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Microbiology

Monitoring of Pathogens Causing Mastitis in Cattle and Use of Bacteriophages for Treatment

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(Presented by Academy Member Tinatin Sadunishvili)

Bovine mastitis, the mammary gland inflammation caused by bacterial pathogens, is a major economic and problematic disease in dairy industry. This study was conducted to investigate the occurrence of pathogens causing bovine mastitis in Georgia, to determine their susceptibility to antibiotics and to create a polyvalent combined phage cocktail effective against these pathogens, respectively. A total of 793 bacterial strains were isolated: 369 strains of *Staphylococcus aureus* (45.2%), 158 strains of *Streptococcus agalactiae* (20%), 80 strains of *Streptococcus pyogenes* (10%), 36 strains of *Staphylococcus epidermidis* (4.5%), 144 strains of *E. coli* (18.2%), 2 strains of *P. aeruginosa* (0.2%), 2 strains of *P. vulgaris* (0.2%), 1 strain of *Enterococcus* (0.1%) and 1 strain of *K. pneumoniae* (0.1%). All cultures isolated from cows were investigated on sensitivity to antibiotics. It was established that most of these strains are resistant to antibiotics generally used for mastitis treatment. Based on the 6 phages selected which showed wide, complementary, non-overlapping host ranges a phage cocktail (polyvalent phage preparation) for mastitis treatment was prepared. In *in vitro* experiments it was shown that this preparation was effective against 95.5% of the tested strains of *Staphylococcus aureus*, 100% of the strains of *Staphylococcus epidermidis*, 98.2% of the strains of *Streptococcus agalactiae*, 96.3% of the strains of *Streptococcus pyogenes* and 95.5% of the strains of *E. coli*. *© 2022 Bull. Georg. Natl. Acad. Sci.*

antibiotic resistance, bacteriophages, bovine mastitis

Bovine mastitis is an inflammatory disease of the mammary gland, affecting all the species of domestic animals and is of great concern to dairy industry [1]. Bovine mastitis can be classified into two types, namely, clinical mastitis and subclinical mastitis (SCM). Clinical mastitis is detected by the changes in the physical appearance of the milk, swelling, redness, and rise in temperature of the

udder whereas animals with SCM do not exhibit any visible changes in milk or udder and the mastitis can only be detected by laboratory tests [2]. Although mastitis is caused by various bacteria, viruses [3], and fungi [4], the most common cause are gram-positive and gram negative bacteria [5]. The immune response of the mammary gland varies towards different bacterial infections [6]. Bovine mastitis disease begins with the invasion and colonization of microorganisms via the teat duct orifice, creating inflammation in the mammary gland that results in a clear productive decline and in unwanted physical and chemical changes in the milk [7].

Coagulase-negative staphylococci (CoNS) have become the most common mastitis causing agents in many countries. They mostly cause subclinical mastitis but have also been isolated from clinical mastitis. It is known that resistance for antimicrobials is in general more common in CoNS than in *S. aureus* [8]. The most common resistance among bovine CoNS is based on the production of the enzyme β-lactamase which confers resistance to benzylpenicillin and aminopenicillins, but also resistance towards aminoglycosides, tetracyclines, and macrolides has been reported [9]. Methicillin-resistant CoNS have been isolated from bovine mastitis which is of special concern due to the risk of spreading the *mec* genes [10].

Spreading of antibiotic-resistant staphylococci and also other groups of microorganisms is caused by unreasonable usage of chemotherapeutics, especially during long-term therapy with the same group of antibiotics and their usage without a prior susceptibility assay of the etiological factor responsible for the infection [11].

Therefore, there is a great need for the development of alternative treatments in infections caused by multi-drug-resistant (MDR). Under these challenging circumstances, bacteriophages have been identified as suitable alternatives to antibiotics, and several advantages compared to antibiotic agents are gaining popularity all over the world. Phage therapy has been found successful against a variety of bacterial infections such as vancomycin-resistant *Enterococci* [12], methicillin-resistant *Staphylococcus aureus* [13], *E. coli* [14], *Pseudomonas aeruginosa* [15], respiratory pathogens [16] and several other bacterial infections in clinical cases.

Materials and Methods

Isolation and identification of mastitis pathogens. A total of 743 quarter-milk samples were collected at three different farms, monthly from March to September 2012-2013 years. CMT analysis was performed on all samples at the farm. 503 samples were collected from cows which had signs of clinical mastitis, such as edema and increased sensitivity, fever, inappetence, dehydration, etc. collected in sterile tubes, kept on ice, and then stored at -20°C during transportation to the laboratory where an analysis was performed. Microbiological examination was performed according to the standards described in the National Mastitis Council guideline [17]. Ten microliters of an individual quarter milk sample were cultured on 5% bovine blood agar plates and MacConkey agar plates. Plates were incubated at 37°C for 24-48 hours. Bacterial colonies were identified based on gross morphology, number of colonies and the hemolytic pattern. Appropriate tests were performed on the colonies isolated to identify the pathogens, including Gram staining and a catalase test to classify *streptococci* and *staphylococci* genera. Gram-negative bacteria were identified using culture morphology on MacConkey agar (Merk, Germany), lactose fermentation, motility and reaction in triple sugar iron.

California Mastitis Test. CMT analysis was performed according to the Schalm [18]. In test chambers, milk samples were evaluated in accordance with the structure and colour change of the reaction after the addition of the CMT solution. The condition without any reaction was evaluated as negative (−); whereas positive reactions were

categorized as weak positive (+), positive (++), and strong positive (+++).

Antimicrobial susceptibility testing. To analyze the resistance of the isolates, the disk diffusion method was employed using 6 commercially available antimicrobial sensitivity discs (Penicillin (Pc), Streptomycin (Sm), Tetracycline (Tc), Kanamycin (Km), Chloramphenicol (Cm), Gentamicin (Gm)). The sensitivity/resistance was interpreted based on the zone of inhibition, inclusive of margins, following the guidelines of the Clinical Laboratory Standards Institute [19].

Bacteriophage isolation. Bacteriophages were isolated from wastewater by filtration, following the addition of broth concentrate and 18h cultures of different strains (test strains) to the filtrate. After 24 h incubation in a thermostat, the mixture was filtered through filters with a pore diameter of 0.45 μm (Millipore, United States), and the filtrates were spot-tested for the presence of phages by application of the filtrate (0.1 mL) on the lawn of the test strain on a solid nutrient medium. The result was considered positive if there was a lysis zone on the lawn in 18–24 h of cultivation at 37°C [20]. The highly specific bacteriophage strains were screened and selected by the plate method according to Gracia [21].

Electron microscopy. The morphology group membership of bacteriophages was investigated by the method of electron microscopy. Phages with a high titer of $>10^{10}$ CFU/mL (CFU- Colony-forming Unit) were negatively stained with 1% uranyl acetate. Pictures were obtained by transmission electron microscopy (JEM 100 SX, JEOL, Japan) with negative contrasting of the preparations by uranyl acetate.

Results and Discussion

From 503 milk samples collected from cows having signs of clinical mastitis, 712 bacterial strains were isolated and identified: 349 strains of *Staphylococcus aureus*, 138 strains of *Streptococcus agalactiae*, 80 strains of *Streptococcus pyogenes*, 28 strains of *Staphylococcus epidermidis*, 121 strain of *E.coli*, 2 strains *P. aeruginosa*, 2 strains of *P. vulgaris*, 1 strain of *Enterococcus* and 1 strain of *K. pneumoniae.*

A total of 240 milk samples were collected from cows with no signs of mastitis. Among the 240 milk samples analyzed, 20 strains were positive for *Staphylococcus aureus*, 20 strains for *Streptococcus agalactiae*, 8 strains for *Staphylococcus epidermidis* and 23 strains for *E. coli*. Totally, from investigated 743 milk samples were isolated 793 bacterial strains: 369 strains of *Staphylococcus aureus*

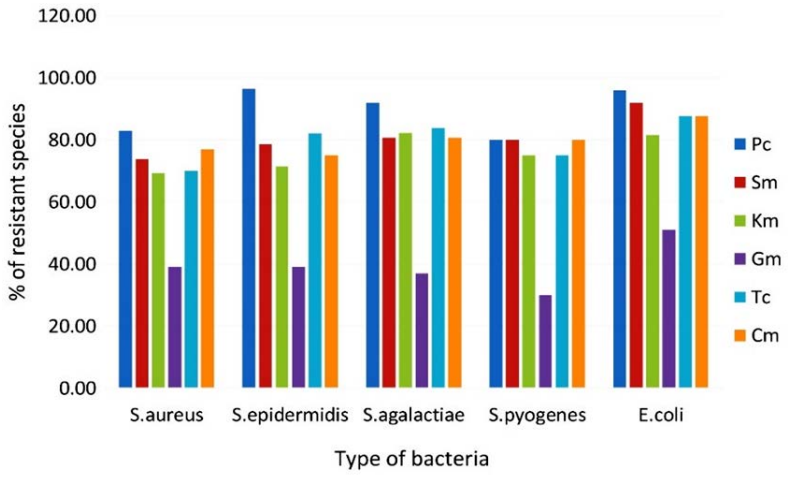

Fig. 1. Sensitivity of bacterial strains to antibiotics isolated from mastitis cows. Pc-penicillin, Sm-streptomycin, Km-kanamicin, Gm-gentamicin, Tc-tetracyklin, Cm-chloramphenicol

(45,2%) 158 strains of *Streptococcus agalactiae* (20%), 80 strains of *Streptococcus pyogenes* (10%), 36 strains of *Staphylococcus epidermidis* (4,5%), 144 strain of *E. coli* (18,2%), 2 strains *P. aeruginosa* (0,2%), 2 strains of *P. vulgaris* (0,2%), 1 strain of *Enterococcus* (0,1%) and 1 strain of *K. pneumoniae* (0,1%).

Antibiotic sensitivity test was performed for all 793 cultures isolated from mastitis cows to ascertain an antibiotic resistance level. Results are presented in Fig. 1.

Isolated *S. aureus* strains showed a certain resistance to Penicillin (83%), while they were sensitive towards Gentamicin (39,2%). *S. epidermidis* strains showed high resistance to Penicillin (96,4%) and Tetracycline (82%). *S. agalactiae* strains were resistant to Penicillin (91,9%). Among the *E.coli* isolates, high resistance was observed with most of the antibiotics used in test. Most of the strains showed sensitivity to Gentamicin.

In the present study we have isolated 3 phages from the sewage and prepared phage cocktail (polyvalent phage preparation) on the base of the newly isolated and laboratory collection bacteriophages for the treatment of mastitis cows (Table 1).

Table 1. Source of isolation of candidate bacteriophages for polyvalent phage preparation

#	Phage clones	Host strains	Source of isolation
1	vB_Sa 4	*S. aureus* # 8	Sewage "Mtkvari"
2	vB_Sa 5	*S. aureus* # 142	laboratory collection
3	vB_Sta 1	*St. agalactiae* # 1	laboratory collection
4	vB_Sta 2	*St. agalactiae* # 3	laboratory collection
5	vB_Stp 3	*St. pyogenes* #44	Sewage "Mtkvari"
6	vB_Eco 32	*E. coli* # 55	Sewage "Mtkvari"

All phage concentrates were tested against different bacterial strains causing mastitis in cows (Fig. 2).

For the selection of effective phages, those from the laboratory collection and also the newly isolated bacteriophages were tested against all

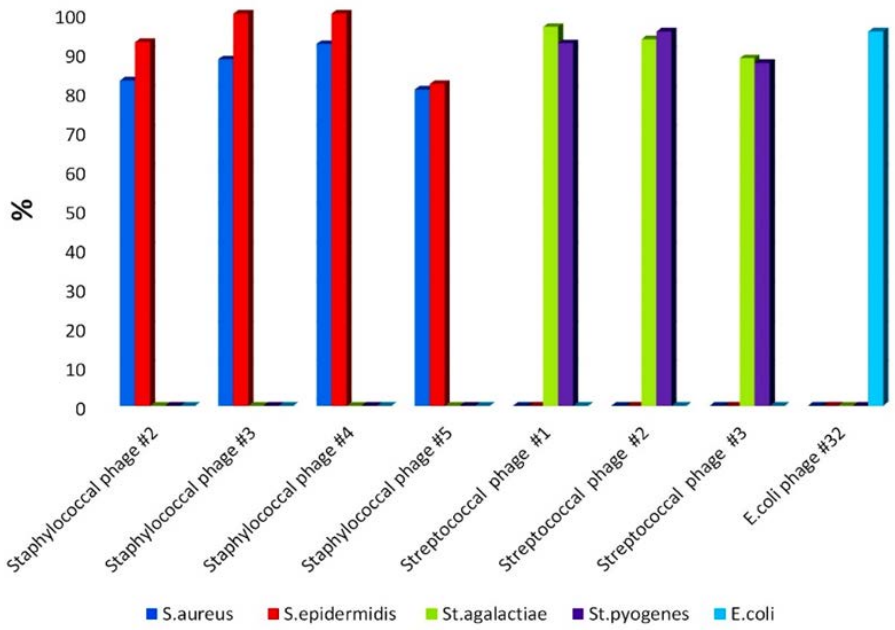

Fig. 2. Activity of different phage clones to the strains causing mastitis in cows.

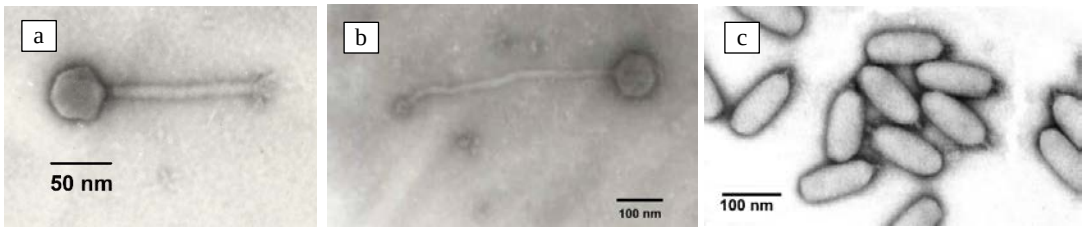

Fig. 3. a) Bacteriophage vB_Sa 4. Description: *Myoviridae*. Size: head: 50 nm X 60 nm; tail: 140 nm X 15 nm.
b) Bacteriophage vB_Stp 3. Description: *Siphoviridae*. Size: head: 95 nm X 95 nm; tail: 340 nm X 15 nm.
c) vB_Eco 32. Description: *Podoviridae*. Size: head: 145 × 44 nm and tail ~ 13 × 8 nm.

isolated bacterial pathogens. Six phages with wide, complementary, non-overlapping host ranges were selected for polyvalent phage preparation. Two *Staphylococcal* phages, three *Streptococcal* phages and one *E. coli* phage were used to prepare the phage cocktail for the treatment of mastitis cows (Table 1). In *in vitro* experiments this preparation was effective against 95.5% of tested strains of *Staphylococcus aureus*, 100% of strains of *Staphylococcus epidermidis*, 98.2% of strains of *Streptococcus agalactiae*, 96.3% of strains of *Streptococcus pyogenes* and 95.5% of strains of *E. coli* (Fig. 2).

Characterization of the phages is very important for assessing their therapeutic potential. Therefore, all six isolates of the phages were characterized and their in vitro lytic activities were assessed. With regard to morphological characteristics, bacteriophages vB_Sa 4, vB_Sa 5 belong to the morphology group of *Myoviridae*, phages vB_Str 1, vB_Str 2 and vB_Stp 3 belong to the morphology group of *Syphoviridae* and vB_Eco 32 belongs to the morphology group of *Podoviridae* (Fig. 3). Further characterization and molecular-biological study of the vB_Eco 32, isolated during our work, was carried out by K. Severinov and his co-workers [22] and was evaluated as possible therapeutic agent of mastitis in cattle. Other selected phages also have considerably more potential for further characterization, especially on the molecular side. Generally, they may be a good candidate for phage therapy against multidrug-resistant strains.

The results demonstrate a high prevalence of bovine mastitis causing pathogens in livestock farms in Georgia. The lytic activity of the bacteriophages assessed in this study revealed considerably more potential for further characterization. Generally, they may be used for therapeutic purposes against antibiotic resistant bacteria causing cow mastitis.

მიკრობიოლოგია

შინაურ ცხოველებში მასტიტის გამომწვევი პათოგენების კონტროლი და მკურნალობისთვის ბაქტერიოფაგების შესაძლო გამოყენება

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(წარმოდგენილია აკადემიის წევრის თ. სადუნიშვილის მიერ)

აღნიშნული კვლევა განხორციელდა საქართველოში მსხვილფეხა რქოსანი პირუტყვის მასტიტის გამომწვევი პათოგენების გავრცელების გამოსაკვლევად, ანტიბიოტიკების მიმართ მათი მგრძნობელობის დასადგენად და ამ პათოგენების მიმართ ეფექტური პოლივალენტური ფაგური კოქტეილის შესაქმნელად. კვლევის ფარგლებში სულ გამოიყო 793 ბაქტერიული შტამი: 369 შტამი *Staphylococcus aureus* (45,2%), 158 შტამი *Streptococcus agalactiae* (20%), 80 შტამი *Streptococcus pyogenes* (10%), 36 შტამი *Staphylococcus epidermidis* (4,5%), 144 შტამი *E. coli* (18,2%), 2 შტამი *P. aeruginosa* (0,2%), 2 შტამი *P. vulgaris* (0,2%), 1 შტამი *Enterococcus* (0,1%) და 1 შტამი *K. pneumoniae* (0,1%). შესწავლილ იქნა ყველა გამოყოფილი შტამის ანტიბიოტიკების მიმართ მგრძნობელობა. კვლევის შედეგად დადგინდა, რომ შესწავლილი შტამების უმრავლესობა ავლენს რეზისტენტობას მასტიტების სამკურნალოდ გამოყენებული ანტიბიოტიკების მიმართ. კვლევის ფარგლებში გამოყოფილი ფაგებისა და ლაბორატორიაში არსებული ფაგებიდან შეირჩა 6 ბაქტერიოფაგი, რომლებიც ხასიათდებოდნენ ფართო ლიზისური სპექტრით და შეიქმნა პოლივალენტური ფაგური კოქტეილი, რომელიც შეიძლება გამოყენებულ იქნეს მსხვილფეხა რქოსანი პირუტყვის მასტიტების სამკურნალოდ. *In vitro* ექსპერიმენტებში აღნიშნულმა პრეპარატმა გამოავლინა ეფექტურობა *Staphylococcus aureus*-ის შესწავლილი შტამების 95,5%-ის მიმართ, 100%-იანი ეფექტურობა *Staphylococcus epidermidis* შტამების მიმართ, 98,2% Streptococcus agalactiae შტამების მიმართ, 96,3% *Streptococcus pyogenes* შტამების და 95,5% *E. coli* შტამების მიმართ.

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