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Environmental persistence and disinfectant susceptibility of *Klebsiella pneumoniae* recovered from pinnipeds stranded on the California Coast

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ABSTRACT

Hypermucoviscous *K. pneumoniae* (HMV) are emergent zoonotic pathogens associated with increased invasiveness and pathogenicity in terrestrial and marine mammals. In this study, HMV and non-HMV isolates recovered from stranded pinnipeds were used to investigate: 1) their persistence in sea and fresh water microcosms at 10 and 20°C, 2) their capacity to form biofilms, and 3) the biocide efficacy of four disinfectants on their planktonic and biofilm phenotypes. Results indicated that although HMV isolates were significantly more mucoviscous, non-HMV isolates displayed significantly greater capacity to form biofilms ($p < 0.05$). Additionally, non-HMV isolates persisted in greater numbers in both sea- and freshwater, particularly at 20°C. These two phenomena could be associated with the greater growth observed for non-HMV isolates in *in-vitro* growth-curve assays ($p < 0.05$). Similar susceptibility to disinfectants was detected in HMV and non-HMV isolates when exposed for 24 h; however, the minimal biofilm disinfectant eradication concentration for HMV isolates was significantly higher than that for non-HMV when exposed to disinfectants for 0.5 h. This information should be taken into consideration when developing biosecurity protocols in facilities holding marine mammals in captivity.

1. Introduction

Klebsiella pneumoniae is a Gram-negative, facultative anaerobic bacterium belonging to the family *Enterobacteriaceae.* Typically found in the environment, and on mucosal surfaces, including oral, nasal and gastro-intestinal tract, it has been associated with a range of infections in humans and animals (Clegg and Murphy 2016; Soto et al., 2017; Whitaker et al., 2018). In both human and veterinary medicine this bacterium is regarded as an emergent and common nosocomial pathogen (Podschun and Ullmann, 1998; Shon et al., 2013; Soto et al., 2012).

A novel, invasive form of *K. pneumoniae* associated with the hypermucoviscosity (HMV) phenotype has emerged since the late 1980′s (Kawai, 2006; Shon et al., 2013. Among marine mammals, it has emerged as a significant pathogen of pinnipeds of the Pacific Basin causing epidemic mortality from septicaemia in both free-living New Zealand sea lions (*Phocarctos hookeri*) and California sea lions (CSL), *Zalophus californianus* (Jang et al., 2010; Roe et al., 2015). For pinnipeds under rehabilitation, HMV *K. pneumoniae* isolates have been reported as the etiologic agent of suppurative pneumonia, pleuritis and abscesses in CSLs (Jang et al., 2010; Seguel et al., 2017; Whitaker et al., 2018), and were recently isolated from mucosal sites from apparently healthy animals such as the intestinal tract and oral and nasal cavities (Whitaker et al., 2018). Since 1996 *K. pneumoniae* infections have been identified in over 200 cases at the Marine Mammal Center, Sausalito, California. Additionally, Thornton et al. (1998) reported *Klebsiella* sp. as one of the most common isolates recovered from lung, liver and brain from animals dying during rehabilitation in a two year study investigating bacterial isolates recovered from CSL (n = 297), harbor seals, (*Phoca vitulina*) (n = 154) and northern elephant seals (*Mirounga angustirostris*) stranded along the California coast.

Marine mammal interactions with humans are frequent particularly under circumstances of managed care such as in aquariums, zoos and rehabilitation centers, and individual animals may live for decades. In those settings, animal caretakers, biologists, veterinarians and in some situations, the general public will interact with the animals and be

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exposed to the same environment (water, air) daily. Moreover, wildlife interactions with humans (direct and indirect) are not infrequent in some regions such as the Pacific northwest of the US, highlighting the importance to better understand the epidemiology of pathogens that have zoonotic importance.

While pinnipeds stranded and rescued along US Pacific coast have been reported with fatal systemic HMV *K. pneumoniae* infection, very little is known about the ecology, diagnosis, prevention and treatment of infection in rescued animals. A better understanding of the persistence of this emerging pathogen in veterinary hospitals, including wildlife rehabilitation centers is important. To address questions around persistence of the bacteria in the environment and their susceptibility to disinfectants, this study was conducted to evaluate the capacity of both HMV and non-HMV *K. pneumoniae* isolates recovered from clinically affected pinnipeds to persist in fresh and marine water at two different temperatures encountered by marine mammals in California. Additionally, the capacity of the isolates to produce biofilms, defined as as naturally formed adherent communities of bacteria within an extracellular polymeric matrix was investigated *in-vitro*. Finally, the susceptibility *K. pneumoniae* in planktonic, defined in this study as free-living bacteria, and biofilm forms to four different disinfectants frequently used in veterinary and human hospitals was investigated.

2. Materials and methods

2.1. Bacteria

Klebsiella pneumoniae were grown on 5% sheep blood agar plates (SBA) (Biological Media Services, UC Davis, Davis, USA) and expanded in Luria Bertani (LB) broth (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C. Five HMV and five non-HMV *K. pneumoniae* isolates recovered from different California sea lions (n = 7) and harbor seals (n = 3) were characterized according to standard clinical microbiologic and molecular methods (Table 1) (Whitaker et al., 2018).

2.2. String test and mucoviscosity testing

Hypermucoviscosity was defined by the formation of viscous strings > 5 mm in length when a loop was used to stretch the colony on the SBA plate (positive string test) (Fang et al., 2004). The mucoviscosity levels were determined by centrifugation as described by Hsu et al. (2011). Briefly, *K. pneumoniae* isolates were cultivated in SBA at 37 °C overnight. The following morning 1.2 mL of optical density OD_{600} normalized bacteria grown in LB broth was centrifuged in microcentrifuge tubes at 2000 g for 5 min. The absorbance of the supernatant was measured at OD_{600}.

2.3. Growth curves

Growth curve comparisons for the isolates were performed at 25 °C. Isolates were harvested after incubation on sheep blood agar media at 37 °C under ambient atmosphere for 24 h and suspended in 1X phosphate buffered saline (PBS) to achieve a turbidity equivalent to that of a 0.5 McFarland standard. This suspension was diluted 1000-fold ($\sim 10^5$ CFU/mL) in LB broth, and 100 µL of each isolate were added to 8 different wells of a NUNC Edge clear flat-bottom 96-well plate with standard microplate lid (Becton Dickinson, Billerica, MA). The Cytation 3 96-well plate reader (Biotek) was used to obtain optical density measurements at 600 nm, every hour for 48 h. Plates were incubated in the plate reader at 25 °C using a 3 mm shaking amplitude. Wells inoculated with broth alone served as the negative control.

2.4. Preparation of microcosms

Sea and fresh water microcosms were utilized in this study. Microcosms as defined by Roeselers et al., 2006 are "constructed, simplified ecosystems that are used to mimic natural ecosystems under controlled conditions". Sea-water and fresh-water were collected from Monterey Bay, California and a freshwater aquaculture farm in California in November 2017. Water samples used for the generation of microcosms were used the same day of collection. Aliquots of 49.5 mL of sea and fresh-water were filtered through 0.22-µm pore-size membranes (Nalgene-Thermo Fisher Scientific, Rochester, NY) and placed in loosely capped 50 mL conical tubes.

Klebsiella pneumoniae was grown aerobically on SBA for 24 h at 37 °C and used to generate a 0.5 McFarland Standard in PBS as described previously. Bacterial suspensions were inoculated into triplicate microcosms per isolate to obtain a final concentration of $\sim 10^6$ CFU/ml, and incubated in a static state at 10 and 20 °C in dark conditions for 44 days. Microcosms were sampled at 0, 7, 14, 21, and 44 d post inoculation. At each time point, tubes were vortexed for 1 min and 180 µL from each microcosm was collected aseptically and was serially diluted 10-fold in PBS. Each dilution was plated in triplicate onto blood agar plates, incubated at 37 °C overnight and quantified the next day.

2.5. Biofilm formation and disinfectant susceptibility

Klebsiella pneumoniae (Table 1) biofilms were formed using the minimum biofilm eradication concentration assay (MBEC™ Assay) For High-Throughput Antimicrobial Susceptibility Testing of Biofilms Version 1.0 (Innovotech Inc., Suite 101–2011 94 St., Edmonton, AB, Canada) with slight modifications. The MBEC™ Biofilm Inoculator consists of a plastic lid with 96 pegs and a corresponding base with 96 individual wells into which a standardized inocula of *K. pneumoniae* was

Table 1
Isolates used in this study.

ID #	Species	Sample Source	Isolate Source	String test	*p-rmpA-2*	*p-rmpA*	*K2 (wzy)*	*wcaG*
Nonhypermucoviscous								
UCD 1	PHS	ear	stranded	–	–	–	–	–
UCD 2	PHS	abscess	stranded	–	–	–	–	–
UCD 3	CSL	lung	stranded	–	–	–	–	+
UCD 4	PHS	feces	stranded	–	–	–	–	–
UCD 5	CSL	pleura	stranded	–	+	+	–	–
Hypermucoviscous			stranded					
UCD 6	CSL	kidney	stranded	+	+	+	+	–
UCD 7	CSL	LN abscess	stranded	+	+	+	+	–
UCD 8	CSL	lung	stranded	+	+	+	+	–
UCD 9	CSL	nasal	stranded	+	+	+	+	–
UCD 10	CSL	nasal	stranded	+	+	+	+	–

UCD; University of California Fish Pathology Lab ID number; PHS; pacific harbor seal, (*Phoca vitulina*).
CSL; California sea lion, (*Zalophus californianus*); LN; lymph node.
Molecular characterization of isolates were performed by Whitaker et al. (2018).

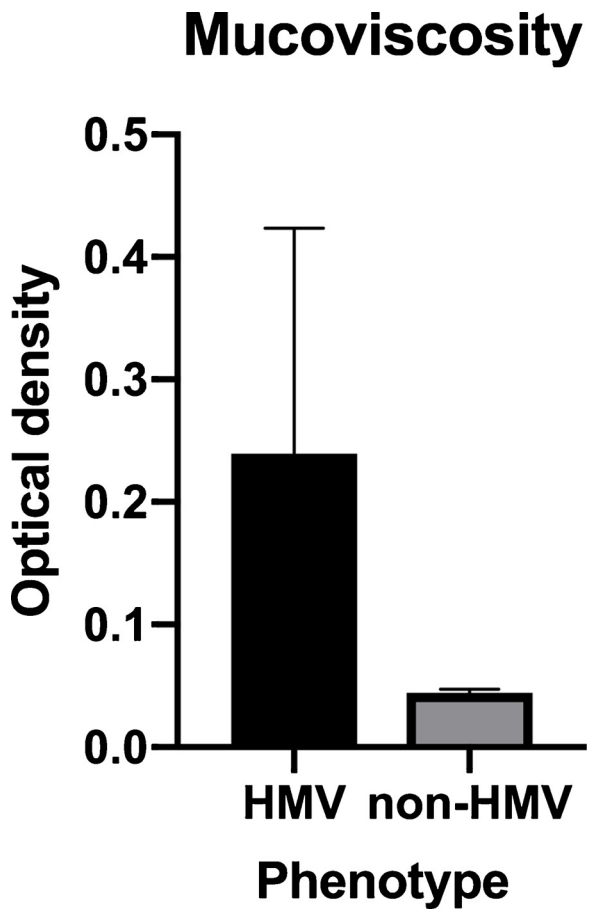

Fig. 1. *Klebsiella pneumoniae* mucoviscosity levels. Mucoviscosity of hypermucoviscous isolates (HMV) and non-hypermucoviscous (non-HMV) was evaluated measuring optical density (OD)600 of normalized bacteria grown in LB broth after centrifugation. The error bars represent standard errors for triplicate samples, and the results shown are representative of three independent experiments.

added. The device is then placed on a rocking platform where a shear force is created against the pegs forming 96 equivalent biofilms. Briefly, overnight bacterial suspensions of HMV isolates were diluted 1:50 and non-HMV isolates were diluted 1:500 in fresh LB broth to normalize inocula and 150 µL were added per well. Sterile LB broth (Becton Dickinson, USA) seeded wells served as negative controls. The plates were incubated for 6, 12, and 24 h at 25 °C on a rocking platform set at 110 rpm. Biofilm growth curve counts were determined by removing three pegs at each time point and placing them in 200 µl of 1 PBS following sonication on a high setting using Bransonic®CPX Ultrasonic Cleaning Bath 2800 (Branson Ultrasonics, Danbury, CT, USA) for 30 min to remove the biofilm, followed by serial dilution of suspended colonies and spot-plating on agar plates to determine colony forming unit (CFU) counts.

The minimum inhibitory concentration (MIC), minimum biocidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) values of four selected disinfectants for *K. pneumoniae* were investigated using the MBEC™ Assay with slight modifications. Briefly, *K. pneumoniae* biofilms were formed in the MBEC™ device as described in the previous section for 24 h at 25 °C prior to treatment with the disinfectants. The day of challenge, working solutions of Chloramine T (20 mg l^{-1}; Axcentive SARL, Chemin de Champause, France), sodium hypochlorite (100 mM; Clorox™, Oakland, California, USA), and ethanol (100%; KOPTEC™, DLI, PA, USA) and Hydrogen Peroxide (3%; Optima™ Fisher Chemical), were prepared in MHB and serial twofold dilutions in triplicate were prepared using a sterile 96-well plate (BD Falcon). Pegs of the MBEC™ device were briefly rinsed in a sterile 96-well plate filled with 200 µL of PBS for approximately 60 s. Biofilm was quantified from four pegs as previously described. Following the rinse step the MBEC™ lid was transferred to the challenge plate prepared with doubling dilutions of disinfectants and incubated at 25 °C for 0.5 or 24 h. A recovery plate was prepared by placing 200 µL of MHB in each well of a sterile 96 well microtiter plate. After the challenge, the MBEC™ lid was transferred to the recovery plate and allowed to stand for 30 min to equilibrate. After the equilibration time, the plate was sonicated as described above to dislodge the biofilm. The MBEC of each disinfectant was determined after 24 h incubation at 25 °C. The Biotek Cytation 96-well-plate reader was used to obtain optical density measurements at 650 nm (OD650) for each well. The MBEC was defined as the minimum concentration of antimicrobial that eradicated the biofilm. Clear wells were evidence of eradication following incubation.

In order to determine the MBC of each disinfectant, 20 µL from each well of the challenge plate was removed immediately after treatment and used to inoculate 180 µL of MHB in corresponding wells of a fresh sterile 96 well microtiter plate. Following incubation for 24 h at 25 °C, the Biotek Cytation 96-well-plate reader was used to obtain optical density measurements at 650 nm (OD650) for each well. The MBC value represented the lowest concentration of disinfectant that killed 99.9% of the population of the dispersed cells that were shed from the biofilm. Clear wells were evidence of effective biocidal concentration following incubation.

MIC results were determined following incubation for 24 h at 25 °C, visually and through using the Biotek Cytation 96-well-plate reader to obtain optical density measurements at 650 nm (OD_{650}) for each well. The MIC was defined as the minimum concentration of antimicrobial that inhibited growth of the dispersed cells from the biofilm. Clear wells were evidence of inhibition following incubation.

2.6. Statistical analysis

The SAS® (Version 9.4, SAS Institute, Cary NC) was used in all comparisons. For growth curves, the general linear model (GLM) procedure was used. Briefly, the mixed procedure was used to conduct a repeated measures analysis of variance with regard to growth curves of the three groups across time (HMV, non-HMV and negative controls). The variance-covariance structure used was an autoregressive single-lagged model in reflection of correlations of adjacent time points. In microcosom studies, GLM least squares procedure was used to conduct an analysis of variance of a factorial arrangement of treatments. Main effects included mucoviscosity, incubation temperature, media salinity and time post-incubation. To investigate disinfectant susceptibility, the GLM procedure was used to analyze the data as a $2 \times 4 \times 3 \times 2$ factorial arrangement of treatments. Factors in the model included mucoviscosity (hypermucoviscous, non- hypermucoviscous), disinfectant (Chloramine T, Ethanol, Hydrogen Peroxide, Sodium Hypochlorite), test (MBC, MBEC, MIC) and time (0.5, 24 h). The Response variable was Value.

In all cases, when overall significance was detected for main effects or interaction effects (all two-way, three-way and the four-way interactions) post hoc comparisons were conducted with pairwise t tests of least-squares means. All comparisons where considered significant at $p \leq 0.05$.

3. Results

Hypermucoviscous isolates produced a significantly more mucoid capsule, than isolates designated non-HMV strains, ($p < 0.05$) (Fig. 1). Growth curve results indicated a significant group*time interaction ($p < 0.0001$). With respect to HMV *versus* non-HMV groups, posterior comparisons showed that non-HMV mean OD was significantly higher than that for HMV beginning 7 h post inoculation and for all time points thereafter (Fig. 2).

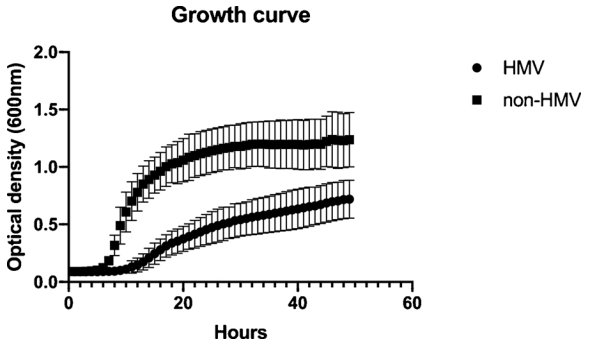

Fig. 2. Growth curve of hypermucoviscous (HMV) and non-hypermucoviscous (non-HMV) *Klebsiella pneumoniae* isolates grown in LB-broth at 25 °C. Absorbance (optical density measurements at 600 nm) was collected every hour. The error bars represent the standard error of six replicate wells.

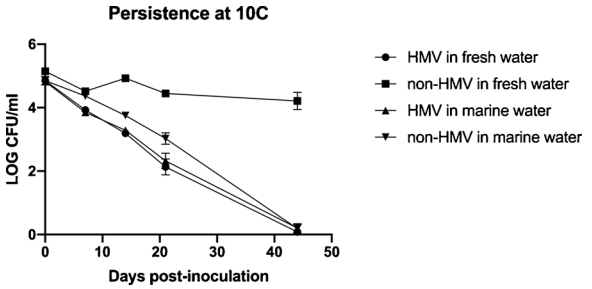

Fig. 3. Culturability of hypermucoviscous (HMV) and non-hypermucoviscous (non-HMV) *Klebsiella pneumoniae* isolates in fresh and marine water microcosms after incubation at 10 °C for a period of 44 d. The error bars represent standard error of triplicate biological replicates each plated in triplicate.

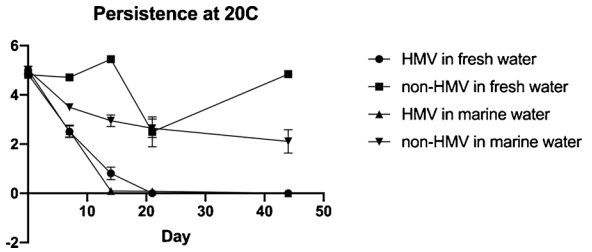

Fig. 4. Culturability of hypermucoviscous (HMV) and non-hypermucoviscous (non-HMV) *Klebsiella pneumoniae* isolates in fresh and marine water microcosms after incubation at 20 °C for a period of 44 d. The error bars represent standard error of triplicate biological replicates each plated in triplicate.

Initial mean CFU count values from working suspensions used to inoculate the water microcosms ranged from 4.85 ± 0.30 LOG CFU/ml to 5.14 ± 0.60 LOG CFU/ml for HMV and non-HMV isolates, respectively. No significant increase in CFU counts were observed in the water microcosms inoculated with HMV isolates, indicating no exponential growth in the absence of added nutrients (Figs. 3 and 4). On the other hand, an increased amount of non-HMV was observed at day 14 post-inoculation in fresh-water microcosms (Figs. 3 and 4). Persistence of culturable non-HMV isolates was greater in fresh water microcosms and when incubated at 20 °C. Significantly lower amounts of culturable non-HMV bacteria were detected 7 d post inoculation when compared to time point 0 in marine microcosms regardless of the temperature ($p < 0.05$) (Figs. 3 and 4). HMV isolate culturability decreased in all microcosoms after 7 d of incubation. Similar to non-HMV isolates, HMV *K. pneumoniae* persisted in greater amounts in fresh water microcosms than in marine microcosms when incubated at 20 °C ($p < 0.005$) (Fig. 4).

Both HMV and non-HMV isolates were capable of forming biofilms.

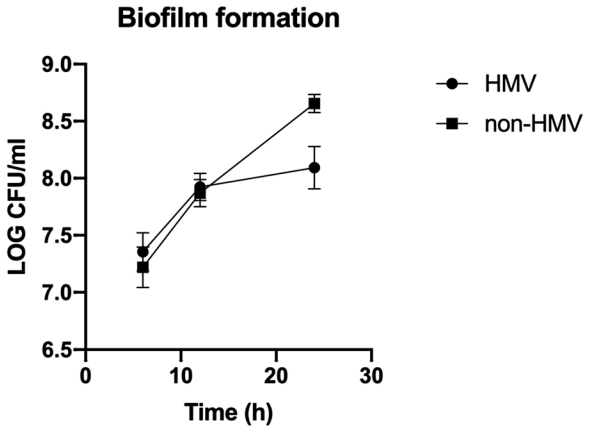

Fig. 5. Biofilm formation of hypermucoviscous (HMV) and non-hypermucoviscous (non-HMV) *Klebsiella pneumoniae* isolates in LB broth, and incubated at 25 °C 24 h. The error bars represent standard error of six replicates and the results shown are representative of three independent experiments.

Biofilm quantification was significantly greater in both groups at 12 h when compared to those at 6 h ($p < 0.0001$); and at 24 h, compared to those at 12 h ($p < 0.0001$) (Fig. 5). Non-HMV isolates produced significantly greater biofilm at time point 24 h (Fig. 5) when compared to HMV isolates.

Similar susceptibility to disinfectants was detected for planktonic HMV and non-HMV *K. pneumoniae* when exposed for 0.5 and 24 h (Tables 2 and 3) with similar MICs and MBCs values regardless of phenotype. The MBECs of chloramine T, ethanol, hydrogen peroxide, and sodium hypochlorite for HMV isolates were significantly higher than those for non-HMV when exposed to disinfectants for 0.5 h ($p = 0.0388$; $p < 0.001$; $p < 0.001$ and $p < 0.001$, respectively). Additionally, MBEC values for HMV and non-HMV isolates were significantly higher than MIC and MBC values when exposed to disinfectants for 0.5 h ($p < 0.05$) (Tables 2 and 3). Similar MBEC values were detected after 24 h exposure regardless of phenotype (Tables 2 and 3).

4. Discussion

Klebsiella pneumoniae infections are frequently encountered in captive marine mammals, particularly in hospitalized animals. Although both HMV and non-HMV *K. pneumoniae* isolates can be associated with diseases, HMV isolates have particularly been associated with poor prognosis and mortality (Jang et al., 2010). However, the association of HMV and hypervirulence remains unclear (Catalán-Nájera et al., 2017) and further work elucidating this relationship is warranted. Although several studies have compared biofilm formation of different phenotypes and genotypes of *K. pneumoniae* isolates recovered from humans (Singh et al., 2019), no comparison amongst isolates recovered from marine mammals has been conducted to the authors' knowledge. Since biofilm formation allows increased resistance to shear stress, increased nutrient uptake, enhanced resistance to antimicrobials and oxidative radicals, and avoidance of phagocytosis, investigating the capability of isolates recovered from marine mammals to form biofilms is warranted to gain a better understanding on their biology (Bjarnsholt et al., 2013; Vuotto et al., 2014).

Most of the characterized HMV *K. pneumoniae* recovered from marine mammals in CA have been found to be string test positive, capsular type 2 (K2) and *rmpA* positive (Jang et al., 2010; Whitaker et al., 2018). Thus, initially we aimed to gain a better understaning on the environmental persistence of this emerging phenotype comparing the capacity of HMV and non-HMV isolates to persist in fresh and marine water at two different temperatures encountered by marine mammals in California. Additionally, since this bacterium is known to

Table 2
Minimum inhibitory concentration (MIC), minimum biocidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) of various disinfectants to hypermucoviscous (HMV) and non-HMV *Klebsiella pneumoniae* isolates after 30 min (0.5 h) incubation. 100 mM sodium hypochlorite = 7130 mg/L available chlorine.

Disinfectant	Isolate Phenotype	MIC		MBC		MBEC	
		Range	MIC 90	Range	MBC 90	Range	MBEC 90
Chloramine-T (mg/L)	HMV	1.25	1.25	2.5	2.5	5	5
	NHM	1.25	1.25	2.5	2.5	2.5	2.5
Available chlorine (mg/L)	HMV	223-445	445	445-891	891	1783-3565	3565
	NHM	445	445	891	891	1783	1783
Ethanol (%)	HMV	6.25	6.25	25-50	50	25-50	50
	NHM	6.25	6.25	25	25	25	25
Hydrogen peroxide (%)	HMV	0.01	0.01	0.02	0.02	> 3	> 3
	NHM	0.02-0.01	0.01	0.02	0.02	0.19-0.38	0.38

produce biofilms in different environments, we compared the two different phenotypes in their biofilm formation capacity. Our results suggest that non-HMV *K. pneumoniae* grow significantly faster ($p < 0.0001$) in broth media, persist longer in fresh and marine water microcosoms at 10 and 20 °C, and produce larger biofilms when compared to HMV *K. pneumoniae* isolates.

Previous studies provided definitive association between specific genes and proteins to biofilm formation (Clegg and Murphy, 2016; Boddicker et al., 2006; Wu et al., 2011). Transcriptional regulators, sugar phosphotransferases, and genes encoding type 1 fimbriae, type 3 fimbriae and capsule have been identified as important genes influencing biofilm formation. Wu et al. (2011) identified the *treC* and *sugE* as not only important genes associated with biofilm formation, but also important influencing capsule production and mucoviscosity. In contrast to our findings, Wu et al. (2011) found positive correlation between HMV, capsule production and biofilm formation. However, Wu et al. (2011) utilized community-acquired pyogenic liver abscess (PLA)-associated HMV isolates when comparing to non-HMV isolates recovered from patients with sepsis but without PLA or other metastatic infections in other tissue (non-tissue-invasive). PLA-associated HMV isolates were previously found to be *magA* positive, a marker of capsular type 1 (K1) HMV *K. pneumoniae* (Fang et al., 2004). Huang et al. (2014) associated lower amounts of capsule to greater amounts of biofilm using two *K. pneumoniae* knockout mutants, the first one with the entire gene cluster responsible for biosynthesis of the extracellular polysaccharide capsule deleted and the second one with the capsule export subsystem deleted. Future studies investigating the specific make-up of the extracellular polysaccharide capsule present in the K2 HMV *K. pneumoniae* isolates recovered from marine mammals and its association with in-vitro and in-vivo biofilms are thus warranted to gain a better understanding of this possible association. This information is also important to gain a better understanding of the pathogenesis of infection in marine mammals, as a greater amount of capsular polysaccharide produced by HMV *K. pneumoniae* has also been associated with greater resistance against the bactericidal activity of complement (Alvarez et al., 2000; Tomas et al., 1986; Merino et al., 1992), increased virulence (Cox et al., 2015; Soto et al., 2016), and even anti-biofilm activity against other microorganisms (Goncalves Mdos et al., 2014).

The final aim of this study was to identify the biocide efficacy of four frequently used disinfectants in aquatic facilities against *K. pneumoniae.* Chloramine T and ethanol at 5 mg/L and 50% v/v for 0.5 h, respectively provided efficient biocide concentrations against the planktonic and biofilm *K. pneumoniae,* regardless of their phenotype (Yanong and Erlacher-Reid, 2012; Rutala et al., 2008). However, chlorine and hydrogen peroxide at the recommended concentration of 650–675 active free chlorine or 3% H_2O_2, for 30 min were ineffective in eradication of the biofilm formed by HMV *K. pneumoniae* (Table 2). For these organisms, the efficacy of H_2O_2 increased with longer exposure time; however, free-chlorine appears ineffective at eradicating *K. pneumoniae* biofilms (Table 3).

Careful interpretation of the results is needed as the disinfectant efficacy against the planktonic and biofilm forms was tested under specific and controlled abiotic conditions. Goel and Bouwer (2004) reported that nutrient media, essential inorganic nutrient concentration and temperature during bacterial growth can have an effect on chlorine susceptibility testing. Additionally, it is well accepted that factors besides disinfectant concentration and contact time, such as temperature, pH, and chemical composition of water can vary the susceptibility of microbes to specific compounds. In our experiments, we tested the disinfectant susceptibility at only 25 °C using MHB. Mueller-Hinton broth was chosen as it is the CLSI recommended medium for susceptibility testing of rapidly growing, facultative aerobic non-fastidious bacteria such as *K. pneumoniae*. Additionally, this medium has good batch-to-batch reproducibility and contains few drug-inhibitors CLSI (Clinical and Laboratory Standards Institute), 2014. Recently, it has been proposed that combinations of oxidizing compounds such as sodium hypochlorite and hydrogen peroxide in the presence of copper sulfate (Olmedo et al., 2015), and novel compounds such as chlorhexidine conjugated gold nanoparticles can be not only used to eradicate *K. pneumoniae*, but to prevent biofilm formation (Ahmed et al., 2016).

Table 3
Minimum inhibitory concentration (MIC), minimum biocidal concentration (MBC) and minimum biofilm eradication concentration (MBEC) of various disinfectants to hypermucoviscous (HMV) and non-HMV *Klebsiella pneumoniae* isolates after 24 h incubation.

Disinfectant	Isolate Phenotype	MIC		MBC		MBEC	
		Range	MIC 90	Range	MBC 90	Range	MBEC 90
Chloramine-T (mg/L)	HMV	1-1.25	1.25	1.25-2.5	2.5	2.5	2.5
	NHM	1.25	1.25	2.5	2.5	2.5	2.5
Available chlorine (mg/L)	HMV	445	445	891	891	891	891
	NHM	891	891	891	891	891	891
Ethanol (%)	HMV	6.25-12.5	12.5	25	25	25	25
	NHM	12.5	12.5	25	25	25	25
Hydrogen peroxide (%)	HMV	0.01-0.09	0.09	0.02-0.19	0.19	0.05-0.19	0.19
	NHM	0.09	0.09	0.09	0.09	0.19	0.19

Based on this, further research investigating disinfectant susceptibility under different abiotic conditions and using different disinfectant combinations or novel drugs appropriate for facilities housing marine mammals or other wildlife, is warranted.

5. Conclusions

Our results suggest that non-HMV *K. pneumoniae* environmental fitness, including faster growth, greater biofilm formation and longer persistence in the environment can be associated with non-host associated persistence and growth. Hypermucoviscous K2 *K. pneumoniae* on the other hand present greater mucoviscosity than the tested non-HMV and are capable of forming biofilms; however, they don't appear as fit as non-HMV isolates to persist in fresh and sea water at the two tested temperatures. Future studies investigating the virulence of the different phenotypes and the presence of an *in-vivo* biofilm in marine mammals are warranted to better understand the pathogenesis of this emergent pathogen. Additionally, the formation of *K. pneumoniae* biofilm in aquariums and veterinary hospitals, and their interaction with other microorganisms should be further studied to improve disinfection protocols.

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