ORIGINAL ARTICLE

LONG TERM BEHAVIOUR OF LYOPHILIZED PHAGES IN RELATION TO ITS MORPHOLOGICAL STABILITY AND TITRE

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ABSTRACT

Bacteriophage therapy is one of the hopeful alternatives to combat the snowballing problem of antibiotic resistance. Lyophilization of bacteriophage could improve their stability in long-term storage. The aim of this study was to assess the stability of lyophilized bacteriophages over the period of 20 years. 6-7 log decrease in the phage titre and morphological changes were observed when lyophilized phages were cultured after 20 years.

Keywords: Stability, Phage titre, Lyophilization,

INTRODUCTION

The emergence and spread of drug-resistant pathogens that have acquired new resistance mechanisms, leading to antimicrobial resistance, continues to threaten our ability to treat common infections.[1] Especially alarming is the rapid global spread of multi- and pan-resistant bacteria that cause infections that are not treatable with existing antimicrobial medicines such as antibiotics. Antibiotics are becoming increasingly ineffective as drug-resistance spreads globally leading to more difficult to treat infections and death. New antibacterials are urgently needed[2] – for example, to treat carbapenem-resistant gram-negative bacterial infections as identified in the WHO priority pathogen list. The clinical pipeline of new antimicrobials is dry. In 2019 WHO identified 32 antibiotics in clinical development that address the WHO list of priority pathogens, of which only six were classified as innovative. The one of the alternative to treat drug resistant pathogen is bacteriophage. Bacteriophages are a sustainable alternative to control pathogenic bacteria in the post-antibiotic era. Despite promising reports, there are still obstacles to phage use, notably phage titre, stability of the isolated bacteriophage[3]. Therefore, it is critical to preserve newly-isolated phages upon isolation to facilitate downstream applications. One of the most common method of preserving bacteriophage is by lyophilisation. The principle of lyophilisation is to remove moisture from microorganisms without passing through intermediate liquid state in order to maintain structural integrity and extend the longevity of the microorganisms stored at either room or refrigeration temperatures. There is a paucity of information on the long-term viability of preserved bacteriophages. Since 2 decades, various phages were isolated against MDR bacteria. The stability and the phage titre was estimated yearly and the results are presented in this article.

MATERIALS & METHODS

The phages were isolated from different sources against MDR bacteria isolated from clinical specimens by the method of Smith and Huggins[3].

In-vitro confirmation of bacteriophage activity of all the phages isolated were done by the standard technique.[3] The bacteriophage isolated were lyophilized and stored at -20°C[4,5]. The morphology of the phages were studied before and after lyophilisation using transmission electron microscopy. The Recovery of lyophilized phages were done by reconstituting phage with 1 mL of buffer and quantifying using double-layer plaque assay. The phage stability and titre was estimated once in a year.

RESULTS

Study period included from October 2001 to September 2021. Over 20 years 124 bacteriophages were isolated against various MDR bacteria. 39 bacteriophages were lyophilized (Approximate concentration 10^9 PFU/ml) and maintained in the stock (Table-1). All the phages isolated had an icosahedral head, measuring about 70-100 nm in diameter, and a 100-120nm long tail. Based on the morphology and the guidelines provided by International Committee on Taxonomy of Viruses (ICTV, Bethesda MD, USA) all the phages are tentatively placed in the *Siphoviridae*, *Myoviridae* and *Podoviridae* family.[5] In-vitro confirmation study revealed all the phages isolated were lytic phages.

An annual titre determination revealed most of lyophilized phages retained original post lyophilisation titres. After five years' retention, 34 phages showed 1log reduction and 5 phages showed approximately the initial concentration. At the end of the 10 years, 32 phages showed 3 log reduction, 7 phages showed 2 log reduction in the phage titre. At the end of 15 years, 27 phages showed 4 log reduction and 10 phages showed 5 log reduction and 2 phages showed 6 log reduction. At the end of 20 years, 19 phages showed 5 log reduction, 16 phages showed 6 log reduction and 4 phages showed 7 log reduction in the phage titre (Table-1). Transmission electron microscopy imaging of the selected phages revealed that at the end of the 1st year, most of the phages retained their morphologies. The minimum damage to morphology was observed at the end of the 5th year and maximum damage to morphology was observed at the end of the 20th year.

DISCUSSION

Phage therapy is one of the promising therapeutic alternatives to combat the problem of antibiotic resistance. The success of phage depends on its readily availability and the technique of storage for long duration. There is a paucity of information on the long-term viability of preserved bacteriophages to corroborate our finding. Around 39 phages were studied for stability and titre for 20 years. At the end of 20 years, 6 to 7 log reduction in the phage titre was seen in the lyophilized phages. Transmission electron microscopic study revealed that the structural damage was observed in long preserved phages. To conclude, long term storage of phages leads to decrease in the phage titre, it is advisable to culture phages regularly to prevent decrease in phage concentration and to prevent morphological damage to phages. But repeated culturing of phages may lead to phage mutation or host becoming resistant to specific phage.

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Table-1: Bacteriophage titre and different time intervals

Phages	Bacteriophage titre (Log10 value)					
	Lyophilized concentration	1 year	5 year	10 year	15 years	20 year
Staphylococcus aureus Ø1	9.544	9.041	8.944	6.964	3.914	2.813
Staphylococcus aureus Ø2	9.431	9.342	8.813	6.653	5.886	3.886
MRSA Ø1	9.602	9.505	8.716	6.505	4.748	3.763
MRSA Ø2	9.491	9.462	8.681	6.763	4.643	3.643
MRSA Ø3	9.255	9.204	8.462	6.342	5.505	3.944
MRSA Ø4	9.322	9.255	8.740	6.556	5.672	3.602
Staphylococcus epidermidis Ø1	9.580	9.556	8.845	6.778	5.653	2.279
Staphylococcus epidermidis Ø2	9.613	9.623	8.869	6.924	4.892	2.881
Enterococci faecalis Ø1	9.591	9.531	8.833	6.991	4.681	3.792
Enterococci faecalis Ø2	9.204	9.146	8.505	6.079	4.255	3.643
Enterococci faecalis Ø3	9.431	9.380	8.763	6.886	4.580	3.771
Enterococci faecium Ø1	9.519	9.491	8.771	6.462	4.833	3.792
Enterococci faecium Ø2	9.613	9.519	8.708	6.531	4.806	3.672
Enterococci faecium Ø3	9.322	9.279	8.623	6.568	4.591	3.886
Enterococci faecium Ø4	9.857	9.732	8.672	6.833	4.602	3.716
Streptococcus pneumococci Ø	9.820	9.653	8.964	6.892	3.991	2.653
E.coli Ø1	9.380	9.342	8.556	6.568	5.663	3.643
E.coli Ø2	9.146	9.041	8.462	7.041	5.255	3.892
E.coli Ø3	9.230	9.079	8.544	7.672	5.505	3.813
Klebsiella pneumoniae Ø1	9.613	9.505	8.763	6.771	5.602	3.944
Klebsiella pneumoniae Ø2	9.653	9.342	8.531	6.826	5.672	4.591
Klebsiella pneumoniae Ø3	9.342	9.301	8.505	6.568	5.681	4.568
Klebsiella pneumoniae Ø4	9.114	9.041	8.398	6.944	5.431	4.431
Klebsiella oxytoca Ø1	9.301	9.279	8.415	6.982	5.301	4.740
Klebsiella oxytoca Ø2	9.398	9.342	8.653	6.851	5.699	4.653
Acinetobacter baumannii Ø1	9.462	9.342	8.690	6.255	5.716	4.681
Acinetobacter baumannii Ø2	9.643	9.462	8.591	6.987	5.477	4.813
Acinetobacter baumannii Ø3	9.580	9.447	8.322	6.892	5.462	4.690
Acinetobacter baumannii Ø4	9.519	9.477	8.568	6.869	5.255	4.580
Acinetobacter baumannii Ø5	9.568	9.491	8.613	6.204	5.505	4.892
Acinetobacter baumannii Ø6	9.813	9.699	8.462	6.041	5.519	4.653
Pseudomonas aeruginosa Ø1	9.643	9.602	8.519	6.591	5.431	4.279
Pseudomonas aeruginosa Ø2	9.519	9.380	8.462	6.447	5.591	4.748
Pseudomonas aeruginosa Ø3	9.491	9.380	8.322	6.898	5.462	4.681
Proteus mirabilis Ø	9.580	9.477	9.447	7.301	5.342	4.672
Proteus vulgaris Ø	9.556	9.380	9.531	7.591	5.447	4.431
Enterobacter cloacae Ø	9.568	9.398	9.519	7.447	5.477	4.255
Citrobacter frundii Ø1	9.580	9.505	9.681	7.813	5.643	4.898
Citrobacter frundii Ø2	9.643	9.447	9.556	7.477	5.531	4.881

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