Pacific Marine Mammal Center

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11-year study of antimicrobial resistant *Klebsiella* spp. in pinnipeds

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Lay Summary

Bacteria in the genus *Klebsiella* have emerged in human and veterinary medicine as important antibiotic resistant "superbugs." We undertook a study of 11 years (2004-2015) of bacteriological data derived from cultures of lesions in distressed pinnipeds at the Pacific Marine Mammal Center. There was an increasing trend in the prevalence of multidrug resistance (MDR). For the fluoroquinolone class of antibiotics, there was a significant trend toward increasing resistance, reaching 37% by 2014-2015. Effluent from wastewater treatment plants is the likely source of contamination of the coastal marine environment. These bacteria have the potential to be a source of infection in humans either by recreational contact with contaminated water, or by foodborne illness from contaminated seafood. MDR *Klebsiella* is considered to be an emerging One Health issue.

Abstract

Multidrug resistant (MDR) *Klebsiella* spp. have become a major challenge in human and veterinary medicine. This study describes the prevalence of these organisms in a series of isolates from lesions in distressed pinnipeds admitted to the Pacific Marine Mammal Center, Laguna Beach, CA from 2004 to 2015. There were 847 gram-negative isolates, of which 112 isolates (13%) were identified as *Klebsiella*: *K. pneumoniae* (101 isolates), *K. oxytoca* (9 isolates), and *K. rhinoscleromatis* (2 isolates). Sensitivity testing was performed to 7 classes of antibiotics and multidrug resistance (MDR) was established by finding non-susceptibility to at least one agent in 3 or more classes. There was a strong and significant statistical association between the years and the number of MDR isolates along with a significant increasing trend in MDR prevalence. Resistance to second-generation fluoroquinolones was examined in detail. There was a significant association between the years and the non-susceptibility of the isolates, together with a significant trend toward increasing prevalence, reaching 37% by 2014-2015. Effluent

from wastewater treatment plants were considered the likely source of contamination to the coastal marine environment, which may constitute a reservoir for these pathogens.

Case Report

On 13 April 2011, a stranded yearling male California sea lion (*Zalophus californianus*) was admitted to the Pacific Marine Mammal Center (PMMC), Laguna Beach, CA. It was markedly underweight (14.5 kg) and displayed a low-grade seizure disorder. The tentative diagnosis was domoic acid toxicosis together with severe mal-nutrition. In addition, there were two abscesses putatively from bite wounds from conspecific animals: one was located in the superficial cervical tissues, and the second was located in the caudal spine with exposure of the spinal canal and spinal cord (figure 1). The animal was euthanized on 15 April and a blood sample was collected from the jugular vein, allowed to clot, and the plasma separated. The sample was stored at -34 C.

This plasma sample was used to assess the oxidative biology of this animal. One aliquot of plasma was sent to Michigan State University Diagnostic Center for Population and Animal Health (Lansing, MI) for assessment of vitamin D status by determining concentrations of 25-hydroxycholecalciferol [25-(OH)D_3] by radioimmunoassay (Van Saun et al. 1996). Another aliquot of plasma was analyzed for biomarkers of oxidative stress (reactive oxygen species and antioxidants) using the FREE System (Diacron International, Grosseto, Italy) and its associated test kits for plasma analytes d-ROM (reactive oxygen metabolites) and BAP (blood antioxidant potential). A measure of oxidative stress (OS) was determined by calculating the ratio between d-ROM and BAP (x1000), with higher values representing higher levels of OS. The details of these tests are summarized by Tangredi, et al. 2014.

Culture swabs were taken from both abscesses and sent to IDEXX Laboratories (Irvine, CA) for bacteriological culture and in vitro testing for sensitivity to antibiotics. Seven classes of antibiotics were tested: Penicillins (amoxicillin, Clavamox®, piperacillin); Cephalosporins (ceftiaxine, ceftiofur, cephalexin); Aminoglycosides (amikacin, gentamicin, tobramycin); Fluoroquinolones (ciprofloxacin, enrofloxacin); Tetracyclines (tetracycline); Phenicols (chloramphenicol); Folate pathway inhibitors (trimethoprim/sulfa). Standardized international terminology was adopted to define and classify organisms resistant to antimicrobials (Magiorakos et al. 2012). Isolates reported as "resistant" or "intermediate" were classified as "non-susceptible." Multidrug Resistance (MDR) was defined as non-susceptibility to at least one antibiotic in 3 or more classes. Extensive Drug Resistance (XDR) was defined as non-susceptibility to at least one antibiotic in 5 or more classes. Pandrug Resistance (PDR) was defined as non-susceptibility to at least one antibiotic in all 7 classes. Since all possible agents were not tested, the MDR/XDR/PDR classifications used in this case report should be understood as "possible MDR", etc.

With respect to the 25-(OH)D_3, d-ROM, BAP, and OS biomarkers, no reference ranges are available. However, the 2014 study by Tangredi, et al. presented data derived from 52 juvenile California sea lions in rehabilitation at PMMC. Some inference is

possible by placing the results of this case in the proper quartiles of the 2014 study's dataset (Table 1).

Table 1. Biomarkers of oxidative biology in a California sea lion (*Z. californianus*)

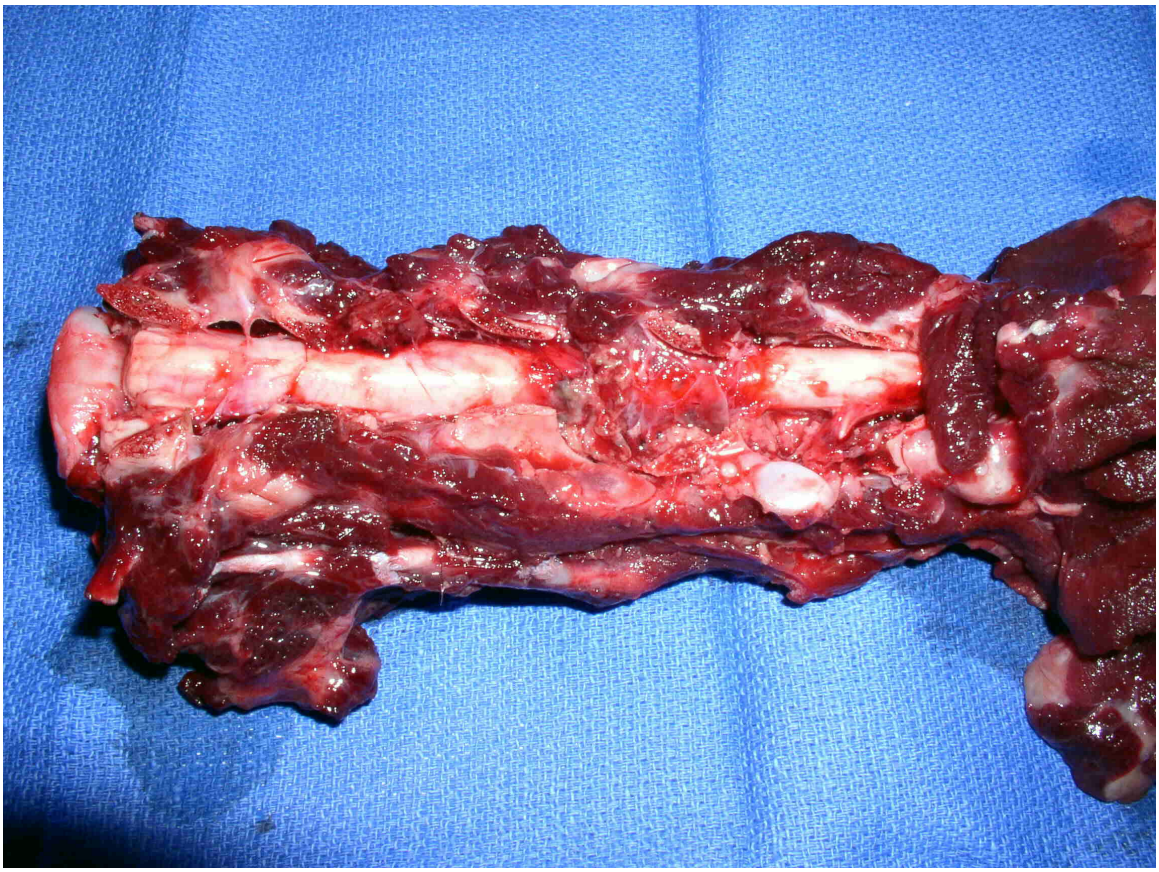
Biomarker	Result	Units	Quartile
25-(OH)D	133	nmol/L	Q1
d-ROM	209	UCARR	Q1
BAP	225	μ mol/L	Q1 (lowest value in dataset)
d-ROM:BAP (x1000)	928	---------	Q4 (highest value in dataset)

Though the d-ROM result appears relatively low, the measure of oxidative stress was the maximum in the dataset due to the relatively low 25-(OH)D_3 and BAP. The generation of ROS in an inflammatory response is part of the mammalian organism's antimicrobial defense (Victor, et al. 2004; Kovacs, et al. 2015). However, antioxidant levels must also be increased to avoid the adverse effects of free radical overproduction. An imbalance can result in OS in the host together with a higher bacterial mutation rate leading to the development of antimicrobial resistance (Kuwahara et al. 2004; Kuwahara et al. 2009). This process may have been at play in this case, as shown in the bacteriological results:

Cervical abscess:
Escherichia coli (MDR)
Klebsiella oxytoca (PDR)
Enterococcus sp. (PDR)
Caudal spine abscess:
Escherichia coli (MDR)
Enterococcus sp. (XDR)
Klebsiella pneumoniae (XDR)

In review of the bacteriological findings derived from 1004 admissions of animals to the PMMC from 2004 to 2015, no other case presented so many exclusively non-susceptible isolates, including two species in the genus *Klebsiella*. This genus constituted the second most common isolate (after *E. coli*) in this 11-year bacteriological dataset. Since *K. pneumoniae* is considered a globally emerging "superbug" (Deris, 2015), a detailed analysis of the 11-year dataset is herewith presented.

Figure 1. Photograph of caudal spine abscess in a California sea lion (*Z. californianus*)



The genus *Klebsiella*

The *Klebsiella* bacillus was named after the 19th century German pathologist Theodor Albrecht Edwin Klebs. It is a member of the family Enterobacteriaceae and can be distinguished from similar species by its lack of motility and inability to decarboxylate ornithine (Koneman et al. 1979). The type species is *Klebsiella pneumoniae* which is an opportunistic human pathogen commonly infecting the urinary and respiratory tracts Struve and Krogfelt 2004). In veterinary medicine, it has been identified as part of the commensal gut microbiota but can be an opportunistic invader of the mammary gland, the reproductive tract, and the urinary tract (Timoney et al. 1988).

The taxonomy of this genus is unsettled, with different schemes identifying anywhere from 4 to 8 species (Bagley 1985, Caputo et al. 2015). Two species other than *K. pneumoniae* were identified in the present study. *K. rhinoscleromatis* is the etiologic agent of rhinoscleroma in humans which is a chronic granulomatous disease of the upper respiratory tract (Botelho-Nevers et al. 2007). *K. oxytoca* is common in the environment, and has been recovered from many tissues in humans and animals in health and disease. It produces a cytotoxin under microaerobic and aerobic conditions (Darby et al. 2014).

Multidrug resistant *Klebsiella* spp. is a global problem in human medicine which has increased over the past three decades (Broberg et al. 2014). Managing this important health threat requires understanding how the ecology and prevalence in wildlife can interact with the clinical diseases.

The 11-year study: Overall non-susceptibility

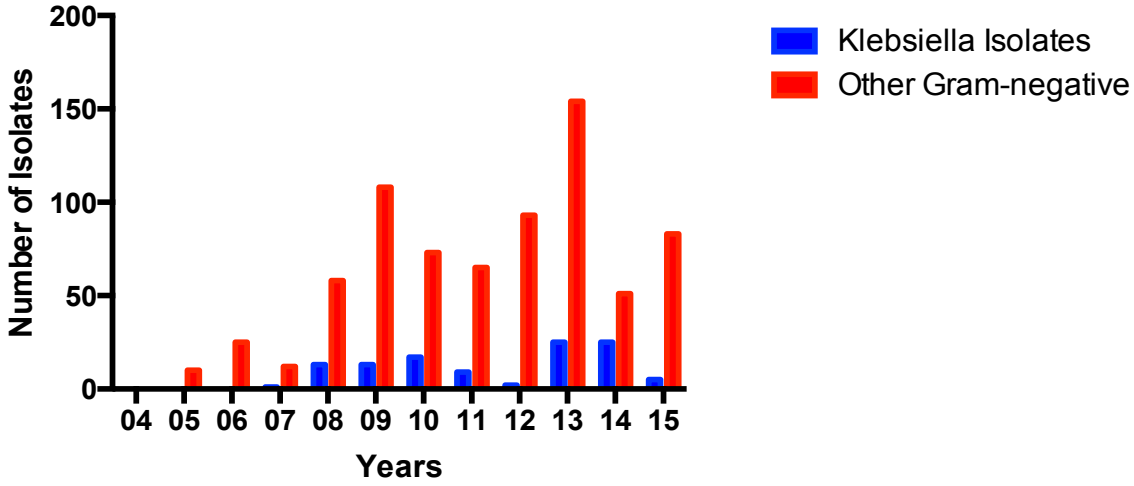
From 2004 to 2015 cultures of lesions in distressed pinnipeds yielded 847 gram-negative isolates. 112 isolates (13%) were identified as *Klebsiella*: *K. pneumoniae* (101 isolates), *K. oxytoca* (9 isolates), and *K. rhinoscleromatis* (2 isolates). 92 isolates derived from California sea lions (*Zalophus californianus*); 11 from northern elephant seals (*Marounga angustirostris*); 9 from harbor seals (*Phoca vitulina*). Statistical analysis was carried out with the chi-square test for independence and for trend using Prism® (GraphPad Software, Inc., La Jolla, CA).

The annual numerical data is presented in Table 2 and depicted graphically in Figure 2. There is strong association between the years and number of isolates (χ^2 = 44.23, df = 1, p = <0.0001) but with no significant overall trend (χ^2 = 0.005; df = 1; p = 0.94)

Table 2. Annual data of *Klebsiella* spp. and all gram-negative isolates

Year	**All Gram-negative Isolates**	***Klebsiella* spp.**	**All Non-*Klebsiella* spp.**	**% *Klebsiella* spp. of All Gram-negative**
2004	5	2	3	40
2005	10	0	10	0
2006	25	0	25	0
2007	13	1	12	8
2008	71	13	58	18
2009	121	13	108	11
2010	90	17	73	19
2011	74	9	65	12
2012	95	2	93	2
2013	179	25	154	13
2014	76	25	51	33
2015	88	5	83	6
TOTAL	847	112	735	13

Figure 2. Histogram of *Klebsiella* spp. versus other gram-negative isolates

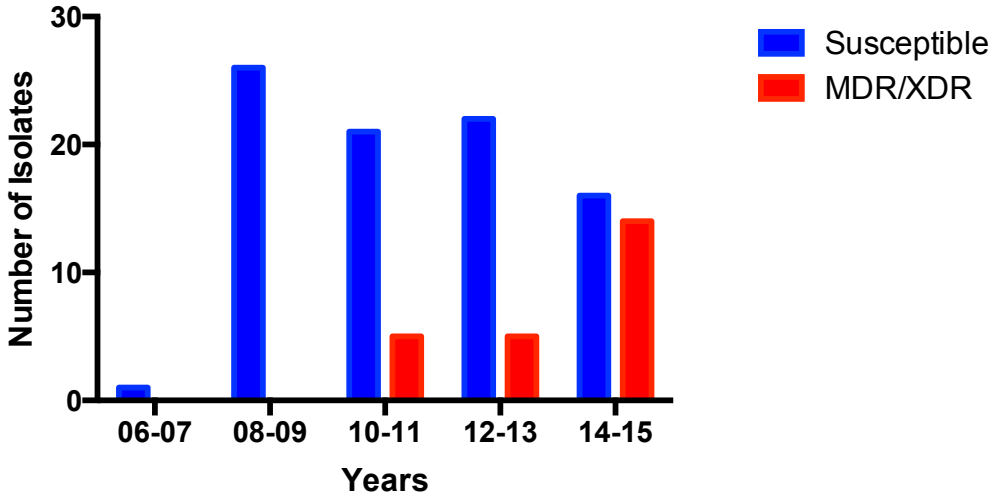


Because of the incomplete testing to all or nearly all possible antibiotics, and because of the relatively low numbers of isolates, maintaining the 3 official resistance categories for analysis was statistically impractical. Therefore, antimicrobial resistance was tabulated for all isolates exhibiting non-susceptibility to at least one agent in 3 or more classes of antibiotics and labeled as MDR/XDR. The annual data is presented in Table 3 and represented graphically in Figure 3.

Table 3. Annual data of non-susceptibility

Year	Susceptible	MDR/XDR	Total Isolates	% MDR/XDR
2004	Not Tested	Not Tested	2	Not applicable
2005	0	0	0	0
2006	0	0	0	0
2007	1	0	1	0
2008	13	0	13	0
2009	13	0	13	0
2010	16	1	17	6
2011	5	4	9	44
2012	0	2	2	100
2013	22	3	25	12
2014	16	9	25	36
2015	0	5	5	100
TOTAL	86	24	112	21

Figure 3. Histogram of susceptible and non-susceptible *Klebsiella* spp. (biennial data)



To obtain valid results with chi-square, data for two consecutive years were combined beginning with 2006-2007. There was a strong and highly significant association between the years and the number of MDR/XDR isolates ($\chi^2 = 18.67$; df = 4; p = 0.0009). There was also a trend toward an increasing prevalence of MDR/XDR ($\chi^2 = 16.34$; df = 1; p = <0.0001).

For the two minority species, their non-susceptibility pattern was as follows. For *K. oxytoca*, 7 of 9 isolates were MDR/XDR, with 2 isolates being possible PDR. For *K. rhinoscleromatis*, both isolates were susceptible.

The prevalence of MDR/XDR by anatomical region of lesions was investigated (Table 4). The results reflect the predominance of bite wound lesions from conspecifics in the juvenile population submitted for care.

Table 4. Prevalence of MDR/XDR isolates by anatomical region

Anatomical Location	Number of MDR/XDR isolates
Abdomen	22
Thorax	28
Head	21
Skin	39
CNS	2

The 11-year study: Fluoroquinolone non-susceptibility

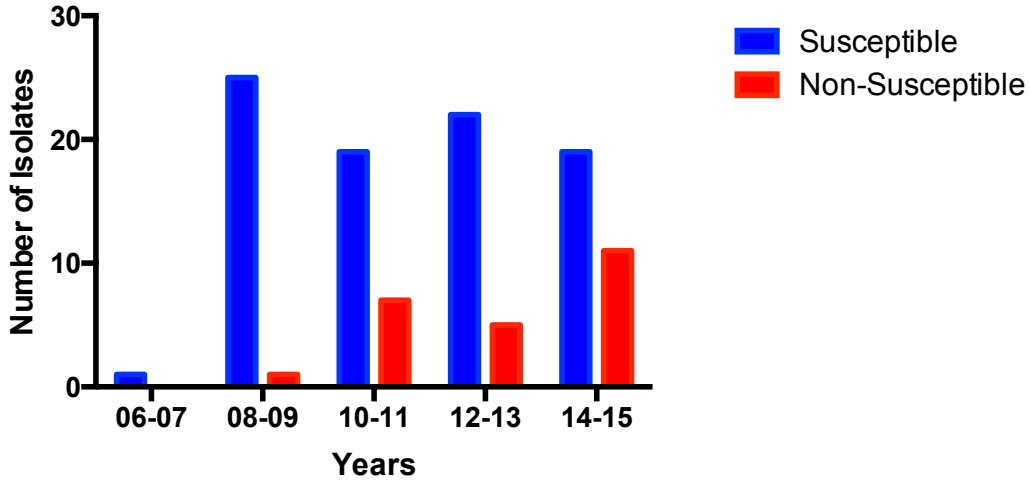
Fluoroquinolone antibiotics came into clinical use in both human and veterinary medicine in the 1980's (Pallo-Zimmerman, et al. 2010), and were especially effective against infection by gram-negative organisms (Piddock 1998). These agents are bactericidal and achieve this effect by inhibiting the activities of DNA gyrase and topoisomerase IV which are necessary for bacterial DNA replication (Yang et al. 2004). Resistance slowly developed due to chromosomal mutations that modified these two enzymes or reduced quinolone accumulation in the bacterial cell (Yang et al. 2004).

However, in 1998, a multiresistant strain of *K. pneumoniae* was discovered in a clinical isolate that was plasmid-mediated and transferable to other species of Enterobacteriaceae (Martinez-Martinez et al. 1998). These plasmid genes (called *qnr*) spread rapidly and coincided with a steep rise in the resistance of these organisms to ciprofloxacin (Strahilevitz J et al. 2007). In the present study, ciprofloxacin and enrofloxacin were tested. The 11-year data is presented in Table 5 and graphically represented in Figure 4.

Table 5. Annual data of fluoroquinolone non-susceptibility

Year	Susceptible	Non-susceptible	% Non-susceptible
2004	Not Tested	Not Tested	Not Applicable
2005	0	0	0
2006	0	0	0
2007	1	0	0
2008	13	0	0
2009	12	1	8
2010	16	1	6
2011	3	6	50
2012	0	2	100
2013	22	3	3
2014	16	9	36
2015	3	2	40
TOTAL	86	24	28

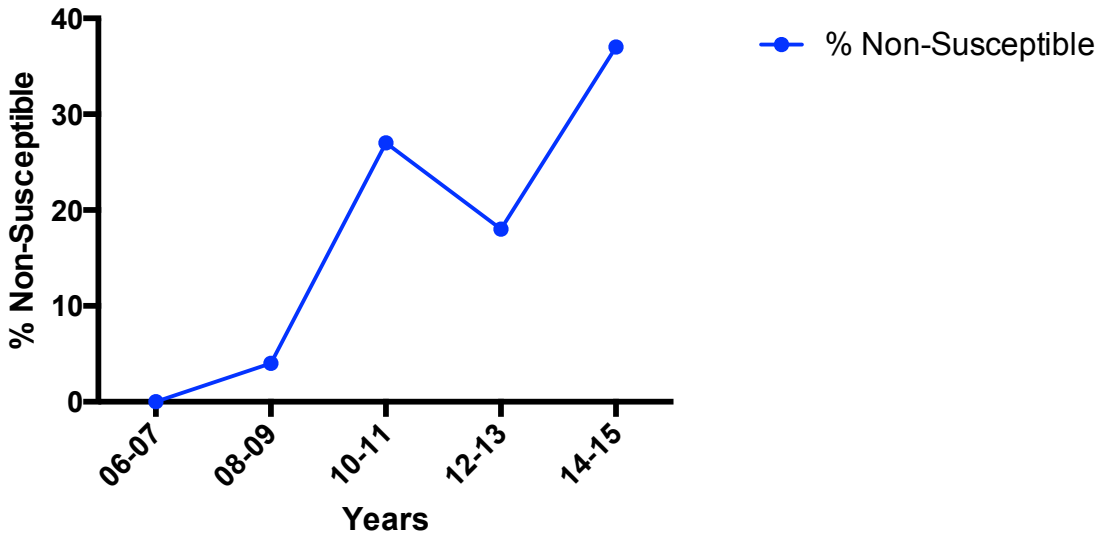
Figure 4. Histogram of susceptible and non-susceptible *Klebsiella* spp. to fluoroquinolone antibiotics (biennial data)



To obtain valid results for the chi-square statistics, data for two consecutive years were combined, beginning with the years 2006-2007. There was a marginally significant association between the years and the susceptibility of isolates ($\chi^2 = 9.7$; df = 4; p = 0.047). There was, however, a significant trend toward increasing rates of non-susceptibility ($\chi^2 = 6.9$; df = 1; p = 0.008).

The trend for fluoroquinolone non-susceptibility is portrayed in figure 5 and shows a peak 37% for the 2014-2015 period.

Figure 5. Time plot of the percentage of fluoroquinolone non-susceptibility



Discussion

Isolation of *Klebsiella* spp. w reported previously in pinnipeds from California (Thornton et al. 1998; Johnson et al. 1998) and Washington (Lockwood et al. 2006). However, none of these studies carried out identification to the species level. Two studies provided data on antibiotic susceptibility. The Johnson paper (1998) identified 7 "resistant" *Klebsiella* spp. out of a total of 129 isolates. These results were not classified in such a way as to allow direct comparison to the results of overall non-susceptibility in the present study. However, regarding fluoroquinolones, only 11% of all Enterobacteriaceae (75 isolates) and 0% of *Klebsiella* were non-susceptible to ciprofloxacin. Lockwood (2006) reported 134 isolates from harbor seals (*Phoca vitulina*), with 87 being gram-negative, of which 8 were identified as *Klebsiella* spp. The sensitivity pattern of the *Klebsiella* isolates was not specified but the overall gram-negative non-susceptibility to enrofloxacin was 11%. Given a non-susceptibility rate to fluoroquinolones of 37% in the present study, there seems to have been a three-fold increase within a decade.

In a recent study of human clinical isolates at Flagstaff Medical Center in 2011-2012, the prevalence of overall MDR *Klebsiella pneumoniae* was 8% (Davis et al. 2015). The trend for ciprofloxacin non-susceptibility in four U.S. studies of human inpatients demonstrates that between 2004 and 2011, the prevalence of non-susceptibility rose to an average of 15.8% (14.2% - 16.8%) (Lockhart et al. 2007; Hoffman et al. 2010; Bouchillon et al. 2013; Sanchez et al. 2013). The prevalence of fluoroquinolone non-susceptibility in the 2014-2015 pinnipeds (37%) is a two-fold difference from these U.S. human inpatients and has reached this peak several years later.

A major reservoir for *Klebsiella* spp. is the GI tracts of humans and terrestrial animals (including livestock) (Bagley 1985; Broberg 2014). Environmental reservoirs are surface waters and soil which receive effluents from wastewater treatment plants (Bagley 1985, Struve and Krogfelt 2004). This includes marine and estuarine waters where these organisms

can survive (Vasconcelos and Swartz 1976) and maintain virulence (Struve and Krogfelt 2004). Moreover, they have been isolated from coastal invertebrates and thus may have entered the marine food chain (Faghri et al. 1984; Choudhury and Kumar 1998). Spill-over into marine mammals is the result. Wastewater treatment plants provide an optimum environment for horizontal gene transfer of genomic elements that confer antimicrobial resistance: high density of gut bacteria, aerobic environment, high nutrient load, and the presence of significant concentrations of antibiotics (Rizzo et al. 2013; Marti et al. 2014). Indeed, there can be an increase in the prevalence of MDR bacteria and resistance genes in the effluent *after* treatment (Czekalski et al. 2012; Berglund et al. 2015), especially resistance to ciprofloxacin (Figueira et al. 2011). According to the California Ocean Wastewater Discharge Report and Inventory (Gordon 2010), 1.35 billion gallons of wastewater is discharged every day directly into the Pacific Ocean from California. The accompanying flow of resistant genetic elements may be one explanation for the higher prevalence of MDR and fluoroquinolone non-susceptibility in the pinnipeds of this study versus the reports involving human inpatients.

Finally, MDR *Klebsiella* spp. have the potential to establish a reservoir in the marine environment and its wildlife (Redhouani H et al. 2014). The presence of bacteria in wastewater effluents is a necessary but not sufficient condition for this. There must also be a fertile medium, as in the spinal tissues of the sea lion presented in the case report, with its particular redox milieu. These pathogens can then spill-back into the human population via contamination of recreational waters (Montezzi et al. 2015) or as a foodborne illness from contaminated seafood (Singh and Kulshreshtha 1992; Nawaz et al. 2012). The prevalence of MDR *Klebsiella* spp. in the marine environment warrants consideration as a One Health issue.

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