Efficacy of bacteriophage in removal of *Pseudomonas aeruginosa* from infectious surfaces

Golnar Rahimzadeh [1], Mohammad Sadegh Rezai [1,*]

[1] Pediatric Infectious Diseases Research Center, Communicable Diseases Institute, Mazandaran University of Medical Sciences, Sari, Iran

*** Corresponding author:**

Mohammad Sadegh Rezai; professor of pediatric infectious diseases

Address: Pediatric Infectious Diseases Research Center, Mazandaran University of Medical Sciences, Sari, Iran.

Mobile: +98 (911) 1551102

E-mail: drmsrezaii@yahoo.com; rezai@mazums.ac.ir

Abstract: Nosocomial infections can be transmitted by contaminated hospital surfaces with resistant pathogens. conventional sanitations are not efficiently contributing to removing resistant pathogens. Bacteriophages suggest as decontaminating agents, safe, their selective ability to kill specific bacteria. This work aimed to assess the efficiency of a phage in removing *pseudomonas aeruginosa* from different hard surfaces. The decontamination ability of phages w was tested *in vitro* against *Pseudomonas aeruginosa* strain. *Cystoviridae* Phages with titer (2×10^{12} PFU/mL) can efficiently reduce viable bacterial cells on contaminated surfaces. The treated surfaces with alcohol 70% and phage showed an evident drop of bacterial cell number from 1 h to 24 h. These results suggest that bacteriophages are biocontrol agents removing nosocomial infection pathogens transmitted by contaminated surfaces in the hospital environment.

Keywords: *pseudomonas aeruginosa*; nosocomial infections; decontaminations; phages

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 April 2021 doi:10.20944/preprints202104.0411.v1

1. Introduction

The presence of microbial agents on the surfaces and medical devices in different units of the hospital is an important detrimental factor for health. Those pathogens play a major role in causing nosocomial infections which they lead to longer duration and dissatisfaction of patients in hospitals. About 3.2 million patients suffer from nosocomial infections each year, with 37,000 dying directly from nosocomial infections [1-2].

The hospital surfaces persistently are contaminated by several drug-resistant pathogens including *Staphylococcus* spp, *Enterobacteriaceae,* and *Pseudomonas* spp [3-5]. *P. aeruginosa* is resistant to disinfectants, antiseptics, and preservatives even can grow in some disinfectants and transmits infection so causes nosocomial infections [5]. *P. aeruginosa* can grow in the environment as a biofilm and survives for as long as one year [5]. The mortality and morbidity rate associated with *P. aeruginosa* is higher than other pathogens [6]. The results are reported from the European Centre for Disease Prevention and Control (ECDC) Register (2016) indicate which it is responsible for infection in the intensive care units (ICUs) 20.8%, ventilator-associated pneumonia (VAP) 14.7%, urinary tract infection (UTI) 11.1% and bloodstream infections 27.8% [7-8].
Up to know, the use of biocides is the most appropriate method against the prevalence of nosocomial infections [10]. However, biocides have shown significant limitation; such as disinfectants can be damage to tissue and cells, lack of preparation a standardized concentration, they evaporate when exposed to air and decreases their concentration, environmental impact, and corrosion of metal surfaces [9-11].
Based on these observations bacteriophages are suggested as a new alternative decontamination. They can contribute to the selection of both disinfectant-resistant and antibiotic-resistant pathogens. They are also devoid of undesirable side effects on eukaryotic cells. They do not need to be diluted. They do not corrode metal surfaces and devices. Furthermore, they are self-replication so they can remain and replicate as long as there exist bacteria. They are economically viable [12-17].
Ho et al 2016, used of ϕAB2 phage with titer of 10^5 PFU/ mL as an environmental biocontrol agent to *Acinetobacter baumannii M3237* in intensive care units (ICUs) [15]. Phages application has been proved effective against foodborne bacteria and food processing surfaces [15-16], based on these data the US Food and Drug Administration (FDA) has approved the use of specific phages as antimicrobial agents against food contamination by *Listeriamonocytogenes* [14-15]. Here, we evaluate the efficacy of bacteriophage as a disinfectant in the removal of *P. aeruginosa* from different hard surfaces at various times.

2. Materials and Methods

2.1. Bacterial species

P. aeruginosa ATCC 27853 and ATCC 51821 were purchased from Pasteur Institute, Iran. Those were cultured on the blood agar, followed by overnight incubation at 37 °C. Then all of the above bacteria were approved by the conventional microbiology tests. The susceptibility to antibiotics was determined by Kirby–Bauer test with antibiotic discs namely ampicillin (10 µg), nitrofurantoin (300 µg), tetracycline (30 µg) meropenem (10 µg), cefotaxime (30 µg), gentamycin (10 µg), cefixime (30 µg), and ciprofloxacin (10 µg), all were obtained from *Baharafshan, Iran* [15].

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 April 2021 doi:10.20944/preprints202104.0411.v1

2.2. Isolation of bacteriophage

The bacteriophage was isolated from *P. aeruginosa* supernatants. All of the above bacteria were incubated in 100 mL Luria-Bertani (Quelab, USA) for 24 hours at 37 ℃ in a shaker incubator. The three samples were centrifuged at 10,000 × g for 10 minutes. The supernatant was filtrated through a 0.22 µm syringe filter at sterile conditions [15-19].

2.3. Double-Layer Plaque Assay (DLA Assay)

900 µl sterilized sodium chloride-magnesium sulfate (SM) buffer (100 mmol/L NaCl, 8 mmol/L $MgSO_4$, 2% gelatin, and 50 mmol/L Tris–HCl [pH 7.5]) was added to 10 sterile tubes. Then 100 µl of phage was added to tube no. 1 and was vortexed. After vortexing 100 µl from tube no. 1 was taken out and added to tube no. 2. This procedure was repeated until tube no. 8. Tube no. 9 and tube no. 10 were selected as the positive and negative control respectively. From each of the diluted phage cocktail, 200 µl was transferred to 200 µl each of the above bacteria ATCC No (1.5×10^8 CFU/ mL). The mixtures were added to the top agar and the top agars were added to the bottom agar then the plates were incubated overnight at 37 °C. The experiment was repeated three times [15-19].

2.4. Transmission Electron Microscopy (TEM)

In order to prepare the phage for TEM, the phage was centrifuged at 20,000×g for 60 min. The phage was deposited on carbon-coated copper grids and was stained by 2% uranyl acetate (pH 4-4.5). The phage cocktail was observed on a Zeiss EM 900 TEM at 130 kV [15-19].

2.5. Determination the host range of phage

The spot test was performed to determine the lytic activity of the phage. The overnight cultured *P. aeruginosa* was inoculated in top agar and were poured into the bottom agar. A certain volume of the phage cocktail supernatant (10 µl) was poured over the solidified agar. The plates were incubated at 37 °C overnight. The formation of the inhibition zone was checked [15-19].

2.6. Phage Stability *in vitro*

The stability of the phage was investigated under various environmental conditions such as temperatures and pH. Phage was incubated at 4, 22, 37, 50 °C for 60 minutes to determine thermal stability. The stability of the phage was determined at pH 3, 5, 7, 9, and 11 for 60 minutes. The lytic activity of the phage was detected by DLA assay.

Also phage stability was measured after 1, 7, 14, 21, and 30 days at room temperature with DLA assay [15-19].

2.7. Decontamination test

The efficiency of lytic activity of phage against *P. aeruginosa* was assessed on different kinds of hard surfaces by *in vitro* decontamination assays. *P. aeruginosa* was grown in tryptic soy broth (TSB, Merck Millipore), then 10 µl of the bacteria suspension (OD_{600nm} =1) was spread on the different types of surfaces as plastic and ceramic (ceramic tiles sterilized by oven previous). The bacteria suspension was seeded and allowed to dry at room temperature. 50 µl of the phage lysate in SM buffer (2×10^{12} PFU/mL), 50 µl of Alcohol 70% separately were spread on the surfaces and

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 April 2021 doi:10.20944/preprints202104.0411.v1

allowed to dry. Two groups as; bacteria suspension (OD_{600nm} =1) , surfaces without bacteria suspension were used as a positive and negative controls respectively. After 15 min, 1, 3, 6, and 24 hours, surfaces were directly sampled by contact plates (Merck Millipore). Each plate, containing samples taken at the different time was incubated for 24 hours at 37°C and bacterial load was evaluated by enumerating plate CFU. Each sample was performed in triplicate [20, 13,14].

3. Results

3.1. Detection of Antimicrobial Resistance

The Kirby-Bauer test showed that *P. aeruginosa* is sensitive to nitrofurantoin, cefotaxime, and cefixime but resistant to ampicillin, tetracycline, meropenem, gentamycin and ciprofloxacin.

3.2. Characterization of phage

Figure 1 shows the lytic activity of phage determined with the formation inhibition zone in spot test. The phage titer was calculated by DLA assay (2×10^{12} PFU/mL). The morphology of the phage was shown by TEM, *Cystoviridae* with spherical shape (80-100 nm) with a lipid membrane around the capsomere (Figure 2).

The viability of phage was considered in various conditions. The phage showed the highest titer at 37 °C, but no active phage was found at 50 °C. The highest and lowest titer of phage was at pH 11 and 3 respectively (Figure 3).

The DLA assay results of incubation of phage after 1, 7, 14, 21, and 30 days at room temperature evidenced that phages efficiently can reduce viable bacterial cells.

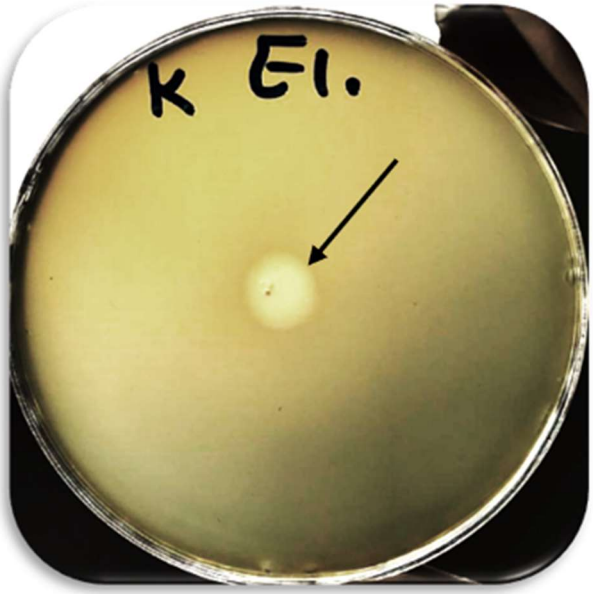

Figure 1. The formation of inhibition zone showed the lytic activity of phage against bacteria.

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 April 2021 doi:10.20944/preprints202104.0411.v1

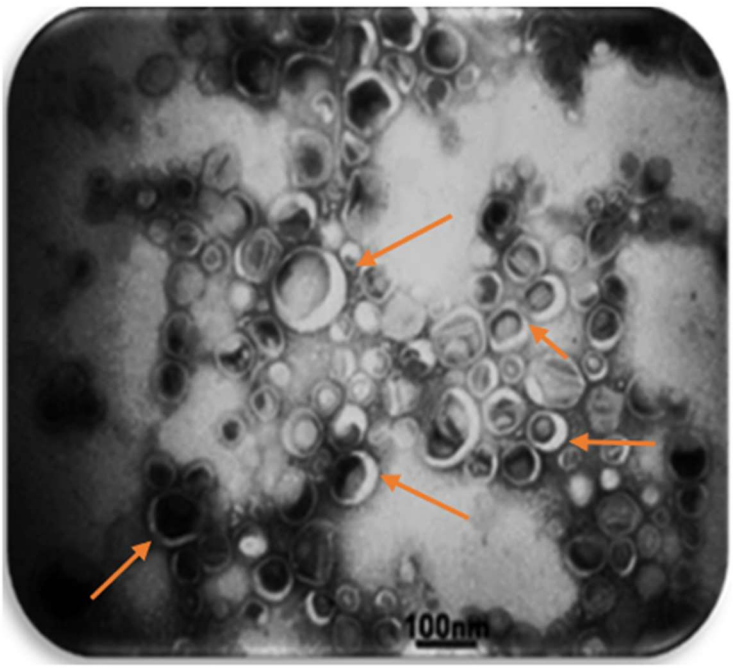

Figure 2. Electron micrographs of phage belong to the *Cystoviridae* family, with 2% uranyl acetate (pH 4- 4.5), Voltage 100Kv, the scale bar 100 nm.

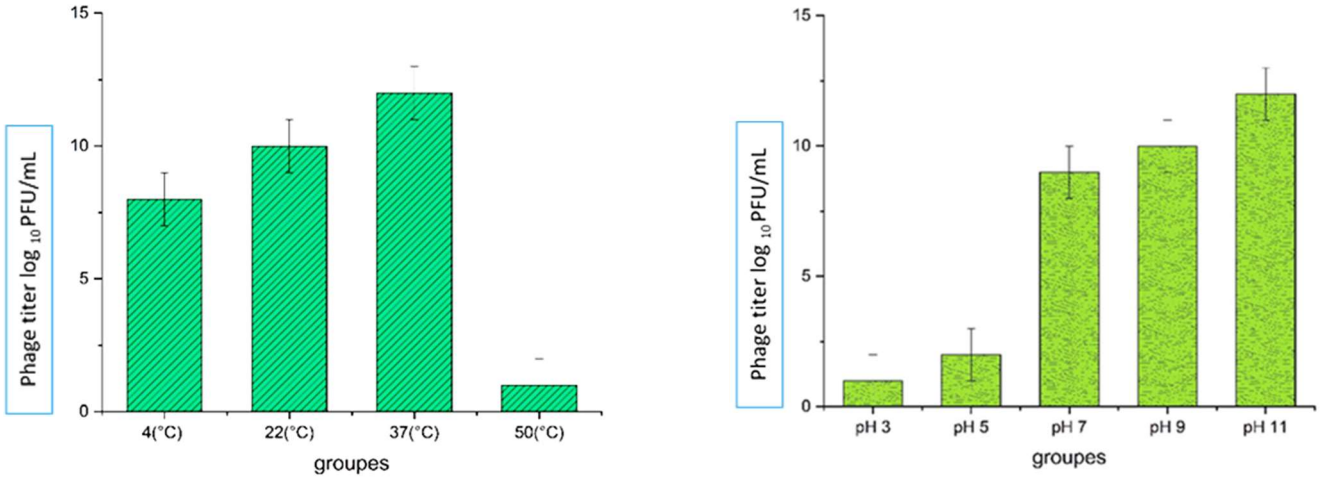

Figure 3. The titer of phage in various temperatures and pH.

3.3. Decontamination potential of phage on the hard surfaces

After 15 min, 1, 3, 6, and 24 h bacteria CFU on artificially contaminated surfaces were measured by enumerating grown colonies after 48 h incubation at 37°C. Phages can efficiently reduce viable

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 April 2021 doi:10.20944/preprints202104.0411.v1

bacterial cells on contaminated surfaces, independently of the surface type (ceramic, plastic). The treated surfaces with alcohol 70% showed an evident drop of bacterial cell number from 1 to 24 hours. Bacterial cell number in positive and negative group was 10^8 CFU, 0 respectively (Figure 4) (Figure 5).

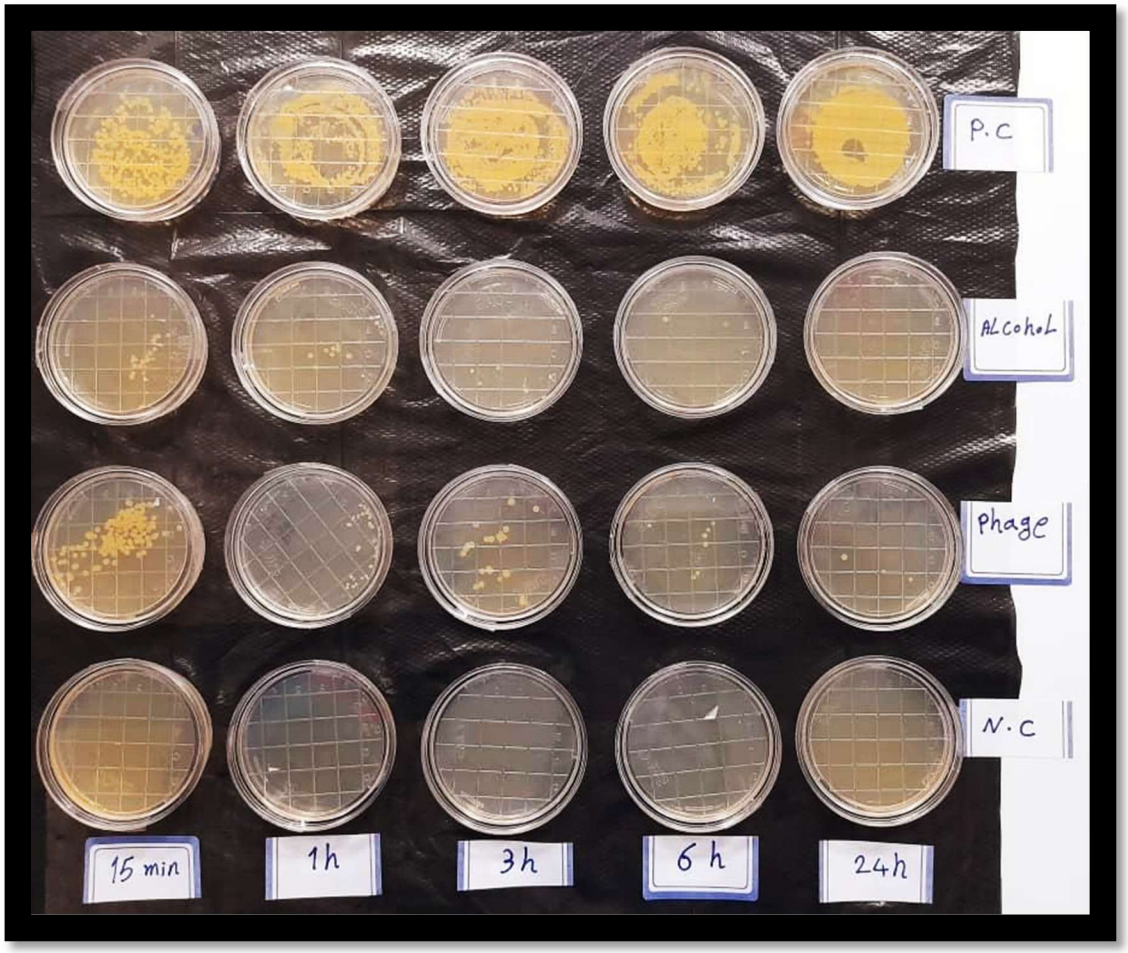

Figure 4. Reduction of *P. aeruginosa* on the hard surfaces treated with phage (2×10^{12} PFU/mL), alcohol (70%), positive control (P.C, 10^8 CFU), negative control (N.C).

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 April 2021 doi:10.20944/preprints202104.0411.v1

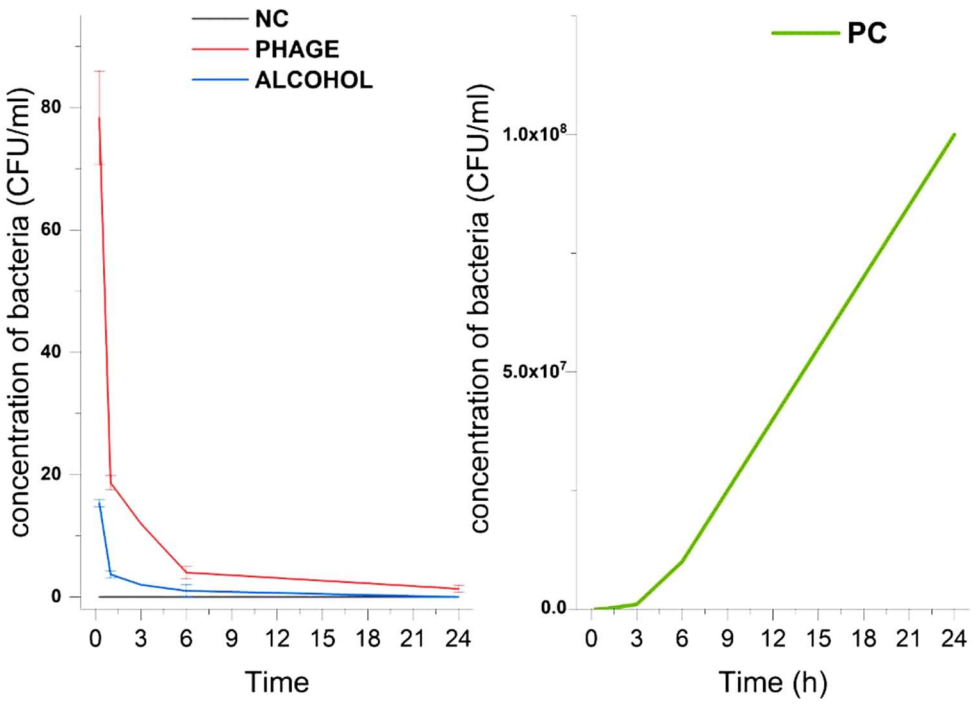

Figure 5. Reduction of *P. aeruginosa* on the hard surfaces treated with phage (2×10^{12} PFU/mL), alcohol (70%), positive control (P.C, 10^8 CFU), negative control (N.C), after 15 min, 1, 3, 6 and 24h.

4. Discussion

In this study, *Cystoviridae* phage against *P. aeruginosa* with a titer (2×10^{12} PFU/ mL) was isolated from a bacterial supernatant. Phage lysate showed lytic activity against *P. aeruginosa* as documented with formation of the inhibition zone in spot test. The efficacy of lytic activity of phage at various hard surfaces (ceramic and plastic) against *P. aeruginosa* confirmed after 1hour with a decrease the number of bacteria (CFU). The highest lytic activity of phage determined at 37 °C and pH 11. We proved phage stability after 1, 7, 14, 21 and 30 days of incubation at room temperature, by PFU titration on the specific bacterial.

In present study phage lysate did not show destructive effects on the hard surfaces. Previous studies were showed phages have not side effect eukarutic cells [17-21]. Many chemical sanitizers have antimicrobials activity but are corrosive and toxic and unacceptable for treating tissue, food, and surfaces [23-24].

Our results showed with increasing the contact time between phage and contaminated surfaces decreased the number of bacteria (CFU). The highest and lowest bacterial concentrations observed in 15 minutes and 24 hours, respectively. The number of released progeny phage from the lysed host bacterium logarithmically increases so increases the lytic activity of phage. Bacteriophages are self-replications so they are economically affordable [17-21]. Gamma irradiation of beef, approved by the U.S. FDA in 1997, produces about a 1,000-fold reduction in the number of viable

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 15 April 2021 doi:10.20944/preprints202104.0411.v1

aerobic bacterial contaminants. However, it is expensive and requires batch processing. In addition, it is not specific [25].

Common antimicrobials used for disinfecting surfaces in healthcare facilities include quaternary ammonium compounds, hydrogen peroxide, and chlorine-based products [24]. Lineback et al 2018, reported significant differences in disinfectant efficacy as bacterial regrowth [24], which compared to our results, phage reduces bacteria over time. Biocides for antimicrobial activity need special conditions as; Glutaraldehyde increases in biocidal efficacy with an increase in pH or some need to be diluted [24].

According to the increased of resistant pathogens such as *P. aeruginosa* to traditional sanitizers including; hypochlorous acid, povidone-iodine, and benzalkonium chloride [26], in this study *P. aeruginosa* was selected and seeded (OD $_{600nm}$ = 1) on the various hard surfaces. Three experimental groups were examined, with the two hard surfaces (ceramic and plate). Surfaces were treated by spreading 50 µl *Cystoviridae* phage with titer (2×10^{12} PFU/ mL). Treated surface with phage, after 15 min did not observe reduction in the number of viable *P. aeruginosa* because the lytic cycle of a bacteriophage, from the phage's attachment to the release of progeny phage from the lysed host bacterium, requires about 40 min. Thus since we used only a 15 min contact between phage and the contaminated surfaces, the significant reductions in *P. aeruginosa* counts was not likely to alcohol 70% group. Treated surfaces with alcohol 70% showed viable bacterial targets dropped very rapidly (within 1 hour) and did not detect of viable bacteria after 24 hours.

D'Accolti et al in 2018, the decontamination with phage was carried out on the plastic, glass, and ceramic surfaces against *Staphylococcus aureus, Escherichia coli, and P. aeruginosa* (OD $_{600nm}$ = 0.4). Their results showed phages targeted efficiently all tested bacteria (up to >90%) after 1 hour [27].

Our results support the idea that *P. aeruginosa* bacteriophage significantly reduce contamination of hard surfaces without any detectable difference between surface types as same Abuladze et al 2008, reported 100µl *E. coli O157:H7* phage with titer 10^9 PFU/ml reduced *E. coli O157:H7* on glass slides and gypsum boards 98% and 95 % respectively [14].

Lytic bacteriophages are alternative approaches to aid the prevention of diseases caused by natural or intentional dissemination of pathogenic bacteria on various surfaces. Several authors [28-29] reported, treatment with bacteriophages significantly reduced the levels of major food-borne pathogens in various foods, with reduction levels ranging from 1.8 to 4.6 logs compared to those of untreated or placebo-treated controls [30-31]. LMP-102 is the first phage based preparation to be approved for a food safety application by a Western regulatory agency [14].

5. Conclusions

Phages potentially with stable lytic activity lead to effective and safe elimination of pathogens from the contaminated hard surfaces. Bacteriophages as biocontrol agents open new perspectives for the development of innovative products aimed at the prevention of nosocomial infections transmitted by contaminated surfaces in the hospital environment.

Author Contributions: Experiment design, G.R, M.S.R; Experimental performance, G. R; Writing and reviewing, G.R, M.S.R. Authors have read and agreed to the published version of the manuscript.

Funding: No funding

Acknowledgments: The authors would like to thank the Pediatric Infectious Diseases Research Center Lab, Bouali Sina Hospital, Sari, Iran.

Conflicts of Interest: The authors declare no conflict of interest.

References

1- Allegranzi, B.; Bagheri Nejad ,S.; Combescure C; et al. Burden of endemic health-care-associated infection in developing countries: systematic review and meta-analysis. *Lancet*. 2011, 377, 228–241.

2- Boyce, JM. Environmental contamination makes an important contribution to hospital infection. *J Hosp Infect*. 2007, 65,50–54.

3- Suleyman, G.; Alangaden, G.; Cecilia Bardossy, A. The Role of Environmental Contamination in the Transmission of Nosocomial Pathogens and Healthcare-Associated Infections. Current Infectious Disease Reports. 2018, 20, 1-12.

4- Weber, DJ.; Rutala, WA. Role of environmental contamination in the transmission of Vancomycin-resistant enterococci. *Infect Control Hosp Epidemiol.* 1997, 18,306–309.

5- Hermandez, J.; Rosa, D. ; Toledano; et al. Biofilm vs. planktonic bacteria mode of growth: which do human macrophages prefer? *Biochem Biophys Res Commun*. 2013, 441,947–52. Kramer A, Schwebke, I.; Kampf ,G. How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. *BMC Infect Dis.* 2006, 6,130-136.

6- European Centre for Disease Prevention and Control. Healthcare-associated infections acquired in intensive care units. In: ECDC Annual epidemiological report for 2016. Stockholm: ECDC; 2018.

7- Ceniceros, A.; Peertega, S.; Galeiras, R.; et al. Predicting mortality in burn patients with bacteremia. *Infection*. 2016, 44,215–22.

8- Bock, LJ.; Wand, ME.; Sutton, JM. Varying activity of chlorhexidine-based disinfectants against Klebsiella pneumoniae clinical isolates and adapted strains. *J Hosp Infect.* 2016, 93,42–48.

9- Wand, ME.; Bock, LJ.; Bonney, LC.; Sutton, JM. Mechanisms of increased resistance to chlorhexidine and cross-resistance to Colistin following exposure of Klebsiella pneumoniae clinical isolates to chlorhexidine. *Antimicrob Agents Chemother*. 2017, 61, e01162-16.

10- Collee, JG.; Duguide, JP.; Fraser, AG. *Practical medical microbiology*. 13th Edition. Vol 2, New York: Churchill Livingstone, 1989,25, 64-90.

11- Young, EC.; Senford, TA. Chaos to comprehension: Cleaning, sterilization, and disinfection. *Urol Nurs* 2003, 23, 329-333

12- Rahimzadeh, G.; Saeedi, M.; Farshidi, F.; Rezai ,MS. Phage Therapy in Treatment of Gram-negative Bacterial Infections: A Systematic Review. *JMUMS.* 2018, 28,203-212.

13- Rahimzadeh, G.; Farshidi, F.; Rezai, MS.; Rezai, S. The Effect of Bacteriophages against Gram Negative Bacteria Infections in Vivo: A Systematic Review. *Journal of Isfahan Medical School*. 2019,37, 427-34.
14- Abuladze, T.; Li, M.; Menetrez, MY.; Dean, T.; Senecal, A.; Sulakvelidze, A. Bacteriophages reduce experimental contamination of hard surfaces, tomato, spinach, broccoli, and ground beef by Escherichia coli O157:H7. *Appl Environ Microbiol.* 2008, 74,6230–6238.
15- Ho, YH.; Tseng, CC.; Wang, LS.; et al. Application of bacteriophage containing aerosol against nosocomial transmission of carbapenemresistant Acinetobacter baumannii in an intensive care unit. *PLoS One*. 2016, 11,e0168380.
16- Tomat, D.; Quiberoni, A.; Mercanti, D.; Balague, C. Hard surfaces decontamination of enter pathogenic and Shiga toxin-producing Escherichia coli using bacteriophages. *Food Res Int.* 2014, 57,123–129.
17- Rahimzadeh, G.; Resch, G.; Rezai, MS.; Nemati Hevelaee ,E. Characterization and Lytic Activity of Isolated Escherichia Coli Bacteriophages against Escherichia Coli in Vitro. *Iranian Journal of Medical Sciences*. 2020, 45, 298-303.
18- Rahimzadeh, G.; Gill, P.; Rezai, MS. Ultra structural characteristics of methicillin resistant Staphylococcus aureus cell wall after affecting with lytic bacteriophages using atomic force microscopy . *Iranian Journal of Basic Medical Sciences*. 2019, 22, 290-295.
19- Rahimzadeh, G.; Gill, P.; Saeedi, M.; Ghasemi, M.; Rahmatpour Rokni, Gh.; Rostamkalaei, S. Evaluation of bacteriophage products against burn wound Methicillin-resistant Staphylococcus aureus (MRSA) infections. *Wound Medicine*. 2020, 28, 100182.
20- Rahimzadeh, G.; Rezai, MS.; Ahmadi, E. Lytic Activity of Isolated Phage from Milk against Extended-Spectrum Beta-Lactamase Escherichia coli. *JMUMS*. 2021, 30, 139-144.
21- Rahimzadeh, G.; Gill, P.; Rezai, MS. Characterization of methicillin-resistant Staphylococcus aureus (MRSA) phages from sewage at a tertiary pediatric hospital. *Arch. Pediatr. Infect. Dis*. 2016, 5 , e39615.
22- Vandini, A.; Timberman, R.; Frabetti, A.; et al. Hard surface bio-control in hospitals using microbial-based cleaning products. *PLoS One*. 2014, 9, e108598.
23- Chesney, J. A.; Eaton, J.; Mahoney, Jr. Bacterial glutathione: a sacrificial defense against chlorine compounds. *J. Bacteriol*. 1996,178,2131–2135.
24- Caitlinn, B.; Lineback ,A.; Nkemngong, C .; Tongyu, Wu .; Xiaobao, Li .; et al. Hydrogen peroxide and sodium hypochlorite disinfectants are more effective against Staphylococcus aureus and Pseudomonas aeruginosa biofilms than quaternary ammonium compounds. *Antimicrob Resist Infect Control*.2018, 7, 154- 161.
25- Brito, M. S.; Villavicencio, A.; Mancini-Filho, J. Effects of irradiation on Trans fatty acids formation in ground beef. *Radiat. Phys. Chem*. 2002,63,337–340.
26- Davidson, P. M.; Harrison MA. Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. *Food Technol*. 2002,56,69–78.
27- D'Accolti ,M.; Soffritti ,I.; Piffanelli ,M.; Bisi ,M.; et al . Efficient removal of hospital pathogens from hard surfaces by a combined use of bacteriophages and probiotics: potential as sanitizing agents. *Infect Drug Resist.* 2018, 11, 1015–1026.
28- Leverentz, B.; Conway,w.; Alavidze,z.; Janisiewicz, w.; et al. Examination of bacteriophage as a biocontrol method for Salmonella on fresh-cut fruit: a model study. *J. Food Prot*. 2001,64,1116–1121.

29- Leverentz, B.; Conway,W.; Camp,M.; Janisiewicz,W.; et al. Biocontrol of Listeria monocytogenes on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Appl. Environ. Microbiol*. 2003,69,4519–4526.

30- Sulakvelidze, A.; Barrow, P. Phage therapy in animals and agribusiness, In E. Kutter and A. Sulakvelidze (ed.), Bacteriophages: biology and applications. *CRC Press, Boca Raton, FL*.2005,23,23-28.

31- Greer, G. Bacteriophage control of foodborne bacteria. *J. Food Prot*.2005, 68,1102–1111.