Emerging Microbes & Infections
2021, VOL. 10
https://doi.org/10.1080/22221751.2021.1902754

Pre-optimized phage therapy on secondary *Acinetobacter baumannii* infection in four critical COVID-19 patients

Nannan Wu [a]†, Jia Dai[a]†, Mingquan Guo[a,b]†, Jianhui Li[a]†, Xin Zhou[a]†, Feng Li[c], Yuan Gao[d], Hongping Qu[e], Hongzhou Lu [f], Jing Jin[g], Tao Li[h], Lei Shi[i], Qingguo Wu[c], Ruoming Tan[e], Mingli Zhu[d], Lan Yang[a], Yun Ling[j], Shunpeng Xing[d], Jianzhong Zhang[k], Bangxin Yao[k], Shuai Le [a,l], Jingmin Gu[a,m], Jinhong Qin [a,n], Jie Li[a], Mengjun Cheng[a], Demeng Tan[a], Linlin Li[a], Yiyuan Zhang[a], Zhaoqin Zhu[b], Jinfeng Cai[b], Zhigang Song[o], Xiaokui Guo[a,n], Li-Kuang Chen [a,p] and Tongyu Zhu [a,q]

[a]Shanghai Institute of Phage, Shanghai Public Health Clinical Center, Fudan University, Shanghai, People's Republic of China; [b]Department of Laboratory Medicine, Shanghai Public Health Clinical Center, Fudan University, Shanghai, People's Republic of China; [c]Department of Respiratory Medicine, Shanghai Public Health Clinical Center, Fudan University, Shanghai, People's Republic of China; [d]Department of Critical Care Medicine, Ren Ji Hospital, Shcool of Medicine, Shanghai Jiao Tong University, Shanghai, People's Republic of China; [e]Department of Critical Care Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China; [f]Department of Infectious Diseases and Immunology, Shanghai Public Health Clinical Center, Shanghai, People's Republic of China; [g]Xituo Biotechnology Co., Ltd, Zhengzhou, People's Republic of China; [h]Department of Tuberculosis, Shanghai public health clinical center, Fudan University, Shanghai, People's Republic of China; [i]Department of Hospital Infection Management, Shanghai Public Health Clinical Center, Fudan University, Shanghai, People's Republic of China; [j]Department of Infectious Disease, Shanghai Public Health Clinical Center, Fudan University, Shanghai, People's Republic of China; [k]Department of Pharmacy, Zhongshan Hospital Fudan University, Shanghai, People's Republic of China; [l]Department of Microbiology, Army Medical University, Chongqing, People's Republic of China; [m]Key Laboratory of Zoonosis Research, Ministry of Education, College of Veterinary Medicine, Jilin University, Changchun, People's Republic of China; [n]School of Global Health, Chinese Center for Tropical Diseases Research, Shanghai Jiao Tong University School of Medicine, Shanghai, People's Republic of China; [o]Department of Pathogen Diagnosis and Biosafety, Shanghai Public Health Clinical Center, Shanghai, People's Republic of China; [p]Department of Laboratory Medicine, Clinical Pathology, Buddhist Tzu Chi General Hospital, Hualien, Taiwan; [q]Shanghai Key Laboratory of Organ Transplantation, Zhongshan Hospital Fudan University, Shanghai, People's Republic of China

ABSTRACT

Phage therapy is recognized as a promising alternative to antibiotics in treating pulmonary bacterial infections, however, its use has not been reported for treating secondary bacterial infections during virus pandemics such as coronavirus disease 2019 (COVID-19). We enrolled 4 patients hospitalized with critical COVID-19 and pulmonary carbapenem-resistant *Acinetobacter baumannii* (CRAB) infections to compassionate phage therapy (at 2 successive doses of 10^9 plaque-forming unit phages). All patients in our COVID-19-specific intensive care unit (ICU) with CRAB positive in bronchoalveolar lavage fluid or sputum samples were eligible for study inclusion if antibiotic treatment failed to eradicate their CRAB infections. While phage susceptibility testing revealed an identical profile of CRAB strains from these patients, treatment with a pre-optimized 2-phage cocktail was associated with reduced CRAB burdens. Our results suggest the potential of phages on rapid responses to secondary CRAB outbreak in COVID-19 patients.

ARTICLE HISTORY Received 24 January 2021; Revised 2 March 2021; Accepted 8 March 2021

KEYWORDS Phage therapy; carbapenem-resistant *Acinetobacter baumannii*; nosocomial infections; COVID-19

Introduction

Bacteriophage therapy is recognized as a promising alternative to antibiotics in treating pulmonary bacterial infections [1–3], however, its use has not been reported for treating secondary bacterial infections during virus pandemics such as coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Severely ill patients with COVID-19 are under the threat of hospital-acquired multidrug-resistant (MDR) bacterial infections, which typically cause longer ventilator occupancy and poorer clinical outcomes than viral infections alone [4–6]. Carbapenem-resistant *Acinetobacter baumannii* (CRAB) is an ubiquitous nosocomial pathogen which ranked in the top critical priority list of MDR bacteria guiding new antibiotics development [7]. Alternative therapeutic modalities, as well as novel methods for rapid

CONTACT Nannan Wu wunannan@shphc.org.cn Shanghai Institute of Phage, Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, People's Republic of China; Li-Kuang Chen likuangchen@gmail.com Department of Laboratory Medicine, Clinical Pathology, Buddhist Tzu Chi General Hospital, Hualien 970, Taiwan; Tongyu Zhu tyzhu@shphc.org.cn Shanghai Key Laboratory of Organ Transplantation, Zhongshan Hospital Fudan University, Shanghai 200032, People's Republic of China
†These authors contributed equally to this work.

Supplemental data for this article can be accessed at https://doi.org/10.1080/22221751.2021.1902754

surveillance of MDR bacterial infections, are in demand. Here, we report findings from a case series study, that assessed the efficacy and safety of compassionate phage therapy on secondary CRAB infections in patients hospitalized with critical COVID-19. While both phage susceptibility testing and multilocus-sequence typing (MLST) determined the same genotype of the CRAB strains from these patients, treatment with 2 successive doses of pre-optimized 2-phage cocktail was associated with reduced CRAB burdens and may have contributed to the clinical improvement of the patients.

Materials and methods

Study design

This study was conducted in an ICU specialized for COVID-19 patients in Shanghai Public Health Clinical Center. The study protocol was modified for critical COVID-19 patients for emergency use from an ongoing case-series study (ChiCTR1900020989, an amendment of the study protocol to shed excess exclusion criteria and make possible the enrolment of critically ill patients, was approved by the Ethics Committee of Shanghai Public Health Clinical Center on 28 March, 2019, Approval No. 2017-S027-08). All patients in the ICU with CRAB positive in bronchoalveolar lavage fluid or sputum samples were eligible for study inclusion if antibiotic treatment failed to eradicate their CRAB infections. Over the study period from 1 March to 28 April 2020, four critically ill men aged 62–81 years were enrolled after their guardians signed to give informed consent. The administered route, dose, and frequency of antibiotic treatment are determined by the clinical need, and phage therapy does not alter antibiotic treatment or other treatments. Assessments of clinical and laboratory data were collected directly from the medical reports. Phage susceptibility tests of isolated CRAB strains were performed in the designated laboratory by professional staff wearing biosafety level III personal protective equipment. The endpoint of the study is the elimination of target bacteria and/or patient discharge from ICU or death.

Phage susceptibility assay (phage-typing)

The susceptibilities of the target bacteria to 124 phages in our library were measured by spot assays [8]. All potential CRAB strains from a single sample distinguished in size, colour, and viscosity of the colonies were analysed. Random selection of 3 bacterial colonies was an alternative if no apparent distinction was observed. The lytic efficacy was assessed by the clarity of plaques, scored as transparent, opaque, or no plaque. Phage ϕAb124 (*Podoviridae,* GenBank ID: MT633129), distinguished by producing a large transparent plaque against all the baseline CRAB isolates from the 4 patients, was selected as the "first-line" phage for therapeutic use.

Next evolution phage-typing (NEPT)

NEPT was introduced to preview the potential phage resistance of target bacteria and screen additional phage(s) for its prevention. Briefly, a 3-mL logarithmic phase culture of the original CRAB isolate in LB medium was infected with 10^7 plaque-forming unit (PFU) of ϕAb124 and cultured for 8 h at 37 °C with aeration. The emergent ϕAb124-resistant CRAB strain(s) was subjected to another phage-typing. Among phages producing transparent plaques against the emergent strain, ϕAb121 (*Myoviridae,* GenBank ID: MT623546) was selected as the "second-line" phage for therapeutic use because of its marked differences from ϕAb124 (phage-typing profile, morphology, and genetic background) and its *in vitro* synergism with ϕAb124.

Phage preparation and administration

Phages were grown on their original host using solid media and recovered by diffusion into SM buffer (5.8 g/L NaCl, 20 mM Tris HCl pH 7.5, 2 g/L $MgSO_4$.-$7H_2O$), yielding lysates with titres of $>1 \times 10^9$ pfu/mL. Phage particles were purified using CIM® Anion-exchange column QA (BIA Separations, Slovenia) according to the manual. The concentrate was dialysed against 0.9% sodium chloride physiological solution (Shandong Qidu Pharmaceutical) 3 times for a minimum of 3 h each [9]. The resulting phage-containing solution was sterilized through a 0.22 μm filter, aliquoted and packaged at the Good Manufacturing Practice (GMP) facility of Zhongshan Hospital of Fudan University, Shanghai, China. ϕAb124 was titrated using the CRAB strain isolated from Patient 1 at 3 days before treatment, while ϕAb121 was titrated using the ϕAb124-resistant strain isolated from Patient 1 the day after ϕAb124 treatment. Single ϕAb124 (1ϕ) or the cocktail (2ϕ) was diluted in saline to 10 mL with a concentration of 10^8 PFU/mL of each phage, and administered via the respiratory or topical route within 20 min, by using a vibrating-mesh nebulizer (Aerogen Solo) connected to the dry side of the humidifier attached to the ventilator (Evita V300, Dräger) or through a gauze pad. For each course of treatment, 2 doses of lytic phages were administered successively with an interval of 1 h.

Primary and secondary indicators

The primary indicators were changes in CRAB load and in bacterial anti-phage resistance from baseline through the first 24 h after treatment, measured by semi-quantitative streak-plate method and phage

susceptibility assay, respectively. The secondary indicators include clinical status on a 7-point ordinal scale [10] (1. dead, 2. hospitalized, on extracorporeal membrane oxygenation or invasive mechanical ventilation, 3. hospitalized, on noninvasive ventilation or high flow oxygen devices, 4. hospitalized, requiring supplemental oxygen, 5. hospitalized, not requiring supplemental oxygen, 6. not hospitalized, but unable to resume normal activities, 7. not hospitalized, with resumption of normal activities) at baseline, 7, 14 and 30 days after treatment, changes in inflammatory indicators including chest radiographies, white blood cells, neutrophils, procalcitonin and hypersensitive C-reactive protein before and after phage therapy.

Multilocus sequence typing (MLST)

MLST was performed to analyse the clonal relatedness of CRAB strains from the studied patients. The fragments of housekeeping genes used by the Oxford and the Pasteur MLST schemes were both amplified, sequenced and analysed following the protocol on the MLST website (http://pubmlst.org/abaumannii/).

Safety

All patients were treated in the ICU setting and safety was assessed using clinical and laboratory parameters, including fever, vital sign monitoring, comprehensive metabolic panels and serial blood examinations (ie, leukocytosis, cytokine storm). Each patient was followed closely by the Shanghai COVID-19 Clinical Treatment Expert Group to assess pathogenic agent, clinical resolution and improvement of infection (i.e. bacterial burden, radiological findings, sputum production), as well as the development of adverse event.

Results

Patient characteristics

From 1 March 2020, through 28 April 2020, four critically ill men aged 62–81 years were enrolled to compassionate phage therapy after their guardians signed the informed consent (Figure 1). Details regarding baseline characteristics before phage therapy can be found in Table 1. Notably, all patients were indicated to be SARS-CoV-2 free by the absence of detectable viral RNA in throat swabs and the positive antiviral IgG/IgM detections in sera. While opportunistic bacterial and fungal infections were sporadically detected, CRAB infections that emerged consecutively in Patient 4, Patient 1, Patient 2, and Patient 3, were the longest existed and the most serious antibiotic-resistance pathogen. CRABs were continuously detected in the sputum and bronchoalveolar lavage fluid, and can be found also in the urine of Patient 4, Patient

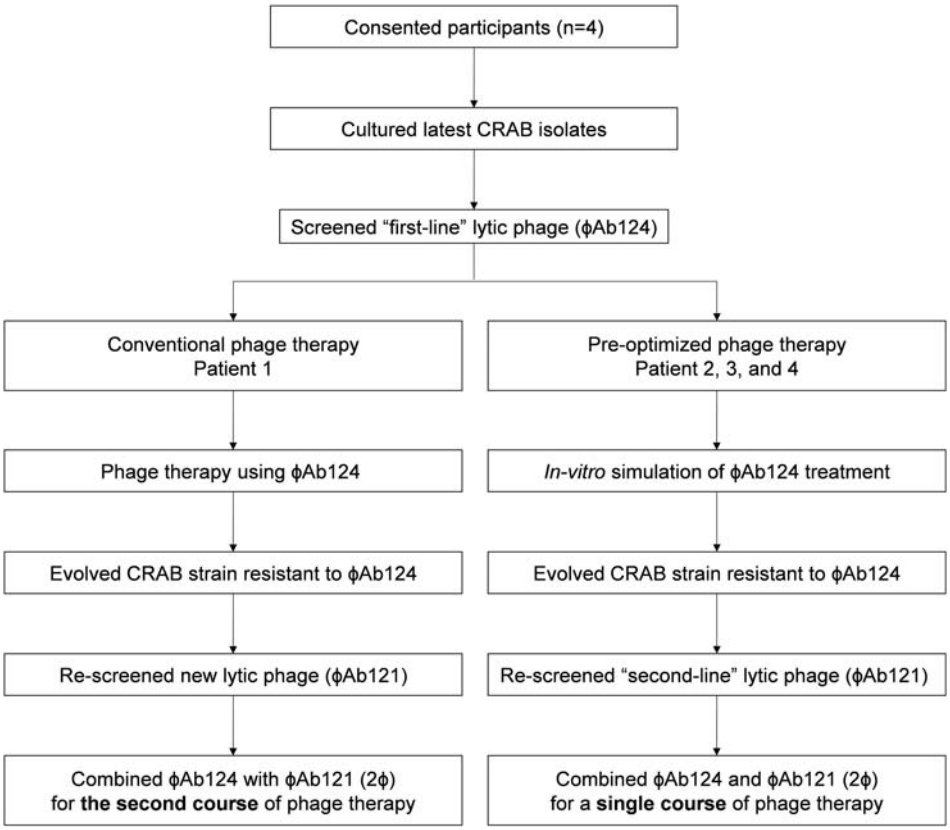

Figure 1. Study diagram. Flow diagram of the conventional phage therapy procedure for Patient 1 and the pre-optimized procedure (via next evolution phage-typing, NEPT) for the rest 3 patients.

Table 1. Baseline characteristics, treatments, and outcomes of the patients*.

	Patient 1		Patient 2		Patient 3	Patient 4
Characteristics at baseline						
Age-year	62		64		81	78
SARS-CoV-2 status (RNA, Antibodies)[a]	(Negative, Positive)		(Negative, Positive)		(Negative, Positive)	(Negative, Positive)
CRAB infections (days after admission to ICU)	Respiratory tract (18)		Respiratory tract (21)	Wound (34)[b]	Respiratory tract (40)	Respiratory tract (18)
Days before phage therapy	11	38	1		6	50
CRAB detected samples	Sputum, BALF	Sputum, BALF	Secretion		Sputum, BALF	Sputum, BALF, Urine
Coexisting infections	*Candida albicans*	*Candida albicans, Ralstonia mannitolilytica*	ND		*Candida albicans, Candida glabrata*	*Candida albicans, CSKP, Sphingomonas paucimobilis*
Antibiotic and life-support treatment						
Principal antibiotics (Susceptibility)	CFP/SUL (S/I)		CFP/SUL (S/I), TGC (S), LEV (S/I)		MPM (R), CFP/SUL (S/I)	TGC (S), IPM (R), CFP/SUL (S/I)
Life-support status	MV, ECMO		MV, ECMO		MV, ECMO, CRRT	MV
Phage therapy						
Therapeutic phages	1ϕ	2ϕ	2ϕ	2ϕ	2ϕ	2ϕ
Administrated route	Inhalation	Inhalation	Inhalation	Wet compress	Inhalation	Inhalation
Primary indicators						
Semi-quantitative CRAB (24 h before, after)[c]	(4+, 2+)	(4+, 2+)	(3+, 2+)	(4+, Negative)	(4+, Negative)	(4+, 2+)
Phage resistance of evolved CRAB	Yes	Yes	Yes	NA	NA	Yes
Adverse event	fever, IL-6&IL-8 storm	ND	ND	ND	ND	ND
Outcome	Discharged from hospital Day 30		Discharged from hospital Day 9		Died Day 10	Discharged from ICU Day 7, Died Day 40

*SARS-CoV-2, Severe acute respiratory syndrome coronavirus-2; CRAB, Carbapenem-resistant Acinetobacter baumanni; ICU, Intensive care unit; BALF, Bronchoalveolar lavage fluid; ND, Not detectable; CSKP, Carbapenem-resistant Klebsiella Pneumoniae; CFP/SUL, Cefoperazone-sulbactam; TGC, Tigecycline; LEV, Levofloxacin; MPM, Meropenem; IPM, Imipenem; S, Susceptible; R, Resistant; I, Intermediate; MV, Mechanical ventilation; ECMO, Extracorporeal membrane oxygenation; CRRT, Continuous Renal Replacement Therapy; 1ϕ, ϕ124; 2ϕ, ϕ124+ϕ121; NA, Not applicable.
[a]SARS-CoV-2 RNA status in throat swab and IgG/IgM status in serum.
[b]Wound infection on the jugular intubation site of ECMO.
[c]Semi-quantitative burden of bacteria was measured by streak-plate method.

2 also acquired a topical infection at the jugular incision of extracorporeal membrane oxygenation (ECMO) intubation. Several high-grade antibiotics had been applied to these patients since the detection of CRAB (6–50 days before phage therapy) but had failed to eliminate it. The latest CRAB isolates from all patients showed an identical profile of phage susceptibility that *podoviral* ϕAb124 was the sole phage with effective lytic activity (Figure 2).

Conventional phage therapy

Patient 1 was the first participant and he firstly received a course of ϕAb124 via nebulization. A decline in semi-quantitative CRAB burden in suctioned sputum was observed, however, a resurgence was soon emerged, which was resistant to ϕAb124 but was susceptible to dozens of other phages in our phage library, among which we selected *myoviral* ϕAb121 to incorporate ϕAb124 for the second course of treatment (Figure 2).

Pre-optimized phage therapy

We adopted the next evolution phage-typing (NEPT) strategy for *in vitro* simulation of bacterial anti-ϕAb124 resistance. We discovered that randomly selected ϕAb124-resistant colonies derived from the original CRAB isolates of the patients displayed similar phage-lysis profiles highly matched with the resistant clone that emerged in Patient 1 after ϕAb124 treatment (Figure 2). Thus, a phage cocktail (2ϕ) that contained ϕAb124 to target the original strain and ϕAb121 tailored to kill the evolved ϕAb124-resistant bacteria, was used for the ensuing phage therapies (Figure S1). The *in vitro* killing curve assay indicated a synergistic effect between ϕAb124 and ϕAb121 in efficiently suppressing the recurrence of target bacteria within 8 h (Figure S3).

Clinical outcomes

Phage therapy was followed by a decline in semi-quantitative CRAB burden in all treatments (Table 1), however, bacterial anti-phage resistance was observed in 4 out of 6 treatments. After a single course of phage inhalations, a minute improvement of the chest radiographs was observed in Patient 1 and 2, while Patient 3, and 4's pulmonary condition was unchanged (Figure S2A). Patient 2's CRAB was also detected at the jugular incision of ECMO intubation and this

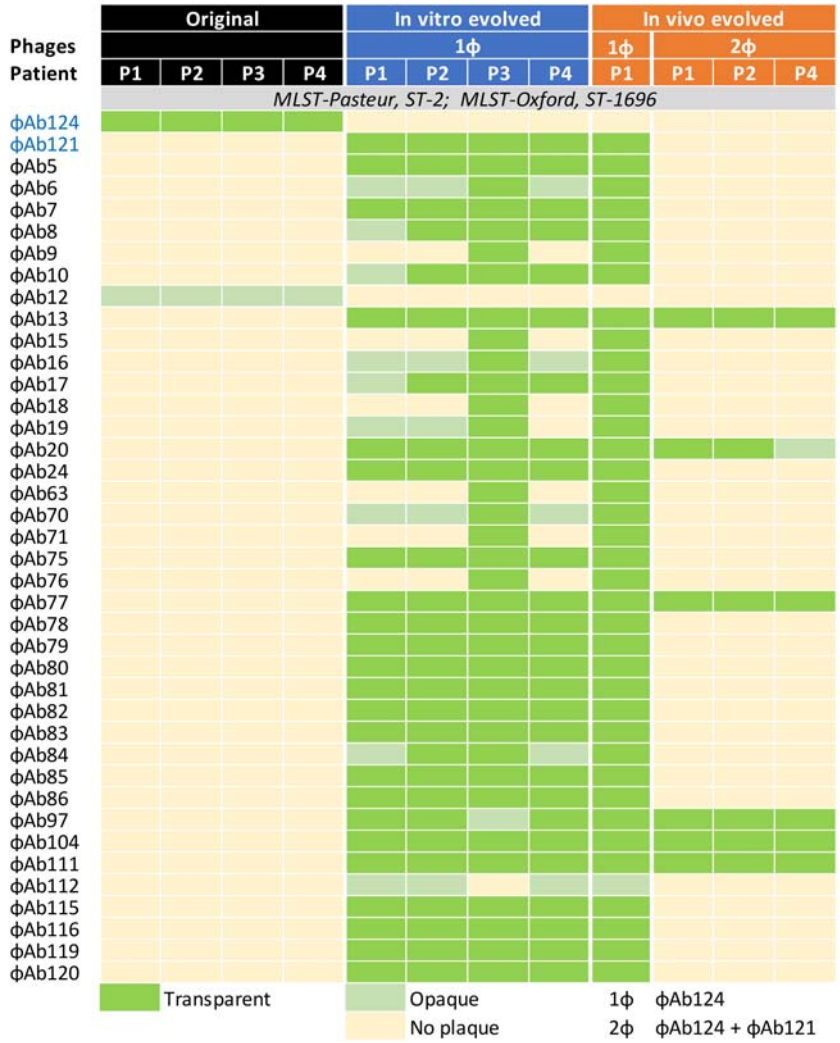

Figure 2. Changes of bacterial phage susceptibility profiles upon *in-vivo* and *in-vitro* phage challenges. Representative CRAB isolates and their phage-resistant derivates induced by phage therapy or *in-vitro* next evolution phage-typing (NEPT) were analysed by Phage-typing and multilocus-sequence typing (MLST) using both the Pasteur and the Oxford schemes.

was categorized as a potentially serious complication because the secretion might approach and invade the jugular vein, thus, 2ϕ was emergently applied via wet compress, in the following days the secretion was absorbing without CRAB detection and the skin converted to normal and dry (Figure S2B).

At the end of this study, Patient 1 and Patient 2 were weaned from ECMO and subsequently discharged from the hospital; Patient 4's illness severity was improved and he was discharged from ICU on day 7 post phage inhalation, however, he died of respiratory failure a month later; Patient 3's CRAB was eliminated but an un-subdued Carbapenem-resistant *Klebsiella Pneumoniae* (CRKP) infection was followed and he died of respiratory failure 10 days after phage therapy (Table 1, Figure 3).

Advert events

Patient 1 had experienced an atypical cytokine storm at 4 h post ϕAb124 administration. This was

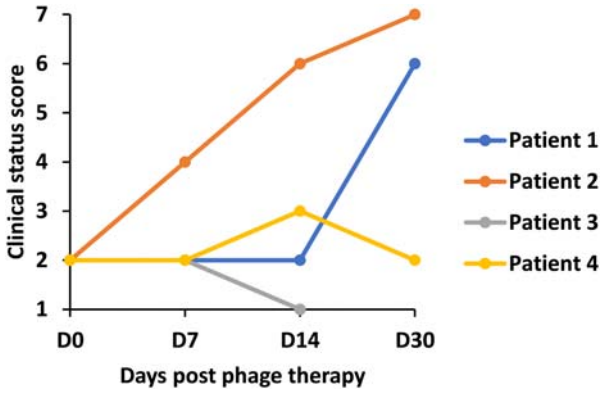

Figure 3. Changes of clinical status within 30 days post phage therapy. Clinical status on a 7-point ordinal scale (1. dead, 2. hospitalized, on extracorporeal membrane oxygenation or invasive mechanical ventilation, 3. hospitalized, on noninvasive ventilation or high flow oxygen devices, 4. hospitalized, requiring supplemental oxygen, 5. hospitalized, not requiring supplemental oxygen, 6. not hospitalized, but unable to resume normal activities, 7. not hospitalized, with resumption of normal activities) was measured at baseline, 7, 14 and 30 days after treatment.

manifested as a transient fever along with a dramatic increase in interleukins 6 and 8 (IL-6 up to 596.32 pg/mL and IL-8 up to 112.05 pg/ mL), but other cytokines have stayed in the reference scope. A day later IL-6 and IL-8 returned to the normal levels (Table S1).

Discussion

To our knowledge, this study describes the first therapeutic use of phages for the secondary bacterial infections in COVID-19 patients and the first clinical application of NEPT strategy to preview bacterial anti-phage resistance.

Bacterial anti-phage resistance is one of the major obstacles for phage therapy [3,11]. In this study, despite the *in vitro* proof of synergistic effect between ϕAb124 and ϕAb121, phage resistance was observed in 4 out of 6 treatments. Nevertheless, bacterial gain of anti-phage resistance might be accompanied by losing advantages in other respects such as virulence [12]. Patient 1 and Patient 2 who saw clinical improvement in chest radiographs were finally discharged from hospital. In contrast, conventional antibiotic treatment had been tried previously and failed to suppress CRAB infection or improve the condition of these patients. Suggesting that phage therapy may have contributed to the recovery of the patients.

Other factors affecting clinical outcome may include phage penetration into the infected site and the host immune state. Phages and the immune system may work synergistically to get rid of bacterial infection [13]. Results from animal model revealed the importance of host immune system in facilitating phage-mediated bacterial elimination [14]. In critically ill COVID-19 patients, the injured respiratory system and impaired immune responses can dramatically reveal the inadequacy of available antibiotic treatments [15,16], may also impair the efficiency of phage delivery and bacterial elimination.

Identifying the source of infection and the route of transmission is crucial for effective disease containment. Conventional bacterial typing methods such as Pulse field gel electrophoresis (PFGE), MLST and whole-genome sequencing are costly and time-consuming [17]. In this report, a single phage-typing can be achieved within 8 h and the NEPT protocol only takes twice the time. Our results also indicate that, on the basis of the epidemiological connections and available phage collections, phage susceptibility assay may serve as a rapid and cost-effective method for bacterial typing, as well as a routine database to monitor nosocomial infections and as a prelude to preparing ready-to-use phages. However, the frequent shift in phage susceptibility should be counted and may be overcome by pre-performed NEPT database. Additional whole-genome alignment can be helpful to validate the bacterial phage-typing but is expensive.

Previous studies have demonstrated that, compared to antibiotic treatment, the rapid lysis of bacteria by phages leads to less endotoxin release from gram-negative organisms and does not increase the innate inflammatory response [18,19]. However, Patient 1 experienced an incident resembling a dramatic wave in IL-6 and IL-8 at four hours post ϕAb124 inhalation, which is distinguished from the previously described patterns of a cytokine storm [20,21]. Risk prevention measures against cytokine storm should be considered during phage therapy.

Our exploratory study is of small size because of the containment of the COVID-19 outbreak in Shanghai as well as the control of CRAB outbreak in our center. Nevertheless, we report here a rewarding application of phages in treating and tracing MDR bacterial infections in ventilator-supported patients. These results may open new opportunities for the prevention and treatment of bacterial secondary infection, a should be an indispensable part of pandemic planning and management.

Acknowledgments

We thank Dr Douglas B. Lowrie and Dr Chen Zhao from Shanghai Public Health Clinical Center for critically proofreading the manuscript. We thank all members of the Shanghai COVID-19 Clinical Treatment Expert Group for thoughtful discussions. We also thank our patients, and the health professionals for providing outstanding patient care at considerable personal risk. This work was funded by the Shanghai Public Health Clinical Center (grant SJTNY to T.Z., grant RCJJ2019-06 to L.K.C.), National Major Science and Technology Projects of China (grant 2020ZX09201001-005-003 to N.W.), and Shanghai Commission of Science and Technology (grant 20Y11900300 to N.W.).

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

This work was supported by National Major Science and Technology Projects of China [grant number 2020ZX09201001-005-003]; Shanghai Municipal Science and Technology Commission [grant number 20Y11900300]; Shanghai Public Health Clinical Center [grant number RCJJ2019-06,SJTNY].

ORCID

Nannan Wu http://orcid.org/0000-0003-1244-0030
Hongzhou Lu http://orcid.org/0000-0002-8308-5534
Shuai Le http://orcid.org/0000-0003-3748-5454
Jinhong Qin http://orcid.org/0000-0001-6712-9472
Tongyu Zhu http://orcid.org/0000-0002-6697-4209
Li-Kuang Chen http://orcid.org/0000-0002-6343-6026

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