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MINIREVIEWS

An Appraisal of Bacteriophage Isolation Techniques from Environment

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Abstract
Researchers have recently renewed interest in bacteriophages. Being valuable models for the study of eukaryotic viruses, and more importantly, natural killers of bacteria, bacteriophages are being tapped for their potential role in multiple applications. Bacteriophages are also being increasingly sought for bacteriophage therapy due to rising antimicrobial resistance among pathogens. Reports show that there is an increasing trend in therapeutic application of natural bacteriophages, genetically engineered bacteriophages, and bacteriophage-encoded products as antimicrobial agents. In view of these applications, the isolation and characterization of bacteriophages from the environment has caught attention. In this review, various methods for isolation of bacteriophages from environmental sources like water, soil, and air are comprehensively described. The review also draws attention towards a handful on-field bacteriophage isolation techniques and the need for their further rapid development.

Keywords Bacteriophage isolation · Methods · Environment · Air bacteriophages · Phage therapy

Introduction

Viruses are ubiquitous microorganisms that are obligate intracellular parasites on all known types of cells – prokaryotes, eukaryotes, protozoa, fungi, yeast, and Archaea. They are the smallest life forms on Earth, ranging in size from ~ 20 to 400 nm [1, 2]. One of the classes of viruses that has held a lot of interest in the scientific community is bacteriophages. Bacteriophages exclusively infect bacteria and do not pose a direct threat to humans or plants. They infect and replicate in bacteria predominantly through lytic and lysogenic life cycles. Virulent bacteriophages lyse the host bacteria soon after infection, while temperate bacteriophages either integrate with the host genome or remain as independent prophages [3].

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Bacteriophages have been instrumental in aiding groundbreaking discoveries in the fundamentals of molecular biology and development of genetic engineering tools since their discovery. They are naturally antibacterial and hence have also been explored for their applications in therapy [4, 5] gene delivery [6], food preservation [7, 8], biocontrol of plant pathogens [9, 10], surface disinfection [11], phage display [12, 13], bacterial biosensors [14, 15], and vaccine carriers [16]. They are increasingly proving to be attractive alternative antibacterials [17–20]. Bacteriophages have also been used in human therapy since 1920s, with clinical trials progressing since 2009 [21–45].

Bacteriophages are isolated from environmental sources like freshwater, marine water, soil, air, and wastewater [46–49]. For therapy, they are commonly isolated from water samples, especially sewage, that are reservoirs of bacteriophages against human pathogens like ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) pathogens [50–56]. An abundance of diverse bacteriophages is also seen in soil and air with possibilities of finding unique bacteriophages suitable for various applications.

Having vast diversity and simpler genomes, bacteriophages hold great potential to be engineered for different applications [57]. While encouraging, the use of genetically engineered bacteriophages may be concerning considering the possible

variations in bacterial community dynamics and microbial genome evolution due to uncontrolled escape of such bacteriophages during various applications [58].

Exploring the natural environment for bacteriophages with properties suitable for different applications is a better alternative. Bacteriophages have their own niche, specificity, protein components, and bactericidal activities [59, 60]. Isolation of specific bacteriophages is the first challenge towards successful application; this step being associated with most variable time requirements and likelihoods of finding success.

In this review, we discuss the known methods of bacteriophage isolation from water, sewage, soil, and air environments.

Approaches to Bacteriophage Detection

Bacteriophages are broadly distributed into two groups – virulent (or lytic) bacteriophages and temperate (or lysogenic) bacteriophages. Lytic bacteriophages start replicating after host infection and form new bacteriophages that burst out of the host cell by rupturing or lysing it. This lysis is responsible for their antibacterial property. Since temperate bacteriophages integrate their genome into the host genome post-infection, they are not preferred as antibacterial agents.

Interestingly, it is the lytic life cycle of bacteriophages that helps in their detection by standard microbiological, culture-based methods. Prevalent methods of detection are culture lysis method, plaque assay, and spot testing [61, 62]. In these methods however, a bias is created towards lytic bacteriophages that are viable or fitter, as against other phages in culture that may be weaker, non-infective, or non-lytic.

Molecular methods like metagenomics and bacteriophage genome analysis can be employed for in silico detection of temperate or under sampled bacteriophages [63]. It is important to understand though that metagenomics can only help detection of phage genomic signatures in the DNA samples and is not really a phage isolation method. Metagenomic approaches detect signature sequences from DNA fragments in a library, which may not be organized completely giving only partial information during analysis [64, 65]. These studies along with culture based assessments can provide a full picture of bacteriophage diversity in a given sample.

Isolation of Lysogenic Bacteriophages by Prophage Induction

Temperate phage genome carried as a lysogen in the host genome is termed as a prophage [66]. Unless obligately lytic bacteriophages are found, many phage scientists prefer to isolate temperate ones and modify them genetically to remove undesired genes. In some cases, for example, that such as *Clostridium difficile*, several prophages are identified phages are temperate [67]. Therefore, temperate phages are also needed to be isolated in many conditions. In nature, prophages are induced to undergo lytic cycle under certain physiological stimuli. The energetic state of the bacterial cell and the growth conditions determines whether the prophage will undergo a lytic cycle [68]. To identify and isolate prophages, protocols involving physical and chemical treatments that attack DNA integrity have been developed. These include use of chemical agents like mitomycin C [69] antitumor drugs [70], antigyrase drugs [71], antifolates [72], fluoroquinolone antibiotics [73], hydrogen peroxide [74], and UV light [75].

A treatment of bacterial culture hosting the lysogen with 0.1–0.5 µg/ml mitomycin C acts effectively in inducing prophages [69]. Another commonly employed technique is irradiation of bacterial culture with short wave UV light in the presence of $MgSO_4$. Post-exposure, the bacterial cells are incubated in double strength media to help them recuperate from UV damage, while the induced prophages grow in titer.

Lab-based Methods of Bacteriophage Isolation

Bacteriophage diversity in the environment is very high; they are globally distributed throughout various environments like aquatic systems, terrestrial systems, deep seas, and air. [65].

The presence of bacteriophages as separate entities was first indicated by Ernst Hankin (in 1896) [75]. Felix d'Herelle described a basic enrichment-based method of bacteriophage isolation that has formed the foundation for all bacteriophage isolation methods [76, 77]. An overview of the various bacteriophage isolation methods from environment is depicted in Fig. 1 below.

Reservoirs of Bacteriophages in Water

Bacteriophages are widely distributed in all aquatic environments [78]. The marine ecosystem hosts about 10 [30] viruses and is considered a big reservoir of bacteriophages [79]. As per Yooseph et al. (2010), members of the family *Vibrionaceae* occur in high abundance in the marine ecosystem with 10^3 to 10^4 cells per ml found in sea water [80]. Typically, *Vibrio* bacteriophages, coliphages, and bacteriophages against *Bacteroides fragilis* have very commonly been isolated from marine environments [81–83]. In marine ecosystems, factors such as ionic environment, hydrostatic pressure, aerobic/anaerobic conditions, temperature, and the dynamics of host bacteria numbers play an important role on the survivability of the bacteriophages and therefore their detection [84].

Rivers too are rich natural sources of bacteriophages. They are highly exposed to anthropogenic microbial

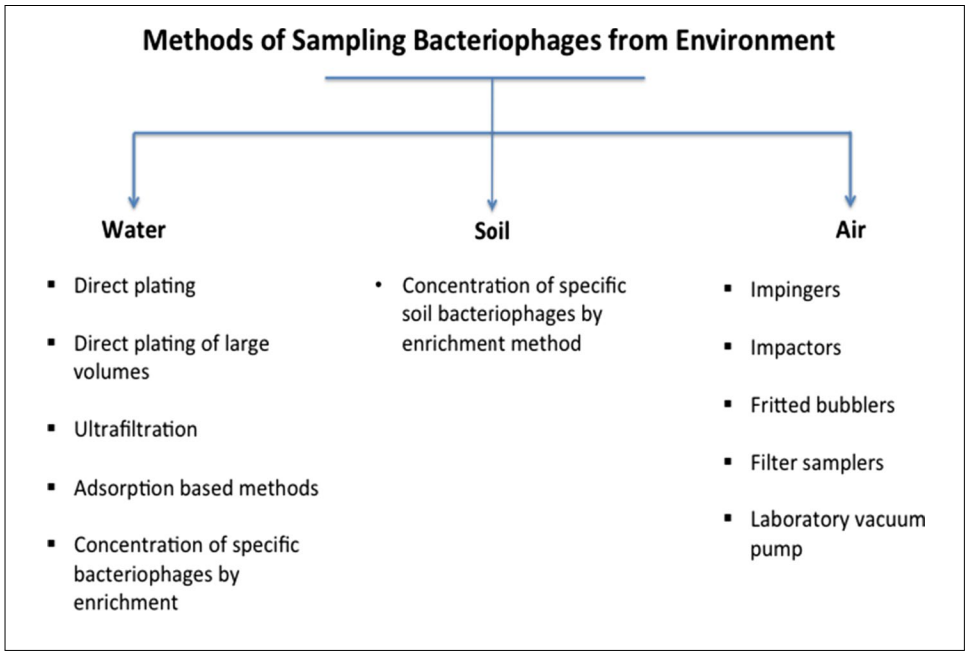

Fig. 1 An overview of bacteriophage isolation methods from environment

pollutants, which increase the recovery of phages like coliphages. Coliphages also serve as indicators of water pollution [85]. High concentrations of somatic and F-RNA coliphages are reported from freshwater samples [86–97]. Freshwater sources have been found to contain bacteriophages against *Klebsiella pneumoniae* and *Salmonella* species [98].

Although not a natural ecosystem, sewage is one of the biggest reservoirs for bacteriophages and forms an important part of the environment with respect to humans today. Due to the high bacterial population in sewage, bacteriophages are commonly isolated from sewage. 10^6–10^8 somatic coliphages per liter of sewage have been reported [99–103]. Sewage samples like cattle wastewater, pig slurry, poultry wastewater, and animal slurry too are rich sources of somatic coliphages and Stx phages [104, 105].

Extreme aquatic environments like hot water springs host bacteriophages against thermophilic bacteria and Archaea [106]. While mostly untapped, studies of some of these thermophilic bacteriophages have given insights into the microbial population dynamics of these environments [106]. Studies on bacteriophages from hot springs have been conducted in different parts of the world [107–112].

Similarly, cold-active bacteriophages have recently been isolated from glacial environments where they influence the bacterial dynamics. Frozen seawaters as well as freshwater ice cover have been shown to support cold-active bacteriophages. Studies have described isolation of active bacteriophages, metagenomic profiling of bacteriophages and viruses, virus-host interactions, and morphological diversity in glacial environments [113–121].

Isolation of Bacteriophages from Water

Direct Plating

The earliest bacteriophage isolation methods involved plating of samples directly with the host of interest without enriching them, followed by observation of plaque formation [122, 123]. Bacteriophage detection and isolation are possible by this method only when the sample has high bacteriophage titer – at least 10–100 bacteriophages per ml must be present for visibility on the plate [124]. Direct plating has been useful in isolating novel bacteriophages from sources like sewage effluents, stool samples, saliva, and dental plaques [125–128]. It is not a commonly preferred method as the probability of missing out on phages due to low titers in samples is very high. Moreover, some bacteriophages are only active in liquid media and may not show up in plaque assays that typically involve solid media.

Direct Plating of Large Volumes

Grabow and Coubrough described direct plaque assays for large volumes of water with agar media in 1986 [129]. One hundred millimeter volume of water samples were mixed with concentrated agar media and poured into 140-mm diameter petri plates along with host culture [129]. A modification in the method involved pouring equal volumes of the mixture into bottom and top as double agar layer. This method substantially increased the yield of bacteriophages and is useful for different kinds of water samples including sewage [130–132]. This method detects bacteriophages at low titers and eliminates losses during recovery steps

considerably [133]. The method suffers from limitations involving laborious handling of large petri dishes and their cost compared to conventional petri dishes. It is useful in isolation of coliphages that are commonly found in water samples. Since bacteriophages are not concentrated in this method at any step, only certain high titer groups of phages may be isolated using this technique.

Non-specific Concentration of Bacteriophages

To improve isolation of bacteriophages, concentration and enrichment protocols were developed. Czajkowski et al. (2016) demonstrated the use of zinc chloride in liquid samples like water and plant or soil extracts in concentrating bacteriophages so as to detect in direct plating without an additional enrichment step [134]. Flocculation of bacteriophages using various salts of metals increased recovery as they form small insoluble aggregates that precipitate out of the suspension [135, 136].

An interesting method of bacteriophage flocculation using casein (at its isoelectric point for flocking) and magnetite has been employed for the recovery of coliphages from freshwater and sewage samples effectively [88, 89]. The technique is rapid, inexpensive, and efficient and works well for concentration of coliphages from highly polluted samples.

Ultrafiltration

Ultrafiltration involves filtering of samples through polysulfonate or related material's membranes with pore size 0.02 µm and weight cut-off limit of 10,000 Da that allow molecules to pass but retain bacteriophages. The method is solely based on physical retention of bacteriophage without involving charges or adsorption phenomenon [137, 138]. Ultrafiltration ensures high efficiency in bacteriophage recovery without exposing it to extreme pH levels or harsh conditions. Bacteriophages have been isolated from large volumes of river water samples and groundwater samples (about 450 l) by ultrafiltration with 30–60% recovery [90, 139]. Recovery efficiencies of even up to 94% have been reported for a variety of phages and other viruses from tap water and ~ 70% from activated sludge effluents [140].

One of the biggest limitations of this method is clogging of the membrane pores that restrict the volume of sample that can be screened. Filtration units with motorized recirculating pumps and stirrers that can prevent clogging and enhance filtration rate have been described [139, 141], but it increases overall equipment cost of the process.

A passive process of membrane retention of bacteriophages was achieved by Padan et al. in 1967, who applied the principle of dialysis for cyanophage isolation from ponds [142]. Briefly, the water sample was poured into a cellulose dialysis bag that was dipped into a hygroscopic liquid material like polyethylene glycol (PEG) that absorbs the water, including microsolutes, through the semipermeable membrane sparing the bacteriophages and macrosolutes.

Although ultrafiltration is least damaging to the bacteriophage during isolation, it is expensive and tedious and not feasible in every microbiological lab. The equipment cost and maintenance is high. It is useful in commercial setups where in a controlled environment, large-scale bacteriophage production is carried out and higher recovery rate is required.

Adsorption-based Methods

Bacteriophages naturally carry predominantly a negative charge at or near neutral pH. A number of isolation techniques involve recovery of bacteriophages based on their adsorption to various matrices. Varying pH levels modify the charges on the bacteriophage allowing them to adsorb to various matrices.

Viruses have been concentrated by adherence to natural adsorbents like bituminous coal, fiberglass, and cellulose nitrate filters [143–146].

Following different principles of adsorption, electronegative microporous filters [147], electropositive microporous filters (1MDS®) [148, 149], and other such adsorbents have been used effectively for bacteriophage isolation. Di- and trivalent cations like calcium and magnesium salts have been added to the samples to improve the adsorption of phages to these membrane by modifying charges on the filter [150], altering the width of charge layer on the filter [151] and developing formation of salt bridges between the membrane filter and viruses [152–155].

In case of electronegative adsorbent filters like glass powder, minerals, fabrics, starch, resins, and alumina gel [156], bacteriophage suspension needs to be adjusted to acidic pH so the bacteriophages are positively charged and adsorb better [157]. The adsorbed bacteriophages are eluted from the matrix using an eluent at alkaline pH [155, 158]. The use of electronegative adsorbent is not usually the best choice for concentration as bacteriophages show poor survival rates at pH extremes ($pH < 3$ and $pH > 12$) [159].

Bacteriophages adsorb naturally to electropositive matrices at neutral pH [90, 147, 160–165]. Electropositive adsorption-elution systems yield better recoveries of bacteriophages compared to electronegative filters although with high variability in their efficiencies [140, 146, 160, 163]. See Table 1 below for examples on adsorption-based bacteriophage detection methods.

The main limitations of chemical adsorption methods for bacteriophage isolation are cost of filters and pH sensitivity [146, 152]. There also is the disadvantage of filters

getting clogged and the non-suitability of the method for marine water [124]. However, such non-enrichment methods are useful in unbiased concentration of bacteriophages from environmental samples and are crucial for large-scale screening and monitoring experiments looking for natural bacteriophage diversity. They allow for large volume screening and can be conveniently performed in simple lab setups.

Concentration of Specific Bacteriophages by Enrichment

The isolation of "specific bacteriophages" was first demonstrated by Guelin in 1948 [166]. Briefly, nutrient broth is added to pre-filtered water samples along with log-phase host bacteria culture and incubated overnight. Post-incubation, the enrichment suspension is centrifuged at 4000 *g* and supernatant filtered through 0.22-µm nitrocellulose membrane filter to get rid of bacteria and debris. While 0.22-µm filters are more commonly used in viral studies, some researchers prefer 0.45-µm filters to improve the isolation of large or jumbo phages or phage aggregates. The filtrate obtained is an enriched bacteriophage suspension that can be used for plaque assays, spot method, or electron microscopy [167–170]. The International Organization for Standardization has included this as a standard bacteriophage isolation method in the year 2000 [171].

The enrichment method helps in detection and isolation of bacteriophages from large as well as small volumes of sample – ranging from 1 to 1000 ml [153, 154, 167, 169, 170] with high accuracy. It has been applied for monitoring of treated drinking water, by detecting the bacteriophages against indicator organisms [170]. Enrichment method is simplest to follow in lab and is, therefore, also the most popular.

Ghugare et al. (2017) recently reported an improved membrane filtration immobilization method for simultaneous isolation and enrichment of specific bacteriophages [172]. Large volumes of pre-filtered environmental water samples are passed through an immobilized layer of bacteria on membrane filter under vacuum and subsequently enriched.

Host-based bacteriophage enrichment method provides an ideal environment for the bacteriophages to bio-amplify without undergoing much stress as opposed to chemical methods like adsorption-elution that modify the bacteriophage structure affecting their functionality.

However, in the enrichment method, even among bacteriophages specific to the host, the ones with higher fitness, i.e., higher infective and reproductive capacities, dominate in number in liquid culture and get selectively amplified to higher titers. A procedure involving extraction and propagation of environmental bacteriophages in dilute and very dilute agarose gels has been described for isolation of under sampled bacteriophages, complicated large-genome bacteriophages, and aggregating bacteriophages [173–175]. The problem is circumvented in semisolid condition (in presence of agar) because fitter bacteriophages are unable to physically infect all the bacterial cells present in the solution due to lower diffusion rate and new/ under sampled bacteriophages are also able to thrive.

Reservoirs of Bacteriophages in Soil and Sediment

The terrestrial soil is divided into several layers and multiple microhabitats creating niches for bacteria-bacteriophage systems to develop. Apart from spatial heterogeneity, the high variability in soil structure, influences of plant root microbiota, fungal microbiota, mineral, and nutritional status render soil a rich reservoir of bacteriophages with high diversity [176–178].

Sediments – composed of organic and inorganic deposits in water ecosystems – adsorb a large number of bacteriophage particles and aggregates offering higher densities of bacteriophages for isolation. Several studies report isolation of bacteriophages from freshwater sediments, mostly river sediments [179–182]. Marine sediments too are important ecological niches for bacteriophages displaying high density and diversity [183–191]. They play an important role in nutrient cycling and sustenance of the benthic food web [192–197].

Some extreme soil environments like deserts too have shown the presence of bacteriophages. The desert environment, characterized by extreme heat, dry sand surface deficient in humidity, and high UV radiation exposure, undergoes considerable shifts in temperature through the day. Bacteriophages in diverse morphologies, genetic makeup, and physical and chemical attributes too have been successfully isolated and characterized from desert soils [198–207].

Similarly, bacteriophages have also been seen to inhabit cold ecosystems like permanently frozen grounds or permafrost where they have been explored for their influence on microbial and community dynamics [208–213].

Isolation of Bacteriophages from Soil and Sediment

Soil is one of environment's richest sources of bacteriophages containing about 10^7–10^9 bacteriophages per gram of soil. Isolation of bacteriophages from soil requires standardization as soil environment varies from place to place. The moisture content of soil, pH, mineral, and microbial composition plays critical roles in isolation of bacteriophage from soil in the lab.

Methods for the isolation of bacteriophages directly from soil have been explored in several studies in the past [214–219]. These techniques primarily focused on the isolation of actinophages or arthrophages. In general, arthrophages have been isolated from soil samples by

Table 1 Adsorption based methods of bacteriophage isolation

Adsorption matrix	Type of charge	Viruses isolated	Advantage	Disadvantage	Example references
Bituminous coal	Electronegative	Poliovirus	Good concentration technique	Preparation of coal expensive	[143, 144]
Cellulose nitrate Polycarbonate Epoxyfiber glass Glass microfiber Virosorb-1-MDS DE	Electropositive	Wastewater phages	High phage adsorption	Expensive	[145]
Zeta plus microporous filters	Electropositive	Coliphages	34–100% efficient	Filter clogging	[147]
Virozorb IMDS AMF Cuno	Electropositive	Poliovirus	AMF Cuno filters show effective recovery of Polioviruses	pH-sensitive process	[148]
Virosorb 1MDS, Posidyne N66, Zeta plus C-30 and Seitz S	Electropositive	Natural phages in water sample	Virosorb IMDS filters adsorb phages without any modification or treatment	pH and ionic dependency of various filters	[149, 160]
Cellulose nitrate membranes	Electronegative	Poliovirus	Concentration of phages from large volumes	Pre-treatment of sample with salts	[150]
Filterite filters	Electronegative	PRD1, MS2, and naturally isolated bacteriophage	Addition of $MnCl_2$ improves by a factor of 4 or 5	Adsorption interference with increasing concentrations of $MnCl_2$	[155]
Glass wool trap	Electronegative	Enteric viruses	Works for viruses that are pH stable	Unsuitable for phages that cannot survive pH extremes	[156]
AP20 glass fiber filter	Electronegative	ZJ/2 MS2 ΦX I74 Ec1 T4	Single step, fast phage concentration	-	[161]
Sand filter	-	ΦX I74, P22, MS2, Φ80, T7, T2, ZJ/2, Lc	Large volume phage isolation	pH adjustment & sample processing required	[162]
Charge-modified AMF Cuno filter, Zeta-Plus filter	Electropositive	MS2, f2	No pH adjustment required, efficient, good recovery	Filters expensive	[163]
Membrane filtration (MF) with new swirling elution (SE)	-	F-RNA coliphages, F-DNA coliphages and somatic coliphages	Highly efficient recoveries from environmental water samples	Limited turbidity range and filter clogging	[165]

incubation along with the host in liquid nutrient broth followed by plaque assay.

For the isolation of actinophages from soil, Lanning and Williams (1982) used sterile soil suspension in nutrient broth as bacteriophage suspension and poured it into petri dishes as basal agar layer, while a spore suspension of streptomycete host was plated over it [220].

Dabbs (1998) described a method for isolation of bacteriophages wherein the soil sample is supplemented with cations, and the 0.22 µm filtrate was used as bacteriophage suspension [221]. Bacteriophages have also been isolated from soil by homogenizing it in Ringer's solution using glass beads and subsequently using the homogenate for plaque detection [222].

A number of elution buffers like 10% beef extract, glycine buffer, 10 mM sodium pyrophosphate, Na/K Sorensen's phosphate buffer, and 1% potassium citrate have been tested for effective isolation of bacteriophages from soil samples with good yield [223–225]. An addition of lysozyme and chloroform followed by ultracentrifugation helped in isolation of an array of bacteriophages of various morphotypes from the rhizosphere in a study conducted by Swanson et al. in 2009 [225].

As a general guideline, a systematic method of isolation of bacteriophages from soil is described in their book chapter in Bacteriophages: Methods & Protocols Volume I [226]. Briefly, liquid nutritive media like Tryptic Soy Broth is added to soil sample and mixed thoroughly. Post a brief incubation, supernatant is filtered and tested for bacteriophages by enrichment with specific hosts and plaque analysis. This is one of the most widely followed and accepted methods of isolation of bacteriophages from soil. In their 2013 study, Williamson and group tested a number of extraction methods for study of viral abundance in soil [227]. Bacteriophages were yielded best when potassium citrate buffer was used for soil suspension and sonication or blending methods were employed for extraction.

Meiring et al. (2012) conducted the first study on isolation of bacteriophages from extreme environment involving lysogenic bacteriophage Psymv2 from the dry valley soil samples of Antarctic desert [208]. Latent prophages were induced by addition of mitomycin C to the soil suspensions.

Reservoirs of Bacteriophages in Air

Most studies on sampling of air bacteriophages until two decades ago involved aerosolization of bacteriophage samples in controlled environment of lab and sampling using commercial air samplers [228–232]. These studies mainly used bacteriophages as surrogates for understanding the behavior of viruses in air and their sampling efficiencies. The open ambient air is an important ecological niche with possibilities of high diversity in bacteriophages. Air being an extremely dynamic system shows great variations in the kinds of bacteriophages isolated with each sampling event. However, studies on the natural bacteriophage diversity in air are sparse currently and may hold greater scope in future.

Bacteriophages have been isolated from air in dairy industries like cheese factories as contaminants in starter cultures [231–238]. Bacteriophage contamination in starter cultures greatly hampers cheese production, and hence, their detection is very important there. Studies on aerosolized bacteriophages in toilets, water treatment plants, and poultries are also important [239–242].

It is to be noted that all studies on bacteriophages in air have aerosols in common. Phages have been isolated either from controlled laboratory environments like laminar hoods and aersolization chambers or from enclosed spaces like dairies, cheese factories, poultries, or bathrooms. Phage isolation from open air systems have not been explored so far.

Isolation of Bacteriophages from Air

Bacteriophage isolation from air is an underappreciated field. Ehrlich and colleagues made one of the earliest attempts on isolation of bacteriophages from aerosolized samples in a bid to study the effects of environmental factors on airborne T-3 coliphages [228]. In 1965, Harstard published his work on comparison of sampling efficiencies of two different kinds of liquid impingers using two kinds of filters and a fritted bubbler [220]. The study concluded that liquid impingers were best for sampling as they cause least destruction to the bacteriophage particles and are relatively more efficient. AGI samplers are therefore the most preferred samplers for bacteriophage-aerosol sampling in most studies that followed due to better recovery of bacteriophages, retention of infectivity, and gentle sampling process [230–232, 243–249]. Among filters, polytetrafluoroethylene (PTFE) filters are studied to be best for sampling of bacteriophages and other viruses with high collection efficiency [250].

Studies describing isolation of bacteriophages from open air are relatively newer. Lactococcal bacteriophages have been detected in the air in dairy industries and sampled using various commercial air samplers and filters [233–235].

Analytical methods other than plaque assays for bacteriophage detection too have been explored [236]. Verrault et al. (2011) detected bacteriophage genomes in aerosols of cheese manufacturing plants by quantitative PCR (qPCR). Five different types of samplers were used for air sampling in this study of which the NIOSH samplers proved most reliable [236]. A detailed review on different types of sampling devices employed for airborne viruses in general is given by Verrault et al. (2008) [237].

Similarly, Espinosa and Pillai (2002) detected male-specific coliphages in confined animal housing operations using impaction-based sampler (SAS-100) within and around

Table 2 Summary of bacteriophage aerosol sampling studies

Sampler	Sampler type	Sampling environment	Bacteriophage sampled	Comments	Example references
AGI-30	Impinger	1600-l plexiglass aerosol chamber	T-3 coliphage	-	[229]
AGI-4, capillary impingers, Chemical Corps Type6 filter papers, MSA 1106BH filter papers, fritted bubblers	Impinger, fritted bubblers	Aerosol chamber with humidifier	T1 bacteriophage	Liquid impingers recovered most viable phages	[230]
AGI-30-humidifier bulb combination	Impinger	Dual Aerosol Transport Apparatus (DATA)	S-13 coliphage T3 coliphage Airborne mengovirus-37A Vesicular stomatitis virus (VSV)	Increased viable recovery of airborne T3 coliphage and S-13 coliphage	[232]
AGI-4 Type 6 filter papers	Impinger	45-l glass carboy	T1 bacteriophage	Phage recoveries of ionized aerosols lesser than non-ionized aerosols. Better recovery with AGI-4	[233]
AGI-30-humidifier bulb combination	Impinger	500-l stainless-steel rotating drums or DATA	T3 coliphage *Pasteurella pestis* bacteriophage	Humidifier bulb improved recovery level and biological decay by 3 logs	[254]
Porton impinger	Impinger	Double-walled static system	T1 bacteriophage	Phage survival better at low initial salt concentrations. Broth protected against aerosol inactivation	[245]
Porton impinger	Impinger	2000-l double-walled static system	MS2 phage	-	[246]
Porton impinger	Impinger	2000-l double-walled static system	T3 coliphage	Peptone broth impingement increases recovery	[248]
Andersen impactor, AGI-30 impinger, gelatin filter, and nuclepore filter	Impactor, impinger, filters	Test chamber	MS2, phi-X174, T7 and phi-6 bacteriophage	Sampling efficiency strongly depends on virus morphology, hydrophilic nature of virus and relative humidity	[249]
AGI-30, SKC BioSampler and frit bubbler	Impinger, bubbler	-	MS2 bacteriophage	Collection efficiencies poor for all samplers below in the 30–100 nm size range	[250]
Polytetrafluoroethylene (PTFE), polycarbonate (PC), and gelatin filters with button inhalable samplers	Filter sampler	Bioaerosol chamber	MS2 bacteriophage	PTFE filters showed very good physical collection efficiencies in the size range of 10–900 nm	[251]
SKC BioSampler, CDC, NIOSH two-stage cyclone bioaerosol samplers	Impinger, cyclone sampler	Controlled chamber	MS2, Φ6, ΦX174, PM2, PR772	SKC BioSampler and NIOSH two-stage bioaerosol sampler show effective recovery of phages for qPCR	[252]
Laboratory vacuum pump	-	Dairy environment	Lactococcal phages	-	[234]

Table 2 (continued)

Sampler	Sampler type	Sampling environment	Bacteriophage sampled	Comments	Example references
MD8 air sampler	Impactor	Dairy environment	Lactococcal phages	Gelatin filters convenient method for lactococcal phage monitoring	[235]
Battery-operated air samplers AirPort MD8, MAS-100	Impactor	Dairy, cheese factory	Lactocccal phages	Both portable air samplers efficient in phage sampling	[236]
PTFE, PC, BioSampler, Coriolis cyclone sampler, NIOSH two-stage cyclone bioaerosol sampler	Filters, cyclone samplers, impinge	Cheese factories	Lactococcal phages	NIOSH sampler highest detectable levels of lactococcal phages	[237]
SAS-100 bioaerosol sampler	Impactor	Poultry broiler houses	Male-specific coliphages	High variability in phage recoveries	[240]
Multi-slit impinger	Impinger	Wastewater treatment facilities	Coliphages	-	[241]
SAS agarized terrain impactor	Impactor	Activated sludge plant, sludge treatment plant, washing station	Enterovirus Reovirus	-	[242]
Laboratory vacuum pump	-	Bathroom, toilet	-	Economical, alternative sampler	[243]

broiler houses for male-specific indicating presence of fecal contaminants [239].

While commercially available samplers are quicker and offer more control, the disadvantages lie with difficulty in device sterilization, high cost, and inactivation of viruses due to sampling [251]. Magare et al. (2017) described an indigenous and economical method for isolation of airborne bacteriophages by impingement by modifying a simple laboratory vacuum filtration unit [242]. All the parts of the system are autoclavable rendering cleaner results. Table 2 provides an overview on the types of sampling devices used for recovering bacteriophages from air samples.

The study of airborne bacteriophages and methods to sample and enumerate them are still unfledged. Standardization in air sampling is a major limitation. The recovery efficiency too is inadequate. The sampling procedures themselves play a role in inactivating the bacteriophages or damaging them physically. All sampling devices have their own shortcomings, but a balanced plan for any study with right analytical methodologies can help generate very useful data.

Field-based Methods of Bacteriophage Isolation

Nearly all techniques of isolation of bacteriophages are confined to the lab in a controlled environment. The instruments required for the isolation process require facilities to maintain sterility, laminar hoods, adsorption-elution columns, or vacuum filtration units that can function only in labs. In most cases where bacteriophages are isolated from various environments, sampling is performed on the field and processed in lab. Sample transport can be costly and time-consuming affecting bacteriophage recovery due to sample deterioration. Therefore, there is a need for developing on-field bacteriophage isolation or entrapment techniques urgently.

In view with designing a method for virus concentration that is low-cost, rapid, efficient and capable of handling large volumes in field, portable devices were devised that could be operated on field and screen large volumes of samples in the range of 500 l [87, 251, 252]. In samples with high number of bacteriophages, up to 75% recovery of bacteriophages was observed. Although efficient in screening large water samples and good bacteriophage recovery, some setups are too bulky and may not be suitable for large-scale monitoring studies of water bodies with a number of sampling sites [252].

An overview of all the methods, their applicability and cost is summarized in the table below (Table 3).

Table 3 Overview of bacteriophage sampling methods

Method	Volume	Type of sample	Aim	Recovery rate	Cost
Direct plating	1–5 ml	Water	Low specificity, low selectivity. Collects group of bacteriophages for a species	Low	Economical
Direct plating of large volumes	100 ml	Water	Low specificity, low selectivity. Collects group of bacteriophages for a species	Moderate	Moderately expensive
Ultrafiltration	100 l	Water	Low specificity, high selectivity. Collects group of non-specific bacteriophages	Moderate	Expensive
Adsorption based methods	10–20 l	Water	Moderate specificity, moderate selectivity. Collects group of non-specific bacteriophages	Moderate	Expensive
Concentration of specific bacteriophages by enrichment	1–10 ml	Water	High specificity, high selectivity. Collects group of bacteriophages for a species	High	Economical
Concentration of specific soil bacteriophages by enrichment method	5–20 ml	Soil, sediment	High specificity, high selectivity. Collects group of bacteriophages for a species	High	Economical
Impingers	-	Air, gas	Low specificity, high selectivity. Collects group of non-specific bacteriophages	Moderate	Moderately expensive
Impactors	-	Air, gas	Low specificity, high selectivity. Collects group of non-specific bacteriophages	Low	Moderately expensive
Fritted bubblers	-	Air, gas	Low specificity, high selectivity. Collects group of non-specific bacteriophages	Low	Moderately expensive
Filter samplers	-	Air, gas	Low specificity, high selectivity. Collects group of non-specific bacteriophages	Low	Moderately expensive
Laboratory vacuum pump	-	Air, gas	Low specificity, high selectivity. Collects group of non-specific bacteriophages	Low	Economical

Conclusions

Bacteriophage-based applications are promising alternative options for tackling various bacterial pathogen-based issues. A resurgence of interest in phage studies in the past few years has been observed, pushing researchers to find newer applications for them in diverse fields. In fact, some phage biocontrol formulations have already been developed and commercialized for purposes like food preservation where they control food-borne pathogens, e.g., EcoShield [254], SalmoFresh [254], ShigActive [254], PhageGuard [255], and ListShield [256, 257]. There also are phage products being used in agriculture for crop protection like AgriPhage [258] and pathogen control in animal feed like BioTector [258]. FASTPlaque TB [258] and FASTPlaque-Response [258] are some rapid diagnostic tools prepared from phages. Phages today are increasingly finding commercial use.

Researchers are now focusing their understanding towards translational use. The primary step towards this end is careful evaluation of requirements and choice of method of isolation. Phage isolation methods have been simplified over the last two decades with enrichment method being the most preferred one. The methodologies have been improvised to retain infectivity and efficiency while also getting pure bacteriophage suspensions. Methods involving extreme pH variations and physical stress on the phages have either gone obsolete or have been modified to do away with physical or chemical stress.

Upcoming fields of high-throughput sequencing and metagenomics too are enabling prediction of bacteriophages from various environmental samples against host of interest with greater precision. Support of powerful computational predictions is helpful in quick screening and selection of appropriate phages for diverse applications. Disciplined approaches and further development of phage isolation methodologies will help leverage the value phage-based applications on the whole.

The numbers of known bacteriophages are increasing with several new bacteriophages being added to the

databases each year. Several dedicated phage banks have been formed around the world to ease the process of finding phages for phage therapy. These dedicated phage banks host thousands of phages against pathogenic hosts that can be screened quickly when needed to avoid the time spent in isolating phages from scratch against a host when needed. In European and Belgian law, commercial magistral phage preparations have been approved so that the time spent in isolating, characterizing, and optimizing the phages is minimized. These preparations will be available for a price for quick development into a phage-based therapeutic product [223].

Needless to say, to realize a future with phage-based applications in the forefront, development of sampling methods that are quicker, more efficient, and economical is still a necessity.

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