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Full Length Research Paper

Stress sensitivity assays of bacteriophages associated with *Staphylococcus aureus*, causal organism of bovine mastitis

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Bacteriophages can provide an alternative measure for the control of *Staphylococcus aureus*, the major causal agent for bovine mastitis. This study looked at the sensitivity of six phages towards simulated environmental and formulation stresses. Phages Sabp-P1, Sabp-P2 and Sabp-P3 showed the most stable replication rates at increasing temperatures (45 to 70ºC), in comparison to phages Sabp-P4, Sabp-P5 and Sabp-P6. The effect of temperature on storage of phages showed that 4ºC was the minimum temperature at which phages could be stored without a significant reduction in their lytic and replication abilities. Furthermore, all phages showed varying levels of sensitivity to chloroform exposure, with Sabp-P5 exhibiting the highest level of reduction in activity (74.23%) in comparison to the other phages. All six phages showed optimal lytic ability at pH 6 to 7 and reduced activity at any pH above or below pH 6 to 7. Exposure of phages to varying glycerol concentrations (5 to 100%) produced variable results. All six phages were most stable at a glycerol concentration between 10 to 15%. Three of the six isolated phages, Sabp-P1, Sabp-P2 and Sabp-P3, performed optimally during the *in vitro* assays and have considerable potential for *in vivo* applications to treat mastitis-infected dairy cattle.

Key words: Bacteriophage, biological control, bovine mastitis, sensitivity assays, *Staphylococcus aureus.*

INTRODUCTION

Staphylococcus aureus-induced bovine mastitis in dairy herds is one of the most widespread and destructive diseases of dairy cows. It has far reaching consequences that affect milk quality and yield, health of the dairy cow, and the economics associated with the processing of milk and milk products. Under optimal conditions, the natural defence mechanisms of the cow itself, in conjunction with cultural control measures, may prove adequate in curbing disease onset and severity. However, the real problem arises when these intrinsic defences and cultural measures are compromised by infection. Antibiotic therapy has traditionally served as the next option to achieve effective control of the disease. Overall benefits of antibiotic therapy include a more rapid elimination of bacterial pathogens than self-cure, a reduced probability of chronic recurrent infections, a reduced depression in milk yield and a more rapid return to an acceptable somatic cell count and hence to saleable milk (Barkema et al., 2006; Nickerson, 2009).

Despite the documented success associated with antibiotic usage, it remains debatable as to whether this therapy is indeed positive in the long term. There are several conflicting views on this (Murchan et al., 2004; Borm et al., 2006; Sandgren et al., 2008; Nickerson, 2009; Blowey and Edmondson, 2010; Vanderhaeghen et al., 2010), many of which revolve around *S. aureus* and its ability to develop antibiotic resistance. In the long term, the cure rate of antibiotic treatments against this

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pathogen is low and, therefore, the disease cannot be effectively eliminated and/or controlled in infected herds by using antibiotics alone (Sutra et al., 1993; Carter and Kerr, 2003; Murchan et al., 2004; Shi et al., 2010).

Antibiotic resistance can be attributed to several factors, ranging from structural features that protect the bacterium, to production of chemicals that can neutralise antibiotics (Almeida et al., 1996; Herbert et al., 2000; Blowey and Edmondson, 2010; Cabrera et al., 2011; Villar et al., 2011). The spread of virulent methicillin-resistant *S. aureus* (MRSA) (Goñi et al., 2004; Murchan et al., 2004; Nickerson, 2009; Shi et al., 2010; Vanderhaeghen et al., 2010), coupled with the development of resistance to two new antibiotics (daptomycin and linezolid) recently approved for clinical use against Gram-positive bacteria (Mangili et al., 2005), has shown that *S. aureus* is indeed a formidable pathogen.

Due to these treatment limitations, research focus into the control of bovine mastitis has shifted to alternative therapies, such as the development of vaccines (Sutra, 1993; Herbert et al., 2000; Pereira et al., 2011) and biological control options, such as the use of botanical extracts (Akinyemi et al., 2005; Fawole, 2009) or phage therapy (O'Flaherty et al., 2005b; Sulakvelidze and Barrow, 2005; Gill et al., 2006a; García et al., 2007; Jones et al., 2007; García et al., 2008; Synott et al., 2009). Considering the wealth of information at our disposal, phage therapy appears to be one of the most sustainable measures for control of bovine mastitis. Several studies have identified various phages with lytic capabilities towards *S. aureus* (O'Flaherty et al., 2005a; Gill et al., 2006a; García et al., 2007; García et al., 2008; Synott et al., 2009). While each study did present noteworthy results, it must be noted that there are limitations which must be overcome. Phage inactivation can be triggered by milk proteins and fats (O'Flaherty et al., 2005b; Gill et al., 2006b), aggregation of *S. aureus* cells within milk (O'Flaherty et al., 2005b), and intrinsic immune factors within the cow itself (O'Flaherty et al., 2005a). While these limitations do exist, the solutions to these problems are merely a product of time. Further studies into the detailed effects of whey proteins on bacterial activity and aggregation, proper delivery of phage cocktails into animal tissues, and the development of phage formulations to facilitate optimal delivery and activity within intramammary tissues, are ongoing.

The primary focus of the current study was to investigate phage sensitivity, *in vitro*, towards simulated environmental and chemical stresses. This study aimed to contribute to phage formulations for *in vivo* application.

MATERIALS AND METHODS

Bacterial host strains and their isolation

Strain SaB1 of *S. aureus* was used for phage isolation and propagation. This strain was isolated from raw bovine milk collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa). SaB1 was initially isolated on blood agar. Strain identification to distinguish *S. aureus* from other staphylococcal strains was confirmed by hemolysis on blood agar, catalase reaction, Gram reaction and arrangement of bacterial cells upon Gram staining. This was followed by culturing on Baird-Parker agar and results were confirmed using a coagulase test (Bactident® Coagulase, Merck). SaB1 was maintained on tryptone soy agar (TSA) or typtone soy broth (TSB).

Phage isolation, propagation and purification

Raw bovine milk samples collected from dairy farms in the province of KwaZulu-Natal, RSA (Republic of South Africa) served as the initial material from which to isolate phages. These milk samples were chosen at random and included milk with both high (>400,000cells.ml^{-1}) and low somatic cell counts (SCC). Phages were isolated using the spot-plate method on double-layer agar (Sambrook et al., 1989; Harley and Prescott, 1993). Overnight SaB1cultures (~1 x 10^8 cfu.ml^{-1}) were incorporated into 7% (w/v) top agar that was supplemented with 1M $CaCl_2$. Raw milk samples were filtered through a 0.45 µm syringe filter. Filtered samples were then spotted onto the surface of the solidified top agar at 10 µl per spot. Plates were allowed to dry for 2 h, followed by incubation for 12 h at 37ºC. Zones of clearing (plaques) were indicative of phage activity. Plaques were removed from top agar and soaked in phage buffer (Sambrook et al., 1989) for 12 h with gentle agitation (150 rpm) at 4ºC; all phages were isolated through five rounds of plating from plaques. The resulting suspension was centrifuged using an Avanti J-26 XPI (www.beckmancoulter.com) at 10,000 g x 10 min at 4ºC. The supernatant was filtered using a 0.45 µm syringe filter and stored as phage stock at 4ºC. Subsequent phage was grown from this stock in liquid broth culture or using the double-layer agar method (Sambrook et al., 1989).

Isolated phages were purified through a modification of standard methods (Sambrook et al., 1989; Harley and Prescott, 1993). Filter-sterilised phage stock isolated from either liquid broth culture or double-layer agar method, were subjected to centrifugation using the Avanti J-26 XPI (Beckman-Coulter) at 75,600 *g* × 3 h × 10°C. The resulting phage pellets were re-suspended in fresh phage buffer and a second centrifugation was conducted (Avanti J-26 XPI (Beckman-Coulter) at 75,600 *g* × 3 h × 10°C). The phage pellets were then re-suspended in fresh phage buffer at 1/10 of the original volume that was processed. All stocks were stored at 4°C. Six phages were isolated and systematically named Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6. Multiplicity of infection (MOI) was calculated for each phage. Phage MOI was determined for each phage: Sabp-P1=3, Sabp-P2=3, Sabp-P3=3, Sabp-P4=0.2, Sabp-P5=0.1 and Sabp-P6=0.2.

Temperature sensitivity

Heat sensitivity

This method was a modification of that described by Harley and Prescott (1993). Optimal phage dilutions of 10^{-5} pfu.ml^{-1} were prepared for each phage for this assay. Phage dilutions were carried out in phage buffer. Diluted phages, Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6, were placed into a water-bath at 45°C. 100 µl aliquots were removed at each of the following time intervals: 0min (control - room temperature, 22°C), 5, 10, 20, 30, 40, 50 and 60 min. Each aliquot was plated using the double-layer plating technique (Sambrook et al., 1989), with an overnight culture of SaB1 as the host bacterium. The experiment was repeated for temperatures 50, 55, 60, 65 and 70°C. Phage diluted in phage buffer was used as a control. All plates were incu-

bated at 37°C for 12 h. After 12 h, the number of plaques was counted and phage titre was calculated for each time and its associated temperature. All assays were carried out in triplicate.

Cold sensitivity (storage feasibility of phages)

This assay was conducted in order to see the opposite effects of heat treatment on phages and to test their storage capability over time. Optimal phage dilutions of 10^{-5} pfu.ml^{-1} were prepared for each phage. Phage dilutions were carried out in phage buffer. This experiment was ongoing over a period of 6mo. Overnight SaB1 was used as the host bacterium. Phages Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6 were screened for stability at various storage temperatures (4, -20 and -80°C) with different durations at each temperature (1 h, 8 h, 12 h, 1 wk, 2 wk, 3 mo, and 6 mo). All phages were stored in sterile phage buffer (supplemented with 5% glycerol) for the duration of the experiment. At the end of each storage duration, at each temperature, a 100 µl aliquot of phage was removed and immediately plated using the double-layer plating technique.

An overnight culture of SaB1 was used as the host bacterium, hence final reaction contained 100 µl phage + 100 µl SaB1 (+ 1M $CaCl_2$). This was followed by double-layer plating as previously described (Sambrook et al., 1989). Fresh phage diluted in phage buffer was used as a control. Plates were incubated for 12 h at 37°C. The total number of plaques was counted and phage titre was calculated for each phage, at each time and at each associated temperature. The same phage sample was stored and screened for the entire duration of the experiment (6 mo). All assays were carried out in triplicate.

pH Sensitivity

This method is a modification of that described by Harley and Prescott (1993). TSB was calibrated (using 1 M HCl) according to the following pH range: 2, 4, 6, 8, 10, 12 and 14, and 900 µl of each was aliquoted into 2 ml Eppendorf vials. Phage stock was added to the calibrated TSB to bring the final phage dilution to10^{-5} pfu.ml^{-1}; that is, approximately 2.91×10^8, 3.04×10^8, 3.12×10^8, 1.9×10^7, 1.76×10^7 and 2.6×10^7 pfu.ml^{-1}, for Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5 and Sabp-P6 respectively. The vials were gently inverted in order to mix the phage within the TSB medium. All vials were left to stand at room temperature (22°C) for 60 min. At 60 min, 100 µl of phage suspension was removed and mixed with 100 µl of an overnight SaB1 culture (+ 1 M $CaCl_2$). This was followed by double-layer plating as previously described (Sambrook et al., 1989). Phage diluted in phage buffer was used as a control. All plates were incubated at 37°C for 12 h. After 12 h, the number of plaques was counted and phage titre was calculated for each pH. All assays were carried out in triplicate.

Chloroform sensitivity

This method is a modification of that described by Harley and Prescott (1993). Optimal phage dilutions of 10^{-5} pfu.ml^{-1} were prepared for each phage. Phage dilutions were carried out in phage buffer (Sambrook et al., 1989). Overnight SaB1 was used as the host bacterium. 1 ml each, of phages Sabp-P1 to Sabp-P6, were treated with 5% (v/v) chloroform. This suspension was gently shaken (100 rpm) at room temperature (22°C) for 15 min, followed by centrifugation at 10,000 *g* × 10 min at 4°C. The aqueous phase was withdrawn and phages were titred using standard double-layer plating. Phage diluted in phage buffer was used as the control. All plates were incubated at 37°C for 12 h. After 12 h, the number of plaques was counted and phage titre was calculated. All assays were carried out in triplicate.

Glycerol sensitivity

This assay was adapted from Santos et al. (2009). Optimal phage dilutions of 10^{-5} pfu.ml^{-1} were prepared for each phage. Phage dilutions were carried out in phage buffer. Overnight SaB1 was used as the host bacterium. Glycerol was made up in sterile distilled water at the following concentrations (v/v): 5, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%. Phage stock was added to the 900 µl of each glycerol solution to bring the final phage dilution to10^{-5} pfu.ml^{-1}. This was left at room temperature (22°C) for 1 h. Incubation was followed by standard double-plating and incubation of all plates at 37°C for 12 h. After incubation, the number of plaques was counted and phage titre was calculated. Phage diluted in phage buffer was used as a control. All assays were carried out in triplicate.

Statistical analyses

Differences between treatments were determined by analysis of variance (ANOVA) using Genstat 14th Edition.

RESULTS

Heat assay

ANOVA of the phage titres showed highly significant ($p<0.001$) differences for phage titers at different temperatures, and a highly significant phage x temperature x exposure time interaction. It was found that an increase in temperature, coupled with increased exposure time to that temperature, reduced phage reproductive activity (Figure 1).

Phage counts dropped significantly from 0 min exposure to high temperature, to 10min exposure to the same temperature (across the complete temperature range of 45 to 70ºC). After 10 min, phage reproductive activity stabilised across all temperature ranges, but remained low (Figure 1). Significant differences were noted between phage activity at each temperature and exposure time (<0.001), as well as between each phage itself (<0.001). In general, Sabp-P1, Sabp-P2 and Sabp-P3 were more stable, and titers remained higher than those for Sabp-P4, Sabp-P5 and Sabp-P6. Exposure to 70°C was the most damaging with a 92 to 96% reduction in phage titers. Overall results show that propagation of these phages is negatively affected by increased exposure to high temperatures.

Cold assay (storage feasibility of phages)

Storage temperature played an important role in influencing phage titre. ANOVA showed highly significant differences ($p<0.001$) in phage counts when the phages were stored at different temperatures for different durations (Figure 2). Storage of phages at temperatures below 4°C reduced phage activity significantly. Sabp-P1,

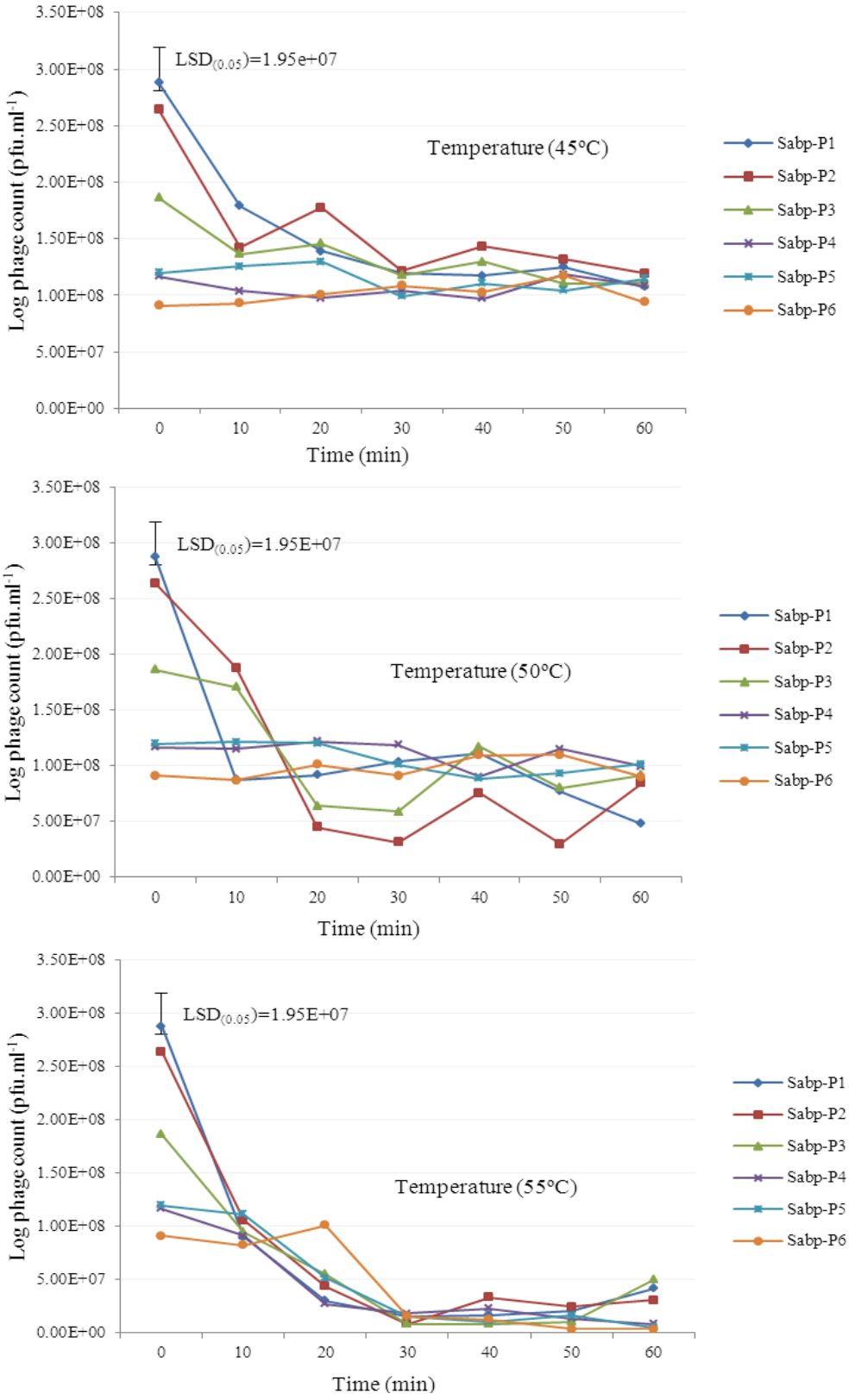

Figure 1. Heat assay screening the titres of six phages over a range of different temperatures. SaB1 was used as the host bacterium for phage propagation. Data points are the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$ when comparing any phage x temperature x time combination.

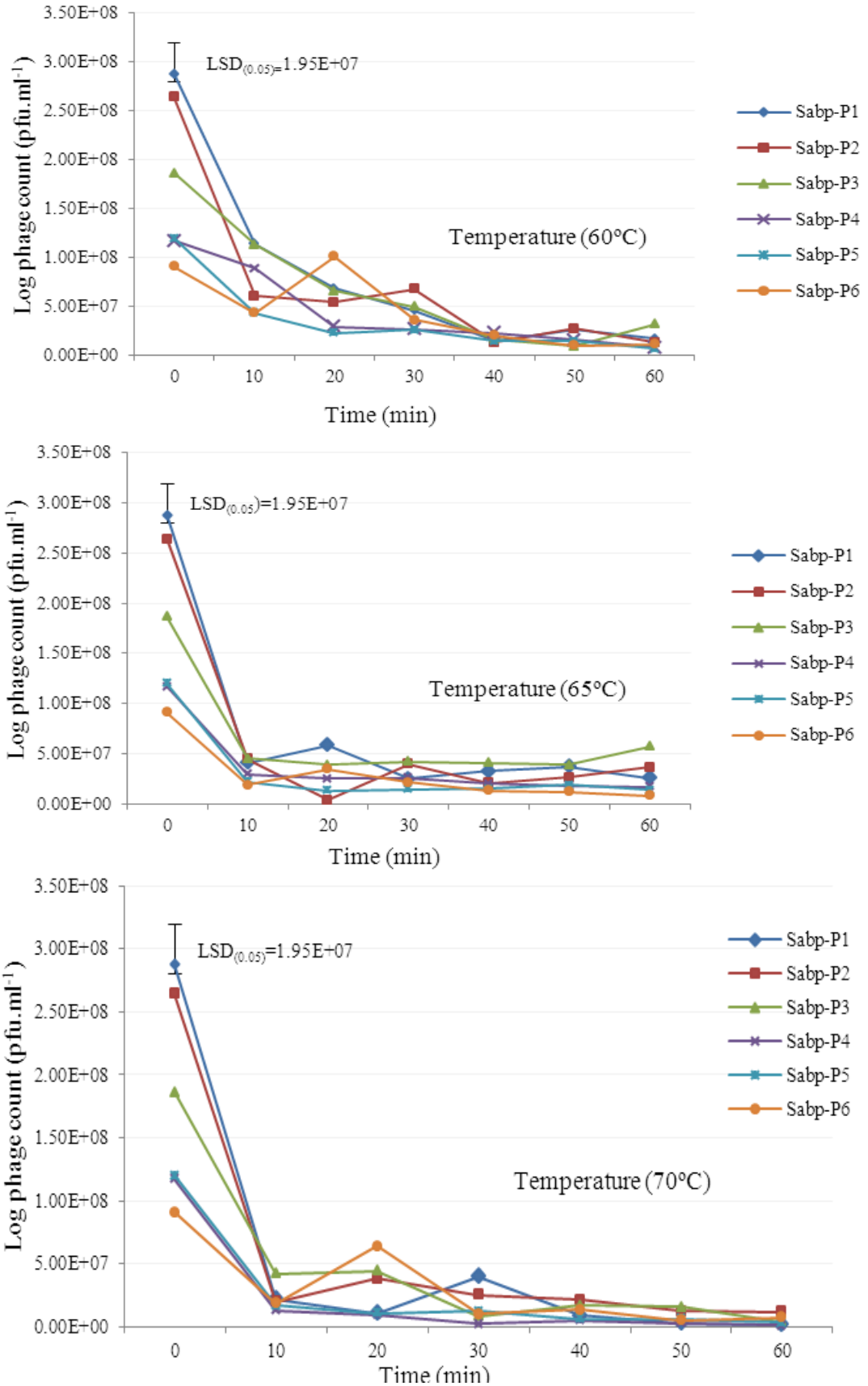

Figure 1. Contd.

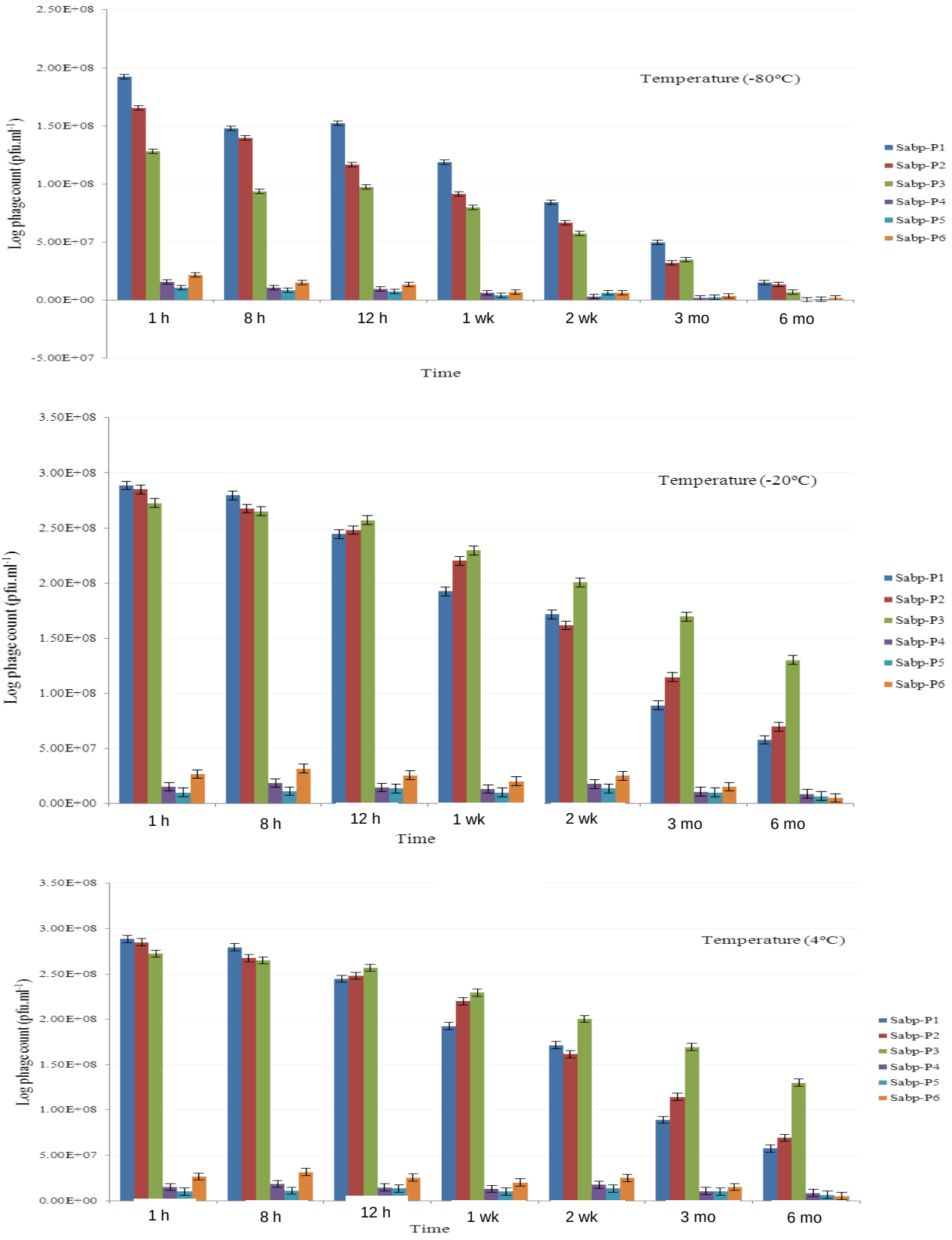

Figure 2. Screening the storage ability of six phages over a range of low temperatures. SaB1 was used as the host bacterium for phage propagation.Each bar represents the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$=3.93E+06 when comparing any phage x temperature combination.

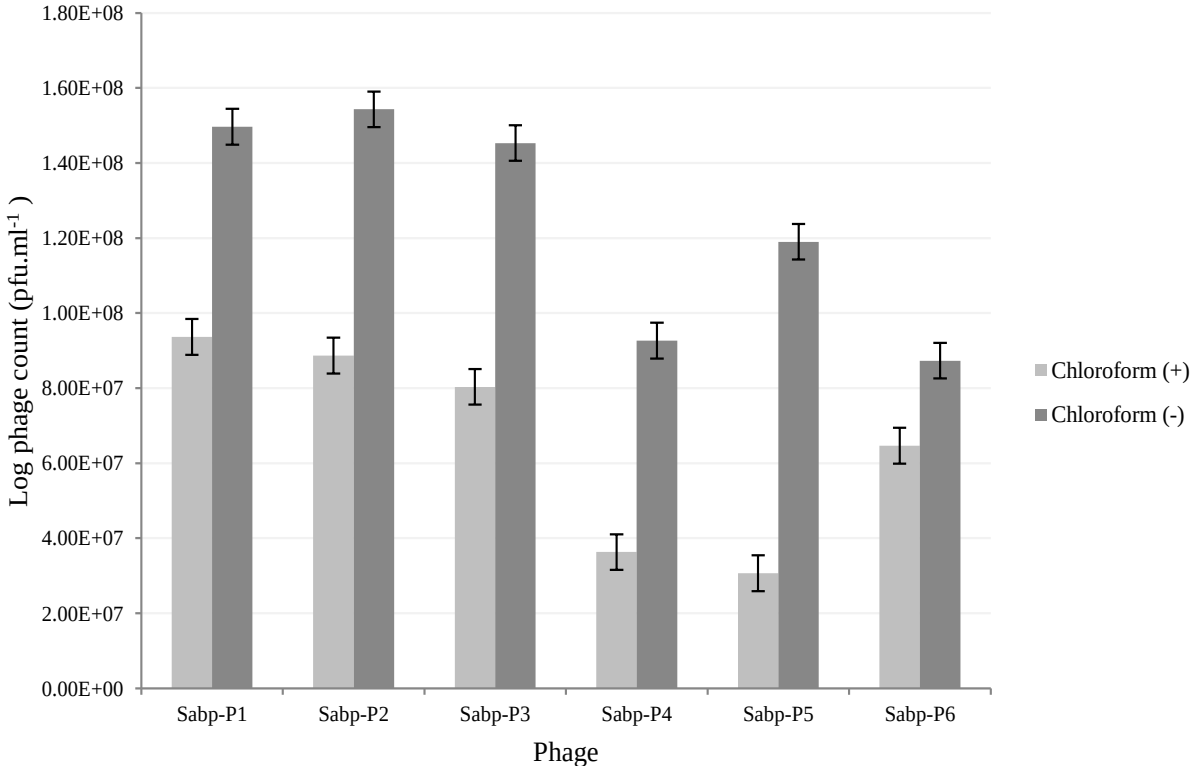

Figure 3. Effect of chloroform on phage titers. *SaB1* was used as the host bacterium for phage propagation. Each bar represents the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$=9.5E+07 when comparing any phage x bacterium combination.

Table 1. Reduction in phage titers upon exposure to chloroform.

Phage	Reduction in phage activity (%)
Sabp-P1	37.42
Sabp-P2	42.55
Sabp-P3	44.73
Sabp-P4	60.80
Sabp-P5	74.23
Sabp-P6	25.95
Mean	47.61
LSD	9.50
CV%	0.4

Sabp-P2 and Sabp-P3 displayed more stability at all three temperature ranges (4, -20 and -80°C), although phage titre did decrease significantly at -80°C. The titres for the other three phages Sabp-P4, Sabp-P5 and Sabp-P6, were generally low throughout these studies. However, these three phages showed stability during storage at all durations and temperatures. While the study did not proceed beyond a screening period of 6mo, it appears that the optimal storage temperature for the isolated phages (in phage buffer) is in the region of 4°C.

Chloroform assay

Chloroform exposure imposed negative effects on phage titers (Figure 3). ANOVA showed that significant ($p<0.001$) differences occurred between the titers of Sabp-P1, Sabp-P2 and Sabp-P3 in comparison to phages Sabp-P4, Sabp-P5 and Sabp-P6. Sabp-P1, Sabp-P2 and Sabp-P3 appeared more stable and titres did not reduce as significantly upon exposure to chloroform, as they did for Sabp-P4 and Sabp-P5. Sabp-P6 showed the most stability over time upon exposure to chloroform. However, a general reduction in phage titers was noted for each phage (Table 1).

pH Sensitivity

All of the screened phages (Sabp-P1, Sabp-P2, Sabp-P3, Sabp-P4, Sabp-P5, Sabp-P6) showed sensitivity to changes in the pH of the surrounding growth media (Figure 4). ANOVA showed significant differences ($p<0.001$) in activity between phages as pH increased, as well as significant difference in interactions between the phages themselves. Sabp-P1, Sabp-P2 and Sabp-P3 showed the highest increase in phage titer between pH 6 to 7. Thereafter, a sharp decline in phage titre was noted

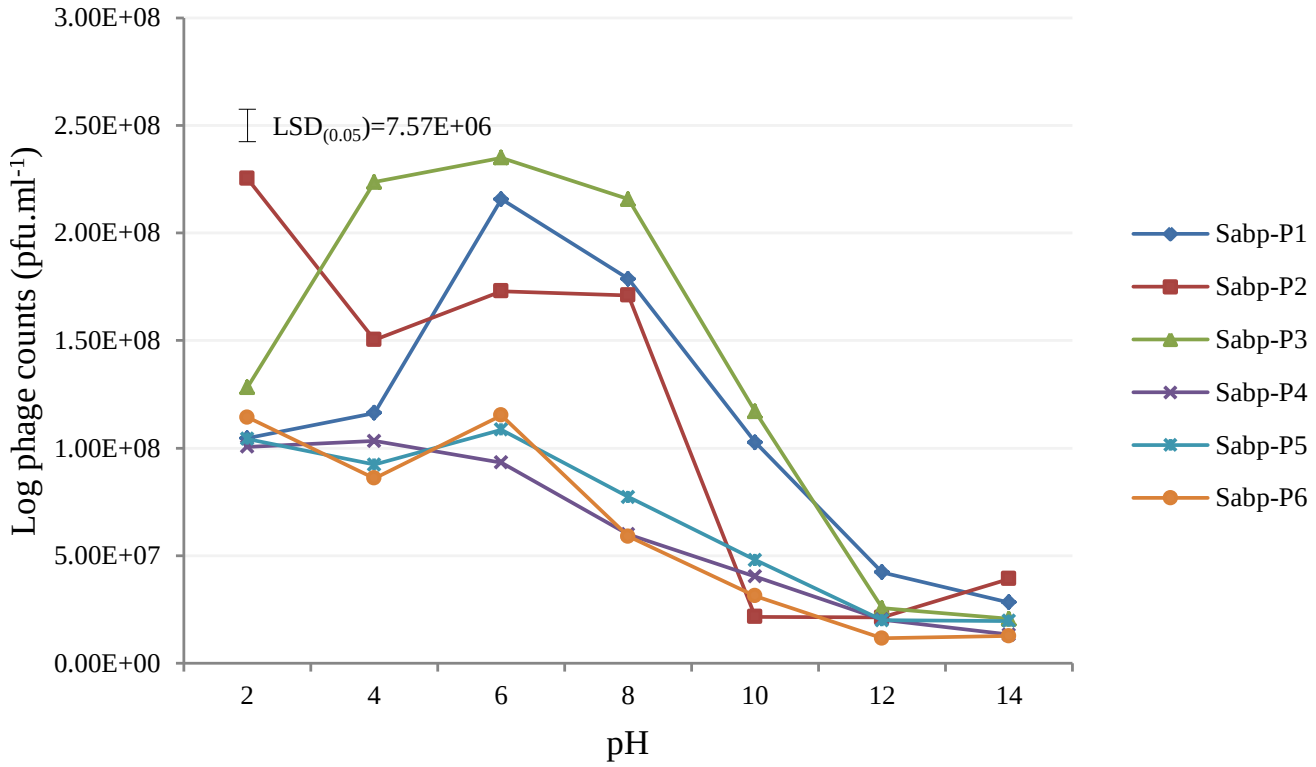

Figure 4. Effect of increasing pH on the titers of six phages. *SaB1* was used as the host bacterium for phage propagation. Data points are the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$ when comparing any phage x pH combination.

from pH 7.2 to 12. Phages, Sabp-P4, Sabp-P5 and Sabp-P6, showed a similar pattern. However, titres were significantly lower from those of Sabp-P1, Sabp-P2 and Sabp-P3. There was no significant difference in activity between Sabp-P1, Sabp-P2 and Sabp-P3, or, between activity of Sabp-P4, Sabp-P5 and Sabp-P6. However, each group differed significantly from each other.

Glycerol assay

A general trend of reduced phage activity was observed with an increasing concentration of glycerol (Figure 5). ANOVA showed significant differences ($p<0.001$) in activity between phages as glycerol concentrations increased from 5 to 100%. Phages Sabp-P2 and Sabp-P3 are stable and able to replicate up to a glycerol concentration of 25%. Thereafter, titres dropped significantly and no further increase was noted. Phages Sabp-P1, Sabp-P4, Sabp-P5 and Sabp-P6 showed a dramatic decline in titre as glycerol concentration increased from 10% upwards. This differed significantly from the activity of Sabp-P2 and Sabp-P3 (<0.001). Exposure of the selected phages to high concentrations of glycerol resulted in a dramatic decrease in titre that ranged from 52 to 94% (Table 2).

DISCUSSION

The onset of antibiotic resistance in *S. aureus* and its implications for the treatment of bovine mastitis have raised awareness of the need for alternative control therapies. Phages provide such an alternative. However, associated with this therapy are several challenges, particularly in terms of developing stable formulations and optimizing storage and *in vivo* application of phage products. The primary objective of the current study was to evaluate phage titers upon exposure to simulated stresses that might be encountered during the formulation of the phage or during *in vivo* application of the formulated phages.

Phage activity was assessed *in vitro*, in a heat assay to determine the temperature at which the selected phages are most stable, for the longest duration. *In vivo* application of phages requires resistance (or tolerance) to high temperatures. Temperature fluctuations in an *in vivo* system could possibly arise from changes in weather patterns, physiological changes in the body of the cow itself, or even changes in the storage conditions of the phage. All six phages that were screened showed reduced lytic ability from as early as 10min exposure to 45°C. This is significant, especially in terms of formulation of phages into commercial products. Certain formulation

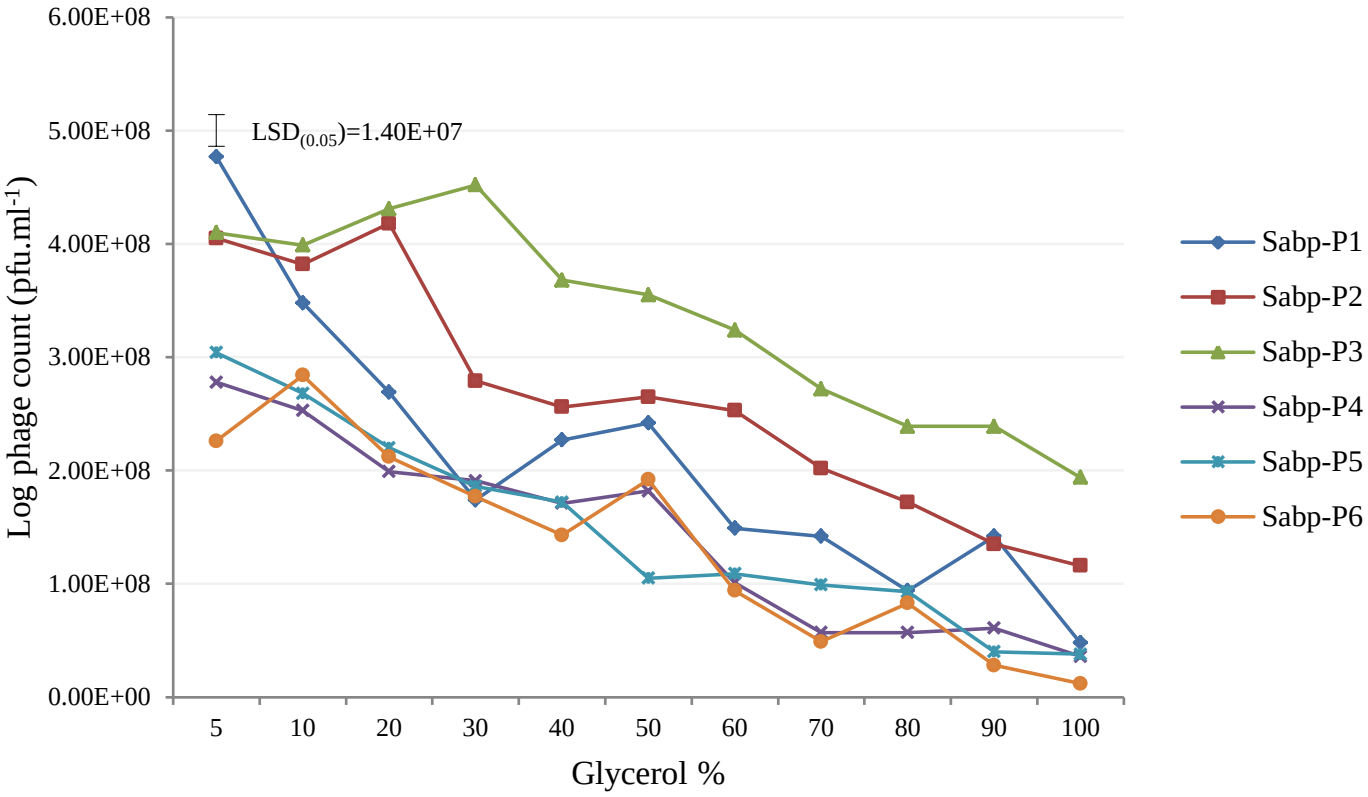

Figure 5. Effects of increasing glycerol concentration on phage titers. *SaB1* was used as the host bacterium for phage propagation. Data points are the means of three independent experiments. The vertical bar represents the LSD $_{(0.05)}$ when comparing any phage x glycerol % combination.

Table 2. Reduction in phage titers after exposure to increasing concentration ranges of glycerol.

Phage	Reduction in phage activity (%)	Range of increase in glycerol concentration that limits phage growth (%)
Sabp-P1	89.94	10 - 100
Sabp-P2	71.36	25 - 100
Sabp-P3	52.68	25 - 100
Sabp-P4	87.05	10 - 100
Sabp-P5	87.5	10 - 100
Sabp-P6	94.69	10 - 100
Mean	80.54	
LSD	7.01	
CV%	1.5	

procedures may require high temperatures during the manufacturing process. This has to be undertaken with caution in order to minimize any reduction in the replication ability of the phages. Phages are primarily composed of protein (Kutter and Sulakvelidze, 2005). Any prolonged exposure to high temperature may cause denaturation of phage proteins responsible for infection of a bacterial host. Compromised physical structure of an otherwise infective phage then reduces the control potential that that phage could have imposed on a target host. These results correlate with previous studies where phage lytic ability was shown to be reduced by prolonged exposure to high temperatures (Da Silva and Janes, 2005; Bryant et al., 2007; Chandra et al., 2011).

The selected phages also showed reduced lytic ability after exposure to temperatures below 4°C. This reduced pathogenicity could be attributed to the effect that freezing/thawing may have on phage ultrastructure. This is particularly relevant for tailed phages from the *Myoviridae* family. Delicate tail and tail fibres could become dissociated from the virus head due to changes in osmotic pressure (Jończyk et al., 2011). This sort of dissociation renders the phage ineffective as a control agent. The results of this study contradict those of Hsieh et al., (2011), where phages were found to have maintained stability even after one year of storage at -85°C. Hsieh et al., (2011) stored all phages in 7% dimethyl sulfoxide, in comparison to phage buffer (+ glycerol) in the current study. The choice of storage medium might therefore influence phage stability over time. For the purposes of an *in vivo* study, long- and short-term storage of phage products at 4°C, in a more protective medium (such as glycerol) seems the most likely way forward.

When phages are grown in large-scale liquid broth cultures, either for laboratory or *in vivo* assays, chloroform is usually added to the growing medium to kill any live bacterial cells that remained un-lysed (Sambrook et al., 1989). The isolated phages from this study showed extreme sensitivity to chloroform treatments. While phages Sabp-P1, Sabp-P2 and Sabp-P3 demonstrated significant tolerance to chloroform treatment, their lytic ability was still compromised. In light of this, an alternative method has to be employed in order to remove host cells from a culture suspension. Micro-filtration serves as one of the least-damaging methods to apply, as phage simply pass directly through the filter while un-lysed bacterial cells as well as large bacterial debris, are retained. The use of micro-filters was implemented throughout the current study.

The pH of the medium in which a phage is propagated is also an area of importance. Depending on where a phage has been isolated from, the pH of its growth medium should ideally mimic that of its natural environment in order to promote optimal phage replication. The pH of milk is between pH 6.6 to 6.7 (Blowey and Edmondson, 2010). Optimal phage replication occurred at pH 6 to 7, followed by a sharp decline at higher pH. This was also noted in a study by Da Silva and Janes (2005), where phages specific to *Vibrio* spp. (infective on oysters) were screened at various pH ranges. The Vibrio phages were most stable at that pH range which best mimicked the pH of the oyster system (pH 7 to 8).

The formulation of phage/s into a product that can be applied in an *in vivo* system requires storage of the phages in a medium that maintains phage stability, is itself stable over time, and does not damage the teat upon application. Dairy farmers implement both pre- and post-dipping disinfectants during the milking process (Blowey and Edmondson, 2010). Teat skin has relatively few sebaceous glands, and continual washing and drying of the teats can remove the limited amount of fatty acids that maintain the skin barrier (Blowey and Edmondson, 2010). This can result in severe cracking/breaking of the teat skin, leaving the teat exposed to entry of pathogens. In an attempt to maintain healthy teat skin, additives are used in post-dipping formulations. Emollients (such as lanolin) and humectants (such as glycerin) are the most commonly applied additives (Blowey and Edmondson, 2010). The current study investigates the effect of glycerol concentrations on phage activity. Phage lytic ability was optimal in a suspension of up to 15% glycerol, but dropped thereafter. Phage suspension in a 10% glycerol solution could be applied as an *in vivo* treatment for the treatment of bovine mastitis. Such an application could have a two-fold benefit: (1) the protective effect of phages against *S. aureus* on teat surfaces; (2) the moisturizing effect of glycerol on sensitive teat skin. It is important to note that phages have not been shown to have any negative effects on either the actual health of the cow (O'Flaherty et al., 2005b; Gill et al., 2006a) or the quality of milk produced (Hudson et al., 2005; García et al., 2008).

Conclusion

This study shows the optimum storage and growth conditions necessary in order to maintain high tire phage cultures. Slight deviations from the standard growth requirements (37°C for optimal growth; storage at 4°C, culture medium pH of 6 to 7) for the selected phages resulted in dramatic reductions in phage activity. Phages Sabp-P1, Sabp-P2 and Sabp-P3 showed more vigour and were more robust than Sabp-P4, Sabp-P5 or Sabp-P6. Sabp-P1, Sabp-P2 and Sabp-P3 would be preferred candidates when investigating *in vivo* applications of the phages as a control treatment against staphylococcal bovine mastitis.

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