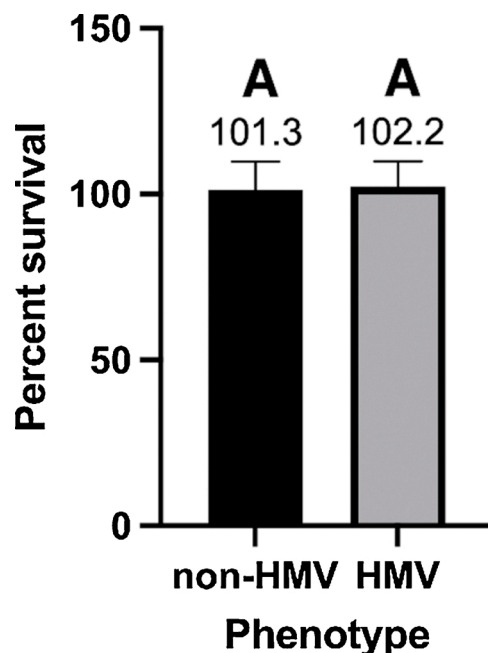


Fig. 6. Percent survival of *K. pneumoniae* strains with defined phenotype in whole blood from California sea lions (*Zalophus californianus*). The error bars represent standard error for triplicate samples, and the results shown are representative of three independent biological replicates. Groups that do not share same letter indicate significance difference $p < 0.05$.

4. Discussion

Our study goals were to investigate virulence gene profiles, *in vitro* virulence of *K. pneumoniae* isolates, and associations between specific lesions seen at necropsy and via histopathology and successful isolation of *K. pneumoniae* and *Klebsiella* spp. from stranded SSOs necropsied at MWVCRC over 24 years. Although precise enumeration is difficult, this amounts to >500 animals where aerobic bacterial culture was attempted from tissues or body fluids at necropsy. During routine necropsies, samples from grossly apparent lesions are typically submitted for bacterial culture; this selection bias may under-represent the actual prevalence of *K. pneumoniae* infections in the SSO population. Over 24 years of necropsy effort, only 18 SSOs were culture-positive for *Klebsiella* spp., with 15 of those isolates (83 %) being *K. pneumoniae*. *Klebsiella* spp. were usually isolated from SSO lymph nodes, heart blood, and peritoneal fluid. Relatively infrequent isolation of *Klebsiella* spp. may reflect relatively low infection prevalence in SSOs and/or postmortem proliferation of other bacteria that may outgrow *Klebsiella* spp. However, *Klebsiella* spp. detection is uncommon in live SSOs. Unpublished microbiological data from assessment of >120 live, ostensibly healthy SSOs sampled during routine capture and tagging activities suggest that *Klebsiella* spp. are uncommon commensals for SSOs (M. Miller, unpub. data).

Klebsiella spp. infection was considered a potential contributor to stranding and death for most culture-positive SSOs in the current study (Supplementary Material, Table 1). Our composite data, although preliminary, suggest that *Klebsiella* spp. isolation in necropsied SSOs is often associated with diagnosis of moderate to severe bacterial pathology (Supplementary Material, Table 1); subsequent studies should examine these associations in greater detail.

Interestingly, all four HMV *K. pneumoniae*-positive SSOs stranded in 2005, and three of the animals stranded within a 57 km stretch of coastline over an eight-week period. Two of these SSOs were recovered on the same day within 4.5 km of each other, suggestive of spatial and temporal clustering of HMV *K. pneumoniae* infection. The four HMV SSOs were of varying age; a similar age range has been reported for klebsiellosis in CSLs from California (Jang et al., 2010; Seguel et al., 2017; Whitaker et al., 2018) and PHSSs (Whitaker et al., 2018), suggesting *K. pneumoniae* does not require age-associated factors (such as a developing immune system in young marine mammals) to be pathogenic. Of note, no HMV *K. pneumoniae* was recovered from CSLs or PHSSs during 2005 (Whitaker et al., 2018; TMMC pers. comm.).

The epizootic potential of *K. pneumoniae* with the HMV phenotype and K2 serotype was first confirmed in marine mammals during multiple mortality events affecting endangered New Zealand sea lions (*Phocartos hookeri*). *Klebsiella pneumoniae* was first detected as the cause of an epizootic in this colony during the 2001–2002 and 2002–2003 breeding seasons, prior to use of the phenotype string test and serotyping to discern HMV and non-HMV strains (Castinel et al., 2007; Roe pers. comm.). HMV *K. pneumoniae* was later identified as the cause of an epizootic that accounted for 58 % of pup mortality on Enderby Island during the 2006–2007 and 2009–2010 breeding seasons (Roe et al., 2015). Subsequent genetic characterization showed clonality of *K. pneumoniae* strains throughout many of New Zealand's offshore islands over multiple years (Pimpimai et al., 2018). In our study, we observed temporal and spatial clustering of three SSOs infected with HMV isolates with identical virulence gene profiles in 2005. These findings are suggestive of clonality and epizootic potential for HMV *K. pneumoniae* infection in SSOs; however, confirmation is not possible due to our small sample and additional genetic work, such as whole genome sequencing, is warranted to assess clonality.

Although virulence gene expression was not investigated in this study, and associations between gene presence and observed pathology were not assessed, confirmation of virulence gene presence can aid our understanding of the pathogenic potential of *Klebsiella* spp. The K1 and K2 capsular serotypes have all been associated with the HMV *K. pneumoniae* phenotype (Yu et al., 2006), and predominate in human

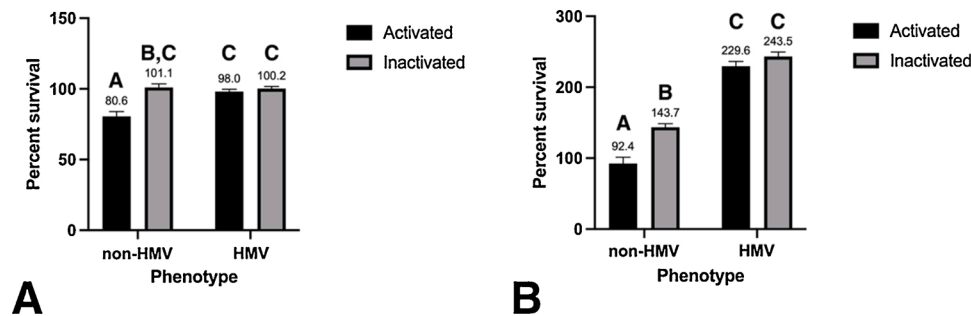


Fig. 7. Percent survival of *K. pneumoniae* strains with defined phenotype in normal (activated) and heat-inactivated (inactivated) serum from California sea lions (*Zalophus californianus*). Survival and growth of *K. pneumoniae* incubated in serum for 3 h (A) and 24 h (B). The error bars represent standard error for triplicate to sextuplet samples. Treatments with different letters are significantly different from one another at $p < 0.05$.

infections (Fang et al., 2007; Yu et al., 2008). In combination with *rmpA*, the presence of these capsular serotypes is considered the best predictor for the HMV phenotype and hypervirulence in humans (Choby et al., 2020). Previous studies have found that the vast majority of marine mammal (CSLs, New Zealand sea lions, and harbor porpoises) HMV *K. pneumoniae* isolates have the K2 capsular serotype (Jang et al., 2010; Roe et al., 2015; Whitaker et al., 2018). All HMV *K. pneumoniae* isolates from SSOs and CSLs in this study had the K2 capsular serotype, while no non-HMV *K. pneumoniae* isolates had this serotype, confirming this serotype predominates in marine mammal HMV *K. pneumoniae* strains. Whole genome analysis and the addition of isolates recovered from California terrestrial mammals and humans may help clarify the significance of this finding.

None of the marine mammal isolates tested here were positive for *magA*, which encodes the K1 capsular serotype (Remya et al., 2018). This is consistent with other reports of HMV *K. pneumoniae* in marine mammals that found no *magA* positive isolates (Jang et al., 2010; Roe et al., 2015; Whitaker et al., 2018). Similarly, all marine mammal HMV *K. pneumoniae* isolates and only one non-HMV isolate were positive for *rmpA*, which supports associations between HMV and *rmpA*. One CSL non-HMV *K. pneumoniae* isolate from a prior study was also found to be positive for *rmpA* and *rmpA2* (Whitaker et al., 2018). The lack of expression of the HMV phenotype could be due to functional mutations or lack of expression of the *rmpA* gene in the non-HMV *K. pneumoniae* isolates (Lin et al., 2020).

The multiplex-PCR methods developed during this study will facilitate future *K. pneumoniae* genotyping efforts and testing for virulence genes in Gram-negative bacteria. Three siderophores, salmochelin, aerobactin, and yersiniabactin, were investigated, all of which help bacteria acquire iron. Iron acquisition is a critical virulence factor for hypervirulent *K. pneumoniae* (Russo et al., 2015). In humans, *K. pneumoniae* strains possessing salmochelin often colonize the nasopharynx and are more virulent than strains lacking this virulence factor (Paczosa and Mecsas, 2016). Although salmochelin is present in only about 2–4 % of human nosocomial *K. pneumoniae* strains, it is much more prevalent in HMV *K. pneumoniae*, with one study reporting >90 % prevalence in HMV strains associated with pyogenic liver abscesses (Hsieh et al., 2008; Paczosa and Mecsas, 2016).

In our study, 3/4 of SSO HMV isolates possessed the salmochelin-specific *iroD* gene, while it was absent in all CSL HMV strains. Other marine mammal HMV *K. pneumoniae* studies have not tested for this gene, so it is challenging to draw conclusions from these data alone (Jang et al., 2010; Roe et al., 2015; Whitaker et al., 2018). Interestingly, all three SSO HMV isolates that were temporally and spatially clustered presented this gene, in contrast with all other marine mammal HMV isolates; this finding further supports the epizootic potential of HMV *K. pneumoniae* in SSO, and the unique clonality of those three isolates. Although salmochelin expression may enhance virulence of HMV strains, it is not critical for phenotype expression or pathogenicity (Paczosa and Mecsas, 2016). In the current study seven SSO and CSL

HMV isolates did not possess *iroD*, while it was detected in a single non-HMV isolate from a CSL.

All marine mammal HMV isolates in this study were positive for the aerobactin specific *iutA* gene, compared with 3/10 non-HMV isolates. The *iutA*-positive non-HMV CSL isolate was also the only non-HMV isolate positive for *iroD*. Aerobactin can facilitate the growth and survival of human *K. pneumoniae* strains *ex vivo* and *in vivo* (Nassif and Sansonetti, 1986; Russo et al., 2015). Disruption of aerobactin synthesis causes significant reduction in siderophore production, decreased growth/survival *ex vivo*, and decreased virulence *in vivo*, thus possessing *iutA* may help enhance virulence.

Disruption of the yersiniabactin specific *fyuA* gene does not decrease virulence of human HMV strains possessing multiple siderophores (despite higher prevalence in HMV strains than non-HMV strains), but may play a minor role in increasing bacterial growth (Hsieh et al., 2008; Russo et al., 2015). Although all SSO HMV isolates possessed the yersiniabactin specific *fyuA* gene, only 50 % (3/6) of CSL isolates were positive. Two SSO non-HMV isolates were positive for *fyuA*; interestingly, these were the same isolates that were also positive for *iutA*. Having multiple siderophores provides functional redundancy due to the importance of acquiring iron for microbes and frequency of siderophore neutralization by hosts and is often linked to virulence (Miethke and Marahiel, 2007; Sheldon and Skaar, 2020). In general, the siderophores, while showing greater prevalence in marine mammal HMV isolates, were not solely associated with HMV strains, supporting that they promote the virulence of HMV strains, but are not restricted to HMV phenotype isolates.

All marine mammal HMV isolates and 9/10 of non-HMV isolates were positive for type 3 fimbriae, which promote biofilm formation (Schroll et al., 2010). As biofilm formation has been shown to enhance survival and persistence *in vivo* and *ex vivo* (Bjarnsholt et al., 2013; Carvalho, 2018), *K. pneumoniae* isolates expressing type 3 fimbriae may have greater survival in the harsh marine environment, possibly increasing opportunities to infect marine mammals and other hosts. Although one study found non-HMV isolates to have greater capacity to form *in vitro* biofilms than HMV isolates, HMV isolates required a higher disinfectant concentration to eradicate the biofilm than non-HMV isolates and comparison of *in vivo* biofilms remains unclear (Soto et al., 2020). Seventy percent (7/10) of marine mammal HMV and 90 % (9/10) of non-HMV isolates were positive for type 1 fimbriae, which mediates adhesion to host epithelial cells, a property that promotes pathogenicity (Schroll et al., 2010). Although type 1 fimbriae may affect pathogenicity, they are neither unique nor critical for HMV virulence. As type 1 and type 3 fimbriae are generally found in *K. pneumoniae*, including environmental isolates (Podschun and Ullmann, 1998), it is not surprising that non-HMV isolates also possessed the fimbriae.

Live-stranded California sea lions that subsequently died that were infected with HMV *K. pneumoniae* often had pleuritis and suppurative pneumonia (Jang et al., 2010; Seguel et al., 2017), while New Zealand sea lions with fatal HMV *K. pneumoniae* infection commonly had

septicemia, polyarthritis, peritonitis, cellulitis/dermatitis, and meningitis (Roe et al., 2015). Like the New Zealand sea lions, some *K. pneumoniae*-positive SSOs also had septicemia, polyarthritis, peritonitis, cellulitis/dermatitis, and meningitis (Table 2 and Supplementary material, Table 1), however not all these sites had swabs submitted for bacterial culture. Evidence of *Klebsiella* spp.-associated septicemia in 89 % of necropsied, *Klebsiella*-positive SSOs suggests that bacterial invasion and dissemination can result from some, but not all *Klebsiella* spp. infections.

One interesting finding was that most *Klebsiella* spp.-infected SSOs (13/18) had suspected or confirmed *Profilicollis* sp. peritonitis at necropsy (Table 2). *Profilicollis* sp. are acanthocephalan parasites that commonly infect the intestinal tract and peritoneum of SSOs, often causing fatal peritonitis. Prior studies have identified acanthocephalan peritonitis as a common cause of death in necropsied SSOs, and septic peritonitis is a common sequela of acanthocephalan peritonitis (Kreuder et al., 2003; Miller et al., 2020). Our preliminary data suggest that co-infection with *Profilicollis* sp. may enhance the risk of fatal *K. pneumoniae*-associated peritonitis and sepsis in SSOs.

Acanthocephalan peritonitis in SSOs is positively correlated with bacterial infection (Miller et al., 2020). Acanthocephalans may alter the enteric environment, facilitating colonization by opportunistic bacterial pathogens (Miller et al., 2020), such as *K. pneumoniae*. Perforations that *Profilicollis* sp. make through the intestinal wall as they migrate may serve as a portal for bacteria to spread regionally and systemically (Fig. 4). Concurrent enteric bacterial infections and acanthocephalan peritonitis may also contribute to formation of larger intestinal perforations and omental abscesses (Figs. 4 and 5A, B) (Miller et al., 2020). Associations between an enteric metazoan parasite and *K. pneumoniae* peritonitis were also noted for CSL pups with hookworm (*Uncinaria* spp.) infection of the small intestine (Spraker et al., 2007).

The most common lesions in *K. pneumoniae* and *Klebsiella* spp.-infected SSOs included septicemia (16/18), acanthocephalan-associated peritonitis (13/18; Figs. 4 and 5B), septic peritonitis (8/18; Fig. 4D), lymphadenomegaly (12/18), suppurative lymphadenitis (9/18; Fig. 2), and suppurative pneumonia, tracheobronchitis and/or pleuritis (9/18; Figs. 3 and 5C) (Table 2 and Supplementary Materials, Table 1). In some cases mixed bacterial infections were detected via culture and histopathology, making the most important causative bacterial groups unclear (Supplementary Materials, Table 1). Although further study is needed, the pathological findings suggest bronchopulmonary aspiration, enteric route following co-infection with enteric *Profilicollis* sp., traumatic wounds, and ascending urinary tract infection as potential routes of *Klebsiella* spp. invasion.

Our study confirmed that HMV *K. pneumoniae* strains isolated from marine mammals can resist CSL complement-mediated bacterial killing more effectively than non-HMV strains. Although HMV isolates did not show greater resistance to CSL whole blood killing than non-HMV isolates, future studies should assess longer incubation times during *in vitro* experimentation, particularly since non-HMV isolates exhibit faster and greater *in vitro* growth in broth media (Soto et al., 2020). Additionally, *in-vitro* challenges using whole blood and serum from various marine mammals, including SSO, will help develop a better understanding of host-pathogen relationships.

5. Conclusions

Our findings represent the first report of HMV *K. pneumoniae* detection and preliminary assessments of *Klebsiella* spp. associated pathology in SSOs. Based on our composite data from 20 years of testing, isolation of *Klebsiella pneumoniae* and other *Klebsiella* spp. is uncommon from SSOs. Although HMV and non-HMV *K. pneumoniae* strains were occasionally isolated at necropsy, no HMV or non-HMV *K. pneumoniae* strains have been isolated from live SSOs. In the current study, many SSO HMV and non-HMV *K. pneumoniae* infections were associated with moderate to severe pathology as a cause of death or sequela, and all

HMV *K. pneumoniae* infections were assessed as a primary cause of death or occurred as a sequela to the primary cause of death.

All SSO HMV *K. pneumoniae* isolates were capsular type K2, the serotype most associated with HMV *K. pneumoniae* infections in CSLs. HMV *K. pneumoniae* isolates were more resistant to marine mammal serum killing *in vitro* than non-HMV *K. pneumoniae* isolates. HMV isolates from both SSOs and CSLs exhibited greater survival when exposed to CSL serum than non-HMV strains, indicating greater pathogenic potential. Temporospatial clustering and apparent clonality of some of HMV *K. pneumoniae* strains isolated in 2005 suggest epizootic potential, although our sample size was too small for confirmation. More virulence genes were detected in marine mammal HMV *K. pneumoniae* isolates when compared to non-HMV isolates, supporting potentially greater virulence as well as previously reported associations between the K2 capsular serotype and HMV phenotype for marine mammal *K. pneumoniae* strains. Future studies investigating bacterial killing by phagocytic cells and sampling additional tissues from SSOs that stranded in 2005 to screen for additional HMV cases are warranted. As more *K. pneumoniae* isolates are recovered from marine mammals, characterizing these isolates will help to clarify connections between HMV and non-HMV *K. pneumoniae* infections and host pathology as well as epidemiologic linkages among marine mammal host species.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors gratefully acknowledge the One Health Institute, Karen C. Drayer Wildlife Health Center, and Students Training in Advanced Research program at University of California-Davis for their support of this study. We thank the California Department of Fish and Wildlife for their financial support for SSO necropsies and microbiology and immense help with organizing and sharing case data, cryopreserved isolates, and histopathology slides. We also thank the many individuals and agencies who helped recover SSO carcasses, including California Department of Fish and Wildlife, Monterey Bay Aquarium, U.S. Geological Survey, The Marine Mammal Center, and U.S. Fish and Wildlife Service. The authors also thank the staff and volunteers of TMMC, including Emily Whitmer and Carlos Rios, for their assistance with sea lion sample collection, and Chrisoula Toupadakis Skouritakis, PhD for creation of the stranding map.

Appendix A. Supplementary data. Metadata associated with Southern sea otter (SSO: *Enhydra lutris nereis*) cases presented in the study.

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetmic.2021.109307>.

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