NOTE *Public Health*

Bacteriophage can lyse antibiotic-resistant *Pseudomonas aeruginosa* isolated from canine diseases

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ABSTRACT. *Pseudomonas aeruginosa* is a pathogen frequently identified as the cause of diverse infections or chronic disease. This microbe has natural resistance to several kinds of antibiotics, because of the species' outer membrane, efflux pumps and growth as a biofilm. This bacterium can acquire increased resistance with specific point mutations. Bacteriophage (phage), however, can lyse these bacteria. Therefore, in the present study, we assessed the host range of phages isolates and their ability to lyse antibiotic-resistant *P. aeruginosa*. Present phages could lyse many strains of *P. aeruginosa* (28/39), including strains with high resistance to fluoroquinolones (4/6). In conclusion, application of phages for antibiotic-resistant bacteria is greatly effective. To avoid pervasive antibiotic-resistant bacteria, further development of phage usage for disease treatment is required.

KEY WORDS: antibiotic-resistant bacteria, bacteriophage, fluoroquinolone, *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a motile gram-negative aerobic bacillus [19]. This pathogen has been isolated as the causative agent in wound or pulmonary infection, urinary tract infection and septicemia in human beings [18]. In dogs, *P. aeruginosa* typically is responsible for otitis externa, chronic deep pyoderma, wound infection and urinary tract infection [5, 12]. The bacterium has high endogenous resistance to many antibiotics, because of the species' outer membrane barrier, multidrug efflux pumps, endogenous antibiotic inactivation and growth as a biofilm [13]. In recent years, *P. aeruginosa* isolates with elevated resistance to fluoroquinolones have emerged, with mechanisms including increased efflux pump expression and specific point mutations in the *gyrA* and *parC* target genes [4, 7, 9, 12, 14, 15, 17]. These isolates confound the clinical treatment of associated infections.

Bacteriophages (phages) are viruses, originally discovered by F. Twort in 1915 and F. d'Herelle in 1917 individually, that specifically infect bacteria [2, 3]. Phages typically are composed of heads with associated tails and tail fibers [1]. At the bacterial surface, the phages with the tail fibers recognize receptor molecules and adsorb to the bacterial surface. After adsorption, the phages inject its DNA, and following replication, phages are released by bacterial lysis [6]. The mechanism of bacterial lysis by phage is distinct from that of drugs, such as antibiotics. Therefore, phage therapy is not expected to be subject to the accumulation of resistance that has reduced the efficacy of most antibiotics [3].

In this study, we assessed 2 phages, isolated from sewage samples, for their ability to lyse various *P. aeruginosa* strains, including those having resistance to fluoroquinolones. These phages lysed a broad range of *P. aeruginosa* strains, notably including isolates with high levels of resistance to enrofloxacin and orbifloxacin.

The bacterial strains used in this study consisted of 39 strains of *P. aeruginosa* isolated from canine skin diseases (otitis externa, chronic deep pyoderma and wound infection). These bacteria were classified using the random amplified polymorphic DNA (RAPD) method (Supplemental Fig. 1). The procedure was performed according to the method of Mahenthiralingam *et al.*, with RAPD primer 272 (5′-AGCGGGCCAA-3′) [10]. Reaction mixtures (20 *µl*) consisted of 40–60 *n*g of *P. aeruginosa* genomic DNA, 16 pmol of primer, 2 U of Taq polymerase (New England BioLabs Ltd., Ipswich, MA, U.S.A.), 2.5 mM (each) deoxynucleoside triphosphate (Takara Bio Inc., Otsu, Japan) and 10× ThermoPol™ Reaction Buffer. Each reaction mixture was amplified using a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, U.S.A.) as follows: (i) 5

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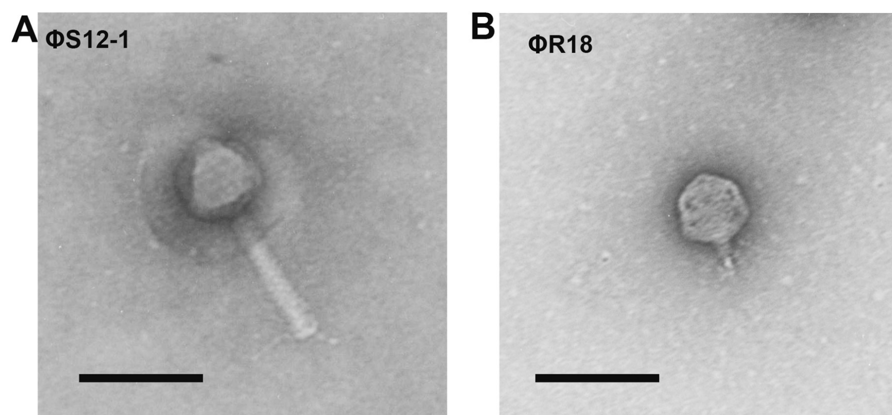

Fig. 1. Morphology of phages: electron microscopy. Bar: 100 nm. (A) ΦS12-1: *myoviridae*. (B) ΦR18: *podoviridae*. ΦS12-1 has an isometrically hexagonal head, 91.7 ± 0.8 nm in diameter and a contractile tail of 154.2 ± 0.8 nm in length (n=3). ΦR18 has an isometrically hexagonal head, 91.7 ± 0.8 nm in diameter, and a short tail of 78.5 ± 1.5 nm in diameter and 29.8 ± 1.6 nm in length (n=10).

min at 94°C; (ii) 27 cycles of amplification, each consisting of 1 min at 94°C, 1 min at 42°C and 2 min at 72°C; and (iii) a final extension at 72°C for 10 min. The products then were separated by electrophoresis in 2% agarose gels. The *P. aeruginosa* strains, Pa12 and Pa18, were used for phage preparation.

Phages were isolated via plaque assay from sewage samples using one of two *P. aeruginosa* strains (Pa12 and Pa18) as host. The phage name designation therefore incorporates the "Pa" number of the host bacterial strain. Sewage samples had been collected from sewage-treatment plants at Sapporo and Ebetsu, Japan. Briefly, 2L of sewage sample was filtered to remove the bacteria and then centrifuged with 10% Polyethylene glycol (PEG) [#6000] and 4% NaCl. The precipitant with phages was dissolved by 4 m*l* of SM buffer (10 mM MgSO4, 100 mM NaCl, 0.01% gelatin and 50 mM Tris-HCl [pH 7.5]).

For electron micrographic imaging, phage samples purified with CsCl density gradient ultracentrifugation (RCF (relative centrifuge force); r_{max} 111,000 × *g*, r_{av} 81,900 × *g* and 25°C, 1 hr) in SM buffer were loaded onto collodion membranes 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 [1] (Fig. 1). ΦS12-1 belonged to family *myoviridae* (Fig. 1A), and ΦR18 belonged to *podoviridae* (Fig. 1B). In addition, these DNAs were submitted to Hokkaido System Science Co., Ltd., for whole genome sequencing. The samples were sequenced as paired-end reads on the Illumina Hiseq 2500 (Illumina, Inc., Hercules, CA, U.S.A.), revealing the following parameters: ΦR18 (accession number; LC102729) is 63.56 kbp in length, with 60.35% GC content, 86 open reading frames and 97% identified with phage KPP25 (accession number; AB910393); ΦS12-1 (accession number; LC102730) is 66.351 kbp in length, with 55.58% GC content, 94 open reading frames and 97% identified with phage vB_PaeM_PAO1_Ab27 (accession number; LN610579).

Characterization of lysis was performed according to the previously reported method [16], which permitted rapid assessment of the degree of bacteriolysis by determination of the morphology of an infected area. Specifically, a volume of 5 μl of a phage suspension (10^{10} plaque forming units/m*l* cultured with each host *P. aeruginosa* strain in Luria-Bertani (LB) media) was dropped onto a double-layer LB agar plate containing 100 μl of an overnight-cultured *P. aeruginosa* strain. This plate was incubated at 37°C overnight. The infected area then was classified as belonging to one of four spot morphologies: clear spots, turbid spots, faint spots or no spots (Table 1, Supplemental Fig. 2). Following scoring, we purified the phage away from bacteriolysis-related material by CsCl density gradient ultracentrifugation (the condition was described above).

Table 1 shows the host range of phages and minimum inhibitory concentrations (MICs) of antibiotics against each *P. aeruginosa* strain. The MICs of enrofloxacin and orbifloxacin were determined as the concentrations required to completely suppress the growth of *P. aeruginosa*. Briefly, serial dilution was employed to generate solutions of antimicrobial agents at 125 to 0.125 μg/m*l* in LB medium, and aliquots (100 μl/well) were transferred to the wells of a 96-well microplate. Samples of *P. aeruginosa* that had been grown overnight were diluted to approximately 10^7 colony-forming units per m*l* and 10 μl aliquots of these suspensions and then were added to each well of a 96-well microplate. After incubation at 35°C for 16–20 hr, the MIC was defined as the lowest concentration at which the antimicrobial agent inhibited any visible turbidity in the well. For MIC determination, each test was performed in triplicate (Table 1). Pa17, Pa22, Pa53, Pa60, Pa61 and Pa63 were highly resistant (>128 μg/m*l*) to both enrofloxacin and orbifloxacin. For enrofloxacin, the MIC_{50} (MIC which prevented the growth of 50% of strains) and MIC_{90} (MIC which prevented the growth of 90% of strains) values were 2 and >128 μg/m*l*, respectively. For orbifloxacin,

Table1. Phage specificity against *P. aeruginosa* isolated from dog disease

Bacterial strains	MIC		Phage	
	ERFX (*μ*g/m*l*)	OBFX (*μ*g/m*l*)	ΦS12-1	ΦR18
Pa1	1	4	C	N
Pa4	1	4	T	C
Pa7	1	4	C	N
Pa8	1	4	N	N
Pa11	2	8	C	F
Pa12	2	8	C	C
Pa14	1	4	C	C
Pa16	4	16	N	N
Pa17	>128	>128	N	N
Pa18	0.5	2	N	C
Pa22	>128	>128	N	N
Pa25	4	16	T	F
Pa26	4	16	N	F
Pa27	4	32	T	T
Pa29	2	8	N	N
Pa34	8	16	N	N
Pa38	1	4	N	N
Pa42	2	4	C	F
Pa43	8	32	T	N
Pa44	0.5	2	N	N
Pa49	8	>128	N	F
Pa50	8	>128	N	T
Pa51	1	4	C	N
Pa52	2	8	N	T
Pa53	>128	>128	N	T
Pa54	2	8	T	C
Pa56	2	8	C	F
Pa57	2	8	C	N
Pa58	2	2	N	C
Pa59	0.5	4	T	N
Pa60	>128	>128	N	C
Pa61	>128	>128	C	C
Pa63	>128	>128	N	C
Pa64	0.5	2	C	T
Pa65			N	T
Pa66	4	8	F	C
Pa67	0.5	8	T	N
Pa68	0.5	2	F	N
Pa70			N	C

Clear spot (C) indicates the highest lysis activity, followed by turbid spot (T), faint spot (F) and no spot (N). Abbreviations: MIC, minimum inhibitory concentration; ORFX, orbifloxacin; ERFX, enrofloxacin.

the MIC_{50} and MIC_{90} values were 8 and >128 *μ*g/m*l*, respectively. Regarding isolation of phage, ΦS12-1 was isolated in a first round of screening using *P. aeruginosa* strain Pa12. In a subsequent round, new phage was isolated from aliquots of the same sewage samples, this time using *P. aeruginosa* strain (Pa18) against which the previous phage isolate did not display lysis. The hosts used in this second round permitted the isolation of ΦR18. Combined results of the experiments to determine the host range of each phage yielded 48.7% (19/39) "clear" spot formation and 71.8% (28/39) "clear" or "turbid" spot formation. Phages in this study were also effective against *P. aeruginosa* strains with high resistance to fluoroquinolones, except for strains, Pa17 and Pa22.

Individual phage isolates are expected to recognize distinct bacterial surface structures, such as outer membrane protein or lipopolysaccharide [6]. ΦR18 appeared to have overlapping host ranges (distinct from those of ΦS12-1), suggesting that ΦR18 was adsorbed to a shared (but distinct) cell wall component that was conserved in the respective host strains. *P. aeruginosa* strains that were not lysed by phages isolated in this study presumably lack the targeted cell wall structure or possess post-infection mechanisms for preventing phages multiplication and/or release [8]. For this reason, new phages that lyse bacteria that fail to serve as hosts for these isolates should be identified, and these various phages then could be used in combination.

Phages employ a variety of mechanisms to lyse host bacteria; some of these mechanisms remain unknown. The first important step of lysis is the adsorption of the phages to the bacterial cell wall. Phages can recognize and bind to distinct protein and/or sugar chains. These related molecules of host and phage mutate in an ongoing "arms race" against each other. Moreover, there are multiple steps leading to the lysis of the host, including insertion of the phage genome, replication of the phage DNA, phage gene expression, assembly of the progeny phages and degradation of the host cell wall by endolysin to permit the release of the progeny phages. Notably, this last step is necessary for killing of the host. Therefore, the overall process of bacteriolysis is not related to serotype. The morphology of the spot partly reflects aspects of the phage lytic process as mentioned above. That is, phages that generate clear plaques generally have high efficiency of bacteriolysis and are expected to show high effectiveness for therapeutic use. However, many factors (including blood and various body fluid components) are known to affect the *in vivo* bacteriolytic activity of phage. There is a possibility that phages will serve better for treatment of surface diseases, such as pyoderma, rather than for treatment of internal diseases. Phage therapy may be more appropriate for treatment of surface diseases, since killing of bacteria in such diseases may evade various physical factors and host immunity. Many further studies will be needed for the application of phages as clinical therapies.

On April 30, 2014, WHO released a statement of concern regarding the emergence of antibiotic-resistant bacteria (http://www.who.int/mediacentre/news/releases/2014/amr-report/en/). Phages used in this study were effective for killing of fluoroquinolone-resistant bacteria, because phage-killing employs a mechanism distinct from that used by antimicrobials. Phage therapy has attracted researchers' attention as a treatment with potential efficacy against antibiotic-resistant bacteria [11].

In conclusion, we isolated phages having strong lytic activities against a broad range of *P. aeruginosa* strains, including highly fluoroquinolone-resistant isolates. To avoid a problem with phage-resistant (or non-adsorbing) bacteria, we must isolate additional phages that lyse diverse *P. aeruginosa* strains, and we will need to investigate more effective usage of phages as anti-bacterial agents.

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