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CLINICAL STUDY

Susceptibility of *Staphylococcus aureus* strains to commercial therapeutic phage preparations

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ABSTRACT

OBJECTIVES: The aim of this study was to determine the susceptibility of *Staphylococcus aureus* strains to commercial phage preparations. The strains were isolated from clinical patients as well as from nasal mucosa of healthy carriers.

BACKGROUND: The elevating number of antibiotic-resistant *S. aureus* strains present a therapeutic challenge, especially in high-risk patients. One of the promising ways to solve this problem is phage therapy.

METHODS: Susceptibility of 111 carrier strains of *S. aureus* (4 strains were methicillin-resistant; MRSA) and 81 clinical isolates from bloodstream or skin and soft tissue infections (28 were MRSA) to four commercial phage preparations was assessed *in vitro* by spot assay. The clonality of *S. aureus* strains was determined by spa typing.

RESULTS: Spa typing revealed 97 distinct spa types. A proportion of 73–80 % of the tested *S. aureus* strains were revealed to have *in vitro* phage susceptibility, depending on the clonal affiliation of the strains and phage preparation tested. The susceptibility to phage preparations was significantly higher in MRSA strains ($p < 0.001$).

CONCLUSIONS: *In vitro* results indicate a promising therapeutic potential of the tested commercial anti-staphylococcal phage preparations. They could be applied to a broad spectrum of bacterial clones, and have an excellent activity especially against MRSA strains *(Tab. 2, Fig. 2, Ref. 43)*. Text in PDF *www.elis.sk*

KEY WORDS: *Staphylococcus aureus*, MRSA, therapeutic phages, *S. aureus* nasal carriage, skin and soft tissue infections, staphylococcal bacteriemia.

Introduction

Staphylococcus aureus is a significant cause of both nosocomial and community-acquired opportunistic infections. It most frequently infects the skin and soft tissue, but the infectious focus may affect any locality of the body, with bloodstream infections being the most severe (1, 2). Humans may also be asymptomatic carriers of this bacterium, especially on their nasal mucosa. Colonized individuals may spread *S. aureus* to other people, which could be especially dangerous if the carriers are healthcare workers (3). Moreover, the treatment of infections caused by this bacterium is often complicated by increasing numbers of strains resistant to methicillin (MRSA) and other anti-staphylococcal drugs (2, 4, 5). Thus, novel ways of therapy are needed as an alternative to conventional antibiotic treatment (6, 7, 8).

Bacteriophage therapy is considered one of the most promising alternative approaches. It employs bacteriophages which are natural predators of bacteria that can lyse bacteria in the infectious site, particularly on the colonized skin or mucosa of the patient (9, 10). Compared with antibiotics, bacteriophages are highly specific to their hosts and the phage therapy has only a negligible effect on the human physiological microbiota equilibrium. Moreover, phages have not shown any significant side effects or toxicity on mammalian cells up to date (11). The activity of phages is not influenced by resistance to antibiotics because their mechanisms for targeting the bacterial cells differ from those of conventional antimicrobial drugs (9). Additionally, phage-resistant bacterial strains are generally of lower fitness. Therefore, phage therapy may be a powerful option in the treatment of infections and also in decolonizing carriers so as to prevent the spread of infectious agents to other people (12). However, their use is restricted to local admini-

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stration due to unknown side effects when given parenterally, especially owing to the potential of undesired immune response. Patients with chronic or repeated infections not responding to antibiotic treatment, patients allergic to antimicrobial drugs, or those infected with polyresistant strains may especially benefit from the phage therapy (9, 10). On the other hand, phages could be potential vehicles that bring virulence or resistance genes to their bacterial hosts (13). This effect must be prevented by a thorough selection of therapeutic phages based on their detailed genome analysis.

Nowadays, phage therapy is still not widely used in most countries of the world because of the lack of efficacy benchmarks and information about well-established safety, approved manufacturing practices and standard protocols for the treatment (10, 14). Nevertheless, there are some eastern European countries with a long tradition of phage therapy in human medicine. Phage preparations are produced, and phages are therapeutically used mainly in Georgia, Russia, Poland and Czech Republic (15, 16, 17, 18, 19). Several commercial therapeutic bacteriophage preparations such as Bakteriofag-Stafilokokovyj, Sextaphag, Staphylococcal Bacteriophage or Pyo-bacteriophage contain lytic phages infecting *S. aureus*. In general, they are recommended for local usage in patients with pyogenic infections of the skin and soft tissues, respiratory tract, eye, and urinary tract, or for decolonizing the nasal cavity of infectious agent carriers (20, 21, 22, 23).

The efficiency of these four commercial anti-staphylococcal phage preparations was assessed in the present study. *Staphylococcus aureus* strains with various antibiotic susceptibility and clonality were isolated from hospitalized patients as well as healthy carriers found among medical students from a geographic area of Slovakia and used as target bacteria.

Materials and methods

Carrier strains of *S. aureus* were obtained from nasal swabs of healthy volunteers, namely from students of the Faculty of Medicine, Comenius University in Bratislava. The clinical strains originated from hemocultures, and skin and soft tissue lesions of patients hospitalized at the University Hospital in Bratislava. *Staphylococcus aureus* strains were isolated and characterized by using standardized culture and identification methods (16). Uncertainly identified strains were submitted to analysis by PCR according to Martineau et al (25). Antimicrobial susceptibility to erythromycin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole and ciprofloxacin was tested by disk diffusion test according to EUCAST recommendations (26). According to Martineau et al, MRSA strains were screened by disc diffusion test using cefoxitin (30 μg) and confirmed by detection of the *mecA* gene by PCR (27). The bacterial strains were preserved in skim milk medium (BioLife, Milan, Italy) at –20 °C and revitalized by overnight cultivation on blood agar before testing.

Molecular characterization of all *S. aureus* strains was done by spa typing based on the sequence typing of the spa gene repeat region (28, 29).

Four commercial phage cocktails of two producers were tested (Tab. 1). According to the producers, these phage preparations are intended for the treatment of bacterial infections of the respiratory tract, skin and soft tissue, surgical site and burn wound infections, urogenital tract, gastrointestinal tract and ocular infections, purulent infections in newborns and other diseases caused by susceptible bacteria (20, 21, 22, 23).

The phage-susceptibility of *S. aureus* strains was determined by spot assay. A standardized inoculum of bacterial strains (McFarland 0.5, corresponding to 1–5x10^8 CFU/mL) was prepared turbidimetrically, using Densitometer DEN-1 (Biosan, Riga, Latvia). Luria-Bertani agar plates were overlaid with 2 ml of standardized bacterial inoculum. The inoculum excess was sucked out by a pipette. After the inoculum had soaked into the agar, phage preparations were point-inoculated in 10 μl volumes of non-diluted phage preparations and preparations diluted 1:10, 1:100 and 1:1000. The incubation took 16 hours at 37 °C. The activity of phage preparations was determined by the detection of confluent bacterial lysis, semi-confluent plaques, or individual isolated plaques in the spot area of inoculated phage suspensions. Any of these reactions at any dilution were considered positive.

Tab. 1. The tested phage preparations.

Phage preparation	Producer	Titer*	Specificity
Bakteriofag stafilokokovyj	NPO Microgen, Perm, Russia	10^5.ml^{-1}	Staphylococcus spp.
Sextaphag®	NPO Microgen, Perm, Russia	10^3.ml^{-1}	Staphylococcus spp., Streptococcus spp., Proteus vulgaris, P. mirabilis, Pseudomonas aeruginosa, enteropathogenic Escherichia coli, Klebsiella pneumoniae
Staphylococcal bacteriophage	Eliava BioPreparations LTD, Tbilisi, Georgia	10^7.ml^{-1}	Staphylococcus spp.
Pyo-bacteriophage	Eliava BioPreparations LTD, Tbilisi, Georgia	10^5.ml^{-1}	Staphylococcus spp., Streptococcus spp., Proteus spp., Pseudomonas spp., Escherichia coli

* Titer of antistaphylococcal phages

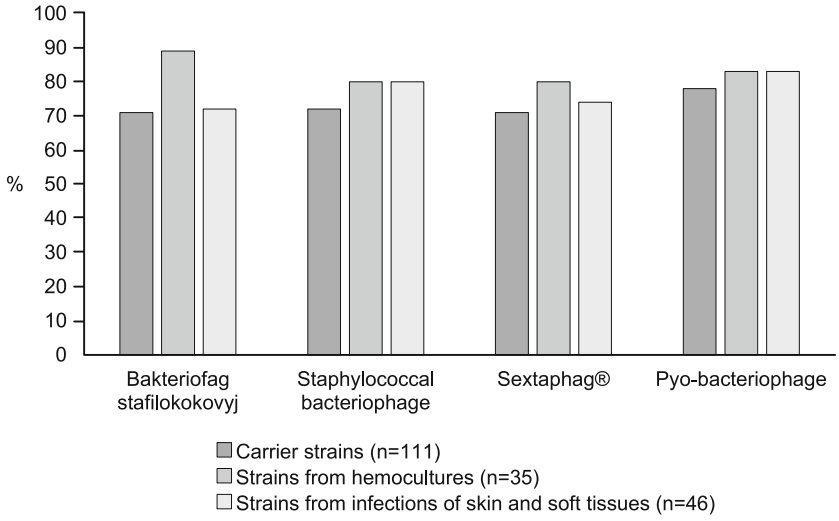

Fig. 1. Susceptibility of Staphylococcus aureus streins to phage preparations.

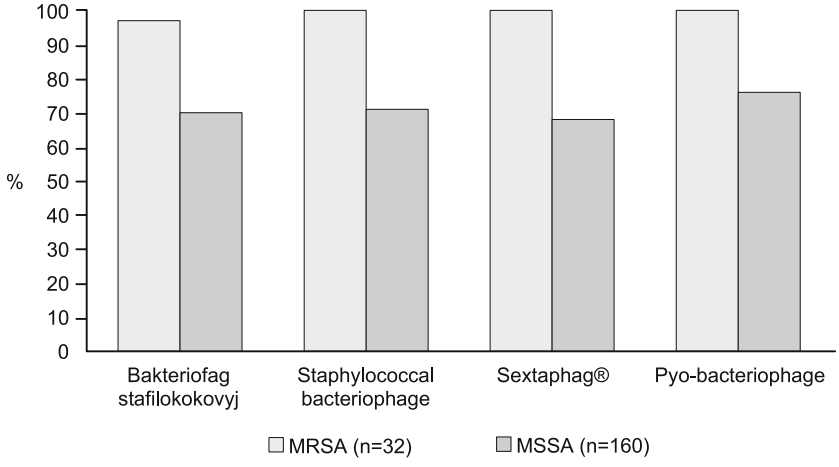

Fig. 2 Susceptibility of methicillin-susceptible (MSSA) and methicillin-resistant (MRSA).

The data were statistically analyzed by Fisher's exact test; the graphics were designed with Microsoft Excel (MS-office 2019; Microsoft Corporation).

Results

Characterization of Staphylococcus aureus strains

In total, 111 carrier strains from nasal mucosa of medical students, 46 strains of patients suffering from infections of the skin and soft tissues and 35 strains from hemocultures were included in the study.

All but four of the 111 carrier strains were sensitive to methicillin. The strains were also well susceptible to trimethoprim-sulfamethoxazole, ciprofloxacin and tetracycline (100 %, 97 % and 100 % susceptibility, respectively). A lower susceptibility to erythromycin and clindamycin was observed (72 % and 74 %, respectively). Inducible resistance of MLSB-type (macrolide-lincosamide-streptogramin B) was present in 28 out of 29 clindamycin-resistant strains.

Out of 46 clinical strains isolated from skin and soft tissue infections, 20 were MRSA (43 %), 28 strains were resistant to erythromycin (61 %), 25 to clindamycin and ciprofloxacin (54 %), and 3 to tetracycline (7 %). No resistance was observed to trimethoprim-sulfamethoxazole in this subgroup of strains.

Eight of 35 hemoculture strains were MRSA (23 %), 13 strains were resistant to erythromycin (37 %), 9 to clindamycin (26 %), 2 to tetracycline (6 %), 12 to ciprofloxacin (34 %) and none to trimethoprim-sulfamethoxazole. The presence of *mecA* gene was confirmed in all strains showing phenotypic resistance to cefoxitin.

Spa typing revealed 97 spa types, while 72 various spa types were identified among carrier strains, 23 in strains from hemocultures, and 25 were identified in strains from the skin and soft tissue infections. Some spa types such as t008, t024, t084 and t160 were present in all three subgroups of *S. aureus* strains (carrier strains, strains from the skin and soft tissue infections and strains from hemocultures). In the whole collection of *S. aureus* strains, the most frequent spa types were t024, t032 and t084. MRSA strains were assigned to spa types t003, t008, t010, t014, t026, t032, t189, t718, t1148 and t4559.

Susceptibility of S. aureus strains to phage preparations

The evaluated commercial phage cocktails were active against 71 % to 89 % of *S. aureus* strains, depending on the strain origin (carrier, hemoculture, skin and soft tissue infection) and phage preparation (Fig. 1). Slight, statistically non-significant differences were observed in the phage susceptibility of clinical and carrier *S. aureus* strains, the former being more susceptible. The highest susceptibility (more than 80 % to each phage preparation) was observed in the subgroup of hemoculture strains.

Pyo-bacteriophage was the most active, namely with 80 % of susceptible *S. aureus* strains. It was followed by Staphylococcal Bacteriophage (76 %), Bakteriofag Stafilokokovyj (75 %), and Sextaphag (73 %) (Fig. 1). Out of 192 *S. aureus* strains, 158 (82 %) were susceptible to at least one of the tested phage preparations. Seventy percent of strains were susceptible to all four phage preparations.

A significantly better phage susceptibility was detected in MRSA strains when compared to strains of MSSA ($p < 0.001$) (Fig. 2). All 32 MRSA strains were susceptible to Staphylococ-

Tab. 2. Susceptibility of identified *Staphylococcus aureus* spa types to the tested phage preparations.

Susceptibility to the tested phage preparations	
Susceptible to all tested preparations	t003, t007, t008, t010, t012, t014, t018, t026, t036, t045, t056, t084, t085, t122, t148, t156, t160, t169, t189, t209, t223, t267, t279, t284, t346, t360, t435, t449, t491, t493, t648, t706, t718, t760, t774, t922, t937, t1148, t1200, t1265, t1491, t1509, t2119, t2124, t2374, t3382, t3732, t3884, t4032, t4559, t4688, t5534, t6943, t12469, t16302, t16466, t18619, t18623, t18626, t18627, t18629
Resistant to all tested preparations	t004, t050, t065, t216, t571, t688, t701, t715, t1040, t1255, t1333, t1646, t2642, t3625, t4545, t6608, t7157, t12588, t18621, t18625, t18628
Variable susceptibility to phage preparations	t002, t015, t024, t032, t091, t179, t289, t342, t362, t728, t1309, t1451, t2448, t2716, t2932

cal Bacteriophage, Sextaphag® and Pyo-bacteriophage, and all strains but one were sensitive to Bakteriofag Stafilokokovyj. On the other hand, the phage susceptibility of MSSA strains varied from 68 to76 %, depending on the phage preparation. Thirty-four *S. aureus* strains were resistant to the tested phage preparations; however, these strains were well susceptible to antimicrobial drugs – all were methicillin-sensitive and 65 % of them were susceptible also to every one of the other tested antibiotic groups.

In many cases, the phage susceptibility patterns correlated with the spa type. Similar susceptibility patterns to the phage preparations were observed in the strains belonging to the same spa type, with only some exceptions. Out of 97 different spa types detected in our *S. aureus* strains collection, 61 contained strains susceptible to all phage preparations. In 21 spa types, all *S. aureus* strains were found to be resistant to the tested phage preparations. Strains from the remaining 15 spa types had variable susceptibility to the phage preparations. Strains from our collection which were resistant to all phage preparations belonged predominantly to infrequent (single and double-membered) spa types (Tab. 2).

Discussion

Staphylococcus aureus is a major causative agent of various human infections. The treatment of staphylococcal infections is often complicated by increasing resistance to antibiotics. *Staphylococcus aureus* can colonize also the skin and mucosa of asymptomatic humans (2). The nasal carriage may be especially dangerous in healthcare facilities, where the matter of concern is the threat of *S. aureus* being spread to risk group patients (3, 30). Medical students during their internship come in contact with hospitalized patients, and students colonized by *S. aureus* represent a potential threat to the susceptible patients (31). Therefore, in the presented phage-susceptibility study, both the carrier strains from the nasal mucosa of healthy medical students and the clinical strains isolated from infected patients were included.

The group of clinical isolates contained *S. aureus* strains from the skin and soft tissue infections and hemocultures. Skin and soft tissue infections are amongst the most frequent staphylococcal infections, and, at the same time, they are excellently accessible to topical treatment, including phage-therapy (32). Bacteremic episodes in patients infected or colonized by *S. aureus* represent a risk of metastatic spread and sepsis. These serious infectious complications can be reduced by patients' monitoring and further therapy or decolonization (33), with phage treatment providing antistaphylococcal efficiency and lack of side-effects.

Antibacterial activity of various bacteriophage preparations has already been proved in several *in vitro* and *in vivo* studies (18, 34, 35, 36, 37, 38), and the high efficiency of two phage preparations tested in our study – Staphylococcal Bacteriophage and Pyo-bacteriophage – was proven in the experimental study also against the methicillin-resistant *S. aureus* in biofilm *in vitro* (38). However, we assumed that *S. aureus* strains might exhibit geographic differences in phage susceptibility. Therefore, 192 *S. aureus* strains of Slovak origin were selected for evaluation of the therapeutic potential of commercial phage cocktails produced abroad. We examined 111 isolates with four (4 %) MRSA strains coming from healthy carriers. MRSA and other antimicrobial-resistant *S. aureus* isolates were preferentially selected in the group of clinical strains. The need for phage preparations covering resistant bacterial strains which represent antibiotic treatment challenges, was taken in account by this selection. Even if the local application of antimicrobial drugs or antiseptic agents is the "classical" option used to reduce *S. aureus* nasal carriage in healthcare workers (3) and among high-risk hospitalized patients (33), and the topical antibiotics suitable for nasal mucosa decolonization, such as mupirocin, bacitracin, neomycin or fusidic acid showed high *in vitro* activity on *S. aureus* strains tested in the study (99% of susceptible strains; data not shown), the application of phage preparations would have many advantages over the local antibiotic application: higher specificity, low toxicity, indifference to antibiotic resistance and zero potential to select antibiotic-resistant strains (11). These advantages have even a higher impact on the therapy in the case of patients infected by antimicrobial-resistant *S. aureus* strains.

In this study, four commercial phage preparations produced in eastern Europe showed a very good *in vitro* efficiency against *S. aureus* strains isolated in Slovakia. These strains were of broad clonality and had various antimicrobial susceptibility patterns. The tested phage preparations were active predominantly against MRSA strains which had a 97 to 100 % phage-susceptibility. Only 34 strains (17.7 %) were resistant to all of the tested phage preparations. In contrast to the phage-sensitive strains, the phage-cocktail-resistant strains were well susceptible to oxacillin and majority of the other tested drugs, including ciprofloxacin. These strains may be potentially used as indicator strains for widening the spectrum of commercial anti-staphylococcal phage preparations.

The relation of clone affiliation to the phage susceptibility of investigated *S. aureus* strains was also analyzed. High clonal diversity was found among the tested *S. aureus* strains. Compared with the clinical strains, even a higher diversity was detected among the strains isolated from healthy carriers. Many spa types in this group

were affiliated only to a single strain. The majority of the spa types were susceptible to all tested phage preparations and 21 spa types showed resistance. Strains from the remaining 15 spa types had various phage responses, which might reflect slight phage receptor changes in some strains from these spa types or may represent the expression of acquired internal resistance of bacterial cells to the infecting phages. Strains within the same spa types (e.g., t003, t032, and t571) with the same susceptibility patterns to antibiotics and phages isolated from the same department during a similar time period indicate a possibility of spreading from the same source. Therefore, it is very important to accomplish measurements to prevent spreading of pathogenic or potentially pathogenic microorganisms in hospital environment, such as aseptic work, and decolonization of carriers and environment.

Phage therapy may be beneficial mainly for the patients infected or colonized by resistant *S. aureus* strains. Several reports on the clinical use of phage therapy in patients with various bacterial infections were already published: phage therapy or antibiotic therapy combined with phage suspension application was successfully used in patients with prosthetic joint infections caused by *S. aureus* (39, 40). Effective co-therapy by phage preparation and linezolid in a patient with diabetic foot infection caused by MRSA was reported by Chhibber et al (41). Kutateladze and Adamia (42), in their survey on phage therapy experience at the Eliava Institute in Tbilisi, Georgia, described a high clinical efficacy of antistaphylococcal phage preparation applied as a monotherapy or in combination with antibiotic treatment in patients with various types of staphylococcal infections such as peritonitis, mastitis, chronic inflammation of the female reproductive system, osteomyelitis or sepsis. In the same survey, the authors informed about the successful therapy of a patient with cystic fibrosis infected by *S. aureus* and *Pseudomonas aeruginosa* by combining antibiotic therapy with commercial Pyo-bacteriophage. However, as it was also supported by our results, the preliminary laboratory determination of the patient's strain to the commercial phage preparation intended to be used, might be essential for a successful outcome of phage therapy (43).

Finally, we can conclude, that the phage preparations evaluated in this study showed a very good *in vitro* activity against *S. aureus* strains of various clonality and antimicrobial susceptibility. A considerable effect was achieved primarily against MRSA strains. Our results indicate a promising therapeutic potential of all tested anti-staphylococcal phage preparations for difficult-to-treat staphylococcal infections.

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