Research Journal of Chemical and Environmental Sciences
Res J. Chem. Environ. Sci. Vol 2 [6] December 2014: 06-10
Online ISSN 2321-1040
CODEN: RJCEA2 [USA]

Website: www.aelsindia.com/rjces.htm

RJCES

REVIEW ARTICLE

Isolation of Bacteriophages Specific to *Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa*

Desalegn Amenu
College of Natural and Computational Science, Wollega University, P.Box, 395, Nekemte, Ethiopia
Email: wadadesalegn@gmail.com

ABSTRACT

Bacteriophages were first discovered in 1915 by bacteriologist Frederick Twort, and a second time independently in 1917 by the microbiologist Felix d'Herelle. This review examine various aspects of bacteriophage ecology, particularly focusing on phages specific to the bacterial species Staphylococcus aureus, Pseudomonas aeruginosa, and Escherichia coli. In addition to its epidemiological threat, S. aureus-associated mastitis incurs an economic cost on the dairy industry as the bacteria itself infects the udder and adversely affects milk production by reducing yield or contaminating the supply with dangerous enterotoxins. Antimicrobial-resistant strains of P. aeruginosa are also highly common, with the reported emergence of strains resistant to antibacterial agents such imipenem, fluoroquinolones and cephalosporins, as well as multidrug-resistant variants. The need to understand the microbial ecology of bacteriophages is an inevitable prerequisite to the successful isolation and acquisition of phages specific against the target bacteria.

Received 12.08.2014 Accepted 21.09.2014

INTRODUCTION

Bacteriophages refer to any virus that infects bacteria as host organisms. Recently, the medical community has become very interested in bacteriophages for therapeutic purposes. The field of phage therapy – bacteriophages as antibacterial agents has advanced considerably including a recent surge of interest in Western medicine as an alternative to antibiotics. The relative ease by which phages can be isolated and produced compared to antimicrobial or antibiotic agents and the increasing resistance of bacterial pathogens to traditional antibiotics both serve as important reasons for the advancement of phage therapy [1, 2, 3]. With the increasing utilization of bacteriophages in phage therapy, an understanding of the ecology of phages would be expected to contribute to the field of bacteriophage therapy by revealing the range of habitats occupied by phages medically useful for treating bacterial diseases of humans. Additionally, bacteriophages represent a more economical and environment-friendly alternative to the environmentally-damaging use of chemical bacteriocides in agricultural industries [1]. Finally, phages are also employed in water treatment techniques as highly effective indicator organisms for the presence of bacteria, being commonly useful when analyzing for possible contamination of potential reservoirs for pathogenic bacteria.

This review examine various aspects of bacteriophage ecology, particularly focusing on phages specific to the bacterial species Staphylococcus aureus, Pseudomonas aeruginosa, and *Escherichia coli*. Of these three bacteria, *S. aureus* and *E. coli* are known to have virulent forms that are responsible for animal-to-human transmissions, either directly via contact with human tenders or through their produced foodstuffs [4]. *S. aureus* and *P. aeruginosa* are known to cause mastitis in dairy cattle as well [5]. Mastitis is an infection of the mammary glands that can result in decreased milk yields, or may contaminate milk supplies with bacterial enterotoxins [6, 7].

As previously mentioned, *S. aureus* has been identified as a common causative microorganism of mastitis in cattle [5, 3]. Epidemiologically, S. aureus are important as common colonizers and infectious agents of humans [8]. Infected individuals may be at risk for surface skin and soft tissue infections, endocarditis, osteomyelitis, meningitis, bacteremia, and pneumonia [8]. Morphologically, *S. aureus* are gram-positive cocci shaped bacteria; Chambers [9] have noted similarities in the recent appearance of methicillin-resistant S. aureus (MRSA) with the spread of penicillin resistant strains during the 1940s and 1950s. Notably, the development of both types of antibiotic resistant S. aureus were strongly correlated with clinical settings, attributable to the prevalence of antibiotic use in such environments and that humans

are a natural reservoir of *S. aureus*. However, the same author [9] notes that MRSA infections are not exclusive to clinical settings, and such resistant strains have also been acquired from other sources as well.

Research on MRSA has revealed evidence that cross-species infections of MRSA from livestock, such as pigs and cattle, to their human tenders are highly common, and a significant portion of MRSA diagnosed in human populations may have originated from non human animal environments [4]. In addition to its epidemiological threat, S. aureus-associated mastitis incurs an economic cost on the dairy industry as the bacteria itself infects the udder and adversely affects milk production by reducing yield or contaminating the supply with dangerous enterotoxins [6,7].

To date, a strong focus that exists is to isolate and identify *S. aureus* from dairy cattle as the bacteria commonly contaminate milk and dairy-based products [10, 5, 3]. In June 2000, over 10,000 cases of food poisoning were reported in Osaka, Japan. The cause of this was a malfunction in the temperature control systems of a plant that produced skimmed milk powder, allowing the proliferation of S. aureus. While the bacteria were killed by pasteurization, heat-resistant staphylococcal enterotoxins remained in the reconstituted milk which eventually reached the consumers [6].

Milk from dairy cattle appears to be the most common source of *S. aureus* and MRSA strains. In non-dairy livestock, successful isolation of *S. aureus* has been reported in beef cattle, chicken and pigs, while MRSA were reported in chickens only [11]. Between May 2001 and April 2003, Lee [11] successfully isolated *S. aureus* from out of 1,913 feces, milk, feed material, joint, trachea, uterus, and meat samples collected from 15 slaughterhouses, seven meat processing facilities, 58 feedlots, and 11 food stores located throughout South Korea. According to this study, *S. aureus* were successfully isolated from dairy and beef cattle, chickens and pigs. MRSA strains were identified via the presence of the mecA gene, testing positive in 12 dairy cattle and three chicken samples. No MRSA strains were detected in the beef cattle or the pigs.

In the investigation conducted by Lee [11], 10 g of feces, feed and meat, or 10 ml of milk, or swabs of joint, trachea and uterus were inoculated in 100 ml of staphylococcus broth or Trypticase soy broth and incubated at 35°C for 20 h. The inocula were transferred to Baird-Parker agar and incubated again at 35°C for 24 to 48 h. S. aureus bacteria were identified using biochemical methods such as Gram staining, colonial morphology, coagulase and urease assays, and the API Staph Ident system [11]. Bacteriophages ΦH5 and ΦA72, specific to *S. aureus*, have been successfully isolated from raw or bulk tank milk from dairy farms [10, 5]. Literature has revealed that the phages may also be found in processed dairy products such as cheese. In 2009, García et al. [5], identified eight different phages, isolated from milk and cheese samples, each collected from 72 different dairy farms and 3 cheese factories in northern Spain. All 75 samples screened positive for phage presence, achieved by culturing onto mastitis milk-derived strains of S. aureus and incubated overnight at 37°C with agitation. Samples were subjected to centrifugation at 13,000 × g for 5 min followed by 5 filtration. The presence of phages was then accessed via plaque assays against various strains of *S. aureus*. All phages isolated in this study were temperate in nature. To demonstrate the temperate nature of the phages, García et al. [5] isolated bacterial colonies, suspected to be resistant to infection, from lysis plaques. These isolated colonies were then exposed to mitomycin C to induce prophage release.

In addition to milk samples, other possible sources of bacteriophages specific to *S. aureus* may include sewage effluent, soil, and straw from cowsheds. In 2011, S. aureus phages were isolated from wastewater collected from a dairy farm in China. Three-step enrichment process using S. aureus grown in Luria-Bertani liquid medium yielded a lytic, tailed phage with morphology matching that of the order Caudovirales. The presence of such phages was confirmed by plaque assay.

Two novel bacteriophages specific to S. aureus were reported to be isolated from sewage, soil and straw samples collected from cowsheds. Phages were screened by culturing onto bacterial hosts, concentrated by centrifugation at 8,000 rpm for 20 min and filtered using 0.45-μm filters, and finally confirmed via plaque assay using S. aureus as host. It is likely that the phages originated from cows, which contaminated the soil, straw and other surfaces through defecation. These phages may eventually be washed into sewage holding tanks.

Most recently Kwiatek et al, (2012) isolated bacteriophages lytic against *S. aureus* from milk via standard enrichment using a mixed culture of three randomly selected bacterial strains; *S. aureus* American Type Culture Collection (ATCC) 43300, ATCC 25923, and *S. aureus* MRSA. Phage lysis was forcibly induced using chloroform, and centrifuged. Phage presence was detected by applying supernatant to bacterial lawn cultures. After a 24 h 6 incubation period, lytic zones were extracted, filtered and subjected to successive single plaque isolations to produce pure phage cultures.

Pseudomonas aeruginosa and associated bacteriophages. *P. aeruginosa* is an aerobic gram negative, rod-shaped bacillus bacterium [12]. Similar to *S. aureus*, *P. aeruginosa* are also identified to be

epidemiologically important human pathogens responsible for pneumonias, urinary tract infections (UTIs), and bloodstream infections, and surface skin infections [12]. Antimicrobial-resistant strains of *P. aeruginosa* are also highly common, with the reported emergence of strains resistant to antibacterial agents such imipenem, fluoroquinolones and cephalosporins, as well as multidrug-resistant variants [12]. Additionally, *P. aeruginosa* have also been identified as causative microorganisms for mastitis in cattle [13].

Compared to *S. aureus*, research literatures on *P. aeruginosa*-caused mastitis infections appear to be much less common. This thesis represented an opportunity to expand on the understanding of the ecology of *P. aeruginosa* and associated bacteriophages in an animal environment. In 1979, a herd of dairy cattle was involved in a mastitis outbreak. During an investigation, *P. aeruginosa* was isolated from milk and udder tissue. It was suspected that bacterial infection was spread via a contamination that was spread during the intravenous administration of antibiotics [13]. One study performed genomic analysis of dairy-based bacteriophages within the family Siphoviridae (phages with long, non-contractile tails) in the order Caudovirales and identified Staphylococcus phage PVL and Pseudomonas phage D3 as dairy phages. The two 7 phages were identified to be specific to the bacterial species *S. aureus* and *P. aeruginosa* respectively.

In addition to *S. aureus*, it is possible to isolate *P. aeruginosa* phages from cow feces and secretions from cows, such as uterine discharges. According to Santos et al, [14], *P. aeruginosa* bacteriophages P2S2 and P5U5 have been isolated from manure and uterine secretions of dairy cows. Samples were centrifuged at 3000 × g for 25 min at 4°C, and then ultrafiltered using membranes with 0.22 μm pore sizes. *Escherichia coli* and associated bacteriophages. In addition, this study will also attempt to isolate E. coli and associated bacteriophages from non-dairy livestock using the same samples. This would serve as an expected positive control to which the results of *S. aureus* and *P. aeruginosa* could be compared, as *E. coli* and *E. coli*-specific phages are found in beef cattle, sheep and goats [15, 16]. *E. coli* are gram-negative, facultative anaerobic bacilli that are generally associated with digestive microflora. However, Shiga toxin-producing *E. coli* (STEC) variants are associated with a spectrum of illnesses including diarrhea, hemolytic uremic syndrome, and acute renal failure (Valcour et al., 2002). Unlike the mastitis-causing *S. aureus*, which constantly threatens the dairy industry via the contamination of milk, the *E.coli* O157:H7 strain affects the beef industry by transmitting to meat products destined for human consumption [16], Cattle have been identified as a natural reservoir for STEC [15, 16].No association could be found between human infections, and sheep and goat density. This was suspected to be due to sheep and goat densities being too low. However, other research has established that sheep and goats are important reservoirs for STEC as well. A study in 1998 by Calci et al. [17] compared the prevalence of bacteriophages lytic to *E. coli* in fecal samples from different animal species. The study reported positive phage isolation in beef cattle, sheep and goats, although it was noted that sheep and goats had the lowest mean density of bacteriophage, based on the number of plaque-forming units per gram of feces.

In 2007, Oot et al. [16] isolated E. coli O157:H7 and associated bacteriophages from fecal samples collected from cattle at a commercial feedlot. One gram of fecal samples was homogenized in 9 ml of phosphate buffer solution (PBS). Aliquots of 300 μl of supernatant were inoculated with 1.2 to 1.5 ml of log phase E. coli O157:H7 culture and incubated overnight at 37°C with agitation. The enrichment culture was centrifuged at 3000 × g for 15 min, and the collected supernatant was then spot tested for phage presence. E. coli O157 bacteria screening was achieved by enrichment and isolated via O157-selective CHROMagar growth media. Serotypes were then determined by Enzyme Linked Immunosorbent Assay (ELISA) tests. E. coli-specific bacteriophages were isolated via enrichment in ~97% of fecal samples but the bacteria were only isolated in ~27% of the samples. This led to the conclusion that a negative correlation between bacteria and phage population which Oot et al. [16] suggested was caused by resident phages preventing colonization of ruminant guts by *E. coli* O157:H7.

More recently in 2009, Niu et al. [15] had successfully isolated bacteriophages specific to *E. coli* O157:H7 from cattle at another commercial feedlot. Similar to Oot et al. [16], Niu et al. [15] attempted to observe correlations between bacteria and phage prevalence while including various temporal and environmental variations. Sources of phages were drinking water from water troughs, dry fecal pats from pen floors, fecal samples from cattle rectum, and fecal slurry from pen floors. Fecal slurry was only available when poor drainage conditions persist. Fecal samples were homogenized in diluent, and filtrate samples were centrifuged at 11,000 × g for 10 min, and filtered through a 0.22-μm syringe filter. Aliquots of filtrate from pats and rectal feces, or centrifuged slurry filtrate, or concentrated trough water were screened for phages. Phage screening was achieved by inoculating 450 μl of processed samples with 50 μl of early log phase E. coli and incubated for 1 h at 37°C. Spot platting were used to observe for phage activity via the appearance or absence of viral plaques. In parallel to phage isolation, Niu et al. [15] also carried out

bacteria isolation of *E. coli*. One gram of fecal samples were enriched in 9 ml of EC broth and incubated at 37°C for 6 h. Immunomagnetic separation followed, by incubating 1 ml of the enrichment culture with 20 µl of anti-*E. coli* 0157 magnetic beads for 30 min. The magnetic beads were washed in PBS thrice and the collected bacterial suspension was then plated onto sorbitol MacConkey agar. Further differentiation was achieved by isolating non-sorbitol-fermenting colonies and testing for the presence of the O157 antigens by agglutination with an E. coli O157-selective latex kit.

Niu et al. [15] noted a significantly greater prevalence of phages in manure slurry compared to fecal pats and rectal feces, achieving successful phage isolation in 94.6%, or 35 out of 37 slurry samples compared to just 26.5% (109 out of 411) of fecal pat samples and 23.8% (76 out of 320) of rectal fecal samples. In contrast, drinking water sources yielded *E. coli*-specific phages in 21.8%, or 19 out of 87 water trough samples. Niu et al. (2009) suggest that phage prevalence is positively correlated with moisture, and shedding of phages and bacteria by cattle might also dictate the probability of successful phage and bacteria isolation.

Comparison of the results of phage and bacteria isolation in rectal fecal samples revealed that 16.9% of samples were positive for E. coli O157:H7-specific phage presence but negative for host bacteria. Only 6.9% were positive for both phage and bacteria. Lastly, the study by Niu et al. [15] demonstrated a negative correlation between E. coli O157:H7 and phage prevalence, thus suggesting the bacteriophage activity reduces the bacteria population. This stands in contrast to the statistically-upheld correlations noted in studies on the use of coliphages as indicator organisms for coliform bacteria such as E. coli [18].

In 1989, Klieve et al. (1989) achieved bacteriophage induction of ruminal bacteria from sheep using mitomycin C. This yielded long, filamentous phage-like particles (PLP) matching that of pyocin 28, a bacteriocin produced by P. aeruginosa. While this study did not analyze the nucleic acids of the filamentous particles, and therefore could not confirm the particles to be pyocin 28, Santos et al. [14] demonstrated that *P. aeruginosa*-specific phages can exist in intestinal tracts of livestock.

Bacteriophage and host ecology in livestock. Given the economic importance of mastitis in dairy industry, most related research is focused on dairy livestock. The primary sources of mastitis-associated *S. aureus* and *P. aeruginosa* bacteria are dairy livestock and their environment [4, 10]. Similarly, dairy cows and their associated environments also appear to be the primary source of bacteriophage specific to mastitis-associated *S. aureus* and *P. aeruginosa* [13]. It should be noted that dairy animals are not the only source of mastitis, as *S. aureus* has been isolated from non-dairy livestock such as beef cattle, chickens and pigs [4].

The impetus for research into bacteriophages associated with such bacteria can be attributed to the increasingly common view of their use as effective biocontrol agents in the dairy industry [1]. Overall, bacteriophages specific to E. coli, S. aureus and P. aeruginosa have been isolated from dairy cattle [10, 5, 3].

In non-dairy livestock, E. coli-specific phages are expected to be isolatable from beef cattle, sheep and goats, which this survey expects to be utilizable as a control to which the isolation of phages specific to S. aureus and P. aeruginosa can be compared [15, 16]. In contrast, P. aeruginosa-specific phages have been reported to be successfully isolated from sheep, *S. aureus*-specific phages have not been isolated from beef cattle, sheep and goats. Possible sources for isolation of bacterial species *S. aureus* and *P. aeruginosa* are milk [6, 11,7,3], and feces. In studies involving dairy livestock, milk and udders were primary sources for samples from which phages were isolated [5, 3]. Alternate sources from which phages could be isolated include sewage or wastewater, and feces [15, 14]. This literature review notes that *P. aeruginosa*-specific phages have not been reported to be isolated from drinking water yet.

The need to understand the microbial ecology of bacteriophages is an inevitable prerequisite to the successful isolation and acquisition of phages specific against the target bacteria. The relative simplicity by which phages can be isolated from the environment can be attributed to the fact that the natural environment of the bacteria of interest is likely to contain the specific phages capable of infecting and lysing the microorganism [2].

REFERENCES

1. Balogh, B., J. B. Jones, F. B. Iriarte, and M. T. Momol. 2010. Phage therapy for plant disease control. Curr. Pharm. Biotechnol. 11:48-57.
2. Gill, J. J., and P. Hyman. 2010. Phage choice, isolation, and preparation for phage therapy. Curr. Pharm. Biotechnol. 11:2-14.
3. Shi, D., Y. Hao, A. Zhang, B. Wulan, and X. Fan. 2010. Antimicrobial resistance of Staphylococcus aureus isolated from bovine mastitis in China. Transbound. Emerg. Dis. 57:221-224
4. Ferber, D. 2010. From pigs to people: the emergence of a new superbug. Science 329:1010- 111.

5. García, P., C. Madera, B. Martinez, A. Rodríguez, and J. E. Suárez. 2009. Prevalence of bacteriophages infecting Staphylococcus aureus in dairy samples and their potential as biocontrol agents. J. Dairy Sci. 92:3019-3026.
6. Ikeda, T., N. Tamate, K. Yamaguchi, and S. Makino. 2005. Quantitative analysis of Staphylococcus aureus in skimmed milk powder by realtime PCR. J. Vet. Med. Sci. 67:1037–1041.
7. Petersson-Wolfe, C. S., I. K. Mullarky, and G. M. Jones. 2010. Staphylococcus aureus mastitis: cause, detection, and control. Virginia Tech, Blacksburg, VA.
8. Frank, D. N., L. M. Feazel, M. T. Bessesen, C. S. Price, E. N. Janoff, and N. R. Pace. 2010. The human nasal microbiota and Staphylococcus aureus carriage. PLoS ONE 5:e10598.
9. Chambers, H. F. 2001. The changing epidemiology of Staphylococcus aureus? Emerg. Infect. Dis. 7:178–182.Dairy J. 17:1232-1239.
10. García, P., C. Madera, B. Martínez, and A. Rodríguez. 2007. Biocontrol of Staphylococcus aureus in curd manufacturing processes using bacteriophages. Int.
11. Lee, J. H. 2003. Methicillin (oxacillin)-resistant Staphylococcus aureus strains isolated from major food animals and their potential transmission to humans. Appl. Environ. Microbial. 69:6489-6494.
12. Driscoll, J. A., S. L. Brody, and M. H. Kollef. 2007 The epidemiology, pathogenesis and treatment of Pseudomonas aeruginosa infections. Drugs 67:351-368.
13. Osborne, A. D., K. Armstrong, N. H. Catrysse, G. Butler, and L. Versavel. 1981. An outbreak of Pseudomonas mastitis in dairy cows. Can. Vet. J. 22:215-217.
14. Santos, T. M., E. C. Ledbetter, L. S. Caixeta, M. L. Bicalho, and R. C. Bicalho. 2011. Isolation and characterization of two bacteriophages with strong in vitro antimicrobial activity against Pseudomonas aeruginosa isolated from dogs with ocular infections. Am. J. Vet. Res. 72:1079-1086.
15. Niu, Y. D., T. A. McAllister, Y. Xu, R. P. Johnson, T. P. Stephens, and K. Stanford. 2009. Prevalence and impact of bacteriophages on the presence of Escherichia coli O157:H7 in feedlot cattle and their environment. Appl. Environ. Microbiol. 75:1271-1278.
16. Oot, R. A., R. R. Raya, T. R. Callaway, T. S. Edrington, E. M. Kutter, and A. D. Brabban. 2007. Prevalence of Escherichia coli O157 and O157:H7-infecting bacteriophages in feedlot cattle feces. Lett. Appl. Microbiol. 45:445–453.
17. Calci, K. R., W. Burkhardt III, W. D. Watkins, and S. R. Rippey. 1998. Occurrence of male-specific bacteriophage in feral and domestic animal wastes, human feces, and human-associated wastewaters. Appl. Environ. Microbiol. 64:5027-5029.
18. Kenard, R. P., and R. S. Valentine. 1974. Rapid determination of the presence of enteric bacteria in water. Appl. Environ. Microbiol. 27:484-487.

CITE THIS ARTICLE
Desalegn Amenu. Isolation of Bacteriophages Specific to *Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa*. Res. J. Chem. Env. Sci. Vol 2 [6] December 2014. 06-10