I. Ozkan¹
E. Akturk¹
N. Yeshenkulov²
S. Atmaca²
N. Rahmanov²
H. I. Atabay¹

¹Department of Medical Microbiology, Faculty of Medicine, Sifa University, Izmir, Turkey

²Sifa University, Faculty of Medicine, Class-3

Lytic Activity of Various Phage Cocktails on Multidrug-Resistant Bacteria

Abstract

Purpose: A bacteriophage is a virus that infects and replicates within a bacterium following the injection of phage genome into the bacterial cytoplasm. They are seen as a possible therapy for multi-drug-resistant strains of many bacteria. The aim of this study is to evaluate the lytic activity of the Pyo, Intesti and Fersisi bacteriophage cocktails on *P. aeruginosa* and *S. aureus*.

Methods: Ten different *S. aureus* and *P. aeruginosa* strains, which were isolated from hospitalized patients in Turkey, were used in the study. The identification and antibiotic susceptibility of the isolates were performed using Vitec 2 system. The identities of the isolates were confirmed by a species-specific Polymerase Chain Reaction (PCR) assay. Lytic activity of the bacteriophage cocktails on bacteria was determined by spot test and plaque assay methods.

Results: The lytic activity of the Pyo phage cocktail was evaluated on *P. aeruginosa* and *S. aureus* strains. It was found that eight isolates of MDR *S. aureus* were susceptible to Pyo phage cocktail and two isolates were resistant. Nine isolates of antibiotic-resistant *P. aeruginosa* were found to be susceptible to this phage cocktail and one isolate was resistant. Thus, the Pyo, Intesti and Fersisi cocktails are very effective in treating clinical strains of multidrug-resistant *P. aeruginosa* and *S. aureus* isolated in Turkey.

Conclusion: The Pyo, Intesti and Fersisi cocktails may prove useful in the treatment of various infections caused by those bacteria.

Clin Invest Med 2016; 39 (6): S66-S70.

Correspondence to:

I. Ozkan

Sifa University, Faculty of Medicine, Department of Medical Microbiology
45 Ankara Street, Bayrakli, Izmir, 35100, Turkey

E-mail: ismail.ozkan@sifa.edu.tr

Antibiotics are frequently used to treat bacterial infections but bacteria are becoming increasingly resistant to a wide range of these antibiotics and antibiotic resistance is becoming a major problem in treating infectious diseases worldwide [1]. Nosocomial infections, which are caused by multidrug-resistant (MDR) bacteria, produce serious problems in hospitals. *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Staphylococcus aureus* (*S. aureus*) are the major bacteria responsible for these hospital infections, causing infections of skin, respiratory airways, urinary tract and other organ systems [2, 3]. In some patients, antibiotics, which were used as a final treatment did not produce the desired results and these cases have triggered the search for alternative therapeutic methods and strategies [4]. Phage therapy is one of the promising alternative therapies to antibiotics in treating infectious diseases caused by *P. aeruginosa* and *S. aureus*. The use of phage treatment started in the George Eliava Institute of Bacteriophages, Microbiology and Virology (GEIBMV), which was founded in 1923, and it has been used actively in the treatment of many diseases [5]. A number of bacteriophage cocktails are now produced commercially and are employed for therapeutic purposes in many countries, especially in Georgia and the surrounding area. GEIBMV was the first center in the world to develop therapeutic phage cocktails against various pathogenic bacteria and efficacy and convenience of those cocktails has been discussed in many studies and articles.

Materials and Methods

Bacterial Strains

Ten strains of each *Pseudomonas aeruginosa* and *Staphylococcus aureus* were used in the study. The strains were isolated from patients in various clinical microbiology laboratories in Aegean region of Turkey. Initial characterization and antibiotic susceptibility profile detection of the bacterial strains were executed by the VITEK 2 system (BioMerieux, France) using GN 21341 card for Gram-negative bacteria in Sifa University Bornova Education and Research Hospital.

Bacterial Growth Conditions and Growth Media

Bacterial cells were grown in Luria-Bertani broth (Merck) at 37°C for 24 h. In addition, Luria-Bertani agar (Merck) was used for phage propagation and spot test. Luria-Bertani soft agar (LB broth + Agar Agar, Merck) was used to mix bacterial strains and poured over an LB agar plate.

DNA Extraction and Molecular Typing

Bacteria were collected directly from the culture plate, washed twice in a solution of 10 mM Tris HCl and 1 mM EDTA, pH 8.0 (TE) and incubated in 50 mM Tris, pH 8.0, containing SDS 0.5% and proteinase K (Gibco-BRL) 200 pg/ml for 2 h at 65°C. Incubation was followed by DNA isolation using Invitrogen PureLink DNA Mini Kit (LOT: 1621175A, CA, USA). DNA isolation was confirmed with 1% agarose gel electrophoresis. The identities of *P. aeruginosa* bacterial strains were verified by PCR with VIC 1 (5'-TTC CCT CGC AGA GAA AAC ATC-3') and VIC2 (5'-CCT GGT TGA TCA GGT CGA TCT-3') primers, which was produced a 520 bp PCR product [6]. The identities of *S. aureus* bacterial strains were verified by SA 1 (5'-AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG-3') and SA 2 (5'-CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3') primers. PCR product was 108 bp and confirmed with 1.5% agarose gel electrophoresis [7].

Small Drop (Spot Test) and Double Agar Plate Assay

In order to determine the phage sensitivity of the bacterial strains, the method of Merabishvili *et. al.* was modified and used in this study [8]. For the spot test assay, 100 µl of *P. aeruginosa* and *S. aureus* overnight culture were added to 3 – 4 ml molten soft LB agar and poured over an LB agar. Subsequently, 10 µl of PYO, Fersisi and Intesti commercial phage cocktails were spotted onto the bacterial culture. For the double agar plaque assay method, 100 µl of bacteria culture and 1 ml phage cocktail were added 3 – 4 ml molten soft agar and poured over an LB agar. The plates were incubated overnight at 37°C for 24 h.

Results

PCR results for *S. aureus* and *P. aeruginosa* are shown in Figures 1 and 2, respectively. Pyo phage cocktail was effective on 80% of *S. aureus* isolates and 90% of *P. aeruginosa* isolates, Fersisi phage cocktail was effective on 100% isolates of *P. aeruginosa* isolates and Intesti phage cocktail was effective on 90% of *S. aureus* isolates and 80% of *P. aeruginosa* isolates. (Table 1 and 2). Thus, Pyo, Intesti and Fersisi phage cocktails showed high lytic activity on all *S. aureus* and *P. aeruginosa* pathogen strains isolated from Turkish patients.

FIGURE 1. Results of polymerase chain reaction (PCR) for the *S. aureus*-specific genes on the agarose gel electrophoresis. The first column depicts bands of the standard markers (Invitrogen). The third and fourth columns contain negative control and positive control bands, respectively. The next ten columns contain bands from strains SUPA_01 to SUPA_10, respectively. Ten out of ten strains were positive for these standard *S. aureus*-specific primers.

FIGURE 2. Results of polymerase chain reaction for the *P. aeruginosa*-specific genes on the agarose gel electrophoresis. The first column depicts bands of the standard markers (Invitrogen). The second and third columns contain negative control and positive control bands, respectively. The next eight columns contain bands from strains SUPA_01 to SUPA_06, SUPA_13 and SUPA_08, respectively. The thirteenth and fourteenth columns contain bands from strains SUPA_09 and SUPA_22, respectively. Ten out of ten strains of *P. aeruginosa* is positive for these standard *P. aeruginosa*-specific primers.

FIGURE 3. Spot test assay of phage cocktails applied onto the bacterial culture.

TABLE 1. Lytic activity of commercial of Pyo, Intesti and Fersisi phage cocktails screening on clinical isolates of *S. aureus*

Isolates	Source	Pyo	Intesti	Fersisi	Antibiotic resistance
SUPA_01	Abscess	R	S	S	OX, P
SUPA_02	Mucus	S	S	S	OX, P
SUPA_03	Wound	S	S	S	RIF, P
SUPA_04	Urine	S	S	S	CIP
SUPA_05	Urine	R	R	S	RIF
SUPA_06	Abscess	I	I	S	TE
SUPA_07	Mucus	S	S	S	CIP, OX, P, FOX
SUPA_08	Abscess	S	S	S	P
SUPA_09	Blood	I	I	S	P, RIF
SUPA_10	Abscess	S	S	S	TE, P

Bacterial isolates are sensitive to phage infection (clean spot; S; light gray), intermediate (blurry spot; I; medium gray), or resistance (No lytic activity at all; R; dark gray)

Antimicrobials: Oxacillin (OX), Penicillin (P), Rifampicin (RIF), Ciprofloxacin (CIP), Tetracycline (TE), Cefoxitin (FOX)

TABLE 2. Lytic activity of commercial of Pyo and Intesti phage cocktails screening on clinical isolates of *P. aeruginosa*

Isolates	Source	Pyo	Intesti	Antibiotic resistance
SUPA_01	Abscess	S	S	SXT, FOX, CXM, AMP, CXA, AMC
SUPA_02	Urine	S	S	CAZ, IPM, CIP, TZP, PRL
SUPA_03	Mucus	R	R	SXT, FOX, CXM, AMP, CXA
SUPA_04	Mucus	S	I	FOX, CXM, AMP
SUPA_05	Mucus	I	I	CXM, AMP, CXA
SUPA_06	Blood	S	S	CAZ, CN, AMP
SUPA_13	Wound	S	R	SXT, FOX, TZP
SUPA_08	Bronchus-alveolar space	S	S	SXT, FOX, CXM, AMP, CXA
SUPA_09	Blood	S	S	SXT, FOX, CXM, CXA, AMC
SUPA_22	Urine	S	S	SXT, FOX, CXM, AMP

Bacterial isolates are sensitive to phage infection (clean spot; S; light gray), intermediate (blurry spot; I; medium gray), or resistance (No lytic activity at all; R; dark gray)

Antimicrobials: Imipenem (IPM), Ciprofloxacin (CIP), Gentamicin (CN) 10 ug, Amikacin (AN), Colistin (CT), Trimethoprim-sulfamethoxazole (SXT), Meropenem (MEM), Cefoxitin (FOX), Cefuroxime (CXM), Ampicillin sulbactam (SAM), Ampicillin (AMP), Cefuroxime axetil (CXA), Piperacillin/Tazobactam (TZP), Piperacillin (PRL), Amoxicillin/clavulanate (AMC), Levofloxacin (LVX), Tigecycline (TGC), Netilmicin (NET), Tetracycline (TE), Cefoperazone sulbactam (SCP)

Discussion

The main causes of bacterial infections in hospitals are *P. aeruginosa* and *S. aureus* and researchers have shown high levels of resistance to antibiotics by these bacteria [9, 10]. Before the sharp rise in the use of antibiotics, some researchers reported the use of bacteriophages as effective antibacterial agents [11]. With the increase in the prevalence of antibiotic-resistant strains, new research into alternatives to conventional antibiotics have again aimed to find methods for generating highly-effective bacteriophages because of their promising therapeutic potential. Our research supported the work in previously published studies and showed that bacteriophages and their cocktails could be used effectively

against antibiotic-resistant clinical strains of *P. aeruginosa* and *S. aureus*.

Our research results showed different patterns of resistance to antibiotics and bacteriophages by the various strains of bacteria. The most resistant *P. aeruginosa* and *S. aureus* strains were resistant to several antibiotics. Fortunately, these resistant strains were sensitive to at least 80% of the bacteriophages. As the results show (Tables 1 and 2), the resistance pattern was different to for the various antibiotics and bacteriophages. This may be because the mechanisms of resistance against bacteriophages are different from those for resistance to antibiotics or from some other mechanisms. All twenty isolated resistant *P. aeruginosa* and *S. aureus* strains, which were resistant to several antibiotics, could be killed by bacteriophages. These bacteriophages are easily isolated from environmental water samples without the need for expensive procedures or obtained commercially as phage cocktails. Similar therapeutic effects of phages to antibiotics *in vivo* suggest that phages may be good alternatives for antibiotics to treat resistant infections - perhaps not just as an alternative therapy but as the main therapy against bacterial infections in both humans and animals.

Financial Support

Sifa University.

References

1. R. Moellering, "MRSA: the first half century.," *Journal of Antimicrobial Chemotherapy*, vol. 67, no. 1, pp. 4-11, 2012.
2. J. Driscoll, S. Brody and M. Kollef, "The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections.," *Drugs*, vol. 67, no. 3, pp. 351-368, 2007.
3. M. Seenivasan and V. Yu, "Staphylococcus lugdunensis endocarditis - The hidden peril of coagulase-negative staphylococcus in blood cultures.," *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 22, no. 8, pp. 489-491, 2003.
4. M. Kutateladze and R. Adamia, "Bacteriophages as potential new therapeutics to replace or supplement antibiotics.," *Trends in biotechnology*, vol. 28, no. 12, p. 591, 2010.
5. B. Weber-Dabrowska, et. al., "Alternative therapies in antibiotic-resistant infection.," 2006.
6. L. Da Silva Filho et. al., "PCR identification of *Pseudomonas aeruginosa* and direct detection in clinical samples from cystic fibrosis patients.," *Journal of medical microbiology*, vol. 48, no. 4, pp. 357-361, 1999.
7. E. Pereira et. al., "Staphylococcus aureus, Staphylococcus epidermidis and Staphylococcus haemolyticus: Methicillin-resistant isolates are detected directly in blood

- cultures by multiplex PCR," Microbiological Research, vol. 165, no. 3, pp. 243-249, 2010.
8. M. Merabishvili, et. al., "Characterization of newly isolated lytic bacteriophages active against *Acinetobacter baumannii*," PLoS one, vol. 9, no. 8, 2014.
 9. R. Bonomo and D. Szabo, "Mechanisms of multidrug resistance in *Acinetobacter* species and *Pseudomonas aeruginosa*," Clinical infectious diseases: an official publication of the Infectious Diseases Society of America, vol. 43, no. 2, pp. S49-S46, 2006.
 10. F. D. Lowy, "Mapping the distribution of invasive *Staphylococcus aureus* across Europe," PLoS Medicine, vol. 7, no. 1, 2010.
 11. H. Smith and M. Huggins., "Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics," J Gen Microbiol, vol. 128, no. 2, pp. 307-318, 1982.