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Short communication

# Pleuritis and suppurative pneumonia associated with a hypermucoviscosity phenotype of *Klebsiella pneumoniae* in California sea lions (*Zalophus californianus*)

Spencer Jang [a], Liz Wheeler [b], Roberta B. Carey [c], Bette Jensen [c], Claudia M. Crandall [d], Kimmi N. Schrader [d], David Jessup [e], Kathleen Colegrove [f], Frances M.D. Gulland [b,*]

[a] William R. Pritchard Veterinary Medical Teaching Hospital, School of Veterinary Medicine, One Shields Avenue, Davis, CA 95616, USA
[b] The Marine Mammal Center, 2000 Bunker Road, Sausalito, CA 94965, USA
[c] Clinical and Environmental Microbiology Branch, Division of Healthcare Quality Promotion CDC, 1600 Clifton Road, MS C-16, Atlanta, GA 30333, USA
[d] Microbial Diseases Laboratory, Division of Communicable Disease Control, Center for Infectious Disease, California Department of Public Health, 850 Marina Bay Parkway, Room E164, Richmond, CA 94804, USA
[e] California Department of Fish and Game, Marine Wildlife Veterinary Care and Research Center, Santa Cruz, CA 95060, USA
[f] Zoological Pathology Program, University of Illinois LUMC, Bld. 101, Rm. 0745, 2160 S. First Ave., Maywood, IL 60153, USA

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ABSTRACT

The aim of this study is to document the isolation of a hypermucoviscosity (HMV) phenotype of *Klebsiella pneumoniae* from 25 cases of suppurative pneumonia and pleuritis and two cases of abscesses in California sea lions (*Zalophus californianus*) from the central California coast, representing the first report of this zoonotic pathogen from the marine environment and only the second report in non-humans. Animals died 2 h to 4 days after first being observed sick on beaches. Clinical signs varied from dyspnoea to coma. Gross post-mortem examination of 25 cases revealed fibrinous pleuritis, copious pus in the pleural cavity and suppurative bronchopneumonia. *K. pneumoniae* isolates obtained from lung and pleural swabs and the hepatic and subcuticular abscesses were highly mucoid on blood agar culture media and were positive to the "string test". Twenty-one of the 27 isolates were examined by PCR and all were positive for *rmp*A and K2*wyz* and negative for K1*magA* genes. Although pneumonia and pleuritis have previously commonly been observed in marine mammals, their association with pure cultures of a zoonotic bacteria, *K. pneumoniae* HMV phenotype, has not. This report provides further evidence of the role marine mammals play as sentinels of health risks to humans from coastal waters.

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## 1. Introduction

*Klebsiella pneumoniae* is a Gram-negative, aerobic, non-motile bacillus that is a common cause of infections in humans and animals (Janda and Abbott, 2005). *Klebsiella* spp. have been isolated from marine mammals from non-specific lesions including poly-arthritis, meningoencephalitis and peritonitis (Castinel et al., 2007), as well as from mixed secondary infections and septicemia (Baker and Ross, 1992; Thornton et al., 1998; Dunn et al., 2001). In humans, the epidemiology of *K. pneumoniae* infections appears to be changing. Traditionally a common enteric organism frequently involved in nosocomial infections, it is emerging as a primary pathogen of humans causing hepatic abscesses (McIver and Janda, 2008). These abscesses are associated with a hypermucoviscosity (HMV) phenotype, but these strains do not appear to be a clonal expansion of one genotype (Ma et al., 2005; Fang et al., 2007). This HMV phenotype has also recently caused outbreaks of liver abscesses in non-human primates, when

* Corresponding author. Tel.: +1 415 289 7344; fax: +1 415 289 7376.
*E-mail address:* gullandf@tmmc.org (Frances M.D. Gulland).

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it was believed to be the first report of the HMV phenotype causing disease in animals (Twenhafel et al., 2008). To date, this HMV phenotype has not been reported as a pathogen in the marine environment.

The HMV phenotype is seen in *K. pneumoniae* capsular serotypes K1 or K2. K1 serotypes of *K. pneumoniae* have two potentially important genes, *rmpA*, a transcriptional activator of colanic acid biosynthesis, and *magA*, which encodes a 43 kD outer membrane protein. K2 serotypes have *rmpA* but do not have *magA*. The relative importance of these genes and capsular serotypes in conferring pathogenicity of *K. pneumoniae* is unclear.

In this paper we characterize the syndrome of *K. pneumoniea* HMV phenotype-associated pleuritis and bronchopneumonia in wild California sea lions.

## 2. Materials and methods

### 2.1. Animals and sampling

Between 1997 and 2008, 5240 California sea lions were found stranded alive along the California coast between San Luis Obispo county and San Francisco and transported to The Marine Mammal Center (TMMC), CA, USA for rehabilitation. Sea lions were considered stranded based on the following criteria: depression, dehydration and/or unwillingness to return to sea (Gerber et al., 1993). Of these, 27 animals were included in this case report due to isolation of *K. pneumoniae* HMV phenotype from their tissues at post-mortem. Details on location of collection, age, and sex of the cases are summarized in Table 1. Clinical signs varied from dyspnoea to coma. All animals died within three days of stranding despite supportive care and were examined post-mortem; samples of all major organs (lung, heart, liver, spleen, kidney, tonsil, lymph nodes, intestines, adrenal, brain) were collected for histology into 5% buffered formalin.

**Table 1**
Characteristics of sea lions and sites sampled for bacterial culture. Isolates from animals marked with * were typed by PCR.

| ID number | Age and sex | Lesions and tissues sampled |
|---|---|---|
| CSL 3458* | Juvenile male | Lung abscess, pleural fluid |
| CSL 3492* | Juvenile male | Lung abscess, pleural fluid |
| CSL 0490* | Yearling male | Pleural fluid |
| CSL 6924* | Yearling female | Pleural fluid |
| CSL 0957* | Subadult male | Pleural fluid |
| CSL 7006* | Subadult male | Pleural fluid |
| CSL 7025* | Subadult male | Inflamed lung |
| CSL 7161* | Subadult male | Inflamed lung |
| CSL 7257* | Juvenile male | Inflamed lung |
| CSL 7393* | Subadult male | Inflamed lung |
| CSL 7406* | Subadult male | Pleural fluid |
| CSL 7503* | Subadult male | Pleural fluid |
| CSL 7513* | Subadult male | Inflamed lung |
| CSL 7536* | Adult male | Inflamed lung, pleural fluid |
| CSL 7669* | Pup male | Inflamed lung |
| CSL 7722 | Yearling male | Abscess in subcutis over shoulder |
| CSL 7725 | Juvenile male | Pleural fluid, lung abscess |
| CSL 7740* | Juvenile male | Inflamed lung, pleural fluid |
| CSL 7757* | Yearling male | Pleural fluid |
| CSL 7769* | Adult male | Inflamed lung |
| CSL 7806* | Juvenile male | Pleural fluid, lung abscess |
| CSL 7894 | Juvenile male | Pleural fluid, liver, intestine |
| CSL 7963* | Yearling male | Liver abscess |
| CSL 7973* | Juvenile male | Pleural fluid, liver, tonsil |
| CSL 8009 | Juvenile male | Pleural fluid, lung abscess, tonsil |
| CSL 8015 | Subadult female | Pleural fluid |
| CSL 8025 | Adult male | Pleural fluid |

### 2.2. Isolation and characterization of Klebsiella spp.

Pleural fluid or abscesses were swabbed and swabs placed in Cary-Blair transport medium (BD Diagnostics, Franklin Lakes, NJ) and held at 4 °C until processing within 48 h of collection. All cultures and antimicrobial sensitivity testing were performed at the Microbiology Laboratory, William R. Pritchard Veterinary Medical Teaching Hospital, School of Veterinary Medicine, University of California, Davis. Swabs received from each specimen were plated directly onto 5% sheep blood agar (Hardy Diagnostics) and MacConkey agar (Hardy Diagnostics) plates incubated at 37 °C in the presence of 5% $CO_2$ in air. Plates were observed daily for 7 days. Colonial and biochemical results were described. A subset of isolates was sent to the Centers for Disease Control and Prevention (CDC) for further identification and characterization. A battery of 49 conventional biochemical tests was performed (Murray et al., 2007) as well as 16S sequencing, and serotyping with capsular antisera to K1 and K2 antigens. Pulsed-field gel electrophoresis (PFGE) was performed using a modified PulseNet *Yersinia pestis* PFGE protocol (http://www.cdc.gov/pulsenet/protocoles.htm) after XbaI digestion of chromosomal DNA. Gel images were analyzed with BioNumerics version 4.01 software (Applied Maths, Austin, TX) and assigned to pulsed-field patterns at 95% relatedness using DICE coefficients and unweighted pair group method using arithmetic averages.

### 2.3. PCR characterization

Twenty-one isolates from 27 discrete animals (see Table 1 for identification of animals from which these isolates were obtained) of *K. pneumoniae* were received at the Microbial Diseases Laboratory, California Department of Public Health from the UC Davis School of Veterinary Medicine. DNA was extracted by heating a suspension of organisms for 5 min at 95 °C and collecting the supernatant following centrifugation at 12,000 × *g* for 5 min. A multiplex PCR assay was performed using the QIAGEN Multiplex PCR Kit (QIAGEN Inc., Valencia, CA) according to manufacturers recommendation and three previously described primer sets: *magA* and *rmpA* (Yeh et al., 2007), and wzy_K2 (Fang et al., 2007). Amplification was performed using an GeneAmp® 9700 thermal cycler (Applied Biosystems, Foster City, CA) and the following amplification parameters: 95 °C for 15 min, 30 cycles of: 94 °C for 30 s, 55 °C for 90 s, 72 °C for 90 s, and a final step at 72 °C for 10 min. The PCR products were subjected to electrophoresis on a 1.5% agarose gel in 0.5% TBE buffer for 35 min at 100 V. The gel was then stained with SYBR® Safe DNA gel stain (InVitrogen, Carlsbad, CA) for 10 min and the bands were visualized in an AlphaImager™ 2200 (Alpha Innotech, San Leandro, CA) using UV light.

## 3. Results

### 3.1. Sea lion lesions

Of the 5240 sea lions stranded, 27 had lesions from which *K. pneumoniae* HMV phenotype was isolated. There was no spatial or temporal clustering of these cases. These animals were mostly male animals in moderate body condition (25/27 male). On post-mortem examination, animals were in good to moderate body condition, indicating a recent disease process. Twenty-five sea lions had between 1 and 5 l of thick yellow purulent fluid exudate in the pleural cavity. The lungs were atelectic and firm with similar yellow exudate in the trachea and bronchi, and often multifocal abscesses in the lung parenchyma. The pleura were often severely thickened and dark red to dark tan. These lesions appeared sub-acute. In these 25 animals, no other gross lesions were observed. Histological examination of tissues from 10 of these animals revealed a suppurative bronchopneumonia with interlobular edema, fibrin accumulation, and markedly thickened and inflamed pleura in each case. Large numbers of intralesional Gram-negative rod shaped bacteria were commonly noted. No lesions in other organs suggestive of meningitis or septicemia were observed.

Two additional animals (2/27) had abscesses, but the lungs appeared grossly normal: one had a hepatic abscess and the other had an abscess in the subcutis and skeletal muscle over the shoulder (Table 1).

### 3.2. Bacterial isolation and identification

*K. pneumoniae* was isolated from pulmonary abscesses, pleural fluid or abscess (Table 1) from each of these 27 sea lions, as well as from the livers, tonsils and intestine of three animals with pleuritis (Table 1). Large numbers of pure growth on blood agar plate were visible after overnight culture as small, non-hemolytic, gray and mucoid colonies. The colonies stained as Gram-negative rods. They were oxidase negative and indole negative by the spot tests, and with a bacteriological loop gave a "string test" of greater than 5 mm; after 48 h the "string" test increased to greater than 40 mm (Fang et al., 2004). On MacConkey agar there was either no growth or very little growth overnight, but by 48 h colonies were more visible and mucoid. The biochemical reactions in conventional tests confirmed the identification of these isolates as *K. pneumoniae*, however, many were negative for lysine decarboxylase, which is usually positive in human isolates of *K. pneumoniae*. All isolates were identified by 16S as *K. pneumoniae* based on NCBI blast in GenBank. Isolates demonstrated a positive slide agglutination reaction with antisera to the capsular antigen K2 and a negative reaction with antisera to K1 capsular antigen. PFGE analysis showed several different PFGE types indicating that these mucoid strains originated from multiple sources

### 3.3. Molecular characterization

All 21 isolates examined by PCR (see Table 1 for identification of sea lions and their lesions from which these isolates were obtained) were positive for *rmpA* and K2*wyz*, and negative for K1*magA*.

## 4. Discussion

The isolation of *K. pneumoniae* HMV phenotype in pure culture from these cases of suppurative pleuritis and pneumonia is, to the best of our knowledge, the first report of the HMV phenotype causing disease in the marine environment. Interestingly, the clinical presentation was similar in most cases, with a severe suppurative pneumonia and pleuritis causing acute mortality. Isolates were obtained from pleural fluid and lung from all cases sampled, and in three cases other organs including liver, tonsil and intestine were also sources of isolates. These tissues were sampled, although they appeared grossly normal, to investigate the potential route of entry of this bacteria. However, the time lapse between death and organ sampling likely altered the distribution of the bacteria, so that it could be cultured from all tissues post-mortem but may not have been so widely spread in vivo.

Bacterial pneumonia is commonly observed in stranded marine mammals: both primary bacterial pneumonias and bacterial infection secondary to viral or parasitic infections with *K. pneumonia* as part of mixed cultures are often reported (Keyes et al., 1968; Baker and McCann, 1989; Baker and Ross, 1992; Gulland et al., 2001). Two successive outbreaks of Hooker sea lion (*Phocarctos hookeri*) pup mortality in New Zealand were attributed to *K. pneumoniae*. In these outbreaks on Enderby Island, south of the New Zealand main islands, clonal expansion of *K. pneumoniae* likely occurred. Molecular comparisons of isolates using pulsed field gel electrophoreisis (PFGE) analysis showed isolates formed a large distinct cluster, with over 90% of them presenting similarity coefficients greater than 0.75 (Castinel et al., 2007). Thus, *K. pneumoniae* is not a novel pathogen for marine mammals, and emergence of the HMV phenotype in California associated with a characteristic pleuritis may be due to increased recognition of this syndrome rather than its novel appearance in marine species.

Genetic typing of these sea lion isolates using PCR showed all the *K. pneumoniae* isolates associated with pleuritis and pneumonia, as well as the isolate from the hepatic abscess, to be negative for the *magA* gene, although demonstrating the HMV phenotype. In reports on the genotypes of HMV *K. pneumoniae* associated with hepatic abscesses in humans, all isolates have been *magA* positive, whether from Asia or the USA (Fang et al., 2004, 2005). However, a more recent extensive analysis of *K. pneumoniae* isolates from humans in Taiwan showed strains carrying *rmpA* were significantly associated with the hypermucoviscosity phenotype. There was a significant correlation of these isolates with purulent tissue infections, such as liver abscess and lung, neck, psoas muscle, or other focal abscess (Yu et al., 2006).

The gene termed *rmpA* (regulator of the mucoid phenotype A) is a regulatory gene for the synthesis of extracapsular polysaccharide and positively controls the mucoid phenotype of *K. pneumoniae* (Nassif et al., 1989). Although the *rmpA* gene is encoded by the chromosome,

the mucoid phenotype is regulated by *rmpA* located in a plasmid. The *K. pneumoniae* isolates in this study were associated with purulent infections in sea lions and all contained the *rmpA* gene, which supports the hypothesis that specific genes such as *rmpA* may be the putative virulence factor causing purulent infections in mammals (McIver and Janda, 2008)

The detection of *K. pneumoniae* HMV phenotype in coastal marine mammals highlights the potential for zoonotic diseases to be acquired by humans swimming in coastal waters, and the importance of screening marine mammals for infectious agents with potential human health implications (Cowan et al., 2001). This bacterium appears to be emerging as a primary pathogen of coastal marine mammals. Further study of the genotypes of *K. pneumoniae* in marine mammals and their association with specific lesions is warranted to improve our understanding of the epidemiology and pathogenesis of this emerging human pathogen.

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