Chapter I

Introduction

Virtually any type of cell is susceptible to virus infection; viruses cause disease in plants and animals, and can also infect procaryotes and unicellular eucaryotes. Viruses that infect procaryotes are known as *bacteriophages,* or *phages,* because when they were first discovered, they appeared to eat bacterial cells, generating a clearing, or *plaque,* on a lawn of susceptible bacteria. Bacteriophages were discovered by British pathologist Twort in1995 by transmissible agent of '*Micrococcus*' colonies (Michael *et al*. 2003). In 1917, French Canadian microbiologist d'Herelle accidentally discovered the lysis formation of *Shigella* culture in the broth, which caused by phage.

The phages are commonly named in reference to their host. Thus, the phage which attack the bacterium *Staphylococcus* is called staphylophage and those attack *Escherichia coli* is called coliphage. Like all viruses, bacteriophages consist of nucleic acid (RNA or DNA) surrounded by a protein coat, or capsid. But, bacteriophages are not enveloped. Some phages have elaborate structures for attaching to the bacterial surface and injecting nucleic acid into the cytoplasm. The capsid can be variable in shape. The head and tail and tail structure seems to be unique to phages (Dimmock *et al*, 2016).

Bacteriophages are detectable almost everywhere where live bacteria exist (Zhan et al. 2015). The environment populated by bacterial hosts such as soil, sewage, and animal secretions are unique source of all types of phages (Naghavi *et al.* 2013). Total phage population in aquatic environment was estimated as above 10^{13} (Perisian *et al*., 2018 and Dublanchet and Bourne, 2007). Many terrestrial ecosystem have been shown to harbor 10^{7} phages per gram of soil and 10^{8}-10^{10} phages per milliliter of sewage (Strauch *et al.,* 2007 and Dabrowaska *et al*., 2005).

There are two types of bacteriophage host range, monovalent and polyvalent. Polyvalent phages are able to attack two or more bacteria species, whereas monovalent phages are specific to one type of bacteria species (Kalmansom and Bronfenbrenner 1942). Phages are highly host specific and they only attack a particular group of bacteria species. The host specificity is dependent on the evolution of recognition system of the viruses, based on 'lock and key' theory (Kutter and Sulakvelidze 2005). The receptors on the bacteria host are recognized by the protein on the phage

(Nester *et al*. 2004). When the phage attacks the bacteria, each of the phage will multiply and release several hundred new viruses, and the bacteria in the area surrounding the phages are destroyed, leaving a clear area or plaque on the agar. Each plaque corresponds to a single infective virus in the initial suspension (Ted and Christine 2004)

Bacteriophages are now-a-days used for a wide range of purposes in both medical and industrial aspects. Phages and phage products are now applied in several infectious diseases, such as Pseudomonas sp. (Ahmed, 2002), vanomycin resistant Enterococci (Biswas *et al*., 2002) and Uchiyama *et al*., 2005), multidrug resistant Klebsiella pneumonia (Vinodkumar *et al*., 2005), multidrug resistant Pseudomonas aeruginosa (Wright *et al.,* 2009), antibiotic resistant strains of Escherichia coli (Vascardi *et al*., 2009) and methicillin resistant Staphylococcus aureus (Mann, 2008).

Long-term use of phages in poultry has proved to be moderately effective in reducing the number of *Salmonella* pathogens colonizing the digestive tract (Sklar *et al.,* 2001). Phage suspensions applied directly to the air sac in 3-day-old birds in a range of titres from 10^6 to 10^3 PFU to treat *E. coli* infections substantially reduced mortality rates to 5% and 25%, respectively. Similar results were obtained after inoculation of a bacteriophage suspension in the drinking water of birds at 1 week of age (10^3 or 10^4 PFU of bacteriophages per mL) followed by air sac challenge with 10^3 CFU of *E. coli* phages. Mortality was decreased to 25% and 5%, respectively. No mortality was observed in chickens treated with 10^8 PFU of an *E. coli* bacteriophage mixture (Huff et al. 2002). One of the first attempts to use bacteriophages against *Campylobacter* bacteria was a study by Wagenaar *et al*., in which colonization by *C. jejuni* was inhibited in 10-day-old chicks and adult birds, first by 2 and then by 1 log unit in broiler caeca.

Bacteriophages are used for identifying specific bacterial strain. This is defined as phage typing. For detection of unknown bacterial strain, its lawn is provided with different phages and if the plaques appear, it indicates that phage has been grown and lysed the bacterial cell, making it easy to identify the specific bacterial strain (Clark and march, 1998). *Mycobacterium tuberculosis, Pseudomonas, E. coli, Salmonella, Listeria* and *Campylobacter* species can be detected by phage amplification assay (Barry *et al*., 1996)

Certain vaccine antigens are expressed on the surface of the bacteriophage and the phages can carry the vaccine. But in case of DNA vaccine, the essentials for vaccine antigen synthesis are

incorporated into the phage genome and the phage would then act as vehicle for the delivery of DNA vaccine (Clark and March, 2004).

Phages could be used as predators of pests (bacteria) found in association with plants fungi or their products (Flaherty *et al.*, 2001). Phages have been successfully employed agonist *Xanthomonas Campestris* which cause spots on tomato. Bacterial blotch of mushrooms caused by *Pseudomonas tolaasii* can be treated with phages (Gilla and Abedon, 2007)

Phage therapy can be used in dermatology, urology, gynecology, ophthalmology and so on. It is now used in surgery and wound treatment and against certain intestinal infections. The use of phage in the treatment of bacterial infections is an attractive alternative to existing therapies (example, antibiotics), because unlike broad-spectrum antibiotics phage target a particular host and are unlikely to illicit resistance in untargeted bacterial strains (Sulakvelidze and Kutter, 2005). Also, unlike chemical therapeutic agents, phages are not susceptible to the onset of bacterial resistance because they have the ability to evolve with their host (Sulakvelidze and Kutter, 2005).

Enteric bacteria are normal inhabitants of the intestines of humans and other animals (Davis, 2005). Sewage contains high numbers of potentially very pathogenic enteric bacteria known as fecal coliforms. Coliforms are characterized as gram-negative, facultative anaerobic bacteria that ferment lactose within 48 h at 35oC. Examples of fecal coliforms include *Escherichia coli*. The objective of this study was to isolate phage (Coliphage) from sewage sludge.

Chapter II

Methods and Materials

Sample collection

Approximately 500ml of sewage water were collected from sewage line of MA Hannan Hall, CVASU. It was sampled using sterile containers. The sample was first filtered through coarse filter paper to remove the debris.

Culture media preparation

Tryptone soft agar media and tryptone hard agar media was prepared and sterilized at 121 °C (15 psi pressure) for 15 minutes in an autoclave.

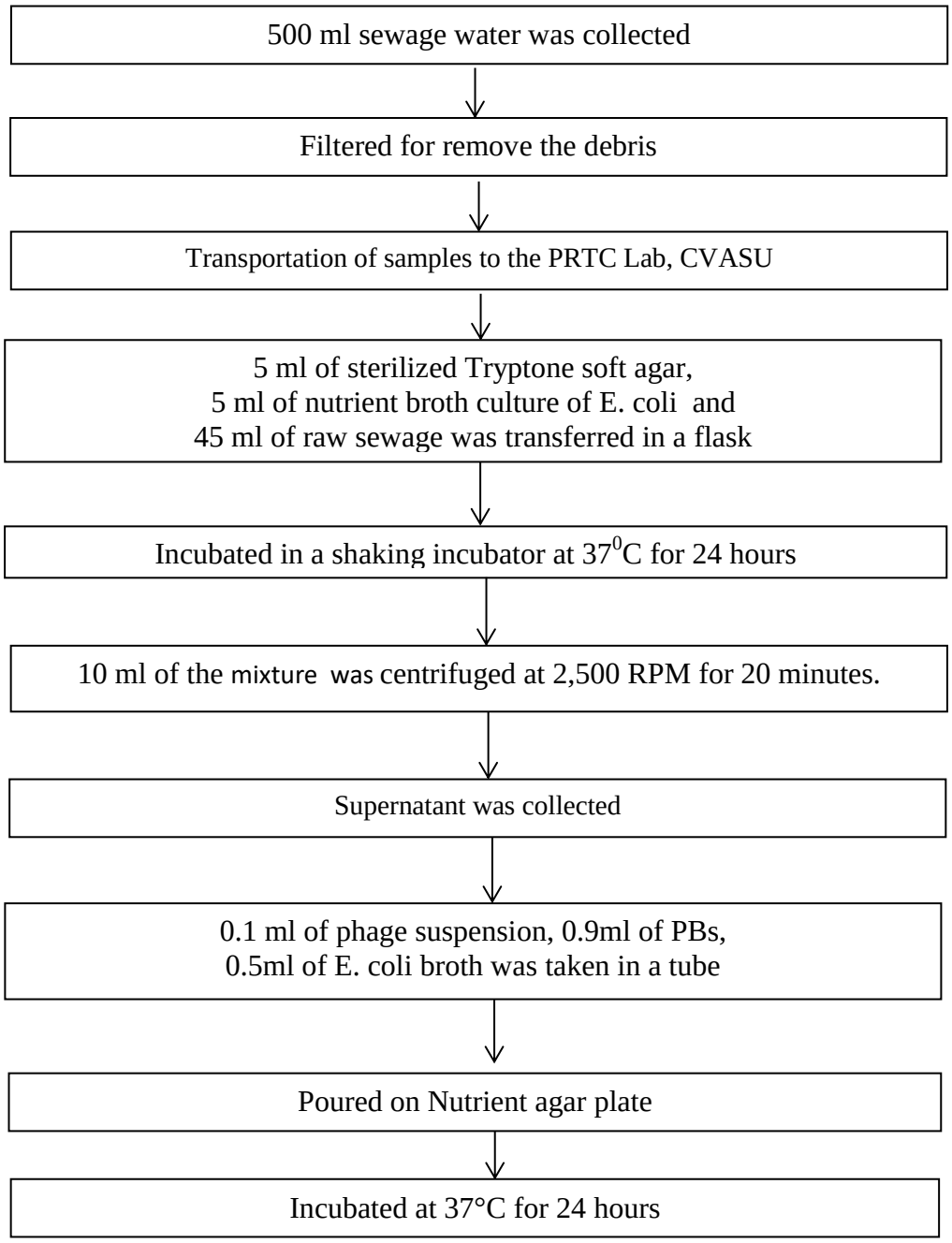
Amplification of Phages

Into a cotton-plugged sterilized empty 250 ml conical flask, 5 ml of sterilized Tryptone soft agar, 5 ml of nutrient broth culture of E. coli and 45 ml of raw sewage was transferred aseptically. This was incubated in a shaking incubator at 37^0C for 24 hours to allow the Coliphage in the sewage to proliferate within the host bacteria cells (cells of E. coli B).

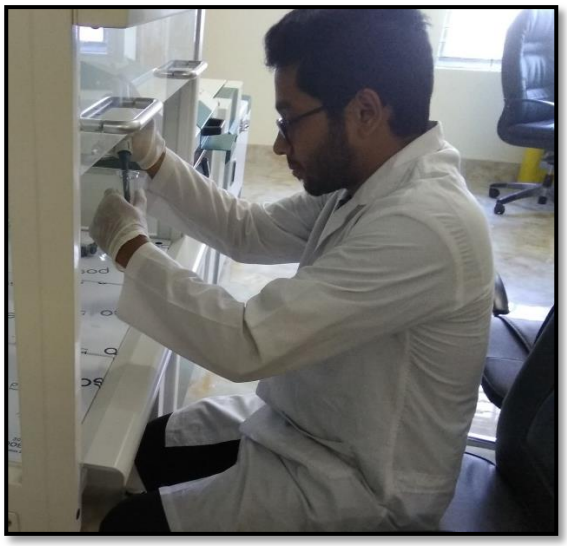
Bacteriophage Isolation and Plating

10 ml of the sewage-bacteria-bacteriophage culture was centrifuged at 2,500 RPM for 20 minutes. The sediment was discarded. The supernatant, rich in Coliphage, was filtered. 0.1 ml of phage suspension was added to 0.9ml of PBS in a sterile tube. 0.5 ml of E. coli broth was added in the tube. It was then incubated at 37^0C for 10 minutes to allow the phage to attach to the bacteria. This was the cell-phage mix. The cell-phage mixture was poured on Nutrient agar plate and incubated the plate, inverted, at 37°C for 24 hours.

WORKING SCHEME



500ml of sewage water from sewage line of MA Hannan Hall, CVASU



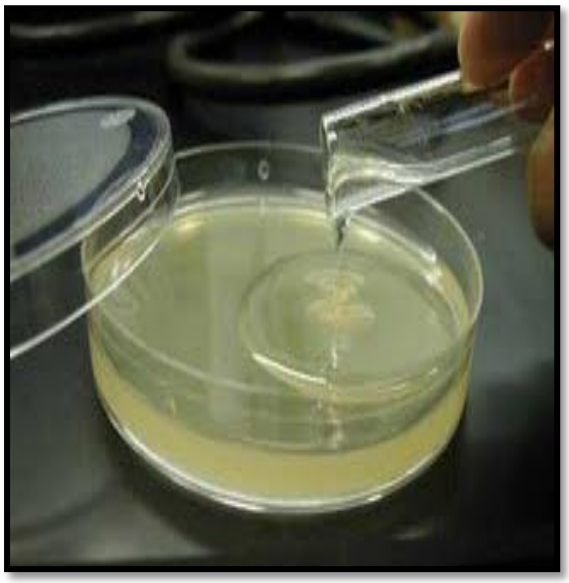
Tryptone soft agar (5ml), Nutrient broth culture of *E. coli* (5ml) and Raw sewage (45ml)

Incubated in a shaking incubator at 37^0C for 24 hours

10 ml of the mixture was centrifuged at 2,500 RPM for 20 minutes

Supernatant was collected

Few drops of suspension, 2 ml Tryptone soft agar, 0.1ml Nutrient broth culture of *E. coli* in a tube



Poured on Tryptone hard agar plate

Incubated at 37°C for 24 hours

Chapter III

RESULT

Enrichment single layer method was followed for the isolation of bacteriophage. Indistinct plaques were observed after 24h incubation at 37oC. A representative culture plate of plaques assay is shown in fig.

Fig: Plaques of bacteriophage

The plaque caused by the phages varies in size and is characterized by the circular zone. The number of plaque forming unit from phage isolates were very few number. The lower plaque forming unit (PFU) observed in this study. It could be due to the number of E. coli in the bacterial culture was less number or other technical issues.

The single plaque is expected to vary in size and morphology because the *E. coli* cells had been infected by different type of phages. There are two common types of phages namely lytic (T-series) and lysogenic phages. The lytic phages normally cause lysis or clear condition to the host cells. On the other hand, the lysogenic phages formed the turbid condition which lysed the cells (Singleton 1992).

Lytic phages multiplied within host cell and lyse them (Singleton 1992). They undergo the lytic infection cycle where they proliferated in the host cells, and emerge from host cells by lysing process. During the process, phages will attach to the bacteria cell wall and inject their DNA into

the cell. Phage DNA will replicate and genes encoding protein coat will be transcribed orderly using the host machinery system. At the end of the cycle, many viruses will be produced and the *E. coli* will burst (Prescott *et al*. 2005).

On the other hand, lysogenic phages integrate their chromosome into the chromosomal DNA of the host or establishing themselves as a plasmid, and enter the host cells in a harmless condition (Prescott *et al*. 2005).

Chapter IV

DISCUSSION

The presence of Cliphage in the sewage line of both human and animal was also detected previously by researchers. Tan *et al.* Isolate and characterize lytic bacteriophages from sewage water. Bacteriophage isolated from sewage eliminates by Ribeiro *et al.* Many workers have isolated phages from sewage of livestock farms (Tiwari *et al*., 2010 and Shukla *et al*., 2011). The bacterial host for the phage isolated in this study was found to be similar to *Citrobacter freudii*, a common enteric bacteria belonging to the family E*nterobacteriaceae*. *C. freudii* is commonly found in sewage and has been associated with nosocomial infections in the urinary, respiratory, and biliary tracts of debilitated hospital patients (Tortora *et al*., 2006).

Higher recovery status of phages in dairy farm waste as compared to buffalo farm waste is in support with report of Tiwari *et al.*, Shukla and Hirpurkar, and Askora *et al.* Higher concentration of phage in bottom and a middle layer of tank were also reported by Carey-Smith *et al.* and Shukla and Hirpurkar. Goyal *et al.* opined that most of the organic matter settles in the deeper layer, thus providing optimum conditions for multiplication of host bacteria in deeper layer, which in turn, improves host range interaction.

Plaque morphology is one of the foremost criteria for characterization of phages (Shukla *et al*., 2011). Variation in the plaque morphology during the present study may correspond to the difference in phage strain and other factors (Ghasemian *et al*., 2017). In contrast, Jothikumar *et al.* found that plaque morphology was not affected by addition of cations. Pedroso and Martins also did not find any relationship between coliphage family and specific plaque morphology.

Wide host range and phage types reported during present study are in conformity to the reports of Bielke *et al.* who observed that phage host range is not always genera restricted, so phages could have wide host range. Present observations are in partial conformity with Carey-Smith *et al.* who had reported narrow range phages restricted to maximum of two bacterial species.

During the last decade, a marked increase in the number of identified phages has been observed. More than 200 lytic *Staphylococcal* phages have been characterized (Ackermann *et al.*, 2007) Present findings are supported by earlier studies also in which phages were recorded against *Streptococci* (Ahmed *et al*., 2012) *E. coli* and other enterobacteria (Marwa *et al*., 2014) *Pseudomonas* (Morello *et al.*, 2011) *S. aureus* (Garcia *et al.*, 2009) and *Bacillus* (Yuan *et al*., 2012).

Chapter V

LIMITATION

Coliphage was not characterized for specific strain of *E. coli.* Plaques of the bacteriophage was not counted.

Chapter VI

CONCLUSION

Sewage contains abundant *Escherichia coli* which serve as susceptible and highly adaptable host for the growth of bacteriophages. Phages were successfully isolated in this study and characterized based on morphology of the plaque, nucleic acid analysis and phage protein composition profile. These isolated phages may be in the range of lytic phages based on the DNA analysis and protein profile study. However, more precise studies using transmission electron microscope (TEM) analysis, DNA sequencing and restriction mapping need to be carried out to further characterize, identify and distinguish the isolated phages to the species level.

Chapter VII

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