Wastewater treatment-adapted organisms as a model to understand environmental AMR

Markus Gänzle[1,2,3], Simon J. G. Otto[1,2,3,4], Candis Scott[1,3], Paul Stothard[3,5], Norman F. Neumann[1,3]

Work Package 9A

Background

- ➔ Wastewater and drinking water treatment are critical public health intervention control measures for infectious disease
- ➔ Recent reports indicate that certain pathogenic bacteria may be evolving resistance to common water disinfection processes such as chlorine, UV and heat, and that many of these pathogens may also carry a large repertoire of antimicrobial resistance genes (ARGs)
- ➔ Zhi et al. (2020) described the presence of extraintestinal pathogenic *Escherichia coli* (ExPEC) from chlorinated sewage that were resistant to antimicrobials and survived water-treatment
- ➔ This project aims to explore whether other bacterial pathogens, such as *Klebsiella pneumoniae,* may be also evolving resistance to water treatment
- ➔ *K. pneumoniae* naturally inhabit a variety of environmental habitats, including wastewater (Perschine et al. 2013)
- ➔ Clinically, *K. pneumoniae* is well known as a nosocomial opportunistic pathogen.
- ➔ Over the last few decades, a "hypervirulent" pathotype of *K. pneumoniae* has emerged, with a distinct clinical presentation and higher virulence compared to classical *K. pneumoniae* (Russo and Marr 2019)
- ➔ Hypervirulent strains are commonly identified by their tendency to display a highly mucoviscous phenotype when plated on agar

Research Questions

- Are *K. pneumoniae* strains from Alberta wastewater treatment plants able to survive and grow after exposure to disinfection methods commonly used in wastewater treatment (specifically chlorine and heat treatment)?
- Do these wastewater *K. pneumoniae* strains display the hypermucoviscous phenotype commonly associated with highly pathogenic 'hypervirulent' *K. pneumoniae*?

Materials and Methods

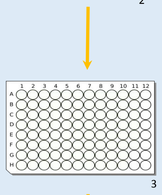
Raw sewage samples were taken from various Alberta WWTPs and treated with sufficient chlorine to reduce *E. coli* populations by 99.99% as described in Zhi et al. (2020). 26 *K. pneumoniae* isolates were biochemically identified from these samples using API 20E test strips (bioMérieux). These 26 chlorine-resistant isolates were tested for heat resistance and hypermucoviscosity.

Heat Resistance Assay

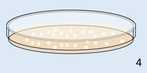
Klebsiella pneumoniae isolates were suspended in autoclaved distilled water (ADW)

Isolates were heated to 58°C for 5 minutes using an ABI 2720 Thermal Cycler.

Heated samples were serially diluted down to a factor of 10^{-6}.



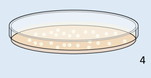
Dilutions were spotted onto TSA plates and incubated at 37°C for 24 hours to determine the growth capacity of treated isolates.



String Test

Klebsiella pneumoniae isolates were suspended in tryptic soy broth

Isolates were streaked onto blood agar plates and incubated at 37°C for 24 hours.



Colonies were stretched vertically using an inoculation loop, with a positive result recorded if strings of length >5mm formed.

Control Strains

E. coli ATCC 25922: Heat–susceptible lab strain, used as negative control

E. coli WW10: Heat-resistant wastewater strain, used as positive control

K. pneumoniae ATCC 700603: Heat-susceptible lab strain, used as negative control

Results

Out of 26 tested wastewater *K. pneumoniae* isolates, 18 (69.23%) displayed some level of resistance to the 58°C for 5-minute treatment, defined as the ability to produce a monolayer of cells at concentrations at least 10-fold lower than the negative control strains (Figure 1). Of these, 8 isolates (30.08% of total) grew at concentrations of at least 10^4-fold lower than the control strains, making them highly resistant to the heat treatment.

5 of the 26 wastewater *K. pneumoniae* isolates (19.23%) had a positive string test for hypermucoviscosity. All 5 of these isolates were at least somewhat heat resistant, and three of them (CL 2301, PC 1208, and BB 193) were highly heat resistant.

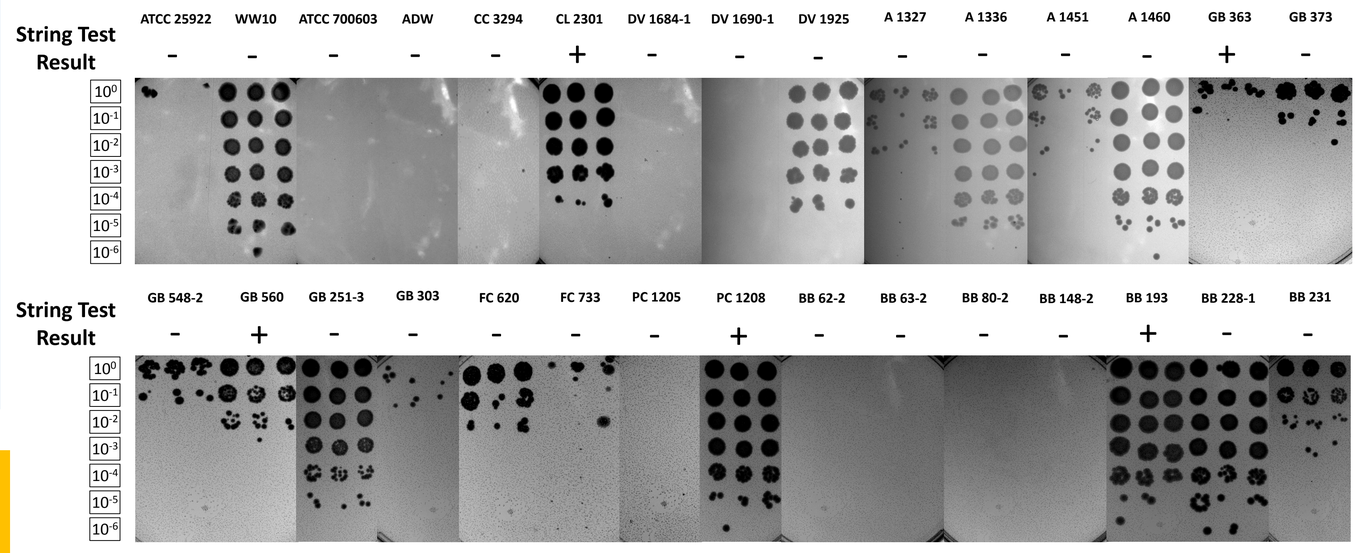


Figure 1: Heat resistances and mucoviscosity phenotypes of wastewater *Klebsiella pneumoniae* isolates as well as *E. coli* and *K. pneumoniae* control strains. Bacterial strains were grown in tryptic soy broth for 24 hours at 37°C, before being washed and diluted by a factor of 10 in autoclaved distilled water (ADW). The samples were then heated to 58°C for 5 minutes using an ABI 2720 thermal cycler, before being serially diluted up to a factor of 10^{-6}. All dilutions were then spotted onto tryptic soy agar plates, incubated at 37°C for 24 hours, and then photographed. The isolates were also streaked onto BAP and incubated at 37°C for 24 hours. BAP colonies were then stretched using an inoculation loop to determine their mucoviscosity. Isolates which could be stretched to a height of >5 mm were recorded as having a positive string test. ATCC 25922 is a heat-susceptible *E. coli* strain. WW10 is a highly heat-resistant *E. coli* wastewater strain. ATCC 700603 is a heat-susceptible *K. pneumoniae*. All other strains are wastewater *K. pneumoniae*.

Discussion

<u>Treatment Resistance</u>:

- ➔ Based on our methods, the 26 strains of *K. pneumoniae* isolated from wastewater represented a chlorine-resistant population
- ➔ Many of the chlorine-resistant strains also displayed a heat-resistant phenotype
- ➔ These results suggest that some strains of *K. pneumoniae,* like *E. coli*, may be well adapted to survive wastewater treatment disinfection processes

<u>Pathogenicity</u>:

- ➔ 5 of the chlorine/heat resistant isolates were hypermucoviscous, suggesting that they possess a 'hypervirulent' phenotype
- ➔ The release of water treatment-resistant, hypervirulent strains of *K. pneumoniae* in the effluents of wastewater treatment plants could pose a significant public health risk

<u>Limitations</u>:

- ➔ Many of the heat resistance results presented here have not yet been replicated in triplicate
- ➔ Although used to presumptively identify clinically-relevant hypervirulent strains, the hypermucoviscosity test is not confirmatory for pathogenicity (Russo et al. 2018), and additional work is needed to address virulence and the pathogenic nature of these strains

Future Directions

- ➔ Screening the wastewater *K. pneumoniae* for their antimicrobial susceptibility will help identify links between treatment resistance and AMR in these strains
- ➔ PCR screening and whole-genome sequencing will allow for the identification of virulence factors and ARGs, which will allow for a more conclusive answer to questions of pathogenicity and AMR in the isolated strains
- ➔ Further replication of the results presented in this poster are also anticipated and will help to reduce random error and enhance reliability in the heat resistance and string test data

Conclusions

K. pneumoniae isolates possessing a chlorine and heat resistant phenotype were isolated from Alberta wastewater treatment plants. Some isolates also presented with a hypermucoid phenotype – a characteristic commonly found in hypervirulent clinical strains of the bacteria. Collectively, the data suggest that like *E. coli*, highly pathogenic *K. pneumoniae* strains may be evolving resistance to wastewater treatment.

References

Russo, T. A., & Marr, C. M. (2019). Hypervirulent Klebsiella pneumoniae. *Clinical Microbiology Reviews*, *32*(3), e00001-19. https://doi.org/10.1128/CMR.00001-19

Russo, T. A., Olson, R., Fang, C.-T., Stoesser, N., Miller, M., MacDonald, U., Hutson, A., Barker, J. H., La Hoz, R. M., Johnson, J. R., & for the Hypervirulent Klebsiella pneumoniae Investigator Group (HVKPIG). (2018). Identification of Biomarkers for Differentiation of Hypervirulent Klebsiella pneumoniae from Classical K. pneumoniae. *Journal of Clinical Microbiology*, *56*(9), e00776-18. https://doi.org/10.1128/JCM.00776-18

Teban-Man, A., Farkas, A., Baricz, A., Hegedus, A., Szekeres, E., Pârvu, M., & Coman, C. (2021). Wastewaters, with or without Hospital Contribution, Harbour MDR, Carbapenemase-Producing, but Not Hypervirulent Klebsiella pneumoniae. *Antibiotics*, *10*(4), 361. https://doi.org/10.3390/antibiotics10040361

Wang, G., Zhao, G., Chao, X., Xie, L., & Wang, H. (2020). The Characteristic of Virulence, Biofilm and Antibiotic Resistance of Klebsiella pneumoniae. *International Journal of Environmental Research and Public Health*, *17*(17), 6278. https://doi.org/10.3390/ijerph17176278

Zhi, S., Stothard, P., Banting, G., Scott, C., Huntley, K., Ryu, K., Otto, S., Ashbolt, N., Checkley, S., Dong, T., Ruecker, N. J., & Neumann, N. F. (2020). Characterization of water treatment-resistant and multidrug-resistant urinary pathogenic Escherichia coli in treated wastewater. *Water Research*, *182*, 115827. https://doi.org/10.1016/j.watres.2020.115827

Image Sources

1. 201105 microtube close2 by DBCLS / CC BY 4.0, https://commons.wikimedia.org/wiki/File:201105_microtube_close2.png
2. Thermal cycler by DBCLS / CC BY 3.0, https://commons.wikimedia.org/wiki/File:Thermal_cycler.png
3. Edited version of 96 well Microplate PNG icon by sewa2k, https://www.iconspng.com/image/22525/96-well-microplate
4. Dish by DBCLS / CC BY 3.0, https://commons.wikimedia.org/wiki/File:Dish.png

All other images were taken by MG