Mini-Review: Biological control of bovine mastitis using bacteriophage therapy

I.H. Basdew and M.D. Laing
Discipline of Plant Pathology, School of Agricultural Sciences and Agribusiness, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, Republic of South Africa

Bacteriophage therapy has been exploited for the control of bacterial diseases in fauna, flora and humans. However, the advent of antibiotic development lead to a cessation of most phage research. Recently, the problem of antibiotic resistance has rendered many commonly used antibiotics ineffective, thereby renewing interest in phage therapy as an alternative source of control. This is particularly relevant in the case of bovine mastitis, an inflammatory disease of bovine mammary glands, caused by *Staphylococcus aureus*. Antibiotic resistance (primarily towards penicillin and methicillin) by staphylococcal strains causing mastitis is regularly reported. Phage therapy can provide a sustainable, organic means of mastitis control with little to no deleterious effect on the surrounding environment or the affected animal itself. Several studies have delved into the field of biocontrol of bovine mastitis using phages. Results are variable. However, some phage-based products have been commercialised and are available on the market. The unequivocal fact that emerges from research findings so far is that phages may be the next avenue of control for bovine mastitis and other problematic bacterial diseases.

Keywords bovine mastitis; bacteriophage therapy; antibiotic resistance; *Staphylococcus aureus*

1. Brief background

Bovine mastitis is an infectious inflammation or irritation of the mammary glands that interferes with the normal flow and quality of milk [1]. Although a range of control measures are used, this disease remains a major cause of economic losses in the dairy industry worldwide. While some diseases, such as foot-and-mouth, can be completely eliminated from a system using culling and vaccination protocols, bovine mastitis is an endemic disease that cannot be completely eradicated. The wide range of microorganisms that can cause this disease, and the ubiquity of these organisms, make complete eradication unlikely. Optimum control therefore lies in first understanding the epidemiology of the disease and the causal agents and then implementing an integrated control strategy.

In recent years, the use of integrated control measures has become paramount, due to the onset of resistance against the antibiotics commonly used to treat the disease. This is particularly relevant to the primary causal bacterium of bovine mastitis, *Staphylococcus aureus*. Numerous strains have developed antibiotic resistance. Resistance was noted to have first developed against penicillin (both β-lactam and non-β-lactam), after which methicillin became the treatment of choice [2]. However, the development of resistance to methicillin shifted treatment to the use of vancomycin, and, as anticipated, some strains of *S. aureus* have now developed resistance towards vancomycin. The financial implications of such resistance within a herd of infected cows can be devastating. Losses can exceed 200USD per cow because culling of infected animals serves as the final control measure [3]. As a result, interest has shifted from the more conventional antibiotic therapies towards the field of biological control of the disease.

One such biocontrol option involves the use of lytic bacterial viruses (bacteriophages) that are specific to *S. aureus*. Phage therapy has been applied to different disciplines, ranging from human and veterinary medicine to agricultural settings. There have been increased applications of phages in the food industry to control major food pathogens such as *Listeria monocytogenes*, *Salmonella*, *Campylobacter* and pathogenic *Escherichia coli* [4]; in human medicine, to treat burns, contaminated wounds, diarrheal diseases [5] and for use against methicillin-resistant *Staphylococcus aureus* (MRSA) [6]. In the United States, in the past two years alone, several phage applications have been approved for use. The most prominent include Listex (an organic anti-*Listeria* phage) [4], an anti-*Escherichia coli* wash, and, an anti-*Salmonella* wash for the treatment of live animals prior to slaughter [7]. In addition to phage therapy, phage use can be extended to the field of phage display, vaccine delivery and pathogen detection. In addition, there has been significant progress, using animal models, in the field of control of *S. aureus* using phages. However, no single phage-based product has yet been commercialized for use against bovine mastitis, despite the clear need for a more sustainable control option to manage the disease.

Bacteriophages are specific for their target bacterium and hence create no negative effects on the surrounding mammary tissues or the environment. This, coupled with their ability to multiply up to 1000-fold within a host cell, makes the phage an ideal candidate for the biocontrol of bovine mastitis [1]. This review covers current control measures used against bovine mastitis and evaluates the use of bacteriophages as an alternative therapy to antibiotics.
2. Mastitis and its control through the ages

Due to the more than 137 recorded organisms that are able to cause mastitis [8] and the varying levels of infection associated with each, there exists a complete lexicon to describe the disease and classify it according to specific organisms. A simple classification method recognizes two distinct forms of the disease, contagious and environmental. With contagious diseases, the mammary glands and teat skin serve as the primary reservoirs of infections with colonies establishing at the teat end and slowly growing through the teat canal over 1-3 days [1]. Among the contagious organisms, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Streptococcus dysgalactiae* have been identified as the major causes of bovine mastitis [1]. Contagious mastitis can be further classified into three major groups based on the symptoms associated with infection: clinical, sub-clinical and chronic mastitis [9]. Environmental mastitis differs in that it is caused by organisms that do not normally live on the surface of the skin or in the udder, but which enter the teat canal when the cow comes into contact with a contaminated environment [9]. These pathogens can normally be found in faeces, bedding material, farmyards and feed and are transferred from the reservoir to the teats between milking [1]. Among the environmental organisms, coliforms (especially *Escherichia coli*) and *Streptococcus uberis* have been identified as the major problematic microbes [1, 9].

The effects of mastitis on milk yield and composition are of economic importance to the dairy farmer. Direct costs can be incurred through discarded milk and drug and veterinary costs while indirect costs can be incurred through penalties as a result of increased somatic cell counts (SCC), decreased milk yield due to udder damage, additional labour requirements for treating infected cows and higher culling and replacement costs [1, 10]. In the United States alone, losses are estimated to approach 2 billion USD and worldwide, it is associated with economic losses of up to 35 billion USD annually [9, 10, 11]. On average, a quarter of an udder infected with a major pathogen will yield approximately 30% less milk than an equivalent uninfected quarter of the same cow [9]. Infected cows are also culled more quickly either because of repeated clinical infections or reduced milk yields. Mastitis has a major effect on the taste and quality of milk, mainly as a result of its effects on the lactose, protein and fat content of milk; typically affected milk is rancid and slightly salty/bitter to the taste [11]. As a result of diminished milk quality, overall profitability is reduced as the farmer receives a lower price (up to 20% less per litre) for such milk [11].

It is of utmost importance when dealing with mastitis, that one first has a grasp of the disease cycle prior to the implementation of any control measures. This understanding is in the best interest of both the farmer and the animal as it will ensure that only the most appropriate control options are applied for specific phases of the disease. Essentially, mastitis occurs in the following sequence: i) arrival of a reservoir of infection (in the form of an infected cow for contagious infections, and, milking facilities, farmyards, housing sheds for environmental infections), ii) transfer of infection from the reservoir to the teat end of the animal usually during, and up to 1 hour after milking (as teat sphincter remains dilated for such time), iii) penetration of the teat canal by the microbe, and, iv) colonisation of mammary tissues by pathogenic bacteria [1]. It must be noted that although the environment serves as one of the primary reservoirs of infection, control of environmental mastitis can be achieved far more successfully than control of the contagious form of the disease by implementing proper environmental hygiene, predipping teats and applying dry period teat sealants [1, 9].

Much of the information required to reduce mastitis incidence has been available for more than three decades. Under optimal conditions, the natural defence mechanisms (self-cure), in conjunction with cultural control measures such as clean bedding, proper milking sanitation and vigilant inspection of lactating cows, may prove effective 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 included 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 cell count and hence to saleable milk [12, 13]. Treatments can be administered both during lactation (lactation cow therapy) and the period during which the cow is dried off (dry cow therapy), via intramammary or parenteral means [1]. However, despite much success with antibiotics, it remains debatable as to whether this therapy is indeed worthwhile. There are several conflicting views on this [1-3, 12, 14-15], many of which revolve around *S. aureus* and its ability to develop antibiotic resistance. Of the large number of organisms that are able to cause bovine mastitis, *S. aureus* is the etiological agent most commonly associated with the disease (clinical, subclinical and chronic forms) [16]. However, the cure rate of antibiotic treatments against this pathogen is low and, therefore, the disease cannot be effectively eliminated and/or controlled in infected herds by using antibiotics [14, 17-19].

3. Antibiotic resistance and *Staphylococcus aureus*

Antibiotic resistance can be attributed to several factors. *Staphylococcus aureus* forms abscesses within the udder that are surrounded by a thick fibrous capsule [20, 21]. This could prevent sufficient concentrations of antibiotic from entering the abscess itself and hence effective destruction of viable bacteria. Some strains of *S. aureus* can live within cells such as macrophages. Most antibiotics are only able to circulate in the body fluids surrounding cells and cannot penetrate within cells themselves, hence these staphylococci are protected from the majority of antibiotics [21, 22].
Many strains of *S. aureus* produce beta-lactamase hence rendering them resistant to certain formulations of penicillin, in addition to other antibiotics that have been deemed more effective (such as certain aminoglycosides, cephalosporins and tetracyclines) [1, 22, 23]. Some strains of *S. aureus* can persist in a state of bacterial dormancy with a mucoid capsule and completely cease all replication [1, 23]. In this state, they are not killed by antibiotics and are able to reactivate once favorable conditions prevail.

The spread of virulent methicillin-resistant *S. aureus* (MRSA) [2, 12, 14, 19, 24], coupled with the development of resistance to two new antibiotics (daptomycin and linezolid) recently approved for clinical use against Gram-positive bacteria [25], has shown that *S. aureus* is indeed a formidable pathogen. Antibiotic resistance is however, not exclusive to *S. aureus*. In the past three decades, we have witnessed a rise in bacteria carrying extended spectrum β-lactamases, which are mutants of enzymes that previously could only inactivate penicillins but now have gained activity against many cephalosporins too [26]; plasmid-mediated (and hence horizontally transmitted) resistance to fluoroquinolone antibiotics [27]; the rise of multi-drug resistant *Neisseria gonorrhoeae* [28]; the emergence and global dissemination of multi-drug resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Enterobacteriaceae* [26, 29]; and, the spread of extensively drug-resistant *Mycobacterium tuberculosis* [26]. This being said however, the development of new antibiotics is still ongoing. Tigecycline, a third-generation, semi-synthetic tetracycline antibiotic approved in 2005, has been shown to have activity against MRSA when used in conjunction with rifampin [30]. In addition, ceftobiprole, a fifth-generation cephalosporin, has shown in clinical trials, to provide significant control over MRSA [31]. The core problem is that the rate of development of new antibiotics is unable to keep up with the pace of microbial evolution and hence resistance easily develops against common antibiotics. As a result, the latest antibiotics being used are themselves the source of the evolutionary pressure that eventually renders them obsolete.

### 4. Alternative therapies for control of bovine mastitis

Due to these treatment limitations, research focus into the control of bovine mastitis has shifted to alternative therapies such as the development of vaccines [16, 22, 18] and biological control options such as the use of botanical extracts [32, 33]. Vaccines for *S. aureus* have produced varied levels of success. This has been a result of the type of vaccine used, adjuvants and other factors such as age of the cow and environmental conditions [16, 34]. A recent study has shown that a commercial bacterin (vaccine comprising killed bacteria) did not result in effective protection from new infections of *S. aureus* [35]. Better results have been obtained with a toxoid produced from three bacterial strains with different haemolysis patterns added to the bacterin which resulted in greater than 50% protection in the experimental challenge [16, 36]. Besides the conventional bacterins, new technologies are currently being used in the development of *S. aureus* vaccines. DNA and recombinant protein vaccines have been tested for the main factors of *S. aureus* virulence that are related to infections in the mammary gland [37]. A DNA vaccine with a booster dose of recombinant protein has proven to significantly increase humoral and cellular immune response and has achieved promising results in protecting mammary glands from new *S. aureus* infections [38]. However, due to the wide variety of vaccines available against *S. aureus* and the variable clinical results associated with each, there remains no general consensus on which immunotherapeutic protocol is the most efficient.

### 5. Phage therapy and bovine mastitis

There remains however, a biological resource for the control of *S. aureus* that has not been thoroughly tapped into. This involves the use of bacterial viruses (bacteriophages) for the biological control of *S. aureus*. Bacteriophages are ubiquitous in the environment - from the oceans, soil, deep sea vents, hot-springs, the water we consume and the food we eat [39, 40]. It is little wonder then that the discovery of these organisms dates back to the early part of the twentieth century [41]. The discoveries by Twort and d’Herelle allowed for phage research to develop, such that phage therapy was applied to different disciplines ranging from human and veterinary medicine to agricultural settings [42], and was initially used to successfully treat a variety of diseases ranging from dysentery, typhoid and paratyphoid fevers, cholera and pyogenic urinary tract infections [4]. A further facet of phage technology is that appropriate phages can control plant bacterial pathogens, which antibiotics never have. Circumstances in which phage therapy of plants or plant products has been attempted include against *Salmonella* associated with fresh-cut fruit [43], to disinfect *Streptomyces scabies*-infected potato seed tubers [44], against bacterial spot of mungbeans [45], against *Xanthomonas pruni* associated with bacterial soft rot of peaches [46], against *X. campestris* infections on peach trees, cabbage and pepper diseases [46], to control *Ralstonia solanacearum* (causal organism of bacterial wilt) [47] and to control soft rot and fire blight associated with *Erwinia* spp. [48]. These findings and more have all supported the continual development of phage therapeutics (Table 1).
Phages have been found to play a fundamental role in the evolution of their host. Whole genome sequencing of bacteria has shown that phage elements contribute significantly to sequence diversity and can potentially influence pathogenicity [49]. Phages can display one of two types of life cycle: lytic or temperate. Lytic phages bring about rapid lysis and death of the host bacterium whereas the temperate form spends part of its life cycle in a quiescent state (prophage) [49]. In this lysogenic cycle, viral DNA is integrated into host cell DNA. Prophage DNA will then be replicated when the host cell genome replicates and hence daughter cells will inherit the viral DNA [50]. This temperate form however, has very little value in terms of phage therapy applications. During a typical lytic cycle, the virus attaches to the host cell via specific receptor sites that may be one of a variety of cell surface components including protein, oligosaccharide, teichoic acid, peptidoglycan and lipopolysaccharide [49]. Phage genetic material is then transferred into the host cell and usually occurs via contraction of the virus tail and formation of a hole within the bacterial cell wall [49, 50]. The viral genome is then transcribed by host cell RNA polymerase, producing early mRNA that has the effect of taking over the metabolic machinery of the bacterium [49]. In so doing, the metabolic processes within the host cell are redirected towards the manufacture of new viral components which are then assembled into new virions [49]. Following construction and assembly of the new virions, there remains the matter of release from the host cell. Nearly all dsDNA phages have developed enzymes that attack bacterial peptidoglycans [49, 50]. These muralytic enzymes (or endolysins) are produced within the cytoplasm and require another enzyme to enable them to cross the cytoplasmic membrane to reach their substrate. This enzyme is a holin that disrupts the membrane, allowing the lysin to degrade the peptidoglycan [50]. In this way, the holin controls the timing of cell lysis and the release of viral progeny.

This intricate network of events was not well understood during the early period of phage discovery. This lack of in-depth knowledge, coupled with the advent of antibiotics, lead to the early demise of phage therapy. However, the surge in antibiotic resistance and the wide pool of information now available on phage biology has proven pivotal in re-establishment of interest in phage therapy. It is easy to see why there is an increased trend towards phages as therapeutic agents because they offer several advantages over the use of antibiotics. Phages target only the pathogens of interest and they have also shown effectiveness against bacteria exhibiting resistance towards multiple antibiotics [49]. As a result, even if they are not used as the first line of defence, phages represent a very useful last line of defence [49]. The effects of phage therapy are all localised at the infection site whereas antibiotics do not necessarily concentrate at the initial site of infection [51]. The pharmokinetics of bacteriophage therapy is such that the initial dose increases exponentially as the virus multiplies within the susceptible bacterial host and is subsequently released [49]. Often all that is required thereafter is a ‘booster’ dose to ensure elimination of any infection. In addition, there is evidence that phages are able to penetrate poorly vascularised tissues and can even cross the blood-brain barrier [52]. Extensive clinical experience in the former Soviet Union and Eastern Europe has revealed very few cases of side-effects or allergic reactions [49, 53]. It is less certain however, what immunological effects might arise upon multiple dosages [49, 53]. In spite of these factors however, it is just part of the evolutionary process for target bacteria, over time, to develop resistance to their respective phages. However, by virtue of their abundance in the environment, it is much simpler to find and develop new bacteriophages than to produce new antibiotics or vaccines. As a result, production costs of phages are significantly lower than those associated with antibiotic manufacture [51].

Considering the wealth of information at our disposal, phage therapy appears to be one of the most sustainable measures for control of bovine mastitis. It is essential when looking at the treatment of bovine mastitis with phages that one has a full grasp of the disease cycle and where phages occur within this cycle (Fig. 1). A limited number of studies have identified various phages with lytic capabilities towards *S. aureus* [54, 55, 56, 57, 58]. In studies by Gill et al. [54],

<table>
<thead>
<tr>
<th>Product</th>
<th>Target organism</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgriPhage™</td>
<td><em>Xanthomonas campestris</em> pv. <em>vesicatoria</em> or <em>Pseudomonas syringae</em> pv. <em>Tomato</em></td>
<td>Omnilytics</td>
</tr>
<tr>
<td>BioTector</td>
<td><em>Salmonella</em> spp. in poultry</td>
<td>CheIlIdang Corporation</td>
</tr>
<tr>
<td>EcoShield™</td>
<td><em>Escherichia coli</em> in foods and food processing facilities</td>
<td>Intralytx</td>
</tr>
<tr>
<td>FASTPlaque-Response™</td>
<td>Detection of rifampicin resistance in <em>Mycobacterium tuberculosis</em></td>
<td>Biotech Laboratories</td>
</tr>
<tr>
<td>FASTPlaqueTB™</td>
<td>Detection of <em>M. tuberculosis</em></td>
<td>Biotech Laboratories</td>
</tr>
<tr>
<td>ListShield™</td>
<td><em>Listeria monocytogenes</em> in foods and food processing facilities</td>
<td>Intralytx</td>
</tr>
<tr>
<td>LISTEX™ P100</td>
<td>Targets <em>L. monocytogenes</em> strains on food products</td>
<td>EBI Food Safety</td>
</tr>
<tr>
<td>MRSA/MSSA blood culture test</td>
<td>Detects <em>Staphylococcus aureus</em> methicillin resistance/susceptibility</td>
<td>Microphage</td>
</tr>
<tr>
<td>MRSA screening test</td>
<td>MRSA</td>
<td>Microphage</td>
</tr>
<tr>
<td>MicroPhage</td>
<td>Differentiation of methicillin resistant (MRSA) and methicillin-susceptible (MSSA) <em>S. aureus</em></td>
<td>Microphage</td>
</tr>
</tbody>
</table>

Table 1 Commercial bacteriophage-based products and companies, adapted from Monk [49]
the effectiveness of phage therapy was evaluated for the control of mastitis during lactation. The study was designed to treat already established intramammary infections in cows housed in a commercial production environment. Infected cows were treated with a 5-day course of intramammary infusions with either the lytic phage (called bacteriophage K) or a saline placebo. The effects of phage infusion into healthy quarters were also examined in order to assess the pharmacokinetics of infused bacteriophages and its impact on milk quality. Results showed that phage infusion was able to elicit a heightened immune response as exhibited by an increase in the SCC of treated udders and that a cure rate of 16.7% was achieved. In addition, phages were detectable in milk for up to 36hrs post-infusion but at significantly lower rates indicating that degradation or inactivation of the infused phage occurred within the gland. While this study did prove that phage was able to control S. aureus in infected udders, several limiting factors were also detected. Phage inactivation in the udder could have taken place as a result of the presence of milk proteins and fats. It has been found that whey proteins attach to S. aureus cell surfaces which inhibit phage binding [59, 60]. In addition, S. aureus has been shown to aggregate when it is grown in milk or milk whey, which could also confer some protection against phage attack [55]. However, another study by O’Flaherty et al. [60] showed that an infusion of a cocktail of three phages at $10^8$ PFU ml$^{-1}$ into live cow teats resulted in no detectable increase in SCC indicating that there was no localised immune response to high numbers of phage. This is in stark contrast to the study by Gill et al. [54] where inflammation of the udder as a result of a high SCC was noted. In vivo studies by Wills et al. [61], showed increased recovery of viable phage from treated animals than was initially administered, implying that phage multiplication occurred successfully within animal tissues. Capparelli et al. [62] also tested phage against S. aureus. Findings included a 97% rescue of infected animals over a 10-day period. In addition, phage delivery into macrophages by S. aureus, was found to kill intracellular staphylococci both in vivo and in vitro. In terms of molecular studies, the genomes of several phages with specificity towards bovine mastitis have been characterised [57, 63-65].

6. Conclusions

While there are several problems associated with the control of bovine mastitis using phage therapy, this is probably a result of the shortage of more in-depth research. Further studies into the detailed effects of whey proteins on bacterial activity and aggregation, proper administration of phage cocktails into animal tissues and the development of phage formulations to facilitate optimal delivery and activity within intramammary tissues, are ongoing. Further studies also need to be conducted on the pharmacokinetics and pharmacodynamics of these organisms in order to properly understand lytic events and exploit them to full potential. While some are of the opinion that phage therapy for bovine mastitis has limited potential [66], the advances made thus far and the scale of antibiotic resistance, has re-opened the doorway for phage therapy.

Fig. 1 Disease cycle of bovine mastitis indicating bacteriophage occurrence.

Acknowledgements The authors would like toPlant Health Products (Pty) Ltd for providing financial support for the project.
A. Méndez-Vilas (Ed.)

Science against microbial pathogens: communicating current research and technological advances

392


