In-vitro virulence and genetic diversity of hypermucoviscous K2 serotype *Klebsiella pneumoniae* isolates from California sea lions (*Zalophus californianus*)

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INTRODUCTION

Klebsiella pneumoniae

- Gram-negative, aerobic, non-motile, encapsulated bacillus within the *Enterobacteriaceae* family[1]
- Opportunistic pathogen with a broad host range
- In humans a hypervirulent strain has been associated with a hypermucoviscous (HMV) phenotype, but this has not yet been established in animal hosts[2]
- Multiple capsular serotypes exist within the HMV phenotype, including K1, K2, and K5 serotypes
- Sudden unexplained increase in the prevalence of HMV K2 *K. pneumoniae* in marine mammals, but especially in California sea lions (*Zalophus californianus*) (CSLs) – from 0.92% in 2017 to 7.2% in 2022 (Fig. 1.)[3]
- Causes klebsiellosis, contributing to disease and standings in marine mammals [4] (Fig. 2. and 3.)

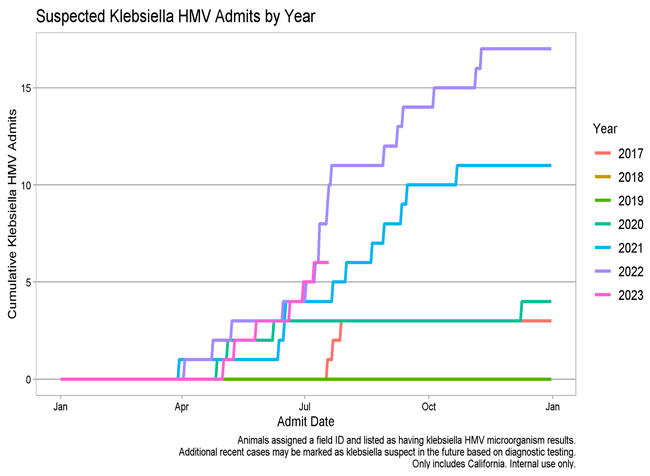

Fig. 1. Suspected HMV klebsiellosis admits to The Marine Mammal Center (TMMC)[3]
Incidence of suspected HMV *K. pneumoniae* admits to The Marine Mammal Center, a marine mammal hospital and rehabilitation facility in Sausalito, CA, from 2017 to 2023. There has been an increase in the number of HMV cases, as well as an admittance of cases earlier in the year, in recent years compared to 2017. Courtesy of Dr. Pádraig Duignan and Carlos Rios.

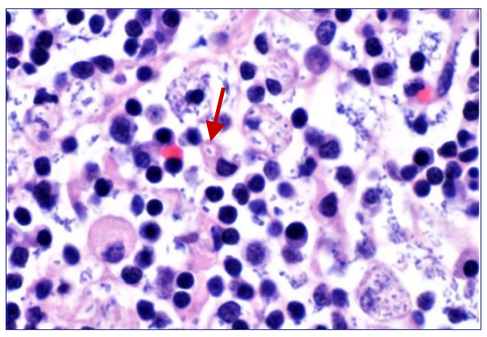

Fig. 2. Lymph node histopathology
Encapsulated bacterial rods within the cytoplasm of inflammatory cells in the lymph node of an affected Southern sea otter (SSO).
Photo courtesy of Dr. Melissa Miller.

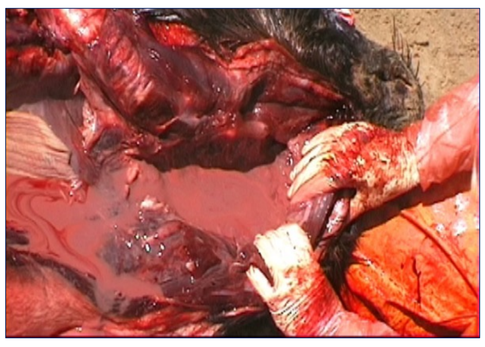

Fig. 3. Perilaryngeal abscess
K. pneumoniae causes widespread infection in marine mammals, resulting in conditions such as pleuritis, suppurative pneumonia, and abscesses.
Photo courtesy of Dr. Pádraig Duignan

AIMS

1. Investigate the genetic diversity and virulence gene profiles of HMV K2 *K. pneumoniae* isolates recovered from diseased CSLs in California between 2020 to 2023.

   Hypothesis: HMV K2 strains isolated from CSLs are genetically diverse following housekeeping genes and virulence factor typing schemes.

2. Investigate the virulence of representative HMV K2 *K. pneumoniae* genotypes through *in-vitro* challenge models of kidney epithelial cells extracted from an African green monkey (*Chlorocebus sabaeus*) (Vero cell line).

   Hypothesis: HMV K2 strains isolated from CSLs are significantly more cytotoxic to mammalian cells (Vero cells) than non-HMV strains recovered from marine mammals.

METHODS

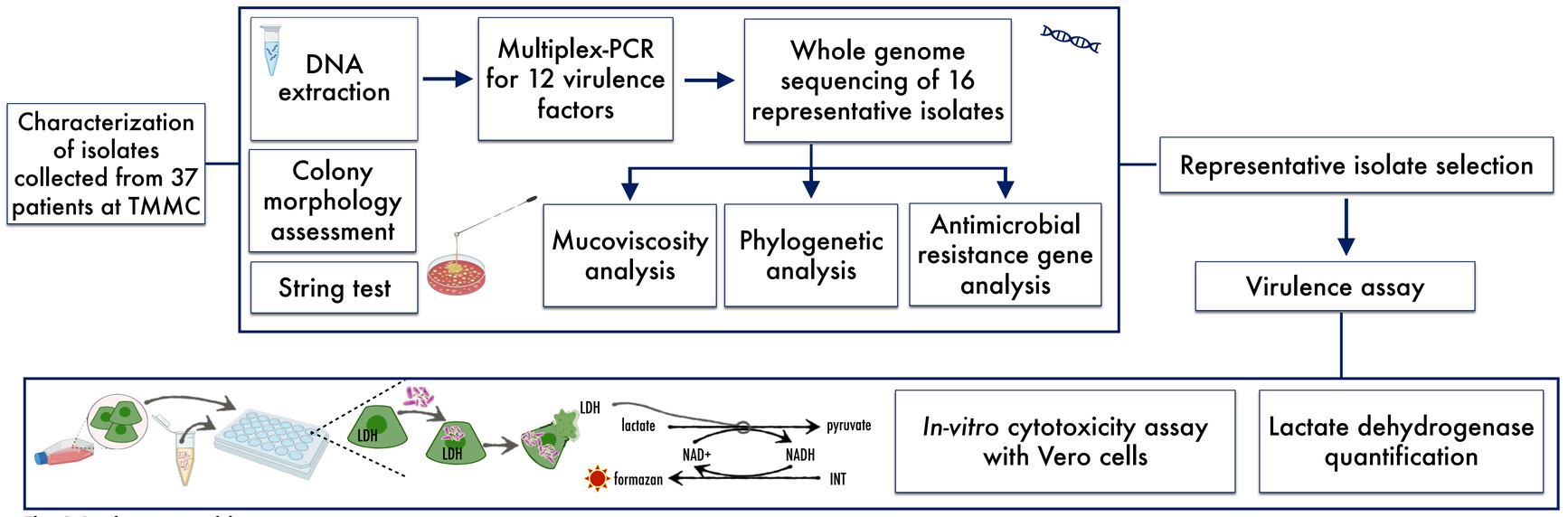

Fig. 4. In-vitro cytotoxicity
Vero cells grown for 24 hours at 37°C to confluence, inoculated with an MOI of 1:100 for 3hrs following the Cytotox96® Non-Radioactive Cytotoxicity Assay protocol by Promega.

ISOLATE CHARACTERIZATION RESULTS

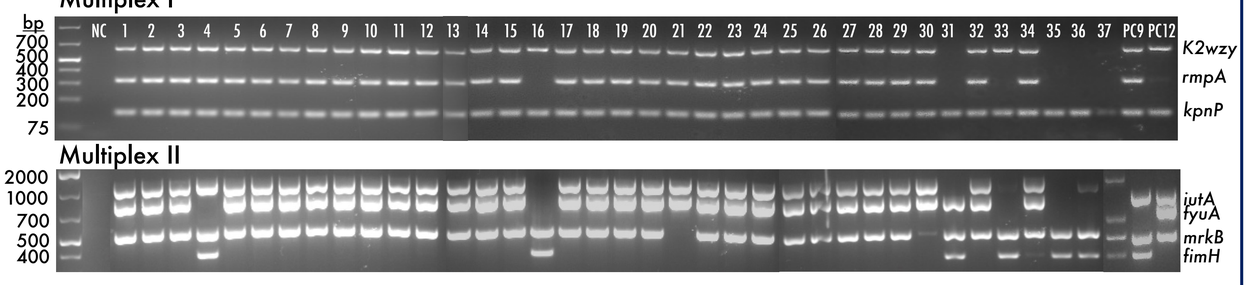

Fig. 5. Multiplex-PCR
Multiplex-PCR showing all isolates positive for the *kpnP* gene, confirming all isolates were *K. pneumoniae*. 31/37 isolates were positive for the *rmpA* gene, a regulator gene for the mucoid phenotype. 33/37 isolates were positive for the *K2wzy* gene, indicating the K2 capsular serotype. Within the K2 serotype, four different virulence profiles were observed and characterized based on the presence of virulence genes: *iutA* (siderophore aerobactin), *fyuA* (siderophore yersiniabactin), *mrkB* (type 3 fimbriae promoting biofilm formation), and *fimH* (type 1 fimbriae promoting adhesion to host epithelial cells)[5]. 28 K2 isolates belong to Clade I (*iutA*$^+$/*fyuA*$^+$/*mrkB*$^+$/*fimH*$^-$), 1 K2 isolate (#4) belongs to Clade II (*iutA*$^+$/*fyuA*$^-$/*mrkB*$^+$/*fimH*$^+$), 2 K2 isolates (#16, #33) belong to Clade III (*iutA*$^-$/*fyuA*$^-$/*mrkB*$^+$/*fimH*$^+$), and 2 K2 isolates (#21, #30) belong to Clade IV (*iutA*$^+$/*fyuA*$^+$/*mrkB*$^-$/*fimH*$^-$). The remaining 4 isolates (#31, #35, #36, #37) were not HMV and did not have capsular serotype K1, K2, or K5. Positive control isolate PC9 belongs to Clade II; positive control isolate PC12 belongs to Clade I.

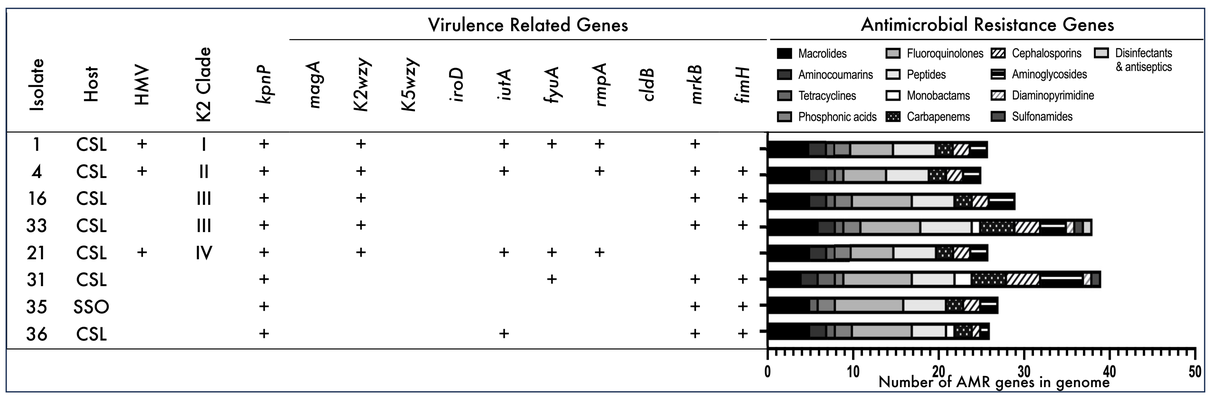

Fig. 6. Isolate Characterization
K2 serotype clades are distinguished by the presence of the following virulence related genes: *iutA* (aerobactin), *fyuA* (yersiniabactin), *mrkB* (type 3 fimbriae), and *fimH* (type 1 fimbriae)[5]. All sequenced isolates had identical AMR gene profiles to the following antimicrobial classes: glycopeptides (*vanG*), nitroimidazoles (*msbA*), elfamycins (*E. coli* EF-Tu mutants), and cephamycins (*H. influenzae* PBP3).

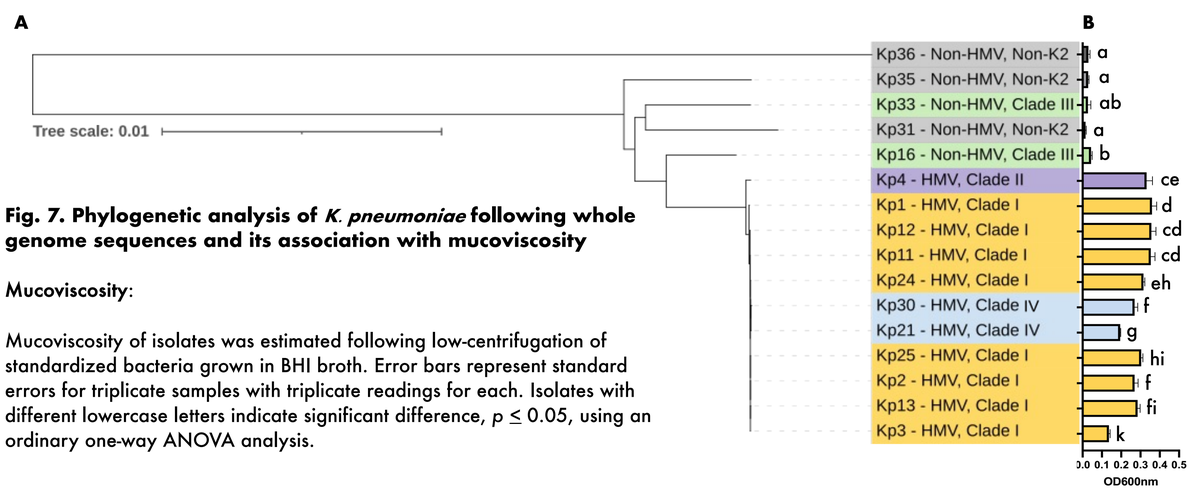

Fig. 7. Phylogenetic analysis of *K. pneumoniae* following whole genome sequences and its association with mucoviscosity

Mucoviscosity:

Mucoviscosity of isolates was estimated following low-centrifugation of standardized bacteria grown in BHI broth. Error bars represent standard errors for triplicate samples with triplicate readings for each. Isolates with different lowercase letters indicate significant difference, $p \leq 0.05$, using an ordinary one-way ANOVA analysis.

CYTOTOXICITY RESULTS

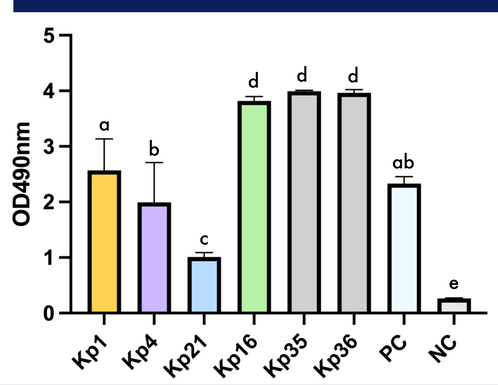

Fig. 8. *In-vitro* Cytotoxicity in Vero cells
Cytotoxicity of *K. pneumoniae* to Vero cells was measured quantifying the release of lactate dehydrogenase (LDH) by challenged Vero cells at a multiplicity of infection of 1:100 (Vero cells:bacteria).

- Non-HMV isolates generated significantly greater cytotoxicity when compared to HMV isolates.
- Error bars represent standard errors for triplicate samples with duplicate readings for each. Isolates with different lowercase letters indicate significant difference, $p \leq 0.05$, using an ordinary one-way ANOVA analysis.

Fig. 9. String Test
31/37 isolates were positive for the string test, indicating the HMV phenotype. All HMV isolates were of the K2 serotype. Image shows a positive string test performed on an HMV K2 Clade I isolate.

CONCLUSION

Multiple clades exist within the HMV K2 serotype of *K. pneumoniae*, with genetic diversity in virulence factors, antimicrobial resistance genes, and mucoviscosity.

Investigation of *in-vitro* virulence of HMV versus non-HMV isolates was hindered by the HMV phenotype's decreased adhesion capacity to mammalian epithelial cells and faster growth of non-HMV strains.

Further studies exploring virulence should employ invasion assays or *in-vivo* methods.

ACKNOWLEDGEMENTS & REFERENCES

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