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Phage Therapy Is Effective in a Mouse Model of Bacterial Equine Keratitis

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ABSTRACT

Bacterial keratitis of the horse is mainly caused by staphylococci, streptococci, and pseudomonads. Of these bacteria, *Pseudomonas aeruginosa* sometimes causes rapid corneal corruption and, in some cases, blindness. Antimicrobial resistance can make treatment very difficult. Therefore, new strategies to control bacterial infection are required. A bacteriophage (phage) is a virus that specifically infects and kills bacteria. Since phage often can lyse antibiotic-resistant bacteria because the killing mechanism is different, we examined the use of phage to treat horse bacterial keratitis. We isolated *Myoviridae* or *Podoviridae* phages, which together have a broad host range. They adsorb efficiently to host bacteria; more than 80% of the ΦR18 phage were adsorbed to host cells after 30 s. In our keratitis mouse model, the administration of phage within 3 h also could kill bacteria and suppress keratitis. A phage multiplicity of infection of 100 times the host bacterial number could kill host bacteria effectively. A cocktail of two phages suppressed bacteria in the keratitis model mouse. These data demonstrated that the phages in this study could completely prevent the keratitis caused by *P. aeruginosa* in a keratitis mouse model. Furthermore, these results suggest that phage may be a more effective prophylaxis for horse keratitis than the current preventive use of antibiotics. Such treatment may reduce the use of antibiotics and therefore antibiotic resistance. Further studies are required to assess phage therapy as a candidate for treatment of horse keratitis.

IMPORTANCE

Antibiotic-resistant bacteria are emerging all over the world. Bacteriophages have great potential for resolution of this problem. A bacteriophage, or phage, is a virus that infects bacteria specifically. As a novel therapeutic strategy against racehorse keratitis caused by *Pseudomonas aeruginosa*, we propose the application of phages for treatment. Phages isolated in this work had *in vitro* effectiveness for a broad range of *P. aeruginosa* strains. Indeed, a great reduction of bacterial proliferation was shown in phage therapy for mouse models of *P. aeruginosa* keratitis. Therefore, to reduce antibiotic usage, phage therapy should be investigated and developed further.

Ulcerative keratitis frequently occurs in racehorses. Horses have large eyes and a laterally prominent position of the globe. In the racehorse, active movement of the head may increase exposure of the corneas to microbes (1). After a race, horses receive a water eyewash and antibiotic eyedrops. Despite this, some horses may develop bacterial keratitis. Although there are regional differences, bacterial keratitis is mainly caused by staphylococci, streptococci, and pseudomonads (2). *Pseudomonas aeruginosa* is a Gram-negative aerobic bacillus having flagella for motility (3). Moreover, since this pathogen secretes proteolytic enzymes, the keratitis caused by *P. aeruginosa* develops rapidly (4). Antibiotic resistance can occur and, in such cases, keratitis could cause corneal perforation or loss of sight. Horses of the Japan Racing Association (JRA) cannot run in races if both eyes become blind (1). Even if horses recover, their eyes could have granulation tissue, and their sight would be limited.

Antimicrobial resistance in bacteria is a concern not only in veterinary medicine but also in general worldwide (5, 6). In 1928, penicillin was developed and soon after that was used globally. Recently, however, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, multidrug-resistant *P. aeruginosa*, and other resistant bacteria have emerged (7). These bacteria can put the lives of humans and animals at great risk; MRSA is especially widespread in human hospitals. Furthermore, MRSA carriage is potentially an occupational risk for veterinary personnel (8). When antibiotic resistance emerged, novel antibiotics to treat these bacteria were developed. However, it is difficult to develop effective new antibiotics every time a new resistance

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mechanism emerges. Now, many pharmaceutical companies are withdrawing from the antimicrobial development business; therefore, to treat bacterial infections, there is a demand for new strategies involving, e.g., predatory bacteria, antimicrobial peptides, phage therapy, phage endolysins, gene-editing enzymes, and metals (9, 10).

Phage therapy is one of these strategies. A bacteriophage (phage) is a virus that specifically infects and kills bacteria. In 1896, Ernest Hankin reported a bactericidal property associated within Indian river water (11, 12). Thereafter, phages were codiscovered by Felix d'Herelle and Frederick Twort (13), and phage therapy was proposed by d'Herelle in 1931 (14). Many phages isolated previously are classified into the order *Caudovirales* (15). Their size is less than 200 nm, and many are composed of a head, a tail, and a long tail fiber. At the bacterial surface, the long tail fiber recognizes receptor molecules and is adsorbed. The host range is partly determined by the interaction of the long tail fiber and specific receptor molecules, such as the outer membrane protein (Omp) or lipopolysaccharide. After adsorption, in the lytic cycle the phage injects its nucleic acid and, through replication, many new phage are released with bacterial lysis (16). After their discovery, the use of phages for therapy had been studied mainly in Eastern European countries but waned because of the triumph of penicillin. Today, we are globally threatened by antibiotic-resistant bacteria, and phage therapy is being reassessed as a new strategy for treatment of infectious disease (13, 17). Indeed, researchers have reported the efficacy of phage therapy for experimental bacterial disease (18–22). Examples include applications for use of the phages ListShield (Intralytix, Inc., Baltimore, MD) for *Listeria monocytogenes* and EcoShield (Intralytix) for *Escherichia coli* O157:H7 to protect against foodborne disease (23, 24). In addition, not only phage but also phage endolysin, which is used at the end of the phage lytic cycle, is being assessed for therapeutic use (25). The quick approval of more applications of phage therapy for bacterial infections that are hard to be treated is strongly expected.

In the present study, *P. aeruginosa* phages that together have a broad host range were isolated from inflow sewage water, and one of them had the ability to adsorb onto the bacterial surface quickly. In *in vivo* experiments using a mouse model of bacterial horse keratitis, we demonstrated highly effective phage therapy.

MATERIALS AND METHODS

Ethical treatment of animals. This study was carried out in strict accordance with the recommendations in the *Guidelines for Proper Conduct of Animal Experiments*, Science Council of Japan (http://www.scj.go.jp/ja/info/kohyo/pdf/kohyo-20-k16-2e.pdf). The protocol was approved by the Committee on the Ethics of Animal Experiments of Rakuno Gakuen University (approval number VH25A3, approved 21 August 2013). Welfare-related assessments and interventions were carried out prior to, during, or after the experiment.

Animals. Specific-pathogen-free 8-week-old C57BL/6 male mice which had the same weight and were healthy were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan), and housed under pathogen-free conditions with three mice per cage at the animal facility of Rakuno Gakuen University (Hokkaido, Japan). All animals were housed in a temperature-controlled room under a 12 h:12 h light-dark cycle for at least 1 week to acclimate to the surroundings and with free access to food and water.

Bacterial strains and culture media. Bacterial strains were isolated from dogs ("Pa" strains, received from the Laboratory of Food Microbiology and Food Safety at Rakuno Gakuen University) and from horses ("NE-" strains, received from the Equine Research Institute, Japan Racing Association). *P. aeruginosa* strains Pa12, Pa18, Pa26, and Pa50 were used for phage preparation, and *P. aeruginosa* strain NE-126 was used for measurement of the adsorption rate and in infection experiments. Luria-Bertani (LB) medium was used for bacterial or phage culture and for counting CFU of *P. aeruginosa* harvested from eye tissue. SM buffer (10 mM $MgSO_4$, 100 mM NaCl, 0.01% gelatin, and 50 mM Tris-HCl [pH 7.5]) was used for phage dilution. To assess phage plaque formation, LB medium containing 1.5% agar or 0.5% agarose ME (Iwai Chemicals Company, Tokyo, Japan) was used to form the lower and upper layers, respectively.

Isolation of phages. In accordance with a previously reported method (26), phages were isolated from sewage samples, obtained from sewage treatment plants at Sapporo and Ebetsu, Japan, using *P. aeruginosa* strains (strains Pa12, Pa18, Pa26, and Pa50) as hosts. This procedure was approved by these plants (27). Of these phages, ΦR18 and ΦS12-1 were sequenced previously (accession numbers LC102729 and LC102730) (28).

Host specificity of phages. Procedure was carried out according to a preciously reported method (27, 29). A 5-μl portion of a phage suspension (10^{10} PFU/ml) was dropped onto a double-layer agar plate containing a *P. aeruginosa* strain. This was then incubated at 37°C overnight. After incubation, the infected area was characterized as one of four categories: clear plaques, turbid plaques, faint plaques, or no plaques.

Large-scale culture and purification of *P. aeruginosa* phages. A fresh LB broth culture of a *P. aeruginosa* strain was mixed with phage and added to 3 ml of soft LB agar (0.5%). This was then uniformly overlaid onto LB agar (1.5%). The plate was incubated at 37°C overnight. After incubation, 3 ml of SM buffer was added to the plate with plaques, and the plates were statically kept for 1 to 2 h at room temperature. The soft agar layer was scraped off and homogenized with SM buffer by a glass spreader. The homogenized soft agar was collected with SM buffer using a truncated 1-ml pipette tip. This was centrifuged at 10,000 × *g* and 4°C for 5 min. The supernatant was transferred to a new tube, and a drop of chloroform ($CHCl_3$) was added; the samples were then stored at 4°C and subsequently purified by density gradient ultracentrifugation using CsCl.

The phage suspension was placed on top of a discontinuous CsCl gradient (ρ = 1.46, 1.55, and 1.63) and purified by CsCl density gradient ultracentrifugation (RCF [relative centrifuge force]: R_{max} = 111,000 × *g* and R_{av} = 81,900 × *g*; 25°C, 1 h). After the phage band collection, the phage was dialyzed against SM buffer overnight. The phage concentration was measured using a plaque assay.

Electron microscopy. The purified phage sample in SM buffer was loaded onto a collodion membrane and stained with 2% uranyl acetate. Electron micrograph images were obtained with a Hitachi H-800 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan) at 75 kV. Phages were classified according to the report by Ackermann (15).

Adsorption rate of a phage on *P. aeruginosa*. Phage ΦR18 in suspension in SM buffer (1.1×10^7 PFU/ml) was mixed 1:1 with NE-126 cells (1.2×10^9 CFU/ml), followed by incubation at room temperature. Aliquots (200 μl) were taken at specific time points: 15 s, 30 s, and 1, 3, and 5 min. Aliquots were diluted 1:100 with SM buffer. The number of free infectious phage virions in the supernatant of the diluted phage-cell mixture was determined with the double-layer method after the mixture was centrifuged (9,800 × *g*, 10 min).

Mouse model of *P. aeruginosa* keratitis. *P. aeruginosa* strain NE-126 cells were grown in 6 ml of LB medium at 37°C, and the cell number was determined by measuring the optical density at 600 nm (OD_{600}) of the medium. When the density was about 2×10^8 CFU/ml, the culture was centrifuged at 10,000 × *g* for 5 min. The cell pellet was washed with 1 ml of phosphate-buffered saline (PBS) and then recentrifuged under the same conditions. The pellet was resuspended and diluted in PBS to a density of 2×10^6 CFU/ml.

C57BL/6 mice were anesthetized 30 min before infection with a mix-

a

	derivation	serotype	ΦR12	ΦS12-1	ΦS12-3	ΦR18	ΦR26	ΦS50
NE-12	cornea	A	C	C	C	C	C	C
NE-15	cornea	G	N	N	F	C	C	F
NE-16	cornea	G	C	C	C	C	C	C
NE-69	cornea	G	C	C	C	C	C	C
NE-94	cornea	D	N	T	T	T	N	N
NE-95	cornea	G	N	F	F	C	N	N
NE-126	cornea	-	C	C	C	C	C	C
NE-149	cornea	A	C	C	C	F	C	T
NE-150	cornea	D	F	T	C	F	C	T
NE-153	cornea	H	N	N	N	N	N	N
NE-167	cornea	G	T	F	F	T	C	C
NE-193	cornea	-	F	F	N	C	N	N
NE-85	pneumonia	G	C	C	C	C	C	C
NE-98	BALF	I	N	F	F	T	F	F
NE-100	BALF	G	C	C	C	C	C	C
NE-102	BALF	G	C	C	C	T	C	C
NE-112	BALF	D	N	T	C	C	C	C
NE-117	sinusitis	C	C	F	F	N	F	C
NE-120	BALF	G	C	C	C	C	C	C
NE-121	BALF	G	C	C	C	C	C	C
NE-124	guttural pouch empyema	H	N	N	N	N	N	N
NE-125	BALF	B	N	C	C	C	C	C
NE-129	empyema	C	C	C	T	C	T	T
NE-132	lung parenchyma	G	T	C	C	C	C	C
NE-137	lung abscess	-	C	T	T	C	T	T
NE-138	BALF	B	N	N	N	N	N	N
NE-158	dermatitis	-	N	T	F	T	C	T
NE-169	BALF	-	N	F	F	C	C	T
NE-188	sinusitis	-	T	C	C	C	C	C

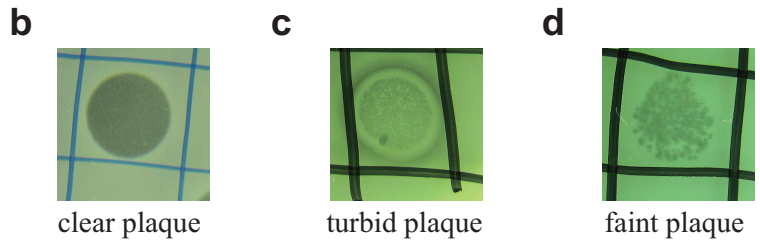

FIG 1 Phage specificity against *P. aeruginosa* isolated from horse lesions. (a) Clear plaques (C, white boxes) indicates combinations resulting in the highest lysis activity, followed by turbid plaques (T, light gray boxes) and faint plaques (F, dark gray boxes). Black boxes indicate combinations yielding no plaque formation (N). (b to d) Representative images of formed spots.

ture of three types of anesthetic agents: 6 mg/kg midazolam (Dormicum; Astellas, Tokyo, Japan), 0.45 mg/kg medetomidin (Dorbene; Kyoritsu Seiyaku Corp., Tokyo, Japan), and 7.5 mg/kg butorphanol (Vetorpale; Meiji Seika Pharma Co., Ltd., Tokyo, Japan). Mouse eyes were infected using a protocol modified from that of Wu et al. (30). Under a stereoscopic microscope, three 1-mm incisions were made in corneas by using a sterile 25-gauge needle. A 5-μl aliquot containing 10^4 CFU of *P. aeruginosa* NE-126 was applied to the corneal surface (control; $n = 11$). Next, 10^9 PFU of phages in 5 μl was applied to the corneal surface 30 min ($n = 16$), 1 h ($n = 10$), 3 h ($n = 9$), 6 h ($n = 10$), or 12 h ($n = 5$) after the initial infection. After these treatments, mice were awakened with 0.9 mg/kg atipamezole (Atipame; Kyoritsu Seiyaku Corp.). The eyes were observed for corneal opacity 24 h after the second infection and then harvested 48 h after the second infection. A multiplicity-of-infection (MOI) study was similarly carried out, except that phage (10^2 PFU [$n = 6$], 10^4 PFU [$n = 6$], or 10^6 PFU [$n = 6$]) were applied 30 min after the application of bacteria (control [$n = 4$]). In a phage cocktail study, the numbers of mice were six for the control group, five for the ΦR18 group, six for the ΦR26 group, and eight for the phage cocktail group.

Determination of the number of viable bacteria in the infected corneas. Individual corneas were homogenized in PBS with a tissue homogenizer. Serial 10-fold dilutions of the samples were plated on

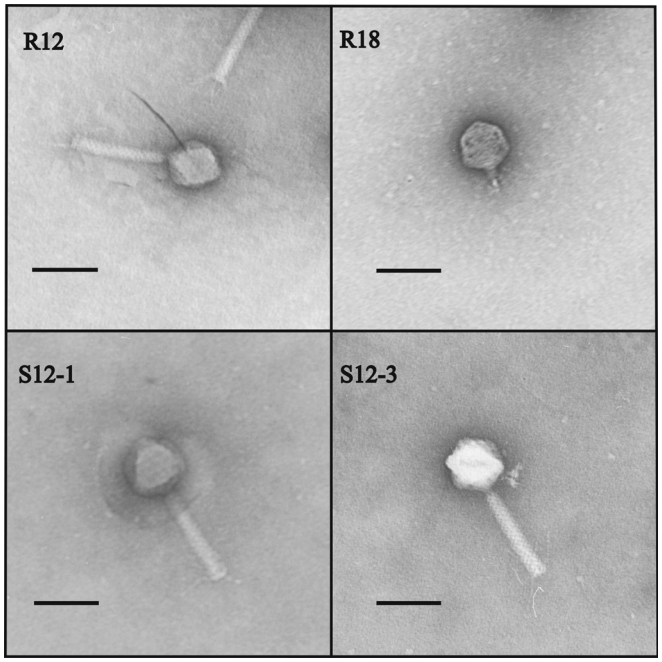

FIG 2 Morphology of phages as observed using electron microscopy. Scale bar, 100 nm. *Myoviridae*: ΦR12, ΦS12-1, and ΦS12-3; *Podoviridae*, ΦR18.

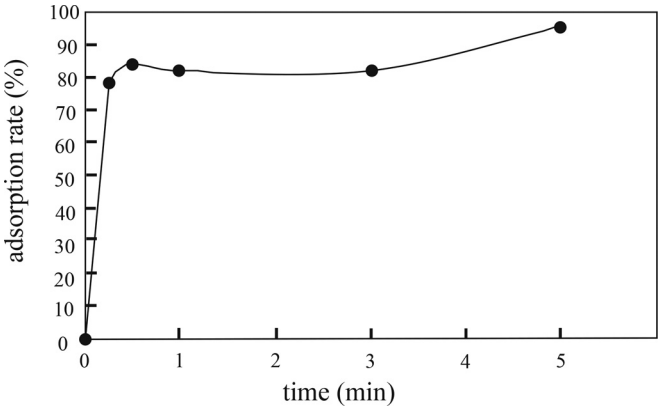

FIG 3 Adsorption rate of ΦR18. The adsorption rate of bacteriophage ΦR18 on *P. aeruginosa* strain NE-126 was measured as described in Materials and Methods.

LB agar and cultured at 37°C for 24 h. The results are reported as CFU per eye.

Coculture of phages and bacteria. The examination was carried out according to the method of Synnott et al. (29) with slight modifications. *P. aeruginosa* strain NE-149 (10^7 CFU/ml) and phages ΦR18 or ΦR26 (10^9 PFU/ml) or cocktailed phages (5×10^8 PFU/ml each) in 4 ml of LB medium were cultured at 37°C using a TVS062CA biophotorecorder (Advantec, Tokyo, Japan) with shaking at 40 rpm. The OD_{660} was monitored at 30-min intervals for a minimum of 24 h. The examination was repeated three times for each.

Analysis of data. All statistical analysis was performed using R software, version 3.2.3 (https://www.R-project.org/), for Windows. Data were analyzed using analysis of variance with Dunnett's multiple-comparison test or the Tukey multiple-comparison test.

RESULTS

Host specificity of phages. First, to collect phages from various *P. aeruginosa* strains, we obtained *P. aeruginosa* isolates that caused disease in dogs and horses. The phages ΦR12, ΦS12-1, ΦS12-3, ΦR18, ΦR26, and ΦS50 were isolated from *P. aeruginosa* strains Pa12, Pa18, Pa26, and Pa50 (see Fig. S1 in the supplemental material). The host range of the phages was examined using various *P. aeruginosa* strains isolated from a horse's lesions on various parts of the body (Fig. 1a). The result of the experiments to determine the host range of each phage was 82.8% (24/29) clear plaque formation. Regarding serotypes, we assumed that phage lytic activity did not correlate. Representative pictures of formed spots are shown in Fig. 1b to d. Of these phages, ΦR18 had a relatively broad specificity. Therefore, we used ΦR18 in the experiments to measure adsorption rate and to determine *in vivo* effects of phage therapy.

Electron microscopy. Of the six phages, ΦR12, ΦS12-1, ΦS12-3, and ΦR18 were observed by using electron microscopy and classified morphologically (Fig. 2). ΦR12, ΦS12-1, and ΦS12-3 had a polyhedral head (78.8 ± 2.0, 91.7 ± 0.8, and 84.0 ± 1.3 nm in diameter, respectively) and a contractile tail (147.5 ± 2.3, 154.2 ± 0.8, and 148.3 ± 1.3 nm in length, respectively). Therefore, they belonged to the family *Myodoviridae*. In contrast, ΦR18 had a polyhedral head 78.5 ± 1.5 nm in diameter and a short tail of 29.8 ± 1.6 nm in length. Therefore, this phage was assigned to the family *Podoviridae*.

Adsorption rate of a phage on *P. aeruginosa*. When phage suspensions are dropped into the eye, they must be adsorbed to bacteria quickly before running out of the eye in tears. Therefore, we measured the adsorption rate of ΦR18 on *P. aeruginosa* strain NE-126, which was isolated from a racehorse exhibiting keratitis. At 30 s, more than 80% of the ΦR18 was adsorbed to the host cells (Fig. 3). This suggested that the phage had adsorbed to the bacteria very effectively, which was promising for its potential use to prevent keratitis.

Effects of bacteriophage in a mouse model of *P. aeruginosa* keratitis. To estimate the efficacy of phage therapy, we carried out an *in vivo* experiment using a mouse model of *P. aeruginosa* keratitis. Mice were treated according to the protocol schedule described above (Fig. 4a). Examination of the eyes showed that phage administration 30 min after infection prevented keratitis (Fig. 4b). Bacterial counts showed that the phage was effective until 3 h after infection (Fig. 4c and Table 1). These results showed that phage treatment was effective using the current schedule for eyewash and administration of antibiotics after races.

Effective MOI range. Phage efficacy was also investigated with regard to the bacterium/phage ratio. The bacterial count decreased depending on the phage number or PFU (Fig. 5). The results suggest that an MOI of 100 was sufficiently effective and that, compared to the control group, bacterial proliferation was suppressed 99.78%.

Efficacy of cocktailed phages. Using a phage cocktail has several advantages. First, because many phages have diverse host ranges, a cocktail of phages could be effective in diverse strains of bacteria. Second, there could be synergism between cocktailed phages (23). Third, the use of mixed phages could prevent the emergence of phage-resistant bacteria (24). Therefore, we investigated whether our cocktailed phages were effective. After considering the practicalities, two phages (ΦR18 and ΦR26) and one bacterial strain, *P. aeruginosa* NE-149, were used. ΦR18

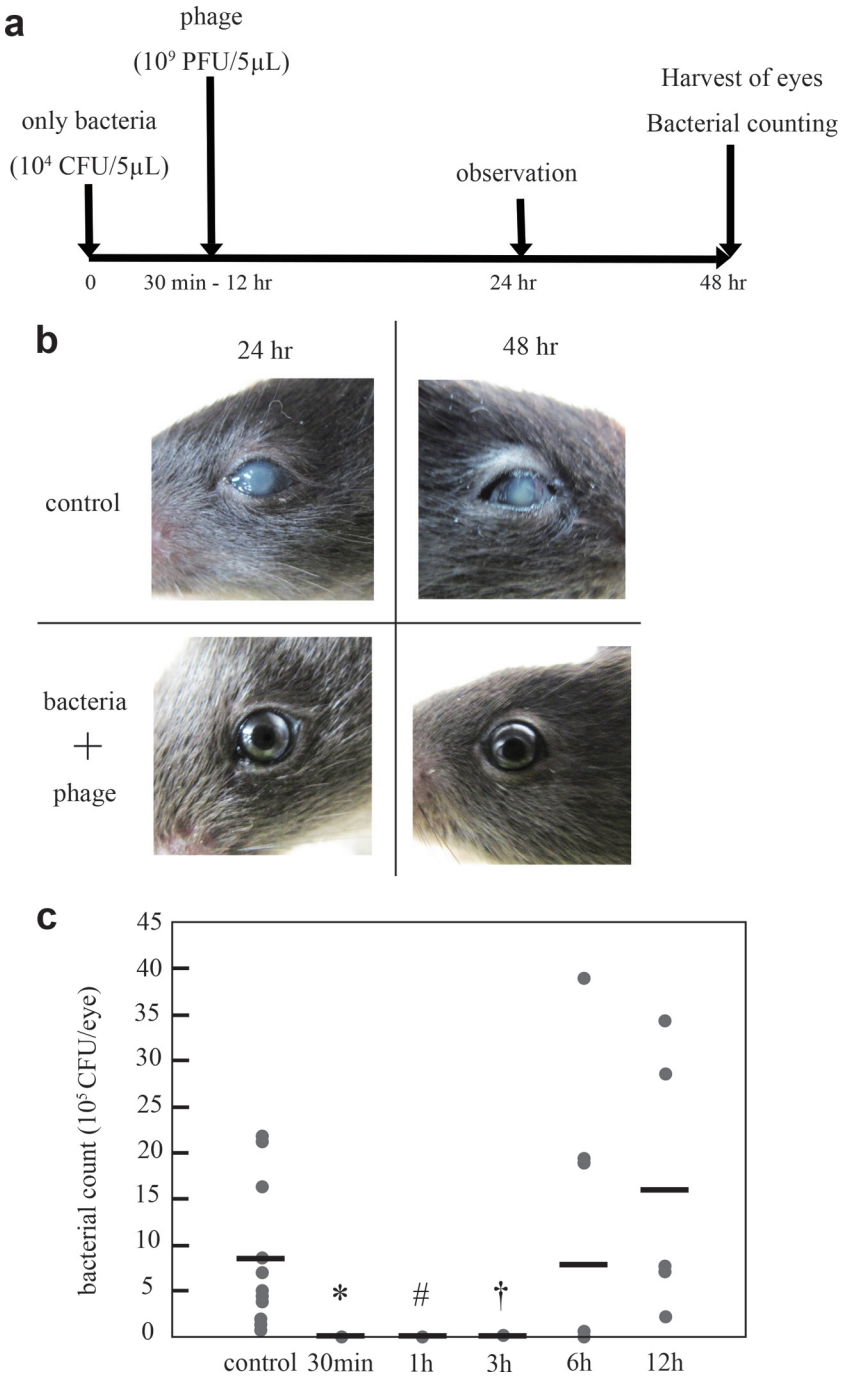

FIG 4 Effects of bacteriophages on mouse model of *P. aeruginosa* keratitis. (a) The experiment was carried out according to this schedule. (b) Typical corneal images 24 and 48 h after infection treated with bacteria only or bacteria/phages. (c) Efficacy of a single dose of phage. Statistically significant differences between control values and phage-treated values are denoted by various symbols: *, $P < 0.05$); #, $P = 0.054$; or †, $P = 0.068$ (determined using Dunnett's test). Circles represent the data obtained from each mouse; horizontal bars represent mean values calculated from all mice in each group.

alone formed only faint plaques, but ΦR26 formed clear plaques, against strain NE-149. The results are shown in Fig. 6: ΦR18 suppressed bacterial multiplication to 20.1% and ΦR26 suppressed bacterial multiplication to 0.22% compared to the control group. The two-phage cocktail suppressed bacterial multiplication to 0.004%.

Coculture of phages and bacteria. We carried out an experiment wherein phages and bacteria were cultured together to confirm the emergence of resistance against phages. The abilities of ΦR18, ΦR26, and the cocktailed phages to lyse *P. aeruginosa* bacteria that cause racehorse keratitis were tested using liquid LB medium cultures (Fig. 7). The OD_{660} of the bacterium-only control increased immediately, followed by that of ΦR18. The OD_{660} of ΦR18 increased slightly more slowly than the control; the peak

TABLE 1 Actual bacterial counts[a]

Control	30 min	1 h	3 h	6 h	12 h
190,000	2,400	0	0	1,890,000	3,430,000
850,000	0	0	100	3,890,000	750,000
1,640,000	0	8,400	100	13,000	730,000
490,000	0	0	63,000	1,000	210,000
710,000	0	0	4,500	0	2,850,000
100,000	0	10	2,390	3,000	
148,000	0	140	4,900	0	
2,180,000	0	360	620	24,000	
430,000	0	10	12,400	1,930,000	
380,000	0	180		12,000	
2,120,000	0				
	0				
	3,000				
	0				
	0				
	5,400				

[a] The actual bacterial counts per eye are shown. The 30-min, 1-h, and 3-h treatment groups showed reduced bacterial counts compared to the other groups. (There is no correspondence between the specific values in each individual row.)

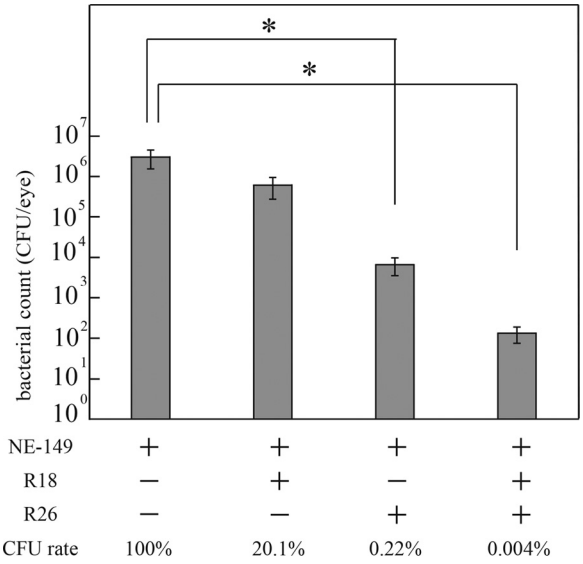

FIG 6 Efficacy of cocktailed phages. The corneas were exposed to 10^4 CFU of *P. aeruginosa strain* NE-149 and 10^9 PFU of phage R18 and/or phage R26. The bacterial counts of the control group and the phage groups were determined. The CFU rate is shown as a percentage of the control CFU. Statistically significant differences between control values and phage-treated values are denoted by asterisks (*, $P < 0.05$ [determined using Tukey's multiple-comparison test]). Bars indicate standard errors of the means.

was at 10 h and then decreased. ΦR26 and the cocktailed phages repressed the proliferation of bacteria until 8 h.

DISCUSSION

One of the main symptoms of the keratitis caused by *P. aeruginosa* is ring abscesses on the corneal surfaces. When the condition worsens, ulcerative keratitis can develop (1, 4). In addition, antibiotic-resistant bacteria have been detected during treatment (1, 31, 32). Novel treatments for infectious diseases are now required. To address this situation, we assessed the potency of phage for prevention of racehorse keratitis. In the present study, phages showed remarkable efficacy against bacterial keratitis caused by *P. aeruginosa*. Phage ΦR18, isolated from sewage samples, prevented bacteria from replicating on the corneal surface if it was administered in eyedrops within 3 h after bacterial inoculation. Furthermore, cocktailed phages showed no interference and covered the other phage. Based on these results in a mouse keratitis model, we demonstrated the possibility that phage therapy for racehorse keratitis may be useful.

First, we isolated six phages that have different host ranges from sewage samples. The phage recognizes bacterial cell wall structures; therefore, these phages could recognize distinct parts of these structures (16). That is to say, a phage which has a broad host range would recognize conserved bacterial structures. In some *P. aeruginosa* strains, our phages produced no clear plaques. Therefore, these strains may not have surface structures in common with most *P. aeruginosa*; alternatively, it is possible that after the phages infected the bacteria, these strains prevented phage replication. Serotype surface antigens may play a role. Group H *P. aeruginosa* might have uncommon structures and, on the other

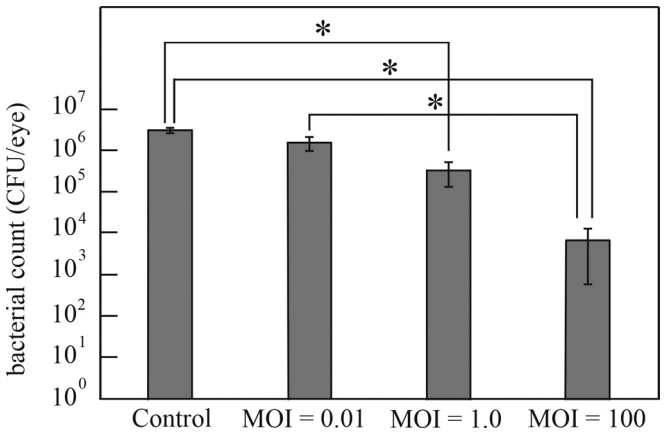

FIG 5 Effective MOI range. The corneas were exposed to 10^4 CFU of *P. aeruginosa* strains NE-126 and 10^2 PFU (MOI = 0.01), 10^4 PFU (MOI = 1), or 10^6 PFU (MOI = 100) of the various phages. The efficacy of the phages was indicated by bacterial counts 48 h after infection. Statistically significant differences between control values and phage treatment values are denoted by asterisks (*, $P < 0.05$ [determined using Tukey's multiple-comparison test]). Bars indicate the standard errors of the means.

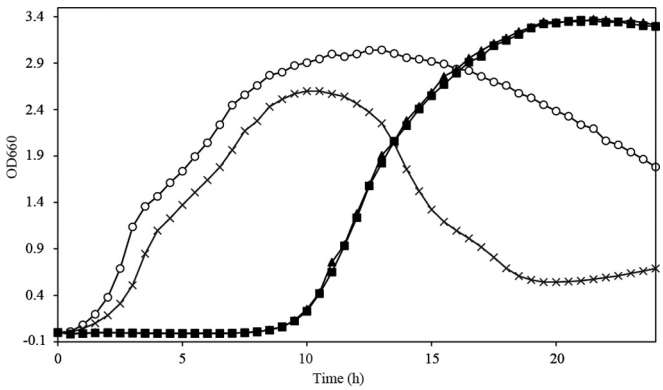

FIG 7 Coculturing bacteriophages and *P. aeruginosa*. The bacterial strain is NE-149. Symbols: ○, bacterium-only controls; ×, ΦR18 added to bacteria; ▲, ΦR26 added to bacteria; ■, both ΦR18 and ΦR26 added to bacteria.

hand, group G *P. aeruginosa* might have relatively conserved structures. In a clinical situation, these bacteria will be a problem for the use of phage therapy. To lyse these bacteria, phages effective for all *P. aeruginosa* strains that we have, at least, should be identified and collected. These phages may recognize important features of the *P. aeruginosa* surface. Using a mixture of phages would lyse more *P. aeruginosa* strains and prevent the development of phage-resistant bacteria (29). The phages used in the present study lysed *P. aeruginosa* from horses exhibiting keratitis and also from animals with other diseases; therefore, phage therapy may potentially be applied widely.

According to the classification by Ackermann (15), phages with contractile tails are *Myoviridae*, those with long and noncontractile tails are *Siphoviridae*, and those with short tails are *Podoviridae*. These tailed phages, the *Caudovirales*, comprise 96% of all phages isolated (15). On the other hand, filamentous phages belonging to the genus *Inovirus* infect Gram-negative bacteria through the bacterial pilus (33). In the present study, electron microscopy showed that ΦR12, ΦS12-1, and ΦS12-3 are *Myoviridae* and that ΦR18 is a member of the *Podoviridae*. To use phage therapeutically, production of phage-neutralizing antibody would not be avoided (34). It has been reported that the production of antibody is associated with host immunity and phage type (35). If phage therapy is applied, whether the genome sequence contains virulence factors should first be determined. The phages examined in the present study have none of the already-known virulence factors (28).

For JRA horses, an eye ointment containing a mixture of erythromycin and colistin is prophylactically used as an eyewash after a race. Generally, eyedrops and eye ointment do not retain an effective antibiotic concentration for a long time. For example, eyedrops are effective for only 30 min because the antibiotics wash out with tears (36). In the present study, the phage used had a high adsorption efficiency. Indeed, phage can proliferate if host bacteria exist. Phage therapy probably could replace the present antibiotic treatment as prophylaxis.

Administration of phage within 3 h after infection mostly eradicated the pathogen. Therefore, if a horse's eyes were washed immediately after a race, phages could replace the antibiotics currently used. On the other hand, 6 and 12 h after infection, the phages did not prevent bacterial growth. It would be reasonable to suppose that since *P. aeruginosa* invaded the deep corneal stromal, phages could not infiltrate there. *P. aeruginosa* are motile and produce proteolytic enzymes, such as elastase B, alkaline metalloprotease (37), protease IV (38), and *P. aeruginosa* small protein (39), and have a type III secretion system (40). These enzymes enable *P. aeruginosa* to rapidly cause keratitis and to invade deep layers. In addition, the mouse model of *P. aeruginosa* keratitis was established, and a similar pathology would occur in racehorse keratitis.

The *in vivo* experiment described above was carried out with 10^9 PFU/5 μl phage and 10^4 CFU/5 μl *P. aeruginosa* suspensions. However, we found that phages are required at 100 times the bacterial concentration, at least, to be sufficiently effective. In a clinical situation, there would be little chance that 10^4 CFU bacteria infect an eye at a given time. Therefore, when phages are used for prevention of keratitis, fewer PFU would be enough for effective therapy.

Cocktailed phages also were very effective in the prevention of bacterial proliferation. Phages forming clear plaques and those forming faint plaques did not interfere with each other. Cocktailing phages with combined broad host ranges would lyse more *P. aeruginosa* strains.

Culturing bacteria with ΦR18 showed resistance to ΦR18 immediately, but the bacterial proliferation was slightly inhibited compared to the control (Fig. 7). ΦR26 and cocktailed phages, on the other hand, prevented resistant bacteria for longer times than did ΦR18. These results were relatively correlated with host specificity of spot test data (Fig. 1) and the *in vivo* keratitis model experiment (Fig. 6). However, the differences in lysis activity between R26 and cocktailed phages *in vitro* experiments were not clear. The bacterial proliferation speed during *in vitro* experiments is much faster than that in the body because of rich nutrition and lack of inhibition. Therefore, the clear effect of cocktailed phage *in vitro* experiments might not be seen. These mechanisms are further issues that should be investigated in the future.

Although antibiotics have been successful for the treatment of various bacterial infections, investigators in Eastern European countries, especially Georgia, have diligently continued to study phage therapy (17). Phage therapy probably will have many advantages for modern veterinary medicine and thus is worthy of continued study. First, since the mechanisms of phage lysis are different from antibiotic mechanisms, phage therapy can be effective for infections caused by antimicrobial-resistant bacteria. Second, if phages are used instead of antibiotics, the amount of antibiotics used decreases. Third, the numbers of veterinarians carrying antibiotic-resistant bacteria will also decrease. As a result, the application of phage therapy may actually restore the effectiveness of antibiotics.

Many researchers have studiously examined phages and their encoded endolysins. Endolysin is produced at the end of the phage lytic cycle. This enzyme cleaves bacterial peptidoglycan, lysing bacteria osmotically. Furthermore, endolysin-resistant bacteria have never been reported. Therefore, the therapeutic application of endolysin is very attractive (41). Previously, we identified endolysins encoded by ΦR18 and ΦS12-1 phages. Both endolysins were composed of lysozyme-like domains (27, 28). Since some investigators study recombinant or designed endolysins (41, 42), we will also examine them.

In summary, we demonstrated that the phages used in this study rapidly adsorbed to *P. aeruginosa* and widely killed *P. aeruginosa* isolates from horse lesions. Furthermore, one of the phages was shown to completely prevent keratitis in a keratitis mouse model. These results lead us to expect a higher efficiency for phage prophylaxis, compared to the current preventive use of antibiotics, for the treatment of horse keratitis. The use of phage may lead to a reduction in the use of antibiotics and may also reduce the numbers of antibiotic-resistant bacteria. Further studies are needed to fully develop methods for phage purification and applications for phage therapy.

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