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Short Communication

Storage of Bacteriophages at 4°C Leads to no Loss in Their Titer after One Year

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ABSTRACT

The storage of bacteriophages is tricky as no ideal common practice is available and the storage conditions may differ from phage to phage. There is disparity among the scientific community regarding bacteriophage storage conditions and various conditions (4 °C, -20 °C, -80 °C and liquid nitrogen freezing at -196 °C) are used by different laboratories. In this study, the storage stability of five bacteriophages, JHP, RLP, RSP, SaPL, and IttPL, at distinct temperatures for complete one year is described. Our results indicated that storage of bacteriophages at 4°C was most appropriate, followed by storage at -20°C and -80°C as no drop in viral titer was witnesses in bacteriophage samples stored at 4°C for up to one year. The results of this research indicated, that a standard refrigeration is suitable and economical means of long term storage with no fall of phage activity.

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Authors' Contributions
IAA and SR designed the study. IAA, MA and RT has performed the experiments. ZA and SR analyzed the data statistically. IAA and MA wrote the article. SR supervised the work.

Key words
Bacteriophage, Titer, PFU, Liquid nitrogen freezing, Storage.

In recent years, the interest in bacteriophages has increased owing to their potential use as a biocontrol agent for antibiotic-resistant superbugs. Use of bacteriophage as a biocontrol agent for plants, animals, humans and even for food pathogens is documented (Doss *et al*., 2017; Tahir *et al.,* 2017). A precise and cost effective method of bacteriophage storage, prior to their practical application, is vital. The dilemma of phage preservation can be traced back to their discovery, as an ideal universal method for bacteriophage preservation has yet to be published. Bacteriophages are typically stored as a free virion; the sensitivity to storage conditions and storage media, however, vary from phage to phage. Storing bacteriophages at 4°C or at chilling temperature may be the most effective for long-term preservation with least impact on virion infectivity (Fortier and Moineau, 2009). Bacteriophage storage at 4°C is the simplest and most cost-effective way, and there are several records of bacteriophages stored at 4°C maintaining their high titer even after 30 years (Ackermann *et al*., 2004; Weber-Dąbrowska *et al*., 2016). Merabishvili *et al*. (2009) found that the titer of *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteriophages, in cocktails did not drop significantly, after 12 months storage at 4°C. They suggested that merely the protection of a bacteriophage lysate from evaporation and bacterial contamination suffices to maintain phage titer with the passage of time during storage. The storage of bacteriophages through freeze-drying is well documented, however, it involves specialized instrument and not all bacteriophages behave in an identical fashion (Ackermann *et al*., 2004; Clark, 1962; Zierdt, 1988). Increasing interest in exploiting bacteriophages as a bio-control agent requires exploring reliable methods for long-term storage of bacteriophages. Different preservation strategies like storage of raw or purified bacteriophage lysate at various conditions (4°C, -20°C and -80°C) are described from different laboratories with fluctuating degree of efficacy (Ackermann *et al*., 2004; Carlson, 2005; Fortier and Moineau, 2009; Zierdt, 1988).

Individual bacteriophage may vary in sensitivity to physical conditions and the composition of storage media, thus none of the aforementioned methods is global. In one study, only 10 out of 19 bacteriophages survived after storage for 10 years without a fall in bacteriophage titer, but the impact of storage in this study was not uniform (Ackermann *et al*., 2004). Thus individual methods for each bacteriophage likely need to be established for long-term storage. In most instances, the bacteriophage titer decreases gradually with time (Ackermann *et al*., 2004). Sometimes a massive drop in titer occurs and the bacteriophage needs to be revived. There are also issues of phage variation with "rescuing" phages as the rescued phage may mutate or otherwise differ from the original, preserved phage (Cuevas *et al*., 2009; Drake, 1991; Santos and Drake, 1994; Wichman *et al*., 2005). Thus, a crucial issue in the choice of bacteriophage storage method is

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minimizing the stock refreshment cycles. However, some bacteriophages do not survive at freezing or even cooling to 4°C (Ackermann *et al*., 2004; Warren and Hatch, 1969).

In the case of large myoviruses, freeze drying may be harmful (Clark, 1962; Clark *et al*., 1962). The initial titer of the phage suspension can also influence the impact that storage may have on phage activity. At high titers, some bacteriophages form aggregates at low temperatures, which attenuates their activity (Carlson, 2005; Serwer *et al*., 2007a, b) and diluting them promotes a more rapid decrease in their titer. In this study, we reported the activity of five tailed bacteriophages storage for a period of one year at varying temperatures and in only one type of storage medium.

Materials and methods

Lauria Bertani (LB) broth was employed as the storage medium of the bacteriophages. For preservation, at -20°C and -80°C, 30% v/v of glycerol (50%), was utilized with a final 15% glycerol concentration. Each sample (1mL) was stored in 1.7 ml Eppendorf tubes in triplicates.

Bacteriophages were stored in triplicate at 37°C, 25°C, 4°C, -20°C and -80°C for 12 months. The titers of bacteriophages JHP, RLP, RSP, SaPL and IttPL before storage was $1.6x10^{13}$, $4.6x10^{11}$, $4.0x10^{17}$, $9.3x10^{15}$ and $2.0x10^{8}$, respectively, for storage at 37 °C, 25 °C and 4 °C, while, the titer for storage at -20 °C and -80 °C for bacteriophage JHP, RLP, RSP, SaPL and IttPL was $1.1x10^{13}$, $3.2x10^{11}$, $2.8x10^{17}$, $6.5x10^{15}$ and $1.4x\ 10^{8}$, respectively.

The titer of the all the bacteriophages before and after storage was counted utilizing double layer agar overlay method (Khawaja *et al*., 2016). In double layer agar overlay method, dilution of bacteriophage, incubated with host bacterium for 10-15 min, was overlaid on the agar plate followed by addition of 3-5 mL of semi solid agar and incubated overnight. The plaques were counted after incubation.

Results

The titer of tested bacteriophages diminished from 8 to 17 logs when the bacteriophages were stored at 37°C and this loss in titer was from 84 to 100 percent. Similarly, a 69-100 percent titer decrease (8-15 logs) was noted when put at 25°C. Interestingly, we detected no decrease in bacteriophage titer of all investigated viruses when the bacteriophages were stored at 4°C. Surprisingly, the bacteriophage titer also decreased (5-12 log; 25 to 70 percent) and (5-12 logs; 45 to 100 %) when stored at -20°C and -80°C, respectively (Table I).

In our investigation, the most reasonable condition observed for bacteriophage storage with minimal titer drop was 4 °C, which is in agreement with others working with tailed bacteriophages (Ackermann *et al*., 2004). At 4°C temperature, no substantial drop in the titer occurred and zero log decrease across. In contrast, freezing conditions of -20 °C and -80 °C appeared to substantially reduce bacteriophage titer. Storage at 25°C or 37°C, however, had the greatest impact on phage titer as few infectious virions were recovered from these phage suspensions after 12 months. Except for SaPL and JHP, zero percent survival was observed in other investigated bacteriophages (RLP, RSP, and ITTPL) at 37 °C, while at 25 °C, zero percent survival was observed in case of SaPL. The reductions in phages titer stored at 37, 25, 4, -20 and -80 °C is presented in Figures 1 and 2.

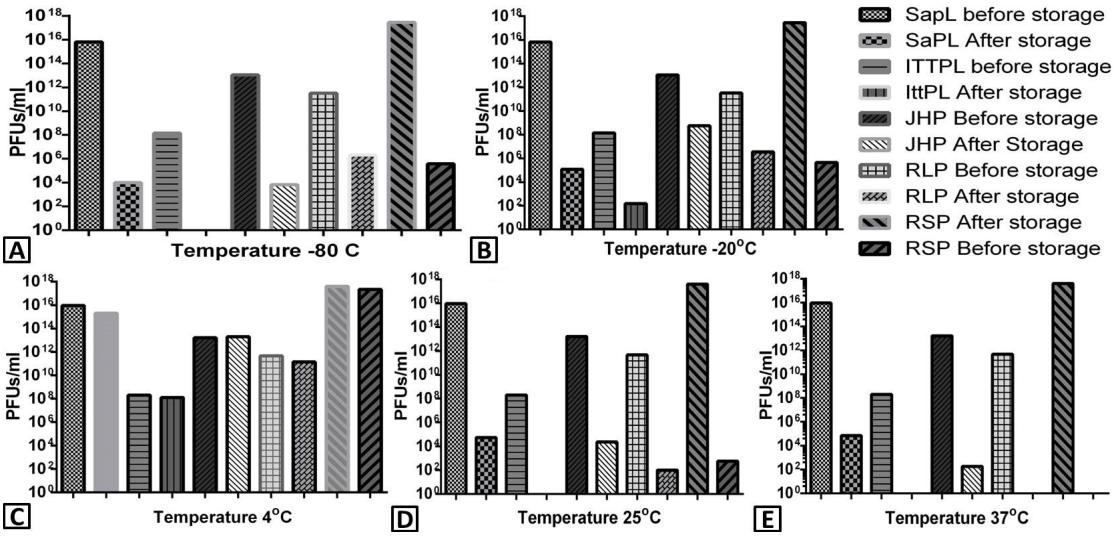

Fig. 1. Effect of specific storage conditions on the viability of acteriophages SaPL, IttPL, JIIP, RLP and RSP, after one year storage. Phage titre before and after storage at -80°C (A), at -20°C (B), at 4°C (C), at 25°C (D) and at 37°C (E).

The comparative detail in terms of percent log decrease at 37, 25, 4, -20 and -80 °C for the 5 bacteriophages under investigation is presented in Figure 2.

Table I.- Titers of bacteriophage lysates before and after (one year) storage at 37, 25, 4, -20 and -80°C for bacteriophage JHP, RLP, RSP, SaPL and IttPL.

Name*	Titer (PFU/ml) of bacteriophage at storage temp.				
	37°C	25°C	4 °C	-20 °C	-80 °C
Before storage					
JHP	1.6×10^{13}	1.6×10^{13}	1.6×10^{13}	1.12×10^{13}	1.12×10^{13}
RLP	4.6×10^{11}	4.6×10^{11}	4.6×10^{11}	3.2×10^{11}	3.2×10^{11}
RSP	4.0×10^{17}	4.0×10^{17}	4.0×10^{17}	2.8×10^{17}	2.8×10^{17}
SaPL	9.3×10^{15}	9.3×10^{15}	9.3×10^{15}	6.51×10^{15}	6.51×10^{15}
IttPL	2.0×10^{08}	2.0×10^{08}	2.0×10^{08}	1.4×10^{08}	1.4×10^{08}
After storage					
JHP	$1.8x10^{2}$	$2.3x10^{4}$	2.0×10^{13}	5.7×10^{08}	$6.53x10^{3}$
RLP	00	$1x10^{2}$	1.4×10^{11}	$3.5x10^{6}$	$2.0x10^{6}$
RSP	00	$5.7x10^{2}$	2.2×10^{17}	$4.45x10^{5}$	$3.8x10^{5}$
SaPL	$6.9x10^{4}$	$5.34x10^{4}$	2.0×10^{15}	$1.2x10^{5}$	$1.0x10^{4}$
IttPL	00	00	1.3×10^{08}	$1.53x10^{2}$	00

* name of bacteriophage.

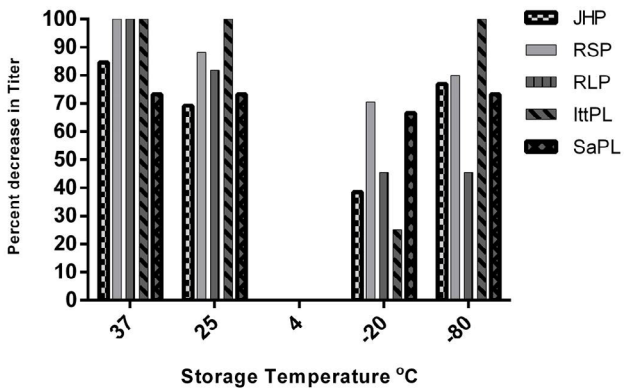

Fig. 2. Decrease (%) in the titers of bacteriophages JHP, RSP, RLP, IttPL and SaPL after one year of storage at different temperatures.

Discussion

Bacteriophages have been studied for over 100 years, which served us to understand many modern biological aspects, however, no common method for their preservation is available and in use. Despite this, there are very few published studies, focusing on this issue. It is perhaps owing to the disparate nature of bacteriophages that fewer efforts on this aspect of the bacteriophage biology are available. In this investigation, the activity of five tailed bacteriophages JHP, RLP, RSP, SaPL and IttPL, isolated from different sewage water sources of Lahore, Pakistan, was examined after their long-term storage. The novelty of this research is that no special preservatives were used in our phage solutions. The medium chosen for long-term storage was standard LB broth, which is regularly employed for the propagation of bacteriophages.

In earlier studies, different conditions for bacteriophage preservation were documented, including storage at 4, -20, -80°C and even Nitrogen freezing at -196°C, however, the suitable storage temperature vary with phage type (Clark, 1962; Clark *et al*., 1962; Warren and Hatch, 1969). In this study, 4°C was found to be the suitable temperature for storage of these bacteriophages, similar to results described by others working with tailed bacteriophages (Ackermann *et al*., 2004). They found that the tailed and filamentous phages without lipids can be stored for over 5-10 years, but their results varied with different bacteriophages. The results of our study are in accordance with another study which revealed no change in titer of bacteriophages *Staphylococcus aureus* and *P. aeruginosa* after one year of storage at 4°C (Merabishvili *et al*., 2009).

There are reports of bacteriophages sensitivity to low temperatures, including seven log reductions in titer observed after only t three months of storage. Interestingly, the *Bacillus* phage CP-54Ber showed the most stability at room temperature (Ackermann *et al*., 2004). None of the bacteriophages under study demonstrated significant viability after one year of storage at room temperature and 37°C, except JHP where a 30 % viability was observed. Sometimes, bacteriophages are also stored at chilling conditions with the addition of glycerol. It has also been documented that freezing with glycerol was not as effective as preservation at 4°C, which is evident in the current study (Clark and Geary, 1973). We observed a 25 to 71% and 45 to 100% decrease in bacteriophage titer, when phages were stored at -20°C and -80°C, respectively for 1year.

Conclusion

Based on this study, it was concluded that 4°C is the best storage temperature for bacteriophages, followed by -20°C and -80°C, in terms of minimally impacting viability suitability. Whereas, the temperature between 25 and 37°C were not suitable for bacteriophage storage. To further enhance our knowledge regarding storage stability of bacteriophages, storage stability studies of more than 1-year are required.

Statement of conflict of interest

The authors declare no conflict of interest.

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