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The Sensitivity of Pseudomonas Agar Plaque Assay in the Isolation of Bacteriophage Φ6 in the Environment: A pilot study

Olasunkanmi Sunmonu

ABSTRACT

The Sensitivity of Pseudomonas Agar Plaque Assay in the Isolation of Bacteriophage Φ6 in the Environment: A pilot study

By

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May 2016

Background: Bacteriophage Φ6 is a lipid-enveloped dsRNA bacteriophage. The limitations in our knowledge of how this bacteriophage occurs in the environment are limited by non-selective isolation techniques. Research on finding phages in the environment in the past has employed the Double Agar Layer (DAL) plaque assay using Tryptic Soy Agar (TSA), a non-selective media. The bacterial host for bacteriophage Φ6 is *Pseudomonas syringae*. In this study, we tested Pseudomonas Agar, a selective media that suppresses the growth of bacteria except *Pseudomonas* species, in the standard double agar layer plaque assay for Φ6.

Methods: DAL plaque assays were performed to determine the sensitivity of both Tryptic Soy Agar (TSA) and Pseudomonas Agar (PA) for determining the titer of pure bacteriophage Φ6 stocks. We used Pseudomonas syringae (HB10Y) as the host, and the plaque formation on both agars was compared. Following the evaluation of PA with pure Φ6 stocks, PA effectiveness for Φ6 isolation from environmental samples was tested in spiked waters obtained from irrigation ponds at an agricultural farm.

Results: Comparison of TSA and PA using pure Φ6 cultured in the laboratory and spiked environmental samples showed that PA agar can detect bacteriophage Φ6 as well as the standard DAL assay using TSA. On PA, formation of clear visible plaques comparable to the plaques formed using TSA was observed.

Conclusions: Pseudomonas Agar can be used for the isolation of bacteriophage Φ6 in environmental samples. This may enhance the detection of these phages in the environment.

The Sensitivity of Pseudomonas Agar Plaque Assay in the Isolation of Bacteriophage Φ6 in the Environment: A pilot study

Olasunkanmi Sunmonu

A thesis submitted to the Graduate Faculty of Georgia State University in partial fulfillment of the requirements for the Master of Public Health degree in the Division of Environmental Health

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APPROVAL PAGE

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Author's Statement Page

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LIST OF ABBREVIATIONS

Φ6	Bacteriophage Phi6
χ^2	Chi Square
DAL	Double Agar Layer
EPA	Environmental Protection Agency
EXP	Experiment
gm/L	Gram per Liter
mg	Milligrams
MPN	Most Probable Number
mL	Milliliter
μL	Microliter
PA	Pseudomonas Agar
PFU	Plaque Forming Units
TNTC	Too Numerous To Count
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth

1. INTRODUCTION

1.1 Background

Bacteriophages are some of the most abundant organisms in the world. They are bacteria predators, which infect the bacterial cells (Serwer, Hayes, Zaman, Lieman, Rolando & Hardies, 2004). Active bacteriophages multiply inside the bacterial host, and in the process result in release of numerous offspring and lysing of the bacterial cells (Abedon, 2009). One of the unique characteristics of bacteriophages is that they are highly host specific, infecting a narrow range of bacterial hosts (Serwer, et al., 2004).

The ability of bacteriophages to lyse bacteria or transfer genes could be the most likely factor that determines the composition of various microbial populations in the environment (Węgrzyn & Węgrzyn, 2015). Bacteriophage Φ6 has RNA genome, and lipid envelops (Węgrzyn & Węgrzyn, 2015). Bacteriophage Φ6 structure is spherical in shape and comprises of three structured layers (Clokie, & Kropinski, 2009). The diameter of the virion is estimated to be about 70 nm (Węgrzyn & Węgrzyn, 2015). The core of bacteriophage Φ6 is about 50nm in diameter and forms an enclosure for the virion's segmented genome (Ktistakis & Lang, 1987). The structural component of bacteriophage Φ6 core is made of a shell forming protein, P1, RNA polymerase P2, nucleoside triphosphate phosphohydrolase P4 and minor protein P7 (Hanlon, 2007;Serwer, et al., 2004). The core of the Φ6 is then surrounded by the second layer composed of protein, which makes up the virion's nucleocapsid (NC), which is about 58nm in diameter (Michel-Briand, & Baysse, 2002). The third layer forms the outermost cover of the Φ6 and it comprises of lipid bilayer envelop, on which 2nm spikes are attached (Węgrzyn & Węgrzyn, 2015). Bacteriophage Φ6 is very common in the environment; they are ubiquitous and have been isolated from leguminous plants, found in sewage and in organic wastes (Abedon, 2009).

Presence of bacteriophage in organic wastes indicates high presence of bacteria in those habitats. This implies that sewage and raw water are importance sources of pathogenic bacteria that cause a variety of human diseases (Abedon, 2009).

The study of bacteriophages is important because of their lytic effects on bacteria in vitro (Michel-Briand, & Baysse, 2002). Bacteriophages demonstrate several common characteristics with both animal and plant viruses. They have a similar size range, ranging from 10nm to 75nm (Vidaver, Koski & Etten, 1973). They also reproduce only within a living host cell and are host specific. However, some phages have a wide host range. Bacteriophages may destroy the host cell or continue inhabiting it in latent state. Moreover, phages are antigenic, producing antibodies that neutralize them (Clokie, & Kropinski, 2009). By examining the properties of bacteriophages, it is therefore possible to learn how human viruses behave.

Bacteriophage Φ6 for example can be applied as a model for studying human respiratory viruses, such as flu virus (Mäntynen, 2016), they are advantageous surrogate viruses for influenza and other enveloped human virus because of their lack of pathogenicity, ease of propagation and assay (Casanova & Weaver, 2015). In human medicine, the application of bacteriophage as antibiotics has been examined. For instance enzymes isolated from GBS bacteriophage NCTC 11261 has been shown to have lytic properties against different pathogenic bacteria affecting humans, such as *Streptococcus agalactaea, Streptococcus salivarius, streptococcus gordonii* and *streptococcus mutans* (Domingo, Georgel, & Bahram, 2016; Mäntynen, 2016). The increasing anti-biotic resistance that has been demonstrated by a variety of bacterial strains has pointed to the need of adopting bacteriophages as alternative to the existing antibacterial drugs (Mäntynen, 2016).

One of the various methods applied in detection and isolation of bacteriophage in environmental samples such as sewage includes the Double Agar Layer (DAL) plaque assay using Tryptic Soy Agar (TSA). The gold standard techniques for isolating bacteriophages from water and sewage are EPA method 1601 and single agar layer EPA method 1602 approved by Environmental Protection Agency (Rodriguez, et al. 2012). However, these techniques are characterized by several shortcomings. Tryptic Soy Agar (TSA) for instance is not selective media, and it promotes the growth of other microorganisms in the environmental sample (Clokie, & Kropinski, 2009). In this regard, it is important to develop an alternative method that is more selective in isolating bacteriophage Φ6 in the environment, the ability to selectively isolate and quantify Φ6 when they exist in environmental samples will help in future phage research, and avoid underestimation in samples where they exist because the growth of other pathogens on non-selective media can interfere with Φ6 quantification, making plaque counts difficult.

There are limited methods of studying and detecting bacteriophage population dynamics in environmental samples.This is due to the challenges associated with high dilution in natural water systems and sorption caused by particulate matter in terrestrial environments (Clokie, & Kropinski, 2009). The common method of isolating bacteriophages from environmental samples such as sewage involves enriching the sample with specific host bacterium as well as plaque assay (Ogunseitan, Sayler, & Miller, 2004). However, this technique is characterized by several shortcomings. The first shortcoming is insufficient quantification, which implies that the limited amount of environmental inocula make it difficult to detect the sparingly distributed bacteriophage (Ogunseitan et al., 2004).

Although these techniques are aimed at detecting bacteriophage particles, there are concerns about the fate of host bacteria that inhabit similar ecosystems (Domingo, Georgel &

Bahram, 2016). Using techniques that rely entirely on plaque forming capability of isolated bacteriophages has been blamed for failure to detect different types of facultative virulent phages in various natural ecosystems (Michel-Briand, & Baysse, 2002). This has resulted in the application of other techniques such as DNA probes in the investigation of dynamics of different bacteriophage distribution in the ecosystem (Michel-Briand, & Baysse, 2002).

1.2 Study Objectives

The primary objective of this study is to develop a selective Double Agar Layer (DAL) plaque assay, the Gold standard recommended by the U.S Environmental Protection Agency for the isolation of bacteriophages in small amounts of environmental samples, for the isolation of bacteriophage Φ6 using a more selective medium for *Pseudomonas*, the host for Φ6. Also, to determine the growth of *Pseudomonas syringae* on Pseudomonas agar, which can be utilized to culture pure colonies of *P. syringae.*

2. LITERATURE REVIEW

The abundance of bacteriophages, which are viruses that infect bacteria species in the environment, has significant effects on the population of the microbes (Serwer, et al., 2004). Also, the ability of bacteriophages to lyse bacteria or transfer genes influences the particular composition and amount of microbial populations in the environment (Serwer, et al., 2004). Bacteriophage Φ6 is an example of bacteriophages with RNA genomes and a lipid envelope (Węgrzyn & Węgrzyn, 2015). Being able to detect Φ6 in the environment can help us understand where enveloped viruses occur in the environment and how they behave, but methods to detect this virus in the environment are needed.

Several studies have commonly utilized Tryptic Soy agar (TSA) and the Double agar plaque assay to determine the presence of phage in environmental samples especially sewage (Węgrzyn & Węgrzyn, 2015). In double agar plaque assay, the virus is grown on a bacterial host, Pseudomonas syringae (Clokie & Kropinski, 2009). TSA is not a selective media and allows for the growth of other microbes in environmental samples, which interferes with the growth of the host and the ability to see the growth of the virus. For example, E. coli is found in abundance in wastewater and sewage and could interfere with the DAL assay if it is used to find Φ6 in wastewater (Clokie, & Kropinski, 2009). Hence it is important to find an alternative method for isolating bacteriophage in environmental samples. Double Plaque assay is one of the commonly used techniques for isolating and detecting bacteriophage Φ6 in an environmental sample such as sewage and wastewater.

Plaque assay was initially applied in counting and measuring the virulent levels of bacteriophages and was later adopted in counting viruses inhabiting mammalian systems (Hanlon, 2007). The technique is one of the most applied methods in detecting, isolating and

purifying phages, including bacteriophage Φ6. Plaque assays is a technique in which dilutions of the phage preparations are combined with a tolerant host bacterium and then spread uniformly on a solid medium (Ktistakis & Lang, 1987). When it is incubated, the host bacterium develops a lawn on the solid medium, but when infectious bacteriophage lyses or prevents the growth of the cells, a plaque, which is a translucent or clear area is formed (Clokie, & Kropinski, 2009). Visualization of plaques facilitates the counting, isolation, and characterization of bacteriophages and their mutants. In this regard, plaque assay depends on the potential of tolerant host bacterium to form a complete lawn on solid media and localized growth of the plaques to an extent they can be seen with the naked eye (Clokie, & Kropinski, 2009).

Development and the subsequent spread of the plaque require adequate growth of reproductive bacteriophages from infected bacterial cells to enable localized infection and lysis of the neighboring bacterial cells. Plaque assay technique has been employed successfully with various microbes, including Pseudomonas, Salmonella, Escherichia and Bacillus (Clokie, & Kropinski, 2009). To enumerate and isolate bacteriophages that require host bacteria that do not form lawns on solid media or visible plaques, plaque assay techniques are ineffective. The most efficient methods in such cases include electron microscopy and fluorescent microscopy (Clokie, & Kropinski, 2009).

Pseudomonas agar plaque assay can be used in the isolation of bacteriophage Φ6 in the environmental samples such as raw sewage and wastewater. To establish the sensitivity of the Pseudomonas agar plaque assay, it is important to look at the structure of bacteriophage Φ6 in detail.

The bacteriophage Φ6 is an enveloped virus from the Cystoviridae family and is known to infect Pseudomonas bacteria species. Hence, they are often used as surrogate viruses for

hazardous viruses in research studies because they are inexpensive and safe to handle for laboratory experiments (Turgeon et al., 2016; Laurinavicius et al., 2004). Bacteriophage Φ6 belongs to Cystoviridae family and this family currently has about ten bacteriophage species. The genetic and structural composition of cystovirus comprises of tripartite dsRNA genome, and a capsid comprising of two concentric, icosahedrally symmetric protein shells and protein rich envelop (Mäntynen, 2016). Bacteriophage Φ6 is the first dsRNA bacteriophage to be isolated with *Pseudomonas syringae* as the host which dates back to 1973, it was first identified and described by Vidaver et al., they described phi6 as a lipid-containing bacteriophage and a three-segmented dsRNA genome. The bacteriophage Phi6 is composed of 25% lipid, 12% RNA, and 62% protein; the lipid membranes found in phi6 is an essential component for its infectivity because it loses its infectivity on exposure to organic solvents, sodium deoxycholate, and phospholipase A. (Vidaver, Koski & Etten, 1973, Yang et al., 2016).

Bacteriophage Φ6 are common in leguminous plants, where the majority were isolated. Bacteriophage Φ6 and other members of the Cystoviridae family are host specific, attacking gram-negative bacteria particularly those that are pathogenic to plants such as *Pseudomonas syringae* species (Abedon, 2009). According to the Pseudomonas Plant Interaction on *P. syringae* Genome Resources (2010), *P. syringae* is a Gram negative, plant pathogenic bacterium strains that are known for their diverse and host-specific interactions with different plant species. There are over 50 known pathovars of *P. syringae* strains based on their ability to infect different plant species and their most favorable growth temperature is around 28 ^{o}C (Abdallah, Hartman, Pletzer, Zhurina & Ullrich, 2016). They are also capable of evolving; modulate their pathogenicity and virulence to adapt to new host plants and environments (Scortichini, Marcelletti, Ferrante, Petriccione & Firrao, 2012)

Bacteriophage Φ6 and other members of Cystoviridae evolve at a very fast rate. This is attributed to their vulnerable error- prone polymerase, making them susceptible to spontaneous mutations (Ktistakis & Lang, 1987).

The structure of Bacteriophage Φ6 is complicated, and it mainly comprises of three concentric layers, which enclose the segmented genome (Węgrzyn & Węgrzyn, 2015). In every genome segment, the genes are set into functional groups. The innermost protein shell or the polymerase complex is made up of four types of proteins, namely MCP P1, RNA-dependent RNA polymerase (RdRP) P2, packaging NTPase P4 and a minor protein, called P7 (Mäntynen, 2016). Polymerase complex is mainly made up of the protein P1. The life cycle of bacteriophage Φ6 is initiated following the dissociation of the layered structure, and the virion passes through the host's bacterial cell envelop (Clokie, & Kropinski, 2009). When the phage Φ6 is attached to the bacterial cell, the pilus retracts, resulting in the interaction of the viral particle with the outer membrane of the bacteria cell (Mäntynen, 2016). There follows a series of biochemical interactions, resulting in the fusion between the viral envelop and the outer membrane of the bacteria. Nucleocapsid of the phage Φ6 then enters into the periplasmic space of the bacteria (Węgrzyn & Węgrzyn, 2015). A series of interactions in the viral envelop of phage Φ6 enables the nucleocapsid to enter the cell membrane of the host bacteria (Węgrzyn & Węgrzyn, 2015). The Nucleocapsid then enters the hosts' cytosol, initiating a series of transcription within the polymerase complex of the bacteriophage Φ6 and subsequent replication inside the host's cell. The replication produces virions inside the host cell, where they mature after the development of p3 spikes. These results to the mature viral particles, which are released into the environment after the rapture of host cell, following the action of lytic enzymes (Mäntynen, 2016).

Viruses are responsible for causing a significant number of infections in human, plants, and animals, leading to loss of productivity and death to the affected organisms. The virulent nature of the viral particles is attributed to their abundance in the environment. Bacteriophages are the most abundant forms of life, estimated at 1031, over ten times more than that of bacterial microbes (Domingo, Georgel, & Bahram, 2016). Viruses can be defined as the simplest biological pathogens that have the ability to evolve, as well as adapt to exist in different environments (Agbandje-Mckenna, 2010).

A very high concentration of phages has been reported to exist mainly in the guts of humans and animals. These phages find their way into the environment by defecation where they remain free and persist for a long time in environmental media such as naturally occurring freshwater, sewage and activated sludge where these phages interact with their bacterial hosts (Muniesa, Imamovic & Jofre, 2011).

Bacteriophages infect and kill the host bacterial cells. This makes phages an important ecological actor. The killing of the infected bacterial cell is accompanied by bursting or lysis of host cells (Domingo, Georgel, & Bahram, 2016). During the infection, the virulent bacteriophages replicate recurrently until the host cell develops bacteriophage resistant mutants. The biology of RNA phages has been poorly investigated till date (Yang et al., 2016). According to the latest report by the International Committee for the Taxonomy of Viruses (ICTV), there are only two official families of RNA bacteriophages; the single-stranded RNA (ssRNA) bacteriophage family Leviviridae and the segmented, double-stranded RNA (dsRNA) family Cystoviridae (Mäntynen, 2016). The dsRNA family contains a single recognized species that is of interest in this study - Bacteriophage φ6 also known as Pseudomonas phage φ6 because it infects the opportunistic Pseudomonas bacteria strains (Clokie, & Kropinski, 2009). The host

specificity of viruses can be valuable for fighting infections caused by pathogenic bacteria using phages as antibacterial, and the hosts are very unlikely to develop resistance in untargeted bacterial strains because of their ability to evolve with their host (Domingo, Georgel, & Bahram, 2016).

3. MATERIALS AND METHODS

The set of experiments for this research involved culturing pure colonies of *P. syringae* on Pseudomonas bottom agar using the streak plate technique, in order to confirm that Pseudomonas agar was suitable for the growth of *P. syringae;* followed by PA-DAL and TSA-DAL assays for recovery of pure stocks of Φ6 prior to using the PA-DAL for Φ6 recovery on environmental samples.

3.1 Isolates

Bacteriophage Φ6 and *Pseudomonas syringae* (HB10Y) were used in these experiments employing the DAL plaque assay, they were obtained from Dr. Lisa Casanova's laboratory at School of Public Health, Georgia State University.

a. Bacteriophage Φ6: The titer of the Φ6 stock used (1.28×10^8) was obtained from the laboratory where it was stored at -80 ^{O}C.
b. *Pseudomonas syringae*: The cultures of *Pseudomonas syringae* used in the DAL were grown for 24 hours in 100mL Tryptic Soy Broth (TSB) on a shaker at room temperature.

3.2 Preparation of Media

All media used for the DAL assays were prepared in the laboratory on the same day before performing the experiments.

a. Tryptic Soy Broth (TSB) Preparation: TSB was prepared from 30g Tryptic Soy Broth powder, added to 1L distilled water and autoclaved at 121 ^{O}C for 20 minutes. It was then allowed to cool before dispensing into microcentrifuge dilution tubes.
b. Tryptic Soy Agar (TSA Bottom Agar): The TSA bottom agar was used for the bottom layer of the petri dishes used for the TSA-DAL assay, it was prepared by addition of 40g TSA powder into 1L distilled water and sterilized by autoclaving at 121 ^{O}C for 20 minutes. The

sterilized solution was then allowed to cool in a water bath set at 50^{O}C before dispensing 15mL onto the agar plates, and allowed to sit for about 5 minutes to cool and form a sticky gel on the plates.

c. Tryptic Soy Agar (TSA Top Agar): The TSA top agar was used to plate the phage and bacterial host solutions for the TSA-DAL assay. It was prepared by measuring 30g Tryptic Soy Broth powder and 7.5g Bacto agar poured into 1L distilled water and autoclaved at 121^{O}C for 20 minutes.

d. Pseudomonas Agar (PA Bottom Agar): This was used for the bottom layer of the plates used in the PA-DAL assay. Prepared by measuring 24.2g PA powder into a media bottle, added 500ml distilled water to the bottle, and 5ml glycerol. The solution was mixed and boiled to dissolve completely, followed by sterilization by incubation at 121 ^{O}C for 20 minutes. After the media is sterilized, it was allowed to cool by placing the bottle in a water bath set at 50^{O}C. Once it became cool, 1 vial of Pseudomonas C-F-C supplement, a culture media supplement produced by OXOID-LTD (Basingstoke, Hampshire England – Product ID 1879425) was then added, mixed thoroughly to allow even dispersion of the supplement before dispensing 15mL of the solution onto agar plates and allowed to sit for about 5 minutes to cool and form a sticky gel on the plates.

e. Pseudomonas Agar (PA Top Agar): The PA top agar was used to plate the phage and bacterial host solutions for the PA-DAL assay. It was prepared by measuring 12.2g PA powder into a media bottle, added 500ml distilled water to the bottle, and 2.5ml glycerol. The solution was mixed and boiled to dissolve completely, sterilized by incubation at 121 ^{O}C for 20 minutes. Followed by cooling in a water bath set at 50^{O}C, half the content of 1 vial of Pseudomonas C-F-C supplement is added to the solution and mixed well.

Table 3.2.1 TSA and PA components

PSEUDOMONAS AGAR COMPONENTS			**TRYPTIC SOY AGAR COMPONENTS**	
1. Typical Formula	**gm/L**		**1. Typical Formula**	**gm/L**
Gelatin peptone	16.0		Pancreatic Digest of Casein	15.0
Casein hydrolysate	10.0		Peptic Digest of Soybean Meal	5.0
Potassium sulphate	10.0		Sodium Chloride	5.0
Magnesium chloride	1.4		Bacto Agar (Top Agar)	7.5
Agar	11.0		Agar	15.0
2. PSEUDOMONAS CFC SELECTIVE AGAR SUPPLEMENT (each vial is sufficient for 500ml of medium)				
Cetrimide	5.0mg			
Fucidin	5.0mg			
Cephalosporin	25.0mg			
3. GLYCEROL (Top Agar– 2.5ml)	5.0 ml			
Final pH 7.3 +/- 0.2 at 25°C				

3.3 Environmental Samples

The environmental water samples used for spiking experiments were obtained from irrigation ponds at an experimental agricultural farm. Two distinct ponds were selected, Pond A and Pond B. Pond A had no animal access and the *E. coli* levels detected in pond A samples were very low (about 2 – 8 MPN). Pond B was a manure application area with animal access to the ponds, higher *E. coli* levels were detected in this pond compared to Pond A (about 130-160 MPN).

3.4 Bacteriophage Isolation and Plating

Preliminary experiments were done to ensure that the host for Φ6, a genetically engineered temperature sensitive mutant strain of *Pseudomonas syringae* that does not grow above $25^{O}C$ would grow successfully on Pseudomonas agar. These preliminary experiments were done by the streak plate technique, as well as a DAL assay using only the host and top agar. The streaking procedure for the streak plate technique was done by using a sterile loop to obtain pure culture of *P. syringae* and streaked over the surface of PA bottom agar in a petri dish. To confirm the growth of P. syringae on Pseudomonas agar using the DAL method, 1ml *P. syringae* added to 6ml diluent TSB was plated and incubated at room temperature.

The DAL assay was performed using PA in both bottom and top agar to determine if the titer of bacteriophage Φ6 stock could be measured. The same stock was titered side by side on TSA and PA. A total of 3 replicate experiments were performed for each media using a 10-fold serial dilution and this allowed for comparison between the two media (PA and TSA), most importantly determining whether the titer of bacteriophage Φ6 on each media was similar.

3.4.1 Spiking Environmental Samples with Bacteriophage Φ6

Irrigation water samples collected for this experiment were initially assayed by DAL for naturally occurring Φ6. Experiments were done to determine if a DAL with PA could be used to isolate Φ6 from environmental samples containing other bacteria. Water samples were spiked with Φ6. For each experiment, 100ml pond samples were spiked with Φ6 (titer 1.28×10^{8}) to ensure that the phage can be recovered when it exists in a natural water sample.

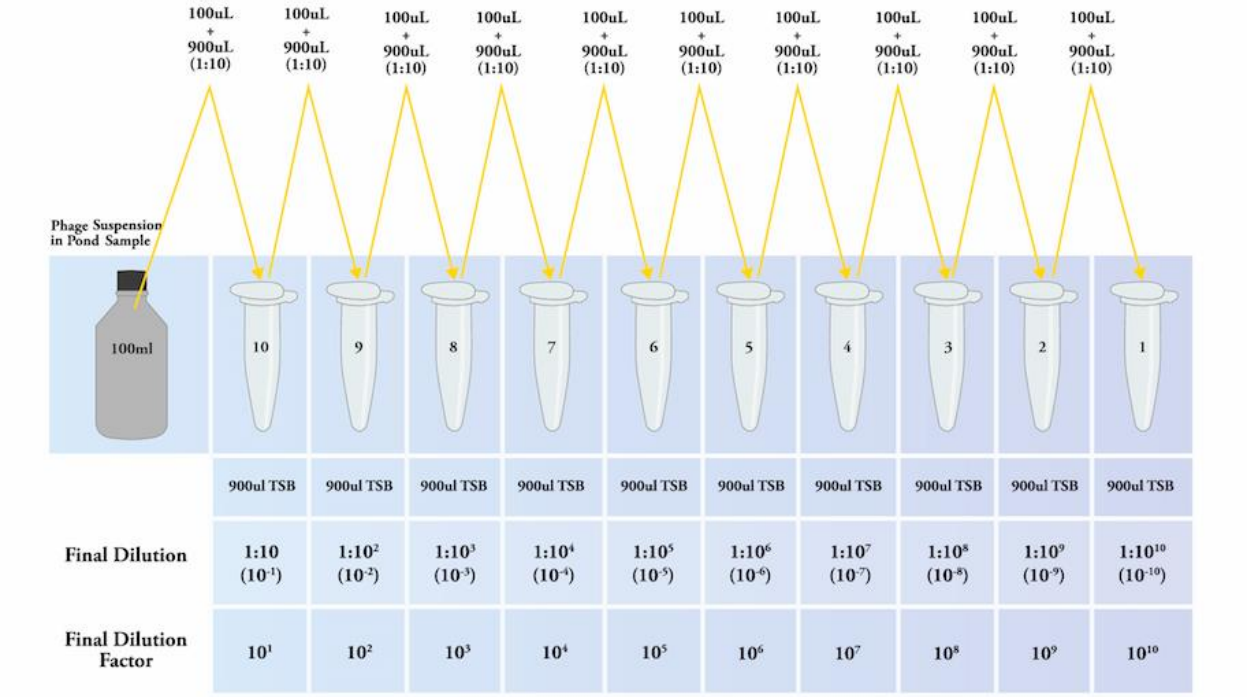
3.4.2 Double Agar Layer Assay - Plating

To pour the plates, 10 warm and dry TSA plates were labeled 1-10 (one for each dilution). 6ml of top agar was dispensed into 10 Nalgene test tubes and each test tube was also

labeled to represent each dilution, then 1ml of *p. syringae* cultured overnight was added to each test tube. The serial dilutions were then transferred into the test tubes. The 10 Nalgene test tubes containing 6mL top agar, 1mL host and cell-phage mixture are then poured onto corresponding TSA plates 1 – 10. The plates are tipped slightly to spread the top agar and allowed to solidify on the bottom agar; the plates are then inverted and incubated at room temperature (25^{O}C) for 24 hours.

For the negative control (NC), the same plating process was followed, but the NC test tube only contained 6mL top agar and 1mL *p. syringae* (host) but no phage.

Fig 3.4.1 Serial Dilutions



Serial dilution of bacteriophage Ø6 suspension in pond sample. First, pipette 90uL of TSB (diluent) into each dilution tube (numbered 1-10). Then transfer 0.1uL of phage suspension in series, mixing each time.

3.4.3 Examination of Bacteriophage Plates

The plates were examined for visible plaque formation after 24 hours of incubation at room temperature. The number of phage particles that were in the original stock phage culture was determined by counting the number of visible plaques counted on each plate. Each of the plaques formed was designated as a plaque-forming unit (PFU) and can then be used to quantify the amount of infective phages in the sample.

4. RESULTS

4.1 Tests for *P. syringae* Growth on Pseudomonas Agar (PA)

Preliminary experiments were done to demonstrate that *P. syringae* could grow on Pseudomonas agar and form confluent lawns in PA top agar, and to observe the typical appearance of *P. syringae* colonies and lawns on this agar.

Streak Plates

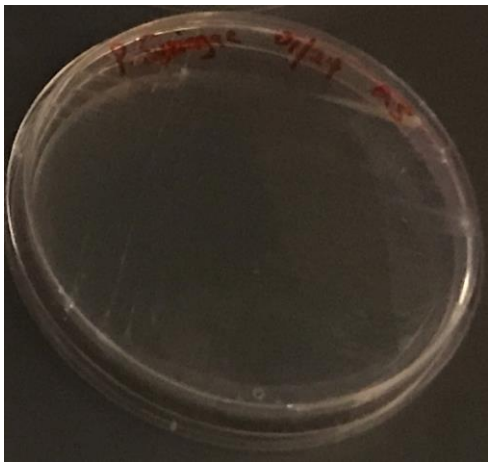
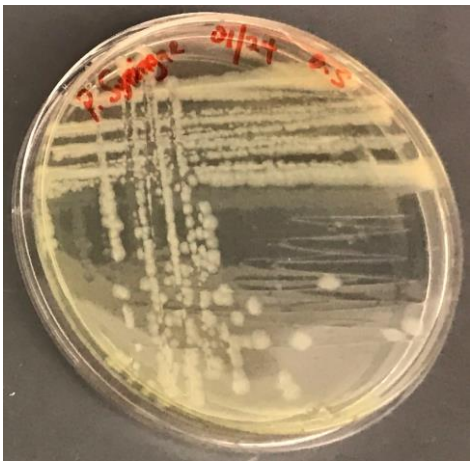
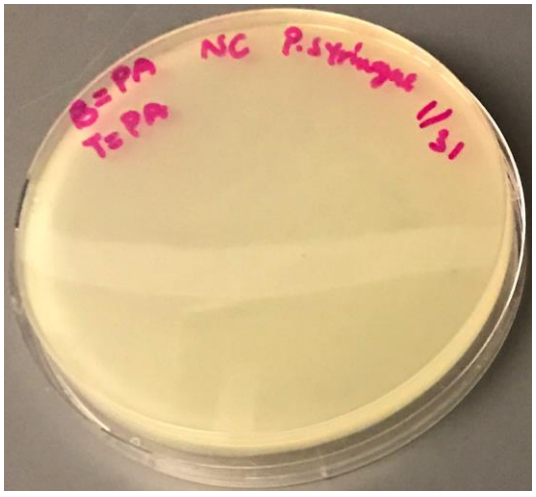
(a)  **(b)** 

Figure 4.1.1. Streak Plates: Fig. (a) on the left shows no visible colonies formed when observed less than 24 hours after streaking. Fig. (b) on the right shows *P. syringae* colonies on the surface of Pseudomonas agar after about 24-48 hours of incubation at room temperature.

DAL Assay: *P. syringae* Host + 6ml PA Top Agar

(a) 

(b) 

Figure 4.1.2. DAL Assay: Both Figures show a confluent lawn, *P. syringae* growth on PA

4.2 Bacteriophage Φ6 Isolation by DAL Assay using Pseudomonas Agar

The ability of pseudomonas agar to quantify bacteriophage Φ6 was first evaluated by DAL plaque assay using pure Φ6 stock, and the titer and plaque formation on PA plates was compared to the titer and plaques formed on TSA plates. A 10 fold serial dilution was done. The formation of plaques on both TSA and PA plates were similar for three experiments performed. This confirmed that a DAL with Pseudomonas Agar as both top and bottom agar could be used to titer Φ6 as much as Tryptic Soy Agar would. The titer of Φ6 recovered using TSA range from 2.43×10^8 to 2.45×10^9 while the titer of Φ recovered using PA range from 9.46×10^7 to 6.06×10^8 as shown in table 4.2.1 below. A non-parametric statistical test (X^2) was used to test for difference between both TSA and PA media; we conclude that TSA and PA are independent of each other and as such there exist a significant difference between both, since the X^2 calculated value is greater than the critical value.

Table 4.2.1 Pseudomonas Agar DAL Assay – PFU/ml formed

	TSA (PFU/mL)	**PA** (PFU/mL)
Experiment		
Exp 1	2.45×10^9	6.06×10^8
Exp 2	2.43×10^8	9.46×10^7
Exp 3	2.00×10^9	9.81×10^7

- $(X^2 = 4031.12, Critical\ Value\ = 5.99, p = 0.05)$

The 95% C.I for the critical value is chi-square (2, 0.95) = 0.10259 and Chi-square (2, 0.05) = 5.991.

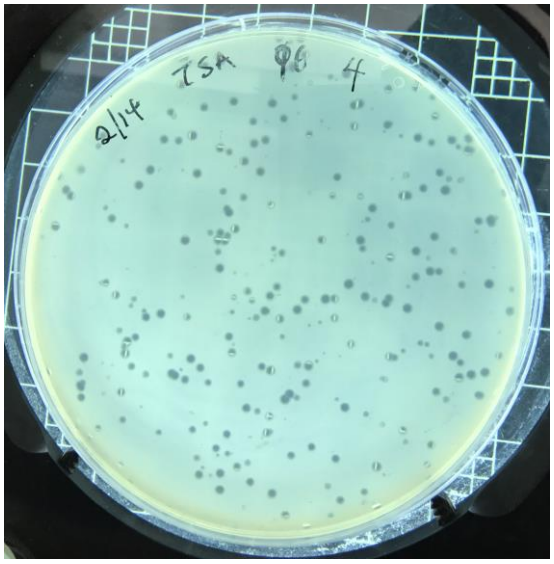
Table 4.2.2 Pseudomonas Agar DAL Assay - Plaques formed

TSA top agar										
DILUTION	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**
PLAQUES										
Exp 1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	238	32	0
Exp 2	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	236	31	3	0
Exp 3	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	201	19	0
Pseudomonas agar										
DILUTION	**10**	**9**	**8**	**7**	**6**	**5**	**4**	**3**	**2**	**1**
PLAQUES										
Exp 1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	603	62	8	0
Exp 2	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	92	12	1	0
Exp 3	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	92	12	4	1

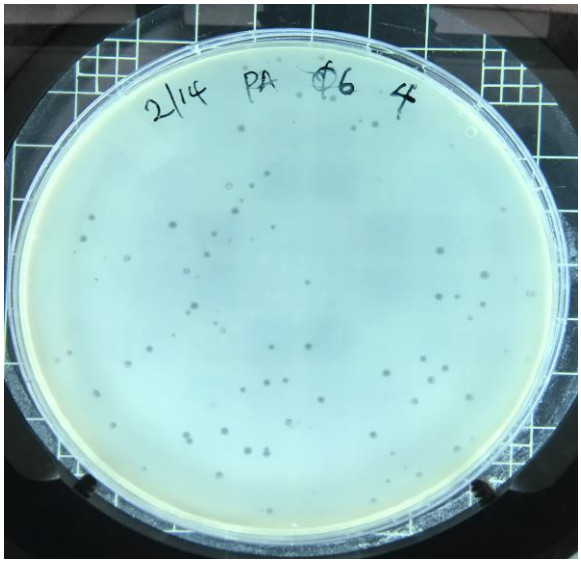
* $(X^2 = 274.11, Critical\ Value\ = 5.99, p = 0.05)$

Table 4.2.2 aboves shows the number of plaques formed on both TSA and PA plates and there there are notable comparisons in the plaque formation, the plaques formed were countable from the 4th plates of the 10-fold serial dilution for both TSA and PA media.

Figure 4.2.1. TSA and PA 4-dilution plates



(a) TSA - Φ6 – 10^7 (n =236)
2.36×10^8 PFU/mL



(b) PA - Φ6 – 10^7 (n =92)
9.20×10^7 PFU/mL

4.3 Bacteriophage Φ6 Isolation in Spiked Pond Samples (DAL Assay)

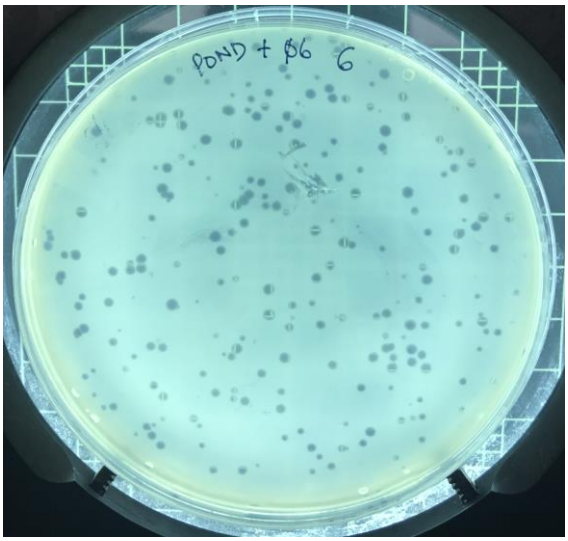
Following the confirmation that a DAL could be performed to titer pure Φ6 stock in a DAL assay using PA agar, pond samples were assayed but there was no Φ6 recovered from the pond samples. The pond samples were then spiked with Φ6 to determine if the DAL using PA could isolate Φ6 from an environmental sample. Φ6 was spiked into 100ml pond water samples, and serial dilutions performed and plated by the DAL assay.

Table 4.3.1 Φ6 Spike Experiment – PFU/mL formed

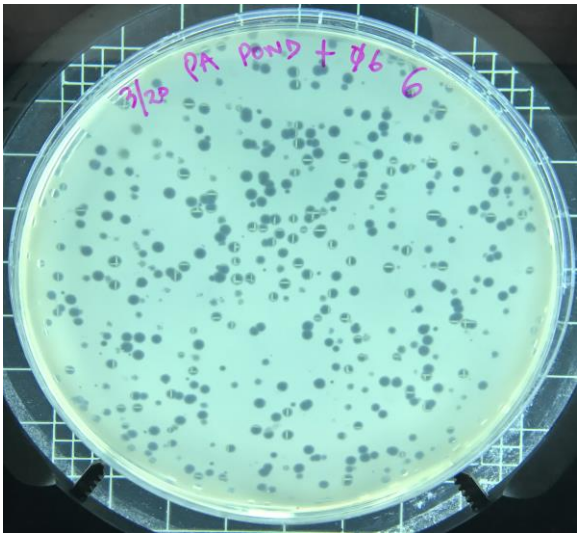
	POND A (PFU/mL)	**POND B** (PFU/mL)
Experiment		
Exp 1	2.27×10^6	3.92×10^6
Exp 2	3.19×10^6	3.71×10^6
Exp 3	4.24×10^6	3.76×10^6

From Pond A, the recovered Φ6 titer was 2.27×10^6 , 3.19×10^6 and 4.24×10^6 respectively. From Pond B, recovered Φ6 titer was 3.92×10^6, 1.26×10^7, 3.71×10^6, and 3.76×10^6

Figure 4.3.2. Serial Dilution – Isolation of Φ6 in spiked Pond Samples



(a) Pond A - Φ6 – 10^5 (n =228)
2.28×10^6 PFU/ml



(b) Pond B - Φ6 – 10^5 (n =368)
3.68×10^6 PFU/ml

Table 4.3.2 Φ6 Spiked Pond Samples Plated with 1ml host + 1ml Φ6

Pond A				*Pond B*			
PLATES	**1**	**2**	**3**	**PLATES**	**1**	**2**	**3**
PLAQUES **Exp 1**	TNTC	TNTC	TNTC	**PLAQUES** **Exp 1**	TNTC	TNTC	TNTC
Exp 2	TNTC	TNTC	TNTC	**Exp 2**	TNTC	TNTC	TNTC
Exp 3	TNTC	TNTC	TNTC	**Exp 3**	TNTC	TNTC	TNTC

* TNTC – Too Numerous To Count.

Table 4.3.2 above shows the result of the DAL assay performed by plating 1mL of the collected pond samples spiked with Φ6, and 1 ml of the *P. syringae* cultured overnight; the plaques formed were too many to be counted. The serial dilutions were more reliable because the plaques formed were countable rather than plating 1ml of the host added to 1ml of the phage solution.

5. DISCUSSION

The preliminary tests performed by the streak plate technique to determine whether the host (*Pseudomonas syringae*) would grow on the media (PA – bottom agar) confirmed the suitability of Pseudomonas agar for *P. syringae* growth, this was confirmed by the growth of distinct colonies on the plates as shown in Fig. 4.1.1(b). The preliminary DAL assay done by plating 1ml of the host added to 6ml diluent TSB was also confirmatory that PA is suitable for *P. syringae* growth by the formation of a confluence lawn. The lawn formation plays an important role in the counting of plaques when phages lyse their host bacterium because of the contrast between the lawn and plaques formed, making the plaques very distinct and visible.

The Double Agar Layer (DAL) plaque assay has been used extensively in phage research to isolate existing phages and also detect new ones; the DAL assay has been in use since the discovery of phages in 1915 by Frederick W. Twort which confirmed the ability of bacteriophages to lyse their host bacterium, hence leading to a plaque formation within a bacteria lawn. This remains the primary aim of performing the DAL plaque assays till date as the interest in phage research has increased in recent times (Santos et al., 2009).

The present study compared plaque formation using TSA and PA media; the TSA and PA DAL assay results shown in Table 4.2.1 allows for comparison between the two media, which shows the difference between the amounts of Φ6 recovered from both media expressed in PFU/ml. The experiment showed comparable results on both TSA and PA DAL assays. Although, a slightly higher amount of Φ6 was recovered from TSA DAL assay ($\sim 2.43 \times 10^8$ to 2.45×10^9 PFU/mL) than PA DAL assay ($\sim 9.46 \times 10^7$ to 6.06×10^8), the PA DAL assay performed well in the recovery of Φ6. Also, to determine if the difference observed between TSA and PA is statistically significant, a chi-square test was conducted which showed that the

calculated chi-square value is greater than Chi-square critical value; therefore we conclude that TSA and PA are independent on each other and there is a significant difference between them. The PA DAL assay can be recommended for use in the isolation of bacteriophage Φ6 from the environment because it is a selective media compared to TSA that is not a selective media. TSA is an all-purpose growth media that provides adequate nutrients for the growth of a wide range of microorganisms that may make quantification of Φ6 titer difficult when used in DAL assays to isolate Φ6. On the other hand, the Pseudomonas Agar (PA) with glycerol, supplemented with cetrimide, fucidin and cephalosporin (C-F-C) tested in this study is a selective biological media, it does not allow for growth of many bacteria; it is only selective for the growth of *Pseudomonas aeruginosa* or *Pseudomonas* spp. at large and this study has confirmed it's suitability for Φ6 phage assays using the DAL technique. Previous studies suggest that the addition of glycerol to both top and bottom PA media before sterilization may be responsible for the effectiveness of the media in the isolation of phages; previous experiments using the combination of glycerol and antibiotics confirmed the formation of larger plaques than when only antibiotics was used, the authors suggest that the mode of action of glycerol is synergic with antibiotics in improving plaque burst size and contrast between the plaques and the turbid bacterial host lawns (Santos et al., 2009).

The abundance of bacteriophages in the environment as well as the limitations in current isolation techniques calls for development of new isolation methods, or the modification of existing research methods to enhance the isolation of bacteriophage Φ6 whenever it exists in the environment. The result of this present study indicates clearly that Pseudomonas Agar with glycerol, supplemented with cetrimide, fucidin and cephalosporin (C-F-C) is a viable media for the isolation of bacteriophage Φ6 from environmental samples. Notably, the plaques formed in

the experiments, as seen in Fig 2 (a & b) were clear and visible to the naked eye, as well as consistency of plaque formation in each serial dilution, therefore enabling better quantification of phages when they occur in environmental samples. This phenomenon as stated earlier, has been observed in previous research where the plaques formed in classical DAL plaque assays yielded clear visible plaques when glycerol and antibiotics were added to growth media used for DAL assay (Santos et al., 2009).

The assays performed using environmental samples chosen for the study (Ponds A & B) did not recover any naturally occurring Φ6, but the spiking experiments performed in this study proves that Pseudomonas Agar would be effective in the recovery of Φ6. The inability of the DAL assay to detect Φ6 in the pond samples chosen for the study may be as a result of the detection limit of Φ6 by the plaque assay or the absence of the bacterial hosts for naturally occurring Φ6 in the pond samples. Hence, this may be considered as a limitation of this study and future research may consider isolation of Φ6 from sewage effluents because previous studies have identified Φ6 in sewage. In conclusion, this study shows evidence that Pseudomonas Agar prepared with glycerol addition of pseudomonas CFC supplement can be used to recover Φ6 in environmental samples using the DAL plaque assay.

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