Utility of Bacteriophage as a Bio-Disinfectant

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Microorganisms present a range of resistances to chemical and enzymatic disinfectants and no single disinfectant is effective in all situations. The aim of the present study is to evaluate the bacteriophage as bio-disinfectant in different conditions. Phage was subjected to a standard battery of tests including the In-Use Dilution Method for Testing Disinfectants and the Germicidal Spray Products Test. Results indicate that aerosolized phage can eradicate or significantly reduce the *Staphylococcus aureus* load on a variety of materials found in the laboratory and hospital-related equipment. Additionally, Bacteriophage was shown to retain full activity under conditions that mimic a hospital environment, i.e., in the presence of non-ionic detergents, hard water, or organic materials. We propose phage as the first biological, narrow-spectrum disinfectant against *Staphylococcus aureus*, which may augment or supplement the use of broad-spectrum disinfectants.

Key words: Bacteriophage, Bio-disinfectant, *Staphylococcus aureus*.

Disinfectants are antimicrobial agents that are applied to non-living objects to destroy microorganisms, the process of which is known as disinfection[1]. Disinfection is defined as: Cleaning an article of some or all of the pathogenic organisms which may cause infection. A perfect disinfectant would also offer complete and full sterilization, without harming other forms of life, be inexpensive, and non-corrosive[2]. Unfortunately ideal disinfectants do not exist. Most disinfectants are also, by their very nature, potentially harmful (even toxic) to humans or animals. They should be treated with appropriate care.

Current broad-spectrum disinfectants belong to one of several chemical categories including alcohols, aldehydes, biguanides, halogens, oxidizing agents, phenols, or quaternary ammonium compounds and enzymes[1]. To various degrees, these compounds have been shown to be flammable, light sensitive, carcinogenic, corrosive to metals, irritating to mucous membranes, and/or toxic to livestock and humans[3]. Additionally, many factors that is often associated with cleaning hospital areas (e.g., hard water, organic load, or detergents) can reduce or even ablate efficacy of chemical disinfectants. Importantly, studies have shown that these commonly used disinfectants can select for mutant bacteria with decreased susceptibility to biocides and antibiotics without compromising virulence[4,5]. In the present study bacteriophage referred to as "Bio-disinfectant," have been investigated as potential disinfectant against pathogenic organisms due to their ability to kill the bacterial. The main objectives of the present study are; To isolate different bacteriophages against most common bacteria,

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to evaluate the feasibility of using bacteriophage as disinfectant and to compare the efficacy of commonly used disinfectant with bacteriophages

MATERIAL AND METHODS

Bacteria isolated

Clinical isolates of *Staphylococcus aureus, Enterococcus faecalis Pseudomonas aeruginosa* and *Klebsiella pneumoniae* were isolated from different clinical specimens[6].

Bacterial growth

Bacterial strains were grown at 37°C and stored at -80°C. all the bacteria were grown in Brain heart infusion broth[6].

Antibiotic sensitivity test

Antibiotic sensitivity by Kirby Bauer's disk diffusion methods revealed that the above organisms were resistance to two or more antibiotics, thus multidrug resistance organisms[7].

Phage isolation

Isolation and purification of phage strains for *Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa, Klebsiella pneumoniae* and *Bacillus* spp.

The source of water samples for phage isolation will be collected from lake, sewage, hospital outlet and open drain. The phages were isolated from raw sewage at a municipal sewage treatment plant, by the method of Smith and Huggins[7]. Sewage water (50ml) was collected in sterile conical flask and treated with a few drops of chloroform. To this equal volume of lactic phage broth and 1 ml of the 24 hrs old broth cultures were added. The sample inoculated with bacterial pathogens was incubated at 37°C for 12-24hr in shaker water bath. After 12-24hrs the lysate was shaken with few drops of chloroform for about 10 min, centrifuged at 10,000 rpm for 10 min and the supernatant was filtered through 0.22m pore size Acrodisc membrane filters (PALL, German Laboratory) to remove the bacteria and subjected to plaque forming unit (PFU) assay using double layer agar method described by Smith and Huggins[8].

Transmission electron microscopic study

Phage Ø CSV solution was filtrated with Acrodisc filter to remove soluble biological macromolecule fragments of host bacteria. After washing three times with 0.1M ammonium acetate solution (pH7.0), the retained phage solution was used directly for negative staining.[8] Photographs were taken with a transmission electron microscope

Spectrophotometric lysis assays

Bacteria were grown overnight at 37°C, washed in sterile PBS (pH 7.4), and resuspended to the desired concentration as predetermined for each strain based on standard curves of the optical density at 600 nm (OD600) versus the number of CFU. For testing against a panel of bacteria, 100µl containing 1×10^7 CFU of each organism was mixed with 100µl of bacteriophage (1×10^9 PFU) in a 96-well plate. The OD600 was measured on a SpectraMax 190 instrument (Molecular Devices) every one hour over a 10 hours time period to monitor OD changes that correlated to lysis of the bacteria. Alternatively, 1×10^9 PFU of bacteriophage was tested in a similar manner against *Staphylococcus aureus* in the presence of detergents [sodium dodecyl sulfate (SDS), hexadecyltrimethylammonium bromide (CTAB) at a final concentration of 1%, EDTA at a 10 mM final concentration, distilled water, fetal bovine serum at final concentrations of 5% and 10%, or synthetic hard water at a calcium equivalent of 200 or 400 ppm. Percent lysis was calculated from endpoint OD600 readings of bacteriophage buffer controls representing 100% lysis in a 60 mins assay. Synthetic hard water was made according to method 960.09 of the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC)[9]. Due to mild clumping of bacterial cells at calcium equivalents of >200 ppm, this experiment was repeated by plate viability assays at 200 ppm and 400 ppm in order to determine the efficacy of bacteriophage in hard water.

Plate viability assays

The bacterial lawn prepared on nutrient agar plates employing 1.0ml (1×10^8 CFU)of 24hr culture by flooding and draining out the excess. Wells were dug into the agar by employing a sterile cork borer and the 20 ml phage suspension (1×10^2 PFU to 1×10^9 PFU) were loaded into each of the well. Sterile distilled water served as the control. The plates were incubated at 37° C for 24 hr. There after the zone of inhibition, if any, was recorded[10].

AOAC Use Dilution test

Modified versions of the AOAC Use Dilution Methods for Testing Disinfectants (official methods 955.15 and 964.02) were used to test bacteriophage efficacy as a hard surface disinfectant. Briefly, a fresh overnight culture of *Staphylococcus aureus* was adjusted to a concentration of 1×10^7 CFU/ml based on absorbance readings. Glass rods, were dipped 2 cm into the culture and allowed to air dry for 30 min. Glass rods (n= 60) were then dipped 2 cm into a bacteriophage solution (1×10^9 PFU/ml) and allowed to air dry for 2 hrs, after which they were transferred to 5 ml of sterile medium (Brain Heart Infusion broth), agitated for 10 s, transferred to another tube containing 5 ml of sterile medium, agitated a second time for 10 s, and removed. Turbidity in either tube after overnight incubation at 37°C constituted a positive result for growth[11].

AOAC Germicidal Spray Products Test

A modified version of the AOAC Germicidal Spray Products Test (official method 961.02)[10,11] was used to test the killing ability of aerosolized Bacteriophage on surfaces. Briefly, pieces of stable equipment were cut into 25.4-mm by 25.4-mm coupons. Coupons were seeded with 2×10^7 CFU of *Staphylococcus aureus* by pipette and allowed to air dry at 25°C for 30 min. Bacteriophage (1×10^9 PFU/ml) was misted 30 cm above coupons in two passes (1 s total time) using a thin-layer chromatography reagent sprayer. Treated coupons were allowed to air dry for 60 min; they were then placed in 50-ml test tubes with 20 ml of sterile PBS and mechanically agitated, and the supernatant was serially diluted and plated on blood agar plates[8]

RESULTS

Staphylococcus aureus, Enterococci feacalis Pseudomonas aeruginosa, Klebsiella pneumoniae were isolated from the clinical materials. Antibiotic sensitivity by Kirby Bauers disk diffusion revealed the bacterial were resistant to commonly used antibiotics.

Designation of Bacteriophage

The Bacteriophage isolated were designated as mentioned in the Table 1.

Electron microscopy

The phage Ø CSV had an icosahedral head, about 65 nm in diameter, and a 100-nm long tail, thus morphologically similar to phages belonging to *Siphoviridae* family

Phage specificity

Phage was tested for lytic activity, measured spectrophotometrically, against a panel of gram-positive and gram negative pathogens. 1×10^9 of phages was sufficient to effect a drop of >0.5 OD600 units in less than 60 mins for all gram negative and gram positive organisms, demonstrating its utility against pathogens. In contrast, phages did not produce lysis when tested against a panel of different organisms, thus exhibiting genus specificity.

Phage lytic properties

Plate viability assays were used to compare the efficacy of phages versus phenol, a common chemical disinfectant used at our laboratory to disinfectant the clinical materials. Both products were tested against 1×10^7 CFU/ml *S. aureus* over a disinfectant final concentration range of 5 mg/ml (0.5% solution) to 10 ng/ml (0.000001% solution) (Fig. 2). Phenol was able to sterilize the Staphylococcal culture within 30 min at 10 mg/ml (1.0% solution) (data not shown) as well as at 5 mg/ml, which is consistent with the effective concentration found in previous reports as well as the concentration recommended by the manufacturer. However, at lower concentrations, the chemical disinfectant quickly lost efficacy. In contrast, Phages at 1×10^9 PFU sterilized the test solution and even produced a >5 log drop in *Staphylococcus* at 60 minutes. Even the other phages isolated against *Enterococci faecalis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae* almost produced similar results (Fig-2) against the individual host bacterium and also when cocktail of phages were used against the clinical samples. Thus, phage was more effective than phenol on a time basis. However, it should be noted that the mass of phage significantly increased, while the affectivity of phenol decreased as the volume of clinical material increased. With the minimal effective concentration determined, we next evaluated the minimal contact time required for complete eradication of *Staphylococcus*. Phage was mixed with 1×10^7 CFU of *Staphylococcus aureus* in a

final volume of 1 ml and allowed to incubate for 0, 20, 40, 60, or 120 min, and survivability was determined via the plate viability assay. The 40- and 60-min incubations achieved complete sterilization (7 log decrease) of *S. aureus*, whereas the 40- and 60-min incubations achieved 3 log and 5 log decrease in CFU respectively

AOAC measurements of Phage bactericidal effects

Phage was subjected to several standardized AOAC disinfectant testing methods. The Use Dilution Method is a carrier-based method that tests the ability of a disinfectant to sterilize a hard surface. twenty duplicate experiments were performed with *Staphylococcus* loaded glass rod penicylinders dipped into a 1×10^9 PFU solution of Phage and allowed to incubate overnight in fresh medium. Sterility was obtained in all twenty carrier sets compared to the phage negative control (data not shown), suggesting a 95% confidence interval, defined by AOAC to be sterility in 19 out of 20 carriers. As a measure of efficacy when phage is used as a potential spray disinfectant, a modified version of the Germicidal Spray Products Test was used to measure the ability of aerosolized phage to eradicate the test organism. One-inch square (25.4 mm by 25.4 mm) coupons of nylon, cotton, polyester, wood, stainless steel, or glass were seeded with 1×10^6 CFU of *Staphylococcus* and misted with a 1×10^9 PFU of phage for 1 s. We estimate that each coupon was exposed to approximately 50µl of aliquot solution, of phage. This amount of phage was sufficient to completely eliminate *Staphylococcus* on nonporous surfaces (glass and stainless steel), medium porosity materials (wood), and highly porous material (nylon, cotton, and polyester) (Table 2). Even with the extremely porous coupon made of weathered wood from a stable wall, phage achieved more than 90% killing of the *Staphylococcus aureus* inoculum (>4 log drop in the number of CFU) at the tested concentration.

Effects of detergent additives or stable conditions on phage eradication of *S. aureus*

In order to mimic a stable, phage synthetic hard water tests were repeated using the plate viability assay. Indeed, no difference was detected between phage buffer controls (data not shown) and phage in hard water up to 200 ppm (Fig. 2).

Table 1. Designation of Bacteriophage isolated against Different Bacterial species

Bacterial isolates	Designation of the bacteriophage
Staphylococcus aureus	Ø CSV-1
Enterococci faecalis	Ø CSV-2
Enterococci faecium	Ø CSV-3
Pseudomonas aeruginosa	Ø CSV-4
Klebsiella pneumoniae	Ø CSV-5
Citrobacter freundii	Ø CSV-6

Table 2. Disinfectant property of aerosolized phage on common laboratory materials

Material	No. of Remaining CFU	Log killing
Glass	0	Sterility
Nylone	0	Sterility
Cotton	0	Sterility
Wood	30	2 log
Steel	0	Sterility
Polyster	0	Sterility

DISCUSSION

Chemicals and enzymes have a long history of industrial use as detergent additives to inactivate, denature or to break down proteins, lipids and carbohydrates[12]. However, no enzyme has yet been described specifically as a disinfectant or environmental bactericide. In both chemical disinfectant and enzymes the approaches, killing is mediated by generation of classical disinfectant chemical entities rather than through direct action[12,13]. We propose phage as the bio-disinfectant with direct action and show here its narrow-spectrum effectiveness against *Staphylococcus aureus*. The ideal disinfectant should have the following characteristics: biocidal against all pathogens, nontoxic to livestock or human beings, environmentally safe and biodegradable, economic and easy to use, noncorrosive or nondestructive to stable surfaces, capable of being used in combination with detergents, and unaffected by organic matter or hard water[12,13]. Clearly, the ideal disinfectant does not exist. However, while phage does not possess

broad-spectrum biocidal activity common to most disinfectants, it nonetheless has most of these attributes. Phage mechanism of action is enzymatic and therefore does not rely on potentially toxic reactive groups utilized by chemical disinfectants[14]. As a live virus, phage is inherently biodegradable and noncorrosive. Due to the receptor specific binding of the phage for the *Staphylococcus* surface, phage binds *Staphylococcus aureus* on contact and begins killing within few minutes at an effective dose of $1X10^9$PFU/ml, which is several orders of magnitude lower than chemical disinfectants. Although disinfectants are susceptible to dilution or being washed away when applied environmentally, but phage once comes into contact with the bacterial surface, it remains tightly bound and is not inactivated by further washing or dilution as are conventional disinfectants. The high-affinity binding of phage for the bacterial surface[15], is a hallmark of phages. Additionally, phage works on a variety of porous and nonporous materials based on availability of target bacteria. While phage and other lysins will never replace the need for broad-spectrum disinfectants, they can nonetheless complement disinfectants under conditions where control of a particular pathogen is desired. Another important component of managing nosocomial infections is the use of detergents, which ideally would be used in conjunction with disinfectants. Detergents serve to disperse and remove soil and organic material from surfaces, thereby allowing disinfectants to reach embedded, otherwise protected microbes. However, chemical disinfectants often have reduced activity (aldehydes) or are inactivated[12]

Additional considerations important in determining optimal disinfectant use result from metal ions in hard water, often found in well water common to rural farm settings, which can bind to either disinfectants or detergents and interfere with their effectiveness. Consequently, metal chelators such as EDTA are commonly used to complex metal ions to combat the negative effects of hard water. Organic materials are also routinely encountered clinical material and could interfere with disinfectant action. Because phage has full bacteriolytic activity in nonionic detergents, EDTA, hard water, and in an organic load (defined by the AOAC)[9], it is well suited for clinical

specimen disinfecting applications. Historically, disinfectants are chemical entities. In fact, all 275 active ingredients from more than 5,000 commercially available disinfectants, sterilizers, sanitizers, antiseptics, and germicides registered and regulated by the U.S. Environmental Protection Agency (http://www.epa.gov) are chemical entities. Thus, our proposed use of bacteriophage, for disinfection/ decontamination of *S. aureus* represents a fundamental shift from convention. We attempted to validate and benchmark phage as a disinfectant using a standard battery of guidelines and tests adopted by the Environmental Protection Agency. However, the intrinsic properties of phage made many of these guidelines difficult to follow directly. For example, most AOAC protocols call for a neutralization solution or dilution step to stop the killing actions of the disinfectant so that remaining bacterial counts can be assessed at discrete time points. Unexpectedly, we could not identify a neutralizing condition to inactivate phage that was not also detrimental to the test organism. For example, temperatures high enough to denature phage also killed *S. aureus* (data not shown). Similarly, cysteine protease inhibitors are known to inhibit the active-site cysteine residue of phage[16], but at the required concentrations, these inhibitors are also lethal to *S. aureus* (data not shown). Even attempts to wash bacteriophage from the surface of *S. aureus* failed due to tight binding of the receptor to the bacterial cell wall, and rapid dilution was likewise unsuccessful, as evidenced by the >1 log drop in the number of CFU when phage was immediately diluted at time zero. While these difficulties underscore the suitability of bacteriophage as a disinfectant, they nonetheless are confounding to standardized disinfectant testing protocols. Clearly, new testing methodologies and validation criteria will be needed to establish the efficacy of narrow-range, enzyme based disinfectants. We have presented here the potential use of phages as a narrow-spectrum bio-disinfectant against *S. aureus*. Full scale testing in hospitals where Staphylococcal infection strangles is nosocomial as well as formulation and stability studies in combination with conventional disinfectants will be required to validate future development of bacteriophage. Although the current testing was limited to

S. aureus, it should be noted that there are a number of other group other nosocomial pathogens which have medical importance that are likewise sensitive to phages. Beyond hospital settings, there are also a number of other settings where a safe, environmentally friendly, narrow-spectrum bio-disinfectant with near-species specificity could drastically reduce disease transmission. For example, disinfectants targeting vancomycin-resistant *Enterococci* may be practical in surgical suites[17], nursing homes, those against *Listeria monocytogenes* would have applications in food-processing facilities; reduction of *S. pyogenes* or *S. pneumoniae* bacterial loads in day care settings or areas of dense living quarters could address cases of bacterial pharyngitis or otitis media, respectively; and additional methods for decontaminating suspected exposures of *Bacillus anthracis* remain a security priority[18]. Notably, bacteriophage can address all of these organisms have been cloned, expressed, and biochemically studied. Further development of these bacteriophage for environmental use as narrow-spectrum disinfectants could provide important alternatives to curtail the acquisition and spread of important pathogens.

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