Isolated Bacteriophage from Calumpang River as an Inhibitor of *Escherichia coli*

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Abstract - Antibiotic resistance has been the problem in medical microbiology for a long time now. Factors contributing to the phenomenon have been identified; however, solution to the problem at hand remains unsolved. Alternative approaches to halt bacterial infection have been thoroughly subjected to researches. Bacteriophages, though highly affected by diversity, have proven to be of beneficial effect in the never ending problem of resistance. Thus, another approach to control harmful bacteria was again ventured years after its discovery. Samples throughout the research were collected from heavily contaminated Calumpang River which has been identified to harbor various coliforms in the previous studies. From the three selected sampling sites, qualitative test for the presence of cocktail of bacteriophage was done through double agar overlay method. Isolated cultured samples were then subjected to electron microscopy for confirmation. Submitted samples were negatively stained and were reported to contain only bacilli with no presence of bacteriophage nor any evidence of bacterial lysis which is the key phenomenon of bacterial phage invasion. In this study, it is therefore concluded that no bacteriophage is isolated from Calumpang River.

Keywords: Calumpang River, bacteriophage, plaque assay, *Escherichia coli*

INTRODUCTION

After a long but successful era of research that had all but eliminated serious threats from bacterial infections, we are now facing this dire problem once again (Friedman, 2012). The emergence of pathogenic bacteria resistant to most, if not all, currently available antimicrobial agents has become a critical problem in modern medicine (Sulakvelidze, Alavidze & Morris, 2001). Antibiotic resistance is a major clinical problem in treating infections (Gales, Jones & Turnidge, 2001). The past two decades have witnessed major increases in

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emergence and spread of multi-drug resistant bacteria and increasing resistance to newer compounds, such as fluoroquinolones and certain cephalosporins (Marshall& Levy, 2004). Resistance to antibiotics is acquired by a change in the gene make up of bacterium, which can occur by either a gene mutation or by transfer of antibiotic resistance gene between bacteria in the environment (Dhanorkar & Tambekar, 2004).

Bacteriophages or phages are bacterial viruses that invade bacterial cells and are amongst the living entities on earth playing important roles in maintaining the natural abundance and distribution ofmicroorganisms (Mathur, Vidhani & Mehndiratta, 2003). Phage therapy for eliminating multidrug resistant bacteria is gaining importance. However, there is a need to carry out further studies on phages as therapeutic agents using specific phage strains against the corresponding bacterial hosts. The potential of phage therapy has been the subject of several recent reviews and the present studies regarding the problem on multi-drug resistance reinforce the view that this potential is worth exploring (Biswas et al., 2002). It appears likely, that phage therapy will regain a role in both medical and veterinary treatment of infectious diseases, especially in the scenario of the emerging antibacterial resistance (Duckworth & Gulig, 2002).

The ability of bacteriophages to survive under unfavorable conditions is highly diversified (Jończyk, Kłak, Międzybrodzki, & Gorski, 2001). In the course of the study, the researchers have identified factors affecting phage's viability when isolated in a laboratory setting. Various external physical and chemical factors, such as temperature, acidity, salinity and ions, determine the occurrence, viability, and storage of bacteriophages and can inactivate a phage through damage of its structural elements, lipid loss and structural changes (Ackerman, Tremblay, & Moineau, 2004).

In the study conducted by (Villanueva, Paala, Ringel, Samson & Villanueva et al., 2012), it was established that Calumpang River was heavily contaminated with fecal and coliform pollution. Fecal contaminants from human and other warm blooded animals implicated that phage. Quality of sample from which bacteriophage would be isolated is an essential factor to consider. Bacteriophage exist in all environments where bacteria grow (Jończyk, et al., 2001); thus, quality of bacteriophage could be well evaluated based on the presence of the specific bacteria phages lyse.

Temperature is a crucial factor for bacteriophage survivability. It plays a fundamental role in attachment, penetration, multiplication, and the length of the latent period in cases of lysogenic phages.

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Moreover, temperature determines the occurrence, viability, and storage of bacteriophages (Jończyk et al., 2001).

Another important factor influencing phage stability is the acidity of the environment. Feng, Ong, Hu, Tan, & Ng (2003) investigated the survivability of coliphages in water and wastewater with regards to the effects of different temperatures and pH on the phages. The phages presented the lowest inactivation rate in the pH range of 6–8 and temperature range of 5–35°C.

Bacteriophages can be resistant to unfavorable physical and chemical factors, such as low and high temperatures, pH, salinity, and ions. Thus, they can settle in extreme environments. Based on the literature, it seems that phage features in that field are highly diversified and may differ not only among families, but also within them. So far, anyone who is going to preserve phages should "know his phage," as suggested by (Ackermann, Tremblay & Moineau, 2004). High bacteriophage resistance for external factors is important for stability of phage preparations.

Hence, in accordance to the statement of (Parisien, Allain, Zhang, Mandeville & Lan, 2007) this study too, did not simply aim to isolate bacteriophage to provide local alternative to antibiotic resistancerather, it was done to contribute to the advancement of phage therapy in the field of microbiology. In addition, it also aimed to establish new understanding of cocktail of bacteriophage from Calumpang River as we test it bacteriostatic or bactericidal effect against *Escherichia coli* ATCC 25922.

MATERIALS AND METHODS

Figure 1. Map of Calumpang River from Tinga,Labac to Barangay Malitam showing the water sample collection sites (Google Maps)

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Water Sample (Villanueva et al., 2012)

River water sample was collected from the three stations of Calumpang River as shown in Figure 1. The three stations identified by the previous researchers were in: Brgy. Malitam, Batangas City; Calumpang Bridge to the Bridge of Promise; and Tinga Labac comprising the upstream, midstream and downstream with the coordinates 13˚ 45' 43.37"N 121˚04'28.12" E (Google Maps). Sample obtained were transferred into 60 mL mason jar (32W-32oz. wide mouth ball mason jar).

Pure Bacterial Strain

E.coli ATCC 25922 acquired from Batangas Medical Center was kept at 37˚C for the rest of the experiment.

Isolation of Potential Bacterial Enteric Host Strains from Calumpang River (Beaudoin, DeCesaro, Durkee, & Barbaro, 2007)

Bacteria were isolated from the collected river water sample by aseptically plating 100 µL of sample onto Tryptic Soy Agar. After 24 hour incubation at room temperature, pure cultures were obtained using streak plate technique.

Gram Staining

Slide was prepared by smearing single bacterial colony from the overnight culture of bacteria on Tryptic Soy Agar. Slide was heat fixed and was subjected to primary staining by crystal violet, then secondary staining with Gram's Iodine. Decolorizing agent used is ethanol, then sample was counterstained using safranin. After rinsing and drying, sample was viewed under light microscope using oil immersion objective (OIO).

Preparation of Viral Suspension (Beaudoin et al., 2007)

Viral suspension was prepared from the river water sample. River water (10 mL) was aseptically transferred to sterile 15 mL conical tube and centrifuged at 2000 rpm for five minutes. Supernatant was obtained and filtered using 0.45 µm pore sized Whattman filter paper to facilitate removal of bacterial cells and bacterial cells. Filtrate was then, aseptically transferred to sterile 15 mL centrifuge tube.

Plaque Assay (Beaudoin et al., 2007)

Sterile underlay Tryptic Soy Agar was poured into petri dishes and was left to harden. Overlay layer was prepared by adding three mL overlay soft Tryptic Soy Agar containing 50% of the original agar

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concentration, three drops of 24 hour Tryptic Soy Broth culture and one mL river water suspension. The mixture was gently inverted and was poured on top of underlay hard agar. Overlay was allowed to harden, then incubated at room temperature for 24 hours.

Plates were checked for plaque formation (clearing zone) within bacterial lawn after 24 hours of incubation. After plaque identification, pure suspension was prepared by removing a portion of plaque using sterile pipette tip was prepared by removing a portion of plaque using the larger end of a sterile Pasteur pipette. Obtained plaque was then transferred to prepared 10 mL Tryptic Soy Broth medium. Residual cells were removed next through centrifugation at 5000 rpm for five minutes. Supernatant was aseptically transferred to sterile 15 mL conical tube which was stored at five degrees Celsius.

Electron Microscopy (Efrony, Loya, Bacharach, & Rosenberg, 2006)

For confirmation of the presence of phage, mixture broth sample was subjected to electron microscopy at the Electron Microscopy Laboratory in the Research Institute of Tropical Medicine. Broth containing bacteriophage were negatively stained with 1% uranyl acetate and examined with JEOL 840A electron microscope at 80 kV.

Statistical Method

No statistical method was used since there was no evidence of Plaque formation in Plaque Assay nor phage existence in Electron Microscopy.

RESULTS AND DISCUSSION

Bacteriophages are the most abundant entities living on earth. They are also highly diversified microorganisms contributing to the microbiological balance in the ecosystem. Isolation of bacteriophage is the most crucial step prior to in vivo phage administration.

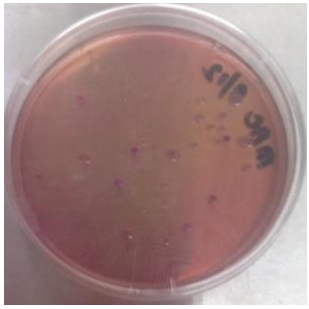

Figure 2. *Overnight culture of river water sample on MacConkey Agar*

River water sample stored at room temperature was tested to have slightly basic pH of 7.4. Determination of pH of water is necessary in assessing if a bacteriophage is more likely to be isolated from the water. Feng et al. (2003) in his research on wastewater phages discovered that phage activity is in the least inactivation in the pH of 6-8 with a temperature

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range of 5-35˚C. Hydrogen ion concentration in the water was described by (Chandran, Pradhan, Heinonen, & Tanski, 2009) to be the factor responsible for phage aggregation.

Figure 2 shows the overnight culture of the water sample on differential media MacConkey Agar. MacConkey Agar is a differential medium that was used to ensure that the cultured bacteria were indeed Gram negative microorganisms. Isolates were then subjected to double agar overlay.

Selection of a single colony from overnight primary culture of water sample was crucial in phage isolation. The bacterial colony was inoculated into Tryptic Soy Broth (TSB) and was incubated for 24 hours. Highly specific phages would be released from the broth culture on the overlay method, thereafter.

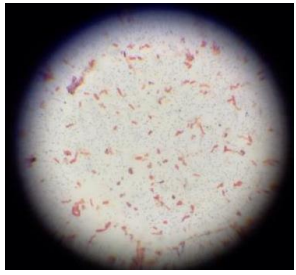

Figure 3. *Red bacilli on Gram staining microscopic examination*

Figure 3 shows the appearance of the gram stained microorganisms under light microscope. To confirm the morphologic appearance of the isolated bacterial strains, gram staining was done. Gram staining plays an important role in defining the microscopic appearance of isolated bacteria. Microscopic examination of gram stained microorganisms revealed the presence of red colored, rod shaped bacteria. According to Villanueva et al. (2012), Calumpang River harbors various coliforms including our target bacteria *E.coli*; thus, cocktail of bacteriophage would be combination of phages from different bacterial strains.

Figure 4 shows no plaque formation on plates in double agar overlay method after 24 hours of incubation at room temperature. Absence of plaques or clearing zones is an indicative that no bacterial lysis has occurred which is the mechanism of action of bacteriophages in multiplication and promotion of their bactericidal effect.

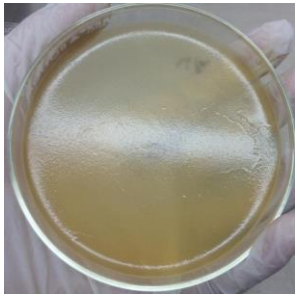

Figure 4. *Double agar overlay plate showing no clearing zone or plaque formation.*

In isolating bacteriophages, too, it is very important to know the nature of the phage concerned. In the review done by Carlton (1999), he addressed the existence of lysogenic phage which might cause

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confusion among those who would isolate phages. In those historic era, he suggested that negative outcome may be due to inadvertent use of phage strains, that being lysogens and could not provide rapid lysis and exponential growth. This being a contributory factor as the study aimed to isolate cocktail of bacteriophages which might be a combination of both lysogenic and lytic or lysogenic phage alone. Furthermore, the presence of this lysogenic or temperate phages as discussed by Thiel (2004) in his review of phage therapy, causes integration into the host chromosome thereby becoming dormant phages in the process.

The whole experiment was done during the rainy season in the country; thus, it is possible that quality of water was highly affected since sample was effluent. Several other methods were employed for the isolation of bacteriophage to validate the negative result and rule out the discrepancy in the original method employed. The Phage Therapy Center in Georgia suggested that the researchers must try amplifying bacteria for greater chance of observing plaque formation in double agar overlay method. However, the several trials done were proven futile since no clearing formation was observed.

Failure to isolate bacteriophages in double agar overlay method could also be due to the heightened host specificity of bacteriophages aside from the presence of dormant phages. This specificity means that a phage can infect only certain bacteria bearing receptors to which they can bind which in turn determines the phage's host range. Moreover, growth conditions also influence the ability of the phage to attach and invade them. In addition, the choice of culture media from which bacteriophages would be forming plaques affects the viability of the host bacteria.

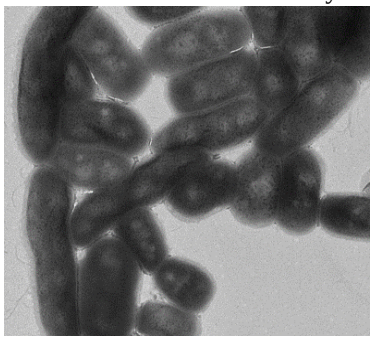

Figure 5. *Electron micrograph of the river water sample reveals gram negative bacilli.*

Bacteriophage target bacteria ratio must also be considered when isolating phages. Since various bacteria were present in the river water sample, bacterial high concentration might have affected the persistence of bacteriophages in forming plaques or clearing zones in the double agar overlay method.

Figure 5 shows the electron micrograph of gram negative bacilli. The liquid broth sample which contains the possible phages

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was subjected to negative staining for bacteriophage to remain untouched while staining its background making it more visible. Bacteria seen in the Transmission Electron Microscope was described to be rod shaped. Bacilli were seen with no sign of lysis which means the absence of phages or presence of phages not specific for the identified bacilli. Thiel (2004) described the lysing mechanism of phages as like any other virus as means of reproduction, that when phages enter a cell, then inject their nucleic acid content inside the cell, thereby making new progeny of bacteriophages to be released when bacterial cell dies. In this reproduction process, bacterial hosts were killed, too, marking the propagation of new bacteriophages.

CONCLUSION AND RECOMMENDATION

On the basis of the present research, therefore, it is concluded that no bacteriophages against *E.coli* were isolated from Calumpang River. Based on the results and observations obtained throughout the study, future researchers can further reconsider and develop bacteriophage as antimicrobial agent. Further improvement in isolation method and exploration of phage sources are also recommended.

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