Phenotypic characterization of marine phage cocktail from Batangas Philippines against Multi-Drug Resistant *Pseudomonas aeruginosa*, Methicillin Resistant *Staphylococcus aureus*, and *Vibrio cholerae*

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***Abstract*— Due to lack of local studies dealing with the morphological characterization of marine phages in the Philippines, the emphasis has been put towards addressing the stated insufficiency. The study sought to phenotypically characterize marine isolate phage cocktail against three microorganisms namely MDR *Pseudomonas aeruginosa*, Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Vibrio cholerae*. The phenotypic characterization was done through Transmission Electron Microscopy (TEM) and stability tests to various physical agents such as the pH, temperature and ultraviolet light . Furthermore, the phage titer for each phage cocktail was initially calculated and presented in the unit Plaque Forming Unit per milliliters (PFU/mL). The TEM micrographs results revealed that the phage cocktail against MDR *Pseudomonas aeruginosa* contains phages belonging to families *Fuselloviridae, Cystoviridae, Leviviridae Siphoviridae, Myoviridae, Guttaviridae* and *Corticoviridae*. On the other hand, the phage cocktail against Methicillin Resistant *Staphylococcus aureus* contains phages belonging to the families *Lipothrixviridae, Cystoviridae, Guttaviridae, Siphoviridae, Myoviridae* and *Podoviridae*. And lastly, the phage cocktail against *Vibrio cholerae* contains phages belonging to the families *Siphoviridae, Myoviridae, Podoviridae* and *Guttaviridae*. Furthermore, stability tests to various physical agents such as the pH, temperature and ultraviolet light was conducted to aid the findings obtained from the TEM micrographs.**

***Keywords*—Antimicrobial resistance, Marine phage, Myoviridae, Siphoviridae**

I. Introduction

Marine bacteriophages, in spite of their ubiquitous nature in pelagic ecosystems have only been extensively studied for about thirty years. Thus, the information about the architectural features of the phages is limited and substantially less than the terrestrial phages (Kuznetsov et al., 2013).

There are numerous purposes of phage characterization and one of the highlights is the identification of phages with industrial and therapeutic applications. There has been a recent resurgence of attention into bacteriophages due to the growing frequency of antibiotic resistant and virulent bacterial pathogens (Lu & Koeris, 2011). Phage therapy makes a good candidate as an antibiotic alternative due to its effectiveness against drug resistant bacteria (Kutter & Sulakvelidze, 2005).

In unexplored aquatic areas in the Philippines, there is a lack morphological analyses of phages and application against antibiotic resistant bacteria (Sulcius et al., 2011; Vital et al., 2014; D.C. Klinzing et al., 2015). Recent phage research and identification focuses on molecular and virus genome analyses, but the size of the genome can provide only an estimate of the rates of ecological interactions between phage and the host, as well as the synergistic or antagonistic relationships among phages. Phages containing the same size of genome may also exhibit different morphological forms. A study of Holmfeldt et al. in 2007 showed that two viruses with a similar genome size, exhibit variation in their morphological forms (Sulcius et al., 2011).

Thus in response to the problem, the researchers isolated and phenotypically characterize a phage cocktail that can infect three pathogenic bacteria namely Multi-Drug Resistant (MDR) *Pseudomonas aeruginosa*, Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Vibrio cholerae*.

II. METHODS

A. Bacterial Strains

The bacterial strains of MDR *Pseudomonas aeruginosa* and MRSA that served as bacteriophage host was obtained from the University's stock culture. It was maintained in nutrient broth medium until further use. The *Vibrio cholerae* which also served as a bacteriophage host, was purchased from UPLB Biotech culture collection. It was maintained in alkaline peptone water until further use also. Colonies of each bacterium was identified according to the standard microbiological techniques. Submit your manuscript

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electronically for review.

B. Phage Isolation

Seawater samples were collected 20 meters away from the coastline and 5 meters deep of Calatagan and Lemery, Batangas, Philippines. The samples were placed in clean glass bottles and were transported immediately into the laboratory for processing. The isolation of bacteriophage was adapted from the University of Pittsburg Phage Hunting Program Protocols. 5mL Seawater sample was mixed with 5mL of Phosphate Buffered Saline (PBS). Using a sterile syringe, the mixture was aspirated. A syringe filter (0.2 µm) was attached to the end of the syringe then the contents were expunged off into a sterile tube. 0.5 mL (500 µL) of each of the bacterial hosts (5-10- hour old MDR *Pseudomonas aeruginosa*, Methicillin Resistant *Staphylococcus aureus* and *Vibrio cholerae*) was added to a separate sterile 15 mL tube. Using a micropipettor, 50 µL of the filtered seawater sample was added to the tube containing each of the bacterial hosts. The suspensions were mixed and were allowed to sit for around 15 minutes to allow any phages that are present to adsorb to the bacteria. The growth of bacteriophage was then attained using Plaque Assay or Double Agar Overlay technique. Formed plaque were then harvested through agar block technique as per Benson, H., J. (2015) and re-suspended into its corresponding bacterial host suspension. After incubation, the suspensions were then filtered using 0.2 µm syringe filters to attain a phage filtrate or lysate.

C. Determination of Phage Titer

Aliquot of 10 µL lysate was serially diluted from 10-1 to 10-9 using PBS. 0.5 mL of bacterial host were then added to each of the dilution and left standing for 20 minutes for phage adsorption. Soft nutrient agar was prepared; it served as the top agar and was maintained in no less than 55°C. 4.5 mL of the soft agar was added to the tube containing the lysate and bacteria. The mixture was poured onto a solidified nutrient agar plate. Immediately, but gently, plates were swirled in a circular pattern to spread the agar evenly across the surface of the plate. Then, the plates were allowed to sit still after swirling until the agar has solidified (generally 15 minutes). It was then incubated at 37°C in ambient air conditions for 18 – 48 hours. After 18 hours, the plates were checked for plaques. The number of plaque was then recorded. The plates containing the plaques were photographed. The phage titer was computed using the formula: (Nouraldin et al., 2015).

$$Plaque\ Forming\ Unit(PFU)/mL = \frac{(Number\ of\ plaques)(Dilution\ Factor)}{Phage\ volume\ plated\ in\ mL}$$

D. Negative Staining for Trasmission Electron Microscopy

Formvar coated grid was picked-up with forceps and the forceps was pushed locking ring down that the grid is firmly held. Forceps were laid down on petri dish lid with tips extending over the edge with grid held coated-side up. A Pasteur pipet was used to add one drop of concentrated particulate suspension to the gridwith. Suspension was removed after 3 to 5 minutes by touching ragged torn edge of filter paper to the edge of forceps jaws until the grid surface is nearly dry. One drop of sodium phosphotungstate (PTA) solution was added to the grid. After 1 minute, the grid was dried as before with the ragged torn edge of filter paper. The grid was dried quickly and completely. The sample side of the grid was immediately touched to a clean piece of filter paper in the bottom of petri dish. A fresh piece of filter paper was slid down between the forceps to remove the dried grid onto the forceps and was placed on dry filter paper in the petri dish. The grid is dried for 15 minutes in the covered petri dish and was examined with a Transmission Electron Microscope model JEM 120.

E. pH Stability Test

To test for the pH stability, the procedure done by Jonczk et al., (2011) was followed with some modifications. A stock of PBS was prepared and the pH for each tubes were adjusted to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 and 13 by adding dropwise of 1 M of HCl or 0.5 M of NaOH until the desired pH was achieved. 10 µL of the phage lysate with 10^8 PFU/mL titer were mixed together with 90 µL pH adjusted PBS and stored at 2-8oC for 24 hours. Each of the lysate exposed to different pH were then subjected to plaque assay and incubated for 18-24 hours at 37^{o}C. After incubation, the number of plaques was counted and phage titer was calculated. Then changes in titer were recorded. All assays were carried out in triplicates.

F. Temperature Stability Test

To test for the temperature stability, the procedure done by Jonczk *et al.,* (2011) was followed with some modifications. Phage lysate aliquot with 10^7 PFU/mL titer were obtained and incubated at various temperature. 1 mL of phage solution was distributed to each of the microcentrifuge tubes labeled with their corresponding incubation temperature. The tubes were incubated for 1 week at the following temperatures: -20^{o}C, 0^{o}C and 2-4^{o}C; 24 hours incubation period: 25^{o}C and 37^{o}C; 10 minutes incubation period for 45^{o}C and 70^{o}C. After incubation, the lysates were subjected to plaque assay and the number of plaques was counted and the phage titer was calculated. All assays were carried out in triplicate.

G. Ultraviolet Light Stability Test

To test for the UV stability, the procedure done by Larcom, L. L., & Thaker, N. H. (1977) was followed with some modifications. Phage lysate with 108 PFU/mL titer were obtained and distributed to each of the 7 microcentrifuge tubes labeled with "30 sec'', "1 min", "15 min", "30 min", "1 hr", "12 hrs", "24 hrs" and "control". Control tube was kept at dark environment for the purpose of eliminating the possible effect of the presence of light. The 6 of 7 test tubes were exposed under UV Light (260 nm) at a prescribed length of time of exposure and at a distance of 25 centimeters. After exposure, lysates were subjected to plaque assay and incubated at 37oC for 18-24 hours. The number of plaques was counted and phage titer was calculated. All assays were carried out in triplicates.

III. Results And Discussion

A. Phage titer

The plaque assay of the phage cocktail against MDR *Pseudomonas aeruginosa* produced a maximum concentration of $3x10^{10}$ PFU/mL at $1x10^{-9}$ dilution. The lowest dilution that produced a titer was $1x10^{-3}$ with $2x10^{4}$ PFU/mL. However, at the dilution of $1x10^{-1}$ and $1x10^{-2}$, no plaque was formed with a concentration of 0 PFU/mL. According to Gratia and his collaborators, the phage lysis activity in general is present at much higher dilutions. Though, no lysis activity is usually detected in dilutions lower than 10^{-3} (Norris, J.R & Ribbons, D.W., 1972).

The plaque assay of the phage cocktail against Methicillin Resistant *Staphylococcus aureus* produced a maximum concentration of $1x10^{9}$ PFU/mL at $1x10^{-8}$ dilution. The lowest dilution that produced a titer was $1x10^{-3}$ with $1x10^{4}$ PFU/mL. Conversely, at the dilution of $1x10^{-1}$, $1x10^{-2}$, $1x10^{-4}$, $1x10^{-7}$ and $1x10^{-9}$ no plaque was formed with a concentration of 0 PFU/mL. According to Y.D. Lee & J.H. Park (2014) report, variation in the results of plaque assay may be due to the presence of several phages having different stability and characteristics (Y.D. Lee & J.H. Park, 2014).

Whereas, The initial plaque assay of the phage cocktail against *Vibrio cholerae* produced a maximum of concentration of $4x10^{10}$ PFU/mL at $1x10^{-9}$ dilution. The highest number of plaques formed was observed at the dilution of $1x10^{-7}$ with a concentration of $6x10^{8}$. The lowest dilution that produced a titer was $1x10^{-2}$ with $4x10^{3}$ PFU/mL. However, no plaque was formed at the dilution of $1x10^{-1}$ and $1x10^{-5}$, with a concentration of 0 PFU/mL.

Likewise, the result may be affected by the temperature, pH and period of incubation used in the procedure since phages have different thermal and pH stability (E. Jonczyk *et al*, 2011; Taj, M. K. *et al*, 2014).

B. Morphological Features of the Phage Cocktail against MDR Pseudomonas aeruginosa, Methicillin Resistant Staphylococcus aureus and Vibrio cholerae through Transmission Electron Microscopy

The TEM micrograph of the phage cocktail against MDR *Pseudomonas aeruginosa,* Methicillin Resistant *Staphylococcus aureus* and *Vibrio cholerae* presents different morphologic forms suggestive of different families under the Order Caudovirales, which involves tailed and tailless phages based on the TEM micrographs in comparison to the modification from Ackermann, H.-W., Bacteriophage taxonomy in 1987 (E. Kutter & A. Sulakvelidze, 2005).

The MDR *P. aeruginosa* phage cocktail is composed of tailless phages belong to the Family of *Fusselloviridae, Cystoviridae, Guttaviridae, Corticoviridae* and *Leviviridae*; and tailed phages under the family *Siphoviridae* and *Myoviridae*. On the other hand, the Methicillin Resistant *Staphylococcus aureus* phage cocktail is consists of tailless phages under the Family of *Lipothrixviridae, Cystoviridae* and *Guttaviridae*; while are tailed phages belong to *Siphoviridae, Myoviridae* and *Podoviridae* Family. Whereas, the phage cocktail against *Vibrio cholerae* is composed of tailed phages belong to *Siphoviridae, Myoviridae* and *Podoviridae*; and tailless phages under *Guttaviridae*.

According to Ackermann (1987), the tailed phages have an average capsid size of 30-160 nm and a tail length that ranges from 10-100 nm. The *Siphoviridae* has long non-contractie tail, the *Myoviridae* has less than 150 nm contractile tail, and the *Podoviridae* has a short tail that can be as small as 10 nm. On the contrary, Tailless phages differs with morphological capsid shape and diameter. *Cystoviridae, Corticoviridae,* and *Leviviridae* Family are all polyhedral-shaped tailless phages with the average capsid size of 23 nm, 60 nm and 75-80 nm, respectively. The Family of *Lipothrixviridae* are filamentous, tailless phages that has a capsid range of 400–2400 × 20–40 nm. While, the *Guttaviridae* Family are pleomorphic, droplet-shaped tailless phages with an average capsid size of 110–180 × 70–95 (E. Kutter & A. Sulakvelidze, 2005).

C. Stability of the Phage Cocktail against MDR Pseudomonas aeruginosa, Methicillin Resistant Staphylococcus aureus and Vibrio cholerae to different physical agents

The acidity, salinity, and ions is used to characterized the phages since pH determines the occurrence and viability of bacteriophages and can inactivate the lytic cycle through damage or changes of the phage structural and genomic elements (E. Jonczk et al., 2011). The pH stability result indicates that the phage cocktail against MDR Pseudomonas aeruginosa is stable at pH 2, 3, 4, 6, 7, 8, 9, 11 and 12, producing a maximum titer of 5x109 PFU/mL and the smallest titer of 1x109 PFU/mL. And as compared to the initial titer, phages when subjected to pH 2, 3, 4, 7, 8, 9, 11 and 12 showed increase in titer. Thus, the result shows that the phage cocktail can elicit lytic activity at both acidic and alkaline conditions with a wide range of pH. The stability to this range of pH is demonstrated by the tailed Families: *Myoviridae, Siphoviridae* and *Podoviridae,* and Tailless Families: *Corticoviridae, Tectiviridae, Leviviridae, Inoviridae, Guttaviridae, Lipothrixviridae, Rudiviridae* and *Fusselloviridae*. Moreover, the pH stability result of the phage cocktail used against Methicillin Resistant *Staphylococcus aureus* shows that the phages are stable at pH 4, 7 and 11 only, producing a maximum titer of $4x10^{9}$ PFU/mL and the smallest titer of $1x10^{9}$ PFU/mL. As compared to the initial titer, phages when subjected to pH 4 and 11, showed increase in titer. This indicates that the phage cocktail can survive at acidic, neutral and alkaline conditions. The pH stability of this cocktail is demonstrated by the tailed Families: *Myoviridae, Siphoviridae* and *Podoviridae*, and Tailless Families: *Cortocoviridae, Tectiviridae, Leviviridae, Inoviridae, Guttaviridae, Lipothrixviridae, Rudiviridae* and *Fusselloviridae*. While, the phage cocktail against *Vibrio cholerae* is stable at pH 5, 6 and 8 only producing a maximum titer of $3x10^{9}$ PFU/mL and the smallest titer of $1x10^{9}$ PFU/mL. As compared to the initial titer, phages when subjected to pH 5 and 6, showed increase in titer. This indicates survival of the phages at alkaline conditions but is more stable at acidic pH. The pH stability of this cocktail is demonstrated by the tailed

Families: *Myoviridae, Siphoviridae* and *Podoviridae,* and Tailless Families: *Tectiviridae, Guttaviridae, Lipothrixviridae, Rudiviridae* and *Fuselloviridae*.

Based on E. Jonczk et al. (2011), Tailed phages from the Families of *Myoviridae, Siphoviridae* and *Podoviridae* may survive at large pH fluctuations. The T2 phage from the *Myoviridae* Family is stable at pH range 5-9 with an optimal pH of 5-6, like the CP-51 phage. The T4 phage from the same Family shows stability at pH 6-7.4 but proven to be unstable at pH less than 5 and 9.2 & above. On the contrary, fgspC can survive in a very high alkaline environment. Moreover, the members under the *Siphoviridae* are generally more resistant to adverse conditions. Most of the phages are stable in a wide range of pH, usually pH 3-11, such as the Lj phage and shows sensitivity at pH of <3. On the other hand, the phages belong to *Podoviridae* prefers alkali conditions, such as T7 that shows stability at pH of 9 and proven to be sensitive to lower pH. Unlike the T7, T3 phage may survive at low acidic conditions (pH 5) up to pH 9. Whereas, Tail less phages like *Corticoviridae* PM2 Phage, *Tectiviridae* PRD1, *Leviviridae* MS2 and QB phages are usually stable at pH 5-6 to 8. QB phage are stable in an alkali environment; while, MS2 are more resistant to acidic conditions. Likewise, the M13 phage under *Inoviridae* has optimal pH of 6 and 9, but can survive at pH 2 for atleast 1 hour. Furthermore, *Guttaviridae, Lipothrixviridae* TTV1 Phage, *Rudiviridae* and phages under *Fuselloviridae* are found in acidic environments. The TTV1 can survive at pH <3, as well as SSV1; but reduces the viability at pH of 5 and above. These phage show sensitivity to pH >11 (E. Jonczk et al., 2011).

Moreover, temperature is the most common external factor that affects the stability of phages. This physical factor may determine the occurrence, viability, inactivity and storage of bacteriophages (E. Jonczk et al., 2011).

The thermal stability shows that the phage cocktail against MDR *Pseudomonas aeruginosa* can survive at a high temperature. Based on the result of the stability test, the phages are stable only at temperature 55^{o}C, producing a titer of 2x10^{8} PFU/mL. Whereas, the phage cocktail against Methicillin Resistant Staphylococcus aureus is stable at temperature 2-4^{o}C, 37^{o}C, 55^{o}C and 70^{o}C, producing a maximum titer of 6x10^{8} PFU/mL and the smallest titer of 1x10^{8} PFU/mL. Thus, the result indicates that the phages can survive at a wide range of temperature. And for the phage cocktail against *Vibrio cholerae*, the phages are stable at temperatures -20^{o}C, 37^{o}C, 45^{o}C, 55^{o}C and 70^{o}C, producing a maximum titer of 2.7x10^{9} PFU/mL and the smallest titer of 1x10^{8} PFU/mL. Therefore, the phage cocktail is stable and can survive at a wider range of temperature. The thermal stability results of the phage cocktail against the three bacterial hosts indicates the presence of tailed Families: *Myoviridae, Siphoviridae* and *Podoviridae,* and Tailless Families: *Guttaviridae, Lipothrixviridae, Rudiviridae* and *Fuselloviridae.*

Recent review on the external factors that influences bacteriophages concluded that the members from the Tailed Families; *Siphoviridae, Myoviridae* and *Podoviridae* family are more resistant to adverse conditions but among these phages only the Siphoviridae TSP4 phage shows stability up to the optimum temperature of 65^{o}C. *Myoviridae* may extremely resistant to a dry environment and may also survive large temperature fluctuations. At 37^{o}C, T4 phage is stable but only 65% survive after freezing at -196^{o}C. Also at low temperature, CP-51 phage is sensitive, but its optimal temperature is 15oC. Likewise, *Podoviridae* shows resistance to dry environment and may be viable even at large temperature fluctuations. There are also tail less phages under *Guttaviridae, Lipothrixviridae, Rudiviridae* and *Fuselloviridae* that are stable at high temperature even up to 85-97^{o}C (E. Jonczyk et al, 2011).

Furthermore, the UV Light stability test shows that the phage cocktail against Multi-drug Resistant *Pseudomonas aeruginosa*, Methicillin Resistant *Staphylococcus aureus*, and *Vibrio cholerae* that the phages can survive UV Light irradiation even at a longer time of exposure, and can elicit lytic activity at a various exposure time. This phage characteristic suggests the presence of tailed phages under the Family of *Siphoviridae, Myoviridae* and *Podoviridae*. According to E. Jonczyk et al. (2011), viruses belong to the Family of Tailed phages, are more resistant to adverse and dry environment (E. Jonczyk et al., 2011). Larcom & Thaker (1977) reported high percentage of survival rate of phages after subjected to UV irradiation. This may be an effect of a mutation leading the phage to enter the lysogenic cycle, making the phage more virulent to adverse conditions (L.L. Larcom & N.H. Thaker, 1977).

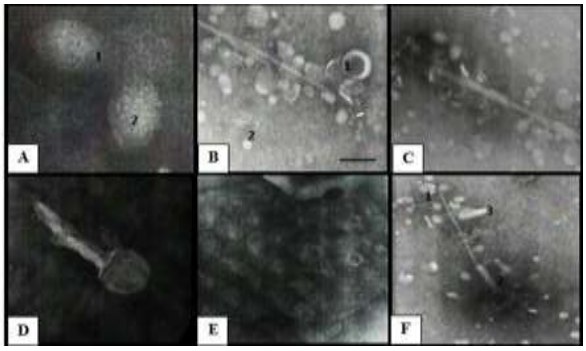

Fig. 1.0 . TEM images of Phage Cocktail against MDR *Pseudomonas aeruginosa.* A. Fuselloviridae, B1. Leviviridae, B2. Cystoviridae, C. Siphoviridae, D. Myoviridae, E. Guttaviridae, F. Corticoviridae

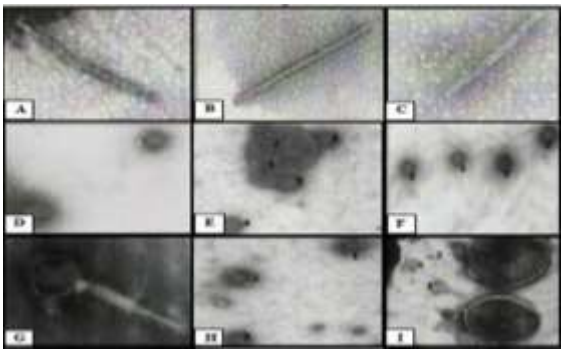

Fig. 2.0 TEM images of Phage Cocktail against Methicillin Resistant Staphylococcus aureus. A, B, C. Lipothrixviridae, D. Cystoviridae, E. Podoviridae, F. Guttaviridae, G. Myoviridae, H. Podoviridae, I. Siphoviridae

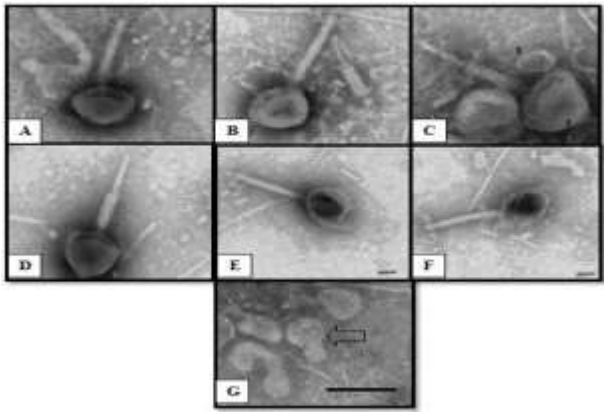

Fig. 3.0 *TEM images of Phage Cocktail against Vibrio cholerae.* A,B,E,F. Siphoviridae, C,D. Myoviridae, G. Podoviridae

TABLE 1.0 MORPHOLOGICAL FEATURES OF THE PHAGE COCKTAIL AGAINST MDR PSEUDOMONAS AERUGINOSA

Phage	Capsid Dimension (nm)	Tail Dimension	Particulars
A1	116 x 46	Tailless	Lemon-shaped
A2	103 x 46	Tailless	Lemon-shaped
B1	78 x 78	Tailless	Not observed
B2	23 x 23	Tailless	Not observed
C	119 x 103	503 x 24	Long, non-contractile tail
D	164 x 173	293 x 62	Contractile tail
E	119 x 92	Tailless	Droplet-shaped
F1	**60 x 60**	**Tailless**	**Not observed**
F2	**208 x 141**	**119 x 32**	**Contractile tail**
F3	**114 x 70**	**Tailless**	**Droplet-shaped**

TABLE 2.0 MORPHOLOGICAL FEATURES OF THE PHAGE COCKTAIL AGAINST MRSA

Phage	Capsid Dimension (nm)	Tail Dimension	Particulars
A	676 x 36	Tailless	Not observed
B	960 x 36	Tailless	Not observed
C	413 x 27	Tailless	Not observed
D	77 x 77	Tailless	Not observed
E1	77 x 77	Tailless	Not observed
E2	145 x 95	Tailless	Droplet-shaped
E3	150 x 118	27 x 27	Short-tailed
E4	136 x 100	27 x 27	Short-tailed
E5	136 x 150	27 x 27	Short-tailed
F1	117 x 85	Tailless	Droplet-shaped
F2	127 x 85	Tailless	Droplet-shaped
G	111 x 118	119 x 27	Contractility not observed
H1	103 x 108	32 x 32	Short-tailed
H2	124 x 135	32 x 32	Short-tailed
H3	102 x 108	16 x 16	Short-tailed
I1	160 x160	266 x 18	Non-contractile tail
I2	71 x 71	Tailless	Not observed

TABLE 3.0 MORPHOLOGICAL FEATURES OF THE PHAGE COCKTAIL AGAINST VIBRIO CHOLERAE

Phage	Capsid Dimension (nm)	Tail Dimension	Particulars
A	102 x 126	184 x 22	Long, non-contractile tail
B	112 x 111	196 x 26	Long, non-contractile tail
C1	113 x 124	91 x 25	Contractile tail
C2	60 x 41	Tailless	Droplet-shaped
D	111 x 128	81 x 31	Contractile tail
E	121 x 121	202 x 23	Long, non-contractile tail
F	134 x 119	208 x 25	Long, non-contractile tail
G	56 x 52	30 x 30	Short tailed

IV. CONCLUSION

The plaque assay of the phage cocktail against MDR *Pseudomonas aeruginosa* can produce a maximum concentration of $3x10^{10}$ PFU/mL at $1x10^{-9}$ dilution. And, the lowest dilution that produced a titer was $1x10^{-3}$ with $2x10^{4}$ PFU/mL. On the other hand, the plaque assay of the phage cocktail against Methicillin Resistant *Staphylococcus aureus* produced a maximum concentration of $1x10^{9}$ PFU/mL at $1x10^{-8}$ dilution. And the lowest dilution that produced a titer was $1x10^{-3}$ with $1x10^{4}$ PFU/mL. Whereas, the initial plaque assay of the phage cocktail against *Vibrio cholerae* produced a maximum of concentration of $4x10^{10}$ PFU/mL at $1x10^{-9}$ dilution. And the lowest dilution that produced a titer was $1x10^{-2}$ with $4x10^{3}$ PFU/mL.

Moreover, the phage cocktail against the three bacterial hosts presents different morphologic forms and stability suggestive of different families which involves tailed and tailless phages belong to the Order Caudovirales. Tailed phages under *Siphoviridae*, *Myoviridae* and *Podoviridae* family are present in the cocktail against MRSA and *V. cholerae*; and only phages under *Siphoviridae* and *Myoviridae* is seen in the cocktail against MDR *P. aeruginosa*. Furthermore, tailless phages under *Fuselloviridae*, *Guttaviridae*, *Corticoviridae* and *Leviviridae* are also present in the cocktail against MDR *P. aeruginosa*. Tailless phages

under *Guttaviridae* are present in the cocktail against the three bacterial hosts. Whereas, phages belong to *Cystoviridae* are present in the cocktail against MDR *P. aeruginosa* and *V. cholerae*. Lastly, *Lipothrixviridae* phages are only present in the cocktail against MRSA.

V. Recommendation

The future researchers may improve and further characterize the phage cocktail against MDR *Pseudomonas aeruginosa*, Methicillin Resistant *Staphylococcus aureus* and *Vibrio cholerae* through molecular analysis.

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