

ELUCIDATE THE HYPOTHESIS FOR EACH EXPERIMENT:

Overall Research Question: Are phages mediating ARG transduction between bacterial cells and contributing to ARG dissemination in the environment, and if so what ARGs are present in water samples collected from the Han River?

Overall Hypothesis: Since bacteriophages in the environment have been known to harbor ARGs (Calero-Cáceres and Balcázar, 2019) and have the ability to transfer these ARGs between bacteria (von Wintersdorff, 2016), if viral samples are collected from the Han River contain ARGs that are being transferred between bacteria by bacteriophages, then sequence analyses of the samples from the Han River should reveal ARGs that are present in both bacteriophages and bacteria, and these ARGs should result in reduced susceptibility to antibiotics.

List of Experiments for each figure:

1. Collection of Han River genome samples that were sequenced and analyzed for their taxonomic distribution of viral and bacterial family/class level
 - a. Descriptive study
2. Sequencing maps of viral contigs structure of H4-C441, H1-C74, H4-C244, and H4-C367, all of which were found to harbor β -lactamase genes HRV-1 or HRVM-1.
 - a. Descriptive study
3. Phylogenetic tree analysis of HRV-1 viral contigs with representative enzymes of classes A, C, and D β -lactamases to determine which group the viral contig belongs to
 - a. Descriptive study
4. Phylogenetic tree analysis of HRVM-1 viral contigs with representative enzymes of subclasses B1, B2, and B3 to see which of these groups the viral contig belongs to
 - a. Descriptive study
5. Minimum inhibitory concentration (MICs) assays of β -lactams for *Escherichia coli* transformants producing HRV-1 or HRVM-1 viral contigs
 - a. Experimental study
6. Genomic maps of the Han River bacterial metagenomic contigs that harbor homologous ORFs to HRV-1 or HRVM-1, as well as a mapping of their sequence similarity to the viral contigs
 - a. Descriptive study

Hypothesis for Each Experiment:

Experiment 1/Figure 1:

Since bacterial and viral genomes, along with ARGs, have been known to be found in all kinds of diverse marine environments (Calero-Cáceres and Balcázar, 2019), if six surface water samples were collected from the Han River and contain ARGs, then the water's composition should comprise of various types of bacteria and viruses harboring ARGs in their genome.

Experiment 2/Figure 2:

Since ARGs have been known to be transferred via phage-related mobile elements (Brown et al., 2015) and phages are known to be found in aquatic environments (Calero-Cáceres and Balcázar, 2019), if viral contig samples from water samples of the Han River contain ARGs, then a genome analysis should reveal sequence regions harboring ARGs.

Experiment 3/Figure 3:

Since sequence analysis of the ORF H4-C441- ORF28 carried the conserved active sites and motifs specific to class A β -lactamases standards (Ambler et al., 1991), if ORF H4-C441- ORF28 does belong to class A β -lactamases, then the ORF should group into a clade of class A β -lactamases in a phylogenetic tree analysis.

Experiment 4/Figure 4:

Since sequence analysis of the ORFs, H1- C74-ORF21, H4-C244-ORF21, and H4-C367-ORF18 revealed conserved active sites and motifs specific to metallo β -lactamases (Ambler et al., 1991), if the ORFs, H1- C74-ORF21, H4-C244-ORF21, and H4-C367-ORF18 do belong to a particular subclass of metallo β -lactamases, then the ORFs should group into a distinct subclass of metallo β -lactamases in a phylogenetic analysis.

Experiment 5/Figure 5:

Since ARGs belonging to the β -lactamases classes have been known to confer resistance to the antibiotic lactamase (Blanco et al., 2020), if HRV-1 and HRVM-1 genes found in the viral contig samples do provide antibiotic resistance, then expressing these genes within *E. coli* should increase the level of resistance to antibiotics when compared to controls.

Experiment 6/Figure 6:

Since ARGs have been known to spread via horizontal gene transfer in microbial ecosystems (von Wintersdorff, 2016), if ARGs are being transferred from phages to bacteria in the Han River of South Korea via horizontal gene transfer, then analysis of the bacterial contigs from Han River Samples should contain ARG sequences with high similarity to ARG sequences found in viral contigs isolated from the same Han River.

References for each of the hypotheses:

- Ambler, R. P., Coulson, A. F., Frère, J. M., Ghuysen, J. M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G., & Waley, S. G. (1991). A standard numbering scheme for the class A beta-lactamases. *Biochemical Journal*, 276(Pt 1), 269–270.
- Blanco-Picazo, P., Roscales, G., Toribio-Avedillo, D., Gómez-Gómez, C., Avila, C., Ballesté, E., Muniesa, M., & Rodríguez-Rubio, L. (2020). Antibiotic Resistance Genes in Phage Particles from Antarctic and Mediterranean Seawater Ecosystems. *Microorganisms*, 8(9). <https://doi.org/10.3390/microorganisms8091293>
- Brown-Jaque, M., Calero-Cáceres, W., & Muniesa, M. (2015). Transfer of antibiotic-resistance genes via phage-related mobile elements. *Plasmid*, 79, 1–7. <https://doi.org/10.1016/j.plasmid.2015.01.001>
- Calero-Cáceres, W., & Balcázar, J. L. (2019). Antibiotic resistance genes in bacteriophages from diverse marine habitats. *The Science of the Total Environment*, 654, 452–455. <https://doi.org/10.1016/j.scitotenv.2018.11.166>
- von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. M., & Wolffs, P. F. G. (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in Microbiology*, 7. <https://doi.org/10.3389/fmicb.2016.00173>