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(54) Title: BACTERIOPHAGE COCKTAIL TO TARGET DIVERSE LINEAGES OF MULTIDRUG-RESISTANT KLEBSIELLA PNEUMONIAE

(57) Abstract: Herein, *Klebsiella pneumoniae* phage cocktails comprising a lytic phage EKq1 newly isolated on *Klebsiella quasipneumoniae* are introduced. A 5-phage cocktail KPM1 comprises five *K pneumoniae* phages including EKq1, EKq2, AFR4, KEN39, KEN42, and the efficacy of KPM1 phage cocktail is demonstrated in carbapenem-resistant *K pneumoniae* strain-infected mice model. Also, a 6-phage cocktail KPM1-1 is introduced, which further comprises EKp148 in addition to the five core KPM1 phages. The cocktails can be used as main ingredients of pharmaceutical compositions either alone or in combination with antibiotics. Also, methods of using the same for the treatment of respiratory infections caused by *K pneumoniae* are presented.



Bacteriophage cocktail to target diverse lineages of multidrug-resistant *Klebsiella pneumoniae*

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invention.

REFERENCE TO ELECTRONIC SEQUENCE LISTING

The application contains a Sequence Listing which has been submitted electronically in
10 .XML format and is hereby incorporated by reference in its entirety. Said .XML copy, created
on October 15, 2024, is named "15969039PC0_seq_listing.xml" and is 740,376 bytes in
size. The sequence listing contained in this .XML file is part of the specification and is hereby
incorporated by reference herein in its entirety.

15 BACKGROUND

1. Field of the Invention

The present invention is directed to the field of antibacterial phages for the treatment of
multidrug resistant *Klebsiella pneumoniae* infection, and in particular to antibacterial phage
cocktails comprising newly isolated *K. pneumoniae* phage Ekq1.

20 2. Background

About 1.27 million people died from drug-resistant bacterial infections in 2019, such as
multidrug-resistant (MDR), extensively drug-resistant (XDR), or pan-drug resistant (PDR)
ESKAPEE infections (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*,
Acinetobacter baumannii, *Pseudomonas aeruginosa*, *Enterobacter spp.*, and *Escherichia coli*),
25 which cause a major threat to public health, especially for people who are immunocompromised
or critically ill.

Klebsiella pneumoniae is a ubiquitous gram-negative bacterium and frequently causes hospital-acquired bacteremia, pneumonia, urinary tract, and other infections, with a high rate of drug resistance. The death rates of *K. pneumoniae* infection are 50% from pneumoniae and 20-40% from bloodstream infection (septicemia), and WHO listed carbapenem-resistant and 3rd generation cephalosporin-resistant *K. pneumoniae* as a critical priority pathogen in 2024. Because of the fast global spread of multidrug-resistant (MDR) and hypervirulent *K. pneumoniae*, there is a growing need for alternative or adjunctive antibacterial treatments.

Bacteriophages (phages) are natural enemies of bacteria and generally safe, and phage therapy has been identified as a valid approach to treat *K. pneumoniae* infection (Herridge et al., 2020, Eskenazi et al., 2022, and Doub et al., 2022).

However, *K. pneumoniae* is a highly diverse pathogen, and *K. pneumoniae* phages usually have limited host ranges (Kęsik-Szeloch et al, 2013). Most of *K. pneumoniae* phages are active against 1-5% of strains, and such narrow specificity of phages and high diversity of bacterial isolates complicate the development of durable phage therapeutics.

SUMMARY

The purpose of the study presented in this disclosure is to design and preclinically test a phage cocktail containing a few phages targeting recent MDR *K. pneumoniae* isolates from military hospitals in the U.S. and other countries. It is intended here to provide a fixed and modularized cocktail of bacteriophages for the treatment of combat trauma-associated infections caused by MDR *K. pneumoniae*.

There are problems when developing anti-bacterial phages. First, broadly phage-susceptible strains cause very limited morbidity and do not cause lethality, which is shown in Examples using a mouse lung model without carbapenems (CP) treatment (phage-neutrophil synergy). Second, hypervirulent *K. pneumoniae* strains are phage-resistant.

However, it has been suggested that phages with broader activity can be isolated on near-neighbor species (Jensen et al., 1998 and Wu et al., 2007). Therefore, in this disclosure, a *K. pneumoniae* phage, EKq1 was isolated using a near-neighbor species of *K. pneumoniae*, i.e., *Klebsiella quasipneumoniae*, and the phage EKq1 was proved capable of lysing some MDR *K. pneumoniae* clinical isolates.

Herein, phage cocktails comprising EKq1 are introduced. Those phage cocktails comprise other 4 or 5 compatible *Klebsiella* phages in addition to EKq1.

In one aspect, a phage cocktail comprising five *Klebsiella* phages (KPM1) is provided, which comprises AFR4, KEN39, KEN42, Ekq2, and Ekq1.

5 In another aspect, a phage cocktail comprising six *Klebsiella* phages (KPM1-1) is provided, which comprises EKp148 in addition to the core 5 phages of KPM1.

In another aspect, another six *Klebsiella* phage cocktail (KPM2) is introduced, which comprises phage KEN18-2-1 instead of EKp148 in addition to the core 5 phages of KPM1

10 In another aspect, a seven *Klebsiella* phage cocktail (KPM3) is introduced, which comprises a recombinant phage 15882-3 generated by *in vitro* phage evolution procedure using a phage mixture comprising KEN22, KEN25, KEN37, and KEN39 *K. pneumoniae* phages as well as phage KEN18-2-1 in addition to the core 5 phages of KPM1

15 The cocktails can be used as main ingredients of pharmaceutical compositions, and as standard- or adjunctive treatment against MDR, XDR, and PDR *K. pneumoniae* infection either alone or in combination with antibiotics. Also, methods of using the same for the treatment of respiratory infections caused by *K. pneumoniae* is presented.

For antibacterial therapeutic purposes, other phage variants comprising a nucleic acid sequence of at least 97% sequence identity to the sequence of those phages aforementioned, or a fragment thereof can also be contemplated for being included in a phage cocktail.

20 In a certain aspect, a phage cocktail comprising a smaller number of phages can be contemplated. That is, a 2-phage cocktail, 3-phage cocktail, or 4-phage cocktail may be considered if they infect the same range of host bacterial strains, which will reduce production cost. Further, each phage can also be used individually as an adjunctive means to antibiotic(s) approach for the treatment of MDR *K. pneumoniae* infections.

25 In one embodiment, EKq1 phage is presented, which comprises a nucleotide sequence of SEQ ID NO: 4, a variant thereof having at least 97% sequence identity, or a fragment thereof.

In another embodiment, EKp148 phage is presented, which comprises a nucleotide sequence of SEQ ID NO: 6, a variant thereof having at least 97% sequence identity, or a fragment thereof.

5 In another embodiment, KEN18-2-1 phage is presented, which comprises a nucleotide sequence of SEQ ID NO: 7, a variant thereof having at least 97% sequence identity, or a fragment thereof.

In another embodiment, phage 15882-3, a recombinant phage of KEN22, KEN25, KEN37, and KEN39 is presented, which comprises a sequence of SEQ ID NO: 8, a variant thereof having at least 97% sequence identity, or a fragment thereof.

10 In a certain embodiment, a 5- phage cocktail (KPM1) is provided, comprising: AFR4 phage comprising a nucleotide sequence of SEQ ID NO:1, a variant thereof having at least 97% sequence identity, or a fragment thereof;

KEN39 phage comprising a nucleotide sequence of SEQ ID NO:2, a variant thereof having at least 97% sequence identity, or a fragment thereof;

15 KEN42 phage comprising a nucleotide sequence of SEQ ID NO:3, a variant thereof having at least 97% sequence identity, or a fragment thereof;

EKq1 phage comprising a nucleotide sequence of SEQ ID NO:4, a variant thereof having at least 97% sequence identity, or a fragment thereof; and

20 EKq2 phage comprising a nucleotide sequence of SEQ ID NO:5, a variant thereof having at least 97% sequence identity, or a fragment thereof.

In another embodiment, the 5-phage cocktail KPM1 further comprises EKp148 phage comprising a nucleotide sequence of SEQ ID NO:6, a variant thereof having at least 97% sequence identity, or a fragment thereof, to make a six-phage cocktail (KPM1-1).

25 In another embodiment, the 5-phage cocktail KPM1 further comprises phage KEN18-2-1 comprising a nucleotide sequence of SEQ ID NO:7, a variant thereof having at least 97% sequence identity, or a fragment thereof, to make another six-phage cocktail (KPM2).

In another embodiment, the 5-phage phage cocktail KPM1 further comprises phage 15882-3 comprising a nucleotide sequence of SEQ ID NO:8, a variant thereof having at least 97% sequence identity, or a fragment thereof to make another six-phage cocktail.

In another embodiment, the 5-phage cocktail KPM1 further comprises phage KEN18-2-1 and phage 15882-3 to make a six-phage cocktail (KPM3).

Also, in some embodiments, pharmaceutical compositions are provided, and the composition comprises any of the phages or the phage cocktails aforementioned. The composition is formulated into a solution for parenteral administration, solution for nebulizer, solution or aerosol for nasal spray, aerosol or dry powder for inhalation, or lotion, cream, emulsion, ointment, or dry powder for topical administration. The pharmaceutical composition may comprise pharmaceutically acceptable carriers or excipients for nasal, parenteral, or topical administration.

In certain embodiments, a spray container containing a pharmaceutical composition of a solution or aerosol for nasal spray, or aerosol or dry powder for inhalation is provided for dispensing the phages or phage cocktail.

In addition, a method is provided for treating a subject having *K. pneumoniae* infection, the method comprising administering an effective amount of the pharmaceutical composition, through nasal, parenteral, or topical route. The subject has *K. pneumoniae* infection in the lung, urinary tract, skin, and/or blood stream, and the subject may have *K. pneumoniae* infection after lung transplant or is suffering from pneumonia, cystic fibrosis, bronchiectasis, bladder infection (cystitis), kidney infection (pyelonephritis), skin infection (cellulitis, burn wounds), and/or sepsis.

The pharmaceutical composition is optionally co-administered with an antibiotic or antibiotics, and the antibiotic or antibiotics are administered through nasal, parenteral, topical or oral route, along with the composition or separately before, after, or at the same time with the administration of the composition.

The spray container aforementioned optionally comprises an antibiotic or antibiotics.

Further, other applications of the phage, phage cocktail, and composition than pharmaceutical use can be contemplated. In one embodiment, a method of disinfecting a surface is provided, comprising applying to the surface the composition comprising any of the phage cocktail, which is formulated into a solution, lotion, aerosol, or powder form. In another embodiment, a method of disinfecting a fish tank or aquafarm is provided, comprising applying to the water the composition comprising any of the phage cocktail, which is formulated into a solution or powder form.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. *Klebsiella* phylogeny.

Figure 2. Phage EKq1 isolated on *K. quasipneumoniae* and its genome.

Figure 3. Testing KPM1 against *K. pneumoniae* lung infection.

5

DETAILED DESCRIPTION

A. Definitions

Embodiments of materials and methods are described herein; any methods and materials similar or equivalent to those described herein can be used in the practice of or testing of the invention. Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. In describing and claiming the present invention, the following terminology will be used. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and it is not intended to be limiting.

Other than in the operating examples, or where otherwise indicated, all numbers expressing quantities of ingredients or reaction conditions used herein should be understood as modified in all instances by the term “about.” The term “about” generally refers to a range of numbers that one of skill in the art would consider equivalent to the recited value (i.e., having the same function or result). In a specific embodiment, the term “about” includes a stated numerical value as well as a value that is +/- 15% of the stated numerical value. For example, about 5.75 M includes 5.75 molar as well as 6.61 M and 4.89 M, and all 1/10 values in between. In many instances, the term “about” may include numbers that are rounded to the nearest significant figure.

The present invention can “comprise” (open ended) or “consist essentially of” the components of the present invention as well as other ingredients or elements described herein. As used herein, “comprising” means the elements recited, or their equivalent in structure or function, plus any other element or elements which are not recited. The terms “having” and “including” are also to be construed as open ended unless the context suggests otherwise. As used herein, “consisting essentially of” means that the invention may include ingredients in addition to those

recited in the claim, but only if the additional ingredients do not materially alter the basic and novel characteristics of the claimed invention.

All ranges recited herein include the endpoints, including those that recite a range “between” two values. Terms such as “about,” “generally,” “substantially,” “approximately” and the like are to be construed as modifying a term or value such that it is not an absolute, but does not read on the prior art. Such terms will be defined by the circumstances and the terms that they modify as those terms are understood by those of skill in the art. This includes, at very least, the degree of expected experimental error, technique error and instrument error for a given technique used to measure a value. Unless otherwise indicated, as used herein, “a” and “an” include the plural, such that, e.g., “a phage cocktail” can mean at least one phage cocktail, as well as a plurality of phage cocktails, i.e., more than one phage cocktail. As understood by one of skill in the art, the term “phage” can be used to refer to a single phage or more than one phage.

Where used herein, the term “and/or” when used in a list of two or more items means that any one of the listed characteristics can be present, or any combination of two or more of the listed characteristics can be present. For example, if a composition of the instant invention is described as containing characteristics A, B, and/or C, the composition can contain A feature alone; B alone; C alone; A and B in combination; A and C in combination; B and C in combination; or A, B, and C in combination. The entire teachings of any patents, patent applications or other publications referred to herein are incorporated by reference herein as if fully set forth herein.

As used herein, the term “bacteriophage” or “phage” refers to bacterial viruses with a DNA or RNA genome, which is usually protected by a membrane or protein shell (capsid). Bacteriophages infect bacteria either to replicate to large numbers and cause the cell to lyse (lytic infection) or, in some cases, to integrate into the bacterial genome without killing the host bacteria (lysogenic infection). In the lytic infection, viral DNA and proteins are rapidly synthesized and packaged into virus particles, leading to the lysis (destruction) of the host bacteria and then sudden release of progeny virus particles (virions). In contrast, in the lysogenic infection, phage DNA is inserted into the host bacterial chromosome (prophage state), replicated together with bacterial chromosome, and transmitted to daughter cells. Lysogeny can sometimes significantly affect the host (lysogenic conversion). Under certain conditions causing DNA damage, the prophage is excised and initiates a lytic cycle, leading to the formation of progeny viruses and lysis of the host.

(Fortier LC, Sekulovic O. Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence* 2013 Jul 1;4(5):354-65.)

As used herein, the term “lytic activity” refers to phage activity leading to cell lysis of a target bacteria as determined by a plaque assay.

5 As used herein, the term “plaque assay” refers to phage titration, which is a process for isolation of phage clones and quantitation of the number of viable phage particles in a sample, which is presented as plaque-forming units (pfu) per unit volume, indicating active phage particle numbers in a unit volume of a phage suspension. For plaque assay, 10-fold serial dilutions of a phage sample are carried out from 10^{-1} to 10^{-6} ; mixing each 10-fold serial dilution of the phage
10 sample with aliquots of phage-sensitive bacteria suspended in bacterial culture medium; incubating the mixture of phage and bacteria for 10 minutes to allow for adsorption (attachment) of the phage to the bacteria; preparing tubes containing top agar (0.3–0.75% agar in bacterial culture medium) warmed at the 50°C water bath; adding certain volume of phage and bacteria mixture (50-500 μ L, usually 100-200 μ L) into a top agar tube, vortex, pouring the top agar onto the appropriately
15 labeled bottom agar plate (1–1.5% agar in bacterial culture medium), one plate for each phage dilution; allowing the top agar to solidify (about 5-10 minutes); and inverting the plates and incubating the plates at 30°C or 37°C. Phage-infected and lysed bacteria release phages that infect and lyse the surrounding bacteria in the top layer, and multiple rounds of infection and lysis continue until the area of the infected bacteria on the plate is cleared, which appears as individual
20 holes, or plaques, in an otherwise confluent bacterial lawn. Since each plaque is caused by a single viable phage when there are much less phages than bacteria, counting the number of plaques can be assumed as the number of plaque-forming units in the original suspension.

In the specification, the term “phage cocktail” means a phage mixture comprising two or more phages of the invention, or variants or progeny thereof, each of which have been isolated
25 from the environment from which they were originally found or have been produced by means of a technical process such as genetic engineering or serial passage techniques.

As understood herein, the term “synergy” is familiar to one of skill in the art, i.e., a combined effect that is greater than the sum of individual effects. Thus, with regard to the methods of the instant invention, the terms, “synergy”, “and like terms refer to a bacterial growth hold time
30 (i.e., demonstrated delay in bacterial growth) and/or lytic activity that is greater than the simple

addition of each individual phage's observed hold-time/growth delay or lytic activity. Thus, one of skill in the art will appreciate that, with regard to a "synergistic phage cocktail", the synergistic therapeutic effect observed is a therapeutic effect greater than the demonstrated sum of the individual effects of the phages in the cocktail. Similarly, with regard to a synergistic combination of phage(s) and an antibiotic, it is understood that the synergistic therapeutic effect is a therapeutic effect greater than the sum of the effect of the phage(s) and the antibiotic on bacterial growth hold times. It is contemplated herein that synergistic cocktails of the instant invention can extend growth hold time long enough or have sufficient lytic activity to have therapeutic efficacy. In addition, synergistic cocktails may prevent any detectable bacterial growth for the entire time course of the assay, e.g., 18 hours or more. Thus, in some cases, a synergistic response can result in a near extinction event, i.e., no bacterial growth occurs at all after exposure to the synergistic cocktail. It is understood herein that such cocktails may display a desirable synergistic delay.

The term "pharmaceutically acceptable" as used herein refers to any material (e.g., carrier, excipient or vehicle) that is compatible for use in a mammalian subject. Such includes physiologically acceptable solutions or vehicles that are harmless or do not cause any significant specific or non-specific immune reaction to an organism or do not abrogate the biological activity of the active compound. For formulation of the composition into a liquid preparation, saline, sterile water, Ringer's solution, buffered physiological saline, albumin infusion solution, dextrose solution, maltodextrin solution, glycerol, ethanol, and mixtures thereof may be used as a pharmaceutically or veterinary acceptable excipient or carrier. If necessary, other conventional additives such as thickeners, diluents, buffers, preservatives, surface active agents, antioxidants and bacteriostatic agents may be added. Further, diluents, dispersants, surfactants, binders and lubricants may be additionally added to the composition to prepare injectable formulations such as aqueous solutions, suspensions, and emulsions, oral formulations such as pills, capsules, granules, or tablets, or powdered formulations.

In the specification, the term "isolated" should be considered to mean material removed from its original environment in which it naturally occurs, for example, in this instance bacteriophage specific for a particular bacterium. The removed material is cultivated, purified and cultured separately from the environment in which it was located. Thus, the purified isolated phage in this instance does not contain any significant amounts of other phages. For the term "progeny"

or “isolated progeny”, the term should be considered to mean replicates of the original phage, including descendants of the phage created by serial passage of the isolated phage or by other means known in the art, that are typically isolated in the same manner as described above, and/or phages having a substantially equivalent RFLP profile to the deposited phage.

5 As used herein, the term "treatment" in the context of pharmacological or medical meaning refers to intervention of disease, disorder, condition or symptoms thereof to obtain a desired physiological or clinical effect. "Treatment" includes: inhibiting the disease, disorder, condition, or symptoms thereof, arresting its development or progression, and also includes relieving, alleviating, or ameliorating the disease, disorder, condition, or reducing one or more symptoms
10 thereof, such as, for example, inhibition or prevention of the growth of *Klebsiella* bacteria and/or *K. pneumoniae* biofilms, ideally in the lungs of an individual suffering from an infection or lung inflammation, or cystic fibrosis. In typical embodiments, the subject has a pulmonary or blood stream or skin, or urinary *Klebsiella* infection.

 As used herein, the term "administering" refers to introducing an agent to a subject, and
15 can be performed using any of the various methods for drug or composition delivery known to those skilled in the art. Routes of administering include, but are not limited to oral administration, parenteral administration (subcutaneous (SC/SQ: <1 mL), intravenous (IV: 1-20 mL), intradermal (ID: <0.2 mL), intramuscular (IM: < 4 mL), intraperitoneal (IP), intraarterial, intracardiac, intraarticular, and intraspinal injection), rectal administration by way of suppositories or enema,
20 local/topical administration directly into or onto a target tissue, nasal administration (nebulizer, nasal spray, inhalation), or administration by any route or method that delivers a therapeutically effective amount of the drug or composition to the cells or tissue to which it is targeted.

 As used herein, the terms “co-administration” refers to the administration of a first active agent before, concurrently, or after the administration of a second active agent such that the
25 biological effects of the two (or more) agents overlap.

 As used herein, the term “dosage form” refers to a pharmaceutical preparation in which a specific mixture of active ingredients (e.g., a phage or phage cocktail) and inactive components (excipients) are formulated in a particular shape or form to facilitated administration and accurate delivery of active ingredients, and/or to be presented in the market. Solid dosage forms include
30 powders, granules, capsules, tablets, cachets, pills, lozenges, gummies, suppositories. Semi-solid

dosage forms include ointment, creams, paste, gels, poultices. Liquid dosage forms include collodions, droughts, elixirs, emulsions, suspension, enemas, gargles, linctuses, lotion, liniments, mouth washes, nasal drop, paints, solutions, syrups. Gaseous dosage forms include aerosols, inhalations, and sprays.

5 As used herein, the term “composition” refers to a pharmaceutical composition, meaning a mixture of substances suitable for administering to an individual, which includes one or more pharmaceutically active ingredients. For example, a pharmaceutical composition may comprise a certain amount of phage particles in solution or lyophilized dry powder as well as suitable pharmaceutical excipients.

10 As used herein, the term “therapeutically effective amount” or “effective amount” refers to an amount a phage or one or more phages, a phage cocktail or composition, resulting in a decrease in the number of *K. pneumoniae* in a subject after treatment when compared with the number of *K. pneumoniae* before treatment.

15 As used herein, the term “subject” encompasses the whole phyla of animal kingdom, including Arthropoda, Mollusca, Chordata, for example, crab, lobster, shrimp, squid, oysters, clams, mussels, or other shellfish, eel, fishes, amphibians, reptiles, birds, and mammals that include domestic-, farm-, zoo- and wild animals, primates, and humans, who may be suffering from *K. pneumoniae* infection, particularly an infection caused by non-MDR or MDR *K. pneumoniae* strains in the lung, urogenital organ, skin or blood stream. For example, a “subject”,
20 interchangeable with “patient” has *K. pneumoniae* infection after lung transplant or is suffering from pneumonia, cystic fibrosis, bronchiectasis, bladder infection (cystitis), kidney infection (pyelonephritis), skin infection (cellulitis, burn wounds), and/or sepsis. In addition, the subject may include mushrooms, algae, or plants that can be infected with non-MDR or MDR *P. aeruginosa* or *K. pneumoniae* strains.

25 As used herein, the term “antibiotics” refers to antimicrobial substance active against bacteria for the treatment of bacterial infections, which usually either kill or inhibit the growth of bacteria. General antibiotics can be classified into to: (1) bacterial cell wall or membrane synthesis inhibitors, e.g., penicillins and cephalosporins; (2) protein synthesis inhibitors (e.g., macrolides, tetracyclins); and (3) DNA synthesis inhibitors (e.g., fluoroquinolones gyrase inhibitors and sulfa
30 antibiotics inhibiting bacterial folic acid synthesis). Standard or traditional antibiotic agents

include, but are not limited to: (1) cell wall or membrane synthesis inhibitor antibiotics, e.g., penicillins or beta-lactam compounds: penicillins (e.g., penicillin G, penicillin V, isoxazolyl penicillins, oxacillin, cloxacillin, flucloxacillin, dicloxacillin, nafcillin, methicillin, ampicillin, amoxicillin, piperacillin, ticarcillin, carbenicillin, aminopenicillins, carboxypenicillins, ureidopenicillins, temocillin, azlocillin, mezlocillin, mecillinam); cephalosporins and cephamycin: (e.g., cefazolin, cephalexin, cephradine, cefadroxil, cefuroxime, cefaclor, cefamandole, cefonicid, cefprozil, ceforanid, cefoxitin, cefmetazole, cefotetan, cefoperazone, cefotaxime, ceftazidime, ceftriaxone, cefepime, ceftaroline fosamil, cephalothin, cephapirin, cefpodoxime, ceftibuten, cefdinir, ceftizoxime, ceftriaxone, cefepime, cefditoren, cefixime, ceftibuten, cefacetrile, cefaloglycin, cefalonium, cefaloridine, cefatrizine, cefazaflur, cefazedone, cefroxadine, ceftezole, cefuzonam, cefcapene, cefdaloxime, cefdinir, cefditoren, cefetamet pivoxil, cefmenoxime, cefteram, ceftiofur, cefoperazone, ceftazidime latamoxef, cefclidine, cecluprenam, cefoselis, cefozopran, cefpirome, cefquinome, ceftobiprole, flomoxef (or oxa-1-cephamycin), ceftobiprole); carbacephems (e.g., loracarbef); carbapenems (e.g., biapenem, doripenem, ertapenem, imipenem, imipenem-cilastatin, meropenem, tebipenem pivoxil, faropenem, panipenem/betamipron, razupenem (PTZ-601), thienpenem (thienamycin)); monobactams (e.g., aztreonam, tigemonam, nocardicin A, or tabtoxinine β -lactam); beta-lactamase inhibitors (e.g., clavulanic acid, sulbactam, tazobactam); glycopeptides (e.g., vancomycin, teicoplanin); lipoglycopeptide (e.g. oritavancin, dalbavancin and telavancin) ; daptomycin, fosfomycin, bacitracin, cycloseine, isoniazid; polypeptide antibiotics (e.g., polymyxin B, or polymyxin E (colistin)); (2) antibiotics targeting bacterial ribosome subunits, the 30S and the 50S subunits; dactinomycin (or actinomycin D), chloramphenicol; tetracyclines (e.g., tetracycline, chlortetracycline, doxycycline, oxytetracycline, demeclocycline, lymecycline, meclocycline, methacycline, minocycline, rolitetracycline, or tigecycline); macrolides (e.g., erythromycin, clarithromycin, azithromycin, fidaxomicin, telithromycin, carbomycin A, josamycin, kitasamycin, midecamycin/midecamycin acetate, oleandomycin, solithromycin, spiramycin, troleandomycin, tylosin, or roxithromycin, dirithromycin, roxithromycin); aminoglycosides: (e.g., streptomycin, rhodostreptomycin, kanamycin, neomycin, amikacin, gentamicin, netilmicin, tobramycin, paromycin, apramycin) apramycin, plazomicin, arbekacin, and streptomycin); lincosamides (e.g., lincomycin, pirlimycin and clindamycin); ansamycins: geldanamycin, naphthomycin, rifamycins (e.g., rifampicin (or rifampin), rifabutin, rifapentine, rifalazil, or rifaximin); quinupristin-dalfopristin, mupirocin,

streptogramins (e.g., streptogramin A, streptogramin B), oxazolidinones (e.g., linezolid, tedizolid), spectinomycin; (3) DNA replication inhibitor antibiotics: fluoroquinolones and quinolones (e.g., nalidixic acid, norfloxacin, ciprofloxacin, levofloxacin, gatifloxacin, moxifloxacin, gemifloxacin, lomefloxacin, ofloxacin, pefloxacin, moxifloxacin, rosoxacin, enoxacin, fleroxacin, nadifloxacin, rufloxacin, balofloxacin, grepafloxacin, pazufloxacin, sparfloxacin, temafloxacin, tosufloxacin, clinafloxacin, garenoxacin, stifloxacin, trovafloxacin, prulifloxacin, cinoxacin, flumequine, oxolinic acid, piromidic acid, pipemidic acid); sulfonamide antibiotics (e.g., sulfacycline, sulfisoxazole, sulfamethizole, sulfadiazine, silver sulfadiazine, sulfamethoxazole, sulphapyridine, sulfadoxine, sulfathalidine, sulfacetamide sodium, mafenide, co-trimoxazole, sulfasalazine, sulfanilamides, sultiame, sulfadimethoxine; pyrimidines (e.g., trimethoprim, pyrimethamine, and more than 200 drugs; <https://go.drugbank.com/categories/DBCAT000349>); others (pyrazinamide, ethambutol, streptomycin, ansamitocin); and any combination thereof can be used.

As used herein, the term “multidrug resistance” refers to antibacterial resistance which happens when bacteria develop the ability to defeat the drugs designed to kill them. That means bacteria are not killed and continue to grow. CDC typically uses this term to refer to an isolate that is resistant to at least one antibiotic in three or more antibiotics classes. Multidrug resistance mechanisms fall into four main categories: (1) limiting uptake of a drug; (2) modifying a drug target; (3) inactivating a drug; (4) active drug efflux. There are two main ways that bacterial cells can acquire antibiotic resistance. One is through mutations that occur in the DNA of the cell during replication. The other way that bacteria acquire resistance is through horizontal gene transfer through plasmids or transposons coding for resistance to a specific agent. Examples of bacteria resistant to antibiotics are methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), multi-drug-resistant *Mycobacterium tuberculosis* (MDR-TB) and carbapenem-resistant *Enterobacteriaceae* (CRE) gut bacteria. Generally, MDR is defined as non-susceptibility to ≥ 1 agent in ≥ 3 antimicrobial categories, extensively drug-resistant (XDR) as non-susceptibility to ≥ 1 agent in all but ≤ 2 categories, and pandrug-resistant (PDR) as non-susceptibility to all antimicrobial agents listed. (Magiorakos AP et al., Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012 Mar;18(3):268-81.)

The term “nucleotide” as used herein refers to a sub-unit of a nucleic acid (whether DNA or RNA or an analogue thereof) which may comprise, but is not limited to, a phosphate group, a 5-carbon sugar group and a nitrogen containing base, as well as analogs of such sub-units. Other groups (e.g., protecting groups) can be attached to the sugar group and nitrogen containing base group. It will be appreciated that, as used herein, the terms “nucleotide” and “nucleoside” will include those moieties which contain not only the naturally occurring purine and pyrimidine bases, e.g., adenine (A), thymine (T), cytosine (C), guanine (G), or uracil (U), but also modified purine and pyrimidine bases and other heterocyclic bases which have been modified (these moieties are sometimes referred to herein, collectively, as “purine and pyrimidine bases and analogs thereof”).

As used herein, the term “nucleotide sequence” or “nucleic acid sequence” refers to any fragment of polyribonucleotide or polydeoxyribonucleotide that may be unmodified RNA or DNA or modified RNA or DNA, among others, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or double-stranded, or a mixture of single- and double-stranded regions. They may comprise artificial nucleic acids including peptide nucleic acids (PNA), Morpholino and locked nucleic acids (LNA), as well as glycol nucleic acids (GNA), threose nucleic acids (TNA) and hexitol nucleic acids (HNA). Each of these is distinguished from naturally occurring DNA or RNA by changes to the backbone of the molecule.

Specifically, the term “variant” as applied to a phage means a phage that exhibits lytic activity towards *Klebsiella* spp (e.g., *K. pneumoniae*), and whose genome has at least 90% sequence identity to the genome of phage described in this disclosure. Typically, a variant of a phage has at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to phage sequences (SEQ ID NOs 1, 2, 3, 4, 5, 6, 7, and 8). The term should be understood to include genetically modified versions of the deposited phage in which the genetic code is manipulated by means of, for example, genetic engineering or serial passage.

As used herein, the term “sequence identity” or “identity” in the context of two nucleic acid or polypeptide sequences refers to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. The term “% identity” in relation to nucleic acid or amino acid sequences designates the level of identity or

homology between the sequences and may be determined by means of computer programs such as BLAST (Basic Local Alignment Search Tool) or other software known per se in the art. With settings adjusted to e.g., DNA sequences, nucleic acid molecules may be aligned to each other using the alignment software available as part of the BLAST program package. The % identity between two sequences designates the identity over the entire length of said sequences.

B. Overview

Several approaches can be employed to expand phage's bacterial coverage activity. For example, diverse *K. pneumoniae* strains can be used for phage enrichment. Also, diverse *Klebsiella* phages can be harvested in various places including USA, Kenya, Thailand and Georgia. Also, *in vitro* directed evolution of phages can be employed, or a near-neighbor species of the target bacteria can be used for phage isolation and enrichment. Figure 1 provides a diagram showing the phylogeny of *Klebsiella*. In this disclosure, a near-neighbor species of *K. pneumoniae*, *K. quasipneumoniae* is used to isolate a phage EKq1 active against *K. pneumoniae*.

A lytic phage EKq1 is an unclassified siphovirus of the class *Caudoviricetes*, similar to *Klebsiella* phages VLCpiS8c, phiKp_7-2, and vB_KleS-HSE3. The 48,244-bp genome has a GC content of 56.43% and 63 predicted protein-coding genes. EKq1 is active against 12/100 superdiverse *K. pneumoniae* strains. Since EKq1 is a phage without virulence factor, it appears to be safe for antibacterial phage therapy.

Using this newly isolated phage EKq1, new *K. pneumoniae* phage cocktails are designed. A phage cocktail KPM1 comprises five core *Klebsiella* phages with broad activity, including EKq1 and other *Klebsiella* phages, AFR4, KEN39, KEN42, and EKq2, which are compatible with EKq1. For the KPM1 efficacy test, five *K. pneumoniae* bacterial strains (MRSN 16233, 111775, 113898, 582610, and 976583) were selected, which caused lethal systemic infection in carbapenems (CP)-treated and untreated mice. The efficacy of KPM1 phage cocktail was demonstrated in carbapenem-resistant *K. pneumoniae* strain-infected mice model (Figure 3).

Next, new phages, which are active against these highly virulent (hv) strains were isolated and genomically characterized. Genomes of 36 new *K. pneumoniae* phages were sequenced and their phylogeny was updated, and some of 11 new *K. pneumoniae* phages showed promise when tested for lysis dynamics against 40 strains of high-risk clones. Next for animal test, mice

were infected with strain MRSN 113898, and treated with a phage cocktail KPM1-1, which comprises phage EKp148 in addition to the core 5-phage cocktail KPM1. When compared with the core 5-phage cocktail KPM1, phage cocktail KPM1-1 showed better efficacy against mouse lung infection caused by a hypervirulent *K. pneumoniae* strain.

5 In addition, another six-phage cocktail KPM2 comprising KEN18-2-1 in place of EKp148 was tested, which showed active *in vitro* against 69% of *K. pneumoniae* strains in the panel. Further, a seven-phage cocktail KPM3 was tested, which comprises a recombinant phage in addition to the 6-phage cocktail KPM2. KPM3 was active *in vitro* against 81% of the 100-strain panel. Overall, KPM2 and KPM3 phage cocktails showed improved antibacterial activity
10 compared with KPM1.

In conclusion, these durable phage cocktails cover 70-95% of MDR clinical isolates.

C. Examples of Embodiments

The present disclosure relates to (a) introducing the whole gene sequence of a bacteriophage EKq1 having lytic activity against broad range of *K. pneumoniae* strains; (b) a
15 *Klebsiella* bacteriophage cocktail KPM1 comprising EKq1 and other four *Klebsiella* phages, i.e., AFR4, KEN39, KEN42, Ekq2; (C) other *Klebsiella* bacteriophage cocktails based on core 5 phages of KPM1, including 6-phage cocktail KPM1-1 and KPM2, and a seven-phage cocktail KMP3, and (d) use of the phage cocktails for medical and non-medical applications, in particular for treatment of MDR *K. pneumoniae* infection in the lung, urogenital organs, skin, and/or blood
20 stream and for prevention of *K. pneumoniae* infection in hospital environment.

According to an embodiment, in order to design a new *K. pneumoniae* phage cocktail, the genome of bacteriophage EKq1 comprising a nucleotide sequence of SEQ ID NO:4 is disclosed. According to another embodiment, the genome of bacteriophage Ekp148 comprising a nucleotide sequence of SEQ ID NO:6 is disclosed. According to another embodiment, the genome of
25 bacteriophage KEN18-2-1 comprising a nucleotide sequence of SEQ ID NO:7 is disclosed. According to another embodiment, the genome of a recombinant phage 15882-3, which is generated by *in vitro* evolution of a phage *Klebsiella* phage mixture comprising KEN22, KEN25, KEN37, and KEN39, which comprises a nucleotide sequence of SEQ ID NO:8 is disclosed. Also, a nucleotide sequence or sequences having at least 97% identity to the aforementioned sequences

as well as isolated fragments thereof or isolated polypeptides encoded by said nucleotide sequences can be contemplated to be included in a phage cocktail.

In certain embodiments, phage cocktails comprise Ekq1 and one, two, three, four, five or six other *Klebsiella* phages. The phage cocktails can be used as active agents in pharmaceutical or veterinary preparations, particularly to treat MDR *K. pneumoniae* bacterial infections or to modify microbial balance in a subject. Embodiments also relate to the use of one or more lytic bacteriophages to improve a subject condition by modifying the microbial flora in said subject. The microbial flora may be modified by correcting, adapting or restoring a proper balance of microorganisms in said flora.

In another embodiment, an antibacterial phage cocktail KPM1 is provided, which comprises EKq1 and four other bacteriophages having lytic activity against various *K. pneumoniae* strains to make a 5-phage cocktail, and said 4 bacteriophages include AFR4 comprising a nucleotide sequence of SEQ ID NO:1 or a sequence having at least 97% identity thereto; KEN39 comprising a nucleotide sequence of SEQ ID NO:2 or a sequence having at least 97% identity thereto; KEN42 comprising a nucleotide sequence of SEQ ID NO:3 or a sequence having at least 97% identity thereto; and Ekq2 comprising a nucleotide sequence of SEQ ID NO:5 or a sequence having at least 97% identity thereto.

In another embodiment, an antibacterial phage cocktail KPM1-1 is provided, which comprises all five bacteriophages of KPM1 and one more bacteriophage EKp148 having lytic activity against various *K. pneumoniae* strains to make a 6-phage cocktail, and the genome of EKp148 comprises a nucleotide sequence of SEQ ID NO:6 or a sequence having at least 97% identity thereto. However, EKp148 may be replaced with other phages.

In another embodiment, an antibacterial phage cocktail KPM2 is provided, which comprises all five bacteriophages of KPM1 and another bacteriophage KEN18-2-1 having lytic activity against various *K. pneumoniae* strains to make another 6-phage cocktail, and KEN18-2-1 comprises a nucleotide sequence of SEQ ID NO:7 or a sequence having at least 97% identity thereto.

In another embodiment, an antibacterial phage cocktail KPM3 is provided, which comprises all six bacteriophages of KPM2 and one more bacteriophage having lytic activity

against various *K. pneumoniae* strains to make a 7-phage cocktail, and said bacteriophage is a recombinant phage 15882-3 comprising a nucleotide sequence of SEQ ID NO:8 or a sequence having at least 97% identity thereto. However, a phage cocktail based on KMP1 including only phage 15882-3 to make a 6-phage cocktail can also be contemplated.

5 In other embodiments, compositions are provided, which comprise at least one bacteriophage comprising the whole phage genome(s) or fragments thereof, for use in the treatment of an infection in an animal or a human, decontaminating a material such as medical devices, or cleaning laboratory and hospital furniture or walls. The compositions described herein may further comprise a pharmaceutically or veterinary acceptable excipient or carrier. They may
10 be liquid, semi-liquid, solid or lyophilized powder.

The targeted subjects of the composition are patients with *K. pneumoniae* infection in the lung, urinary tract, skin, and/or blood stream, and in particular patients who has *K. pneumoniae* infection after lung transplant or are suffering from pneumonia, cystic fibrosis, bronchiectasis, bladder infection (cystitis), kidney infection (pyelonephritis), skin infection (cellulitis, burn
15 wounds), and/or sepsis.

According to other embodiments, provided are method(s) of treating an infection in an animal or a human, comprising the administration to them of a composition comprising at least one bacteriophage having a lytic activity to a *K. pneumoniae* strain through parenteral, topical or nasal route. A spray container comprising a composition as defined above is introduced for
20 topically or nasally applying the same to a subject or surface.

The composition may be used in any vertebrate and invertebrate animals, preferably mammals, and more preferably in human beings, but it can also be applied to plants and fungi contaminated or suspected to be contaminated with *K. pneumoniae*.

As described in Example 1, phage EKq1 may be isolated using *K. quasipneumonia* and tested
25 on *K. pneumoniae*. However other Klebsiella phages in Fig. 1 can be contemplated for *K. pneumoniae* phage isolation and enrichment, including *K. quasivariicola*, *K. variicola*, *K. Africana*, *K. aerogenes*, *K. oxytoca*, *K. michiganensis*, and Cronobacter species. According to another embodiment, provided is another method that involves isolating a new *K. pneumoniae* phage to obtain a *K. pneumoniae* phage having broad antibacterial activity against MDR, XDR, or

PDR *K. pneumoniae* subspecies, wherein the method comprises steps of collecting phages from the environment water or human blood, isolating the phage by performing plaque assay with other *Klebsiella* species than *K. pneumoniae*, and testing lytic activity on *K. pneumoniae*. In a specific example, the *Klebsiella* species other than *K. pneumoniae* is *Klebsiella quasipneumoniae*, and optionally MRSN 829456.

D. Formulations and Administration

For the treatment of bacterial infection, an effective amount of the phage or phage cocktail is administered to a patient through nasal (nebulizer, nasal or inhalation spray), parenteral (intravenous, intramuscular, intraperitoneal, subcutaneous, etc.) or topical route. Pharmaceutical composition for each administration route is formulated into a proper dosage form and comprises necessary excipients. For example, the phage or phage cocktail can be dissolved or suspended in saline or other buffers to be manufactured as a solution for nebulizer mist, nasal spray, or intravenous injection, and each of solutions for different route administration may have similar or different excipients.

In certain embodiments, the composition is formulated into a solution for parenteral administration. In certain embodiments, the composition is formulated into a solution for nebulizer. In certain embodiments, the composition is formulated into a solution or aerosol for nasal spray. In certain embodiments, the composition is formulated into aerosol or dry powder for inhalation. In certain embodiments, the composition is formulated into a lotion, cream, gel, an emulsion, ointment, or dry powder for topical administration.

In addition, such compositions may further comprise pharmaceutically acceptable excipients for nasal, parenteral, or topical administration. As used herein, the term “excipients” refers to substances that are added to therapeutic products to improve stability, bioavailability, and manufacturability. Examples of pharmaceutical excipients can be found in Remington: The Science & Practice of Pharmacy 23rd Edition, Elsevier.

For example, parenteral administration, the composition may comprise lyoprotectants (e.g., human albumin, lactose monohydrate, maltose/trehalose/sucrose, mannitol, dextran, inulin, fructose), micro-encapsulating agents (e.g., aliphatic polyester such as polyglycolide, polylactide, and their copolymers, phospholipids/lecithin, phosphatidic acids, phosphoglycerol,

phosphoserine, phosphorethanolamine, phosphocholine, PEGylated phospholipids, hydroxypropylcyclodextrin, Betadex sulfobutyl ether sodium), solubilizers and emulsifiers (e.g., N-methyl 2-pyrrolidone, PEG, polysorbates, polyoxyl 35 castor oil, polyoxyl-15-hydroxystearate, polyvinyl pyrrolidone, propylene glycol, sodium cholesteryl sulfate, sorbitan esters, poloxamer, 2-pyrrolidone, diacylglycerols, monoglycerol), tonicity agents (e.g., dextrose, glycerin, mannitol, NaCl, KCl, sorbitol/sorbitol solution), solvents and cosolvents (water miscible such as propylene glycol, PEG low molecular weight, glycerin, ethanol, 2-pyrrolidone, and N-methyl-2-pyrrolidone and water immiscible such as ethyl oleate, benzyl benzoate, vegetable oil, soybean oil, sesame oil, peanut oil, castor oil, almond oil, and cottonseed oil), viscosity-building agents (e.g., sodium carboxymethylcellulose (Na CMC), methylcellulose, gelatin, polyvinyl pyrrolidone), antioxidants (e.g., ascorbic acid, acetylcysteine, sodium ascorbate, sodium metabisulfite, sodium bisulfite, and tocopherol), chelating agents (e.g., EDTA), preservatives (e.g., methyl paraben, ethyl paraben, propyl paraben, butyl paraben, benzoic acid, sodium benzoate, EDTA, cetrimide, benzyl alcohol, benzalkonium chloride, thimerosal, and phenylmercuric salts), buffering agents (e.g., acetate, citrate, tartrate, phosphate, triethanolamine (TRIS) buffer).

For topical administration, the composition may comprise gelling agents (e.g., carbomer, carrageenan, chitosan, gelatin, gellan gum, pectin, poloxamer, poly(ethylene)oxide, polycarbophil, pullulan, HEC, HPMC, MC, alginates, Na CMC, xanthan gum, acacia, agar, guar gum, tragacanth, modified starch, povidone), humectants (e.g., corn syrup, glycerin, lactic acid, PEGs, propylene glycol, sodium lactate, sorbitol, trehalose, xylitol), cream and ointment bases (e.g., cetostearyl alcohol, cetyl palmitate, fatty alcohols, hard fat, lanolin, lanolin alcohol, hydrogenated castor oil, mineral oil, petrolatum, glyceryl behenate, hard paraffin, soft paraffin, stearic acid, beeswax (white or yellow), carnauba wax, emulsifying wax, microcrystalline wax), solubilizers and emulsifiers (e.g., cocoylcaprylocaprate, decyl oleate, diethylene glycol monoethyl ether, dimethyl isosorbide, glyceryl monooleate, isopropyl myristate, medium chain triglycerides (MCT), octyldodecanol, oleyl alcohol, oleyl oleate, polyoxyethylene alkyl ethers, polyoxyethylene stearates, propylene glycol monocaprylate, propylene glycol monolaurate, sodium cetostearyl sulfate, lecithin, cyclodextrins, docusate sodium, glyceryl monostearate, hydrogenated vegetable/cottonseed/palm kernel oil, MCT, N-methyl-2-pyrrolidone, poloxamer, PEG, polysorbate, PEG castor oil derivatives, propylene glycol, polyoxyglycerides, sodium lauryl sulfate, sucrose esters), suspending agents and thickeners (e.g., magnesium aluminum silicate, MCC and Na CMC,

propylene glycol alginate, acacia, carbomer, carrageenan, colloidal silicon dioxide, gellan gum, HEC, HPC, HPMC, maltitol, MC, pectin, PEGs, polyvinyl alcohol, povidone, Na CMC, sorbitol, sucrose, xanthan gum, tragacanth, gelatin, guar gum, kaolin, phospholipids), preservatives (e.g., methyl paraben, ethyl paraben, propyl paraben, butyl paraben, benzoic acid, sodium benzoate, EDTA, cetrimide, benzyl alcohol, benzalkonium chloride, thimerosal, and phenylmercuric salts), and stabilizers including pH modifiers (e.g., lactic acid, citric acid, tartaric acid, ascorbic acid, and their sodium salts), antioxidants referred to above, and chelating agent EDTA.

For nasal administration, the composition may comprise a suspending agent and thickener (e.g., colloidal carboxymethyl cellulose (CMC) and microcrystalline cellulose (MCC), MC, HPMC, pectin, PEGs), preservatives (e.g., methyl paraben, ethyl paraben, propyl paraben, butyl paraben, benzoic acid, sodium benzoate, EDTA, cetrimide, benzyl alcohol, benzalkonium chloride, thimerosal, and phenylmercuric salts), penetration enhancer (e.g., polysorbates, poloxamers, PEGs, propylene glycol, EDTA), tonicity agents (e.g., dextrose, glycerin, mannitol, NaCl, KCl, sorbitol/sorbitol solution), buffering agents (e.g., acetate, citrate, tartrate, phosphate, triethanolamine (TRIS) buffer). A composition for nebulizer solution may comprise buffering agents (e.g., phosphate buffer, citrate buffer), EDTA chelating agent, ethanol cosolvent, pH modifier (e.g., HCl, NaOH, sulfuric acid, tartaric acid, citric acid), preservatives (e.g., methyl paraben, propyl paraben, benzalkonium chloride), surfactant (e.g., polysorbate 20, polysorbate 80, sorbitan laurate), and tonicity agent (e.g., NaCl). A pressurized metered-dose inhaler composition may comprise propellants (e.g., hydrofluoroalkanes), surfactants (e.g., oleic acid, sorbitan trioleate, lecithin), pH modifier (e.g., citric acid), lubricant (e.g., PEG 1000, PEG 600), cosolvent (e.g., ethanol, glycerol), suspending agents (e.g., Povidone K25, K30). A dry powder – optionally lyophilized - inhaler composition may comprise carriers (e.g., lactose monohydrate), surfactants (e.g., dipalmitoyl phosphatidylcholine, distearoyl phosphatidylcholine), lubricant (e.g., magnesium stearate).

Further, a spray container, which is pressurized or not, containing the composition for nasal spray or inhalation spray can be contemplated, and the spray container optionally comprises an antibiotic or antibiotics in the dosage form of liposome, solution, or lyophilized dry powder, for example, liposomal ciprofloxacin, aztreonam solution, colistin solution or dry powder, tobramycin solution or dry powder. In a preferred embodiment, the spray container comprises ceftazidime.

In other embodiments, methods of treating a subject having *K. pneumoniae* infections are provided, and the method comprises administering an effective amount of the composition described above, through nasal, parenteral, and/or topical route.

5 The composition comprising at least one phage described in this disclosure can be administered alone or co-administered with antibiotics, especially antibiotics against gram-negative bacteria. Antibiotics can be administered through oral, parenteral, topical, and/or nasal route together with the composition or separately before, after, or at the same time with the administration of the composition. In addition, some antibodies or fragments thereof against the surface antigens of the target bacteria may be contemplated for co-administration with the
10 composition herein.

The composition can be administered once a day, twice a day, thrice a day for one day, couple of days, three days, four days, five days, six days, a week, two weeks, three weeks, a month or a longer period. Daily dose (pfu) is to be determined empirically, depending on the severity of *K. pneumoniae* infection. For example, patients can be administered with 10^5 - 10^{15} PFU,
15 optionally 10^9 - 10^{11} PFU of a single phage or combination of at least two phages intravenously twice daily; or with the same dose by inhalational nebulization. Patients can also receive antibiotic treatment with the phage treatment against the target bacteria. Initial duration of phage treatment can be 1-6 months, but it can be shorter or longer courses of treatment directed by clinical and microbiologic responses. Phage regimens can be adjusted by the treating clinicians as needed
20 based on tolerability to phage-containing pharmaceutical formulation and lab tests during phage treatment.

Although the main subject of this invention is human, any animals including both vertebrates and invertebrates, and even some mushrooms, algae and plants, which can be infected by *K. pneumoniae*, can benefit from treatment with the phages listed above or phage cocktail
25 comprising any of them. For example, the composition comprising the phages or phage cocktail against non-MRD or MRD strains of *K. pneumoniae* can be scattered as dry powder or solution to the fish tank or aquaculture for oysters, clams, mussels, or other shellfish, or algae farm.

Considering the aspect of bactericidal effect of phages, the composition can also be developed into hygiene control products. For example, the composition can be utilized as a
30 disinfectant for cleaning the surface of medical device and instruments, and/or hospital furniture

or walls, by applying a liquid, aerosol, or powder composition to the surface directly or to wiping cloth.

EXAMPLES

5 Example 1. Materials and Methods

1.1. Bacterial Strains

K. quasipneumoniae MRSN 829456 is an isolate from human blood. *K. pneumoniae* strains were obtained from the MRSN and the American Type Culture Collection (ATCC, Manassas, VA, USA). Bacteria were grown in Heart Infusion Broth (HIB) with shaking (200 rpm) or on HIB agar plates at 37°C overnight. Twenty-two bacterial strains used in this study including hv Kp ST11, ST15, ST34, and ST485;

1.2. EKq1 Phage Isolation and Propagation

EKq1 was isolated from sewage water collected in Montgomery County, Maryland, using *K. quasipneumoniae* MRSN 829456. The environmental water was filtered using sterile 0.45 µm filters and then sterile 0.22 µm filters (MilliporeSigma, Bollington, MA, USA) prior to phage enrichment.

To enrich Ekq1 phage, 5×HIB was mixed with the filter-sterilized environmental water sample at a 1:5 ratio, and overnight broth culture of *K. quasipneumoniae* MRSN 829456 was added. The enrichment mixture was incubated overnight at 37°C with shaking at 200 rpm. Then, the mixture was centrifuged, and the supernatant was filtered using a sterilized 0.22 µm filter. The resulting lysate was assessed for phage activity against *K. pneumoniae* by plating the phages on double-layer HIB agar plates, and phage plaques were detected.

Phage purification was performed by three rounds of sequential single plaque isolation until uniform plaque morphology was achieved. Plaque morphology was analyzed using ImageJ software v. 1.53 (National Institutes of Health, Bethesda, MD, USA).

High titer phage lysates were obtained by liquid culture propagation of the phage on bacterial strain in HIB supplemented with 0.5 mM CaCl₂, 2 mM MgCl₂, and 0.1% glucose. Phages

were harvested after centrifugation, supernatant filtration with a sterilized 0.22 μ m filter, and ultracentrifugation to obtain phage particle pellet, and resuspended and stored in SM buffer (50 mM Tris-HCl, pH 7.5, 99 mM NaCl, 8 mM MgSO₄, 0.01% gelatin) (Teknova, Hollister, CA, USA) at 4°C protected from light. Plaque-forming units (PFUs) per ml were determined through 10-fold
5 dilution in SM buffer and plating on double-layer agar plates.

1.3. Host Range Testing

The host range was determined against the *K. pneumoniae* strains using a micro-spot dilution assay on square grid double-layer agar plates. Lytic activity was determined positive if plaque formation was observed after overnight plate incubation at 37°C. Host range testing was
10 performed in two separate replicates with representative results reported.

1.4. Bacterial Strains Lytic Properties Assay

The dynamics of EKq1 lysis against *K. pneumoniae* was determined. Briefly, phages were mixed with a mid-log phase bacterial culture (OD₆₀₀ = 0.5) at a multiplicity of infection (MOI) of 1, 0.1, 0.01, 0.001, and 0. The mixed culture was incubated at 37°C with shaking at 200 rpm for
15 the duration of the experiment. OD₆₀₀ readings were taken every 10–20 min for 3 h. Experiments were repeated independently three times and analyzed using GraphPad Prism v. 9 (GraphPad Software, San Diego, CA, USA).

1.5. Determination of Optimal Multiplicity of Infection

The optimal MOI of EKq1 was determined. Briefly, phage particles at various MOIs were
20 added to mid-log phase bacterial culture (OD₆₀₀ = 0.5) and incubated for 4 hours at 37°C with shaking at 200 rpm. The samples were centrifuged for 10 min and the supernatant was filtered using a sterilized 0.22 μ m filter. Phage titer for each sample was determined by plating on double-layer agar plates. Experiments were repeated independently three times and analyzed using GraphPad Prism v. 9 (GraphPad Software, San Diego, CA, USA).

1.6. DNA Extraction and Whole Genome Sequencing

DNA extraction was performed using a modified QIAamp DNA Mini Kit (Qiagen, Germantown, MD, USA) protocol. Briefly, a high-titer phage sample was treated with 2.5 U/mL DNase and 0.7 mg/mL RNase for 1.5 h at 37°C, after which 20 mM EDTA was added to the

sample. Next, the sample was treated with 3 μ L of proteinase K and incubated for 1.5 h at 56°C. An equal volume of Buffer AL was added to the treated phage lysate and QIAamp DNA Mini Kit instructions were followed to complete the DNA extraction.

Whole genome sequencing libraries were constructed from extracted EKq1 DNA using the KAPA HyperPlus Library preparation kit (Roche Diagnostics, Indianapolis, IN, USA). The prepared library was sequenced using MiSeq Reagent Kit v3 that produced 300-bp paired-end reads (600 cycles 2×300 bp) (Illumina, San Diego, CA, USA). Whole genome sequencing analysis and phylogenetic analysis was performed. Paired-end sequences (1,864,294 reads total) were assessed for quality using FastQC 0.11.9 (Andrews, 2010) and trimmed with Trimmomatic v0.39 (Bolger et al., 2014). Phage EKq1 genome was assembled de novo using Unicycler 0.4.8 (Wick et al., 2017), its termini and DNA packaging mechanism were determined using PhageTerm (Garneau et al., 2017), and lifestyle was predicted using BACPHLIP (Hockenberry and Wilke, 2021). Protein-coding sequences (CDSs) were annotated using the Pharo pipeline (Bouras et al., 2023; Laslett and Canback, 2004; Bland et al., 2007; Steinegger and Söding, 2017; McNair et al., 2019; Alcock et al., 2020; Chen et al., 2005; Chan et al., 2021; Terzian et al., 2021; Cook et al., 2021, and Ondov et al., 2016). Amino acid sequence similarity searches were performed in Diamond (Buchfink et al., 2015 & 2021) against the nr database. All tools were run with default parameters. The average read coverage was 977 $\times$.

Example 2. Phage EKq1 isolated on *K. quasipneumoniae*

Klebsiella phage, Ekq1 was isolated from sewage water and enriched using a human blood isolate *K. quasipneumoniae* MRSN 829456. EKq1 genome is 48,244 bp long, with G + C content of 56.43%, contained 63 predicted CDSs (Fig. 2) and direct terminal repeats (DTR) of 8,798 bp.

Mash alignment to the INPHARED database (Cook et al., 2021; Ondov et al., 2016) placed EKq1 among *Klebsiella siphophages* currently classified as class Caudoviricetes, with highest DNA identity, >98%, to VLCpiS8c (GenBank ON602734), phiKp_7-2 (LC768468), and vB_KleS-HSE3 (Peng et al., 2020; MT075871). Figure 2 shows the genome organization of Ekq1.

Phage vB_KleS-HSE3 was lytic against one of four tested MDR *K. pneumoniae* strains (Peng et al., 2020). Nucleotide identity higher than 95% indicates that phages EKq1, VLCpiS8c,

phiKp_7-2, and vB_KleS-HSE3 belong to the same species (Adriaenssens and Brister, 2017). These phages contain a putative Cas4-like exonuclease. A similar exonuclease in *Campylobacter* phages stimulated acquisition of host-derived spacers by the bacterial CRISPR-Cas system that might be a decoy to prevent phage DNA acquisition and, therefore, an anti-CRISPR measure
5 (Hooton and Connerton, 2014).

BACPHLIP scored EKq1 genome at 89%, while the threshold for high-confidence lytic lifestyle is 95% (Hockenberry and Wilke, 2021). Additionally, nucleotide BLAST search (Altschul et al., 1990) against the non-redundant (nr) database found a region of EKq1 DNA with 78%–89% identity to bacterial chromosomes, within prophage genes (e.g., MKK01_09025 and
10 MKK01_09030 in *Klebsiella variicola*, GenBank CP092632). However, these genes encode a carbohydrate-binding domain protein and a tail fiber protein and have no relation to lysogenicity.

EKq1 putative proteins showed no homology to products related to lysogenic lifestyle, gene transfer, and bacterial proteins including antibiotic resistance determinants (Alcock et al., 2020) nor virulence factors (Chen et al., 2005). Thus, EKq1 appears to be a lytic phage and a
15 candidate for therapeutic use.

EKq1 as well as EKq2 covers 22/100 Kp strains in the panel: 22 STs, including hv Kp ST11, ST15, ST34, and ST485; six of them are not covered by other phages

DATA AVAILABILITY: The EKq1 genome BioProject, BioSample, GenBank, and the NCBI Sequence Read Archive accession numbers are PRJNA1016341, SAMN37380043, OR555718,
20 and SRR26063729, respectively.

Example 3. KPM1 phage cocktail

Phages were isolated from sewage using >100 diverse *K. pneumoniae* strains (including hypervirulent isolates), and 15 strains of other *Klebsiella* spp. were used for phage enrichment. All candidate phages were characterized for genomic properties, lysis dynamics, host ranges, and
25 pairwise mix stability.

Overall, 187 lytic phages were isolated and classified into 25 genera. These phages were able to lyse 161/206 (78%) diverse MDR *K. pneumoniae* isolates, but only a few phages had

relatively broad host ranges, varying within 17-36%. Therefore, it was pursued discover a phage to cover as many strains as possible with a limited number of phages.

First, *K. pneumoniae* panel was optimized, focusing on MDR epidemic and hypervirulent clones. In order to establish a lethal *K. pneumoniae* lung infection model, five MDR epidemic and/or hypervirulent strains (MRSN 16233, 111775, 113898, 582610, and 976583) were selected and tested in mice. The selected strains caused lethal systemic infection in carbapenem (CP)-treated and untreated mice.

Then, phages were selected to make a phage cocktail. With the selected five core *Klebsiella* phages (AFR4, KEN39, KEN42, EKq1, and EKq2), which are highly lytic and broadly active and compatible in a mix, a new phage cocktail was developed and named KPM1. The KPM1 phage cocktail included one *K. pneumoniae* podophage that belongs to the family *Autographiviridae* (genera *Teetrevirus*, e.g., KEN42), one myophage (Straboviridae, Jiaodavirus, e.g., KEN39), two classified siphophage (Drexlerviridae, Webervirus, e.g., AFR4, and Ekq2), and one unclassified the siphophage isolated on *Klebsiella quasipneumoniae* described above, Ekq1.

KPM1 covers 48/94 sequence types in the diversity strain panel, including high-risk clones ST11, ST14, ST15, ST20, ST37, ST45, ST101, ST107, ST147, ST258, ST322, ST336, ST340, ST394, and ST512.

Next, four groups of BALB/c mice, eight each, were infected intranasally (IN) with a single dose (10^7 CFU) of *K. pneumoniae* MRSN 113898. All mice in PBS-treated group died by Day 2, while phage cocktail KPM1-treated mice survived until Day 4 or Day 5 (extension of time to death by 100-150%). The phage cocktail showed efficacy against a lethal mouse lung infection caused by a hypervirulent strain of *K. pneumoniae* and outperformed standard-of-care antibiotic gentamicin. These data suggest the strong promise of phage therapy to address severe MDR *K. pneumoniae* infections.

Example 4. KPM1-1 phage cocktail

However, there are two problems. One is that broadly phage-susceptible strains did not cause lethality and caused very limited morbidity in a mouse lung model even without carbapenem (CP) treatment (phage-neutrophil synergy). The other is that hypervirulent *K. pneumoniae* strains were phage-resistant.

Next, new phages active against the hv strains were isolated, genomically characterized and evaluated for lysis dynamics and compatibility with the five core phages of KPM1, and tested in MRSN 113898-infected mice. Among the phages active against hv MRSN 113898 strain, phage EKp148 (family Autographiviridae, genera Drulisvirus), was selected and added to the phage cocktail KPM1 so as to make it a 6-phage-containing phage cocktail, KPM1-1 (Table 1).

Table 1. *K. pneumoniae* 6-phage cocktail KPM1-1

Phage ID	Genome size, bp	Family	Subfamily	Genus	Host range
AFR4	48,962	Drexlerviridae	NA	Webervirus	17%
KEN39	166,254	Straboviridae	Tevenvirinae	Jiaodavirus	27%
KEN42	37,932	Autographiviridae	Studiervirinae	Teetrevirus	18%
EKq1	48,244	Siphoviridae	NA	Unclass.	12%
EKq2	?	Drexlerviridae	NA	Webervirus	10%
EKp148	43,127	Autographiviridae	Slopekvirinae	Drulisvirus	0%

KPM1-1 phage cocktail comprising 4 families and 5 genera overall shows 50% host range for the 100-strain panel, and covers 49/94 MLSTs, 32 K serotypes, and 9 O serotypes. Most of STs are global epidemic, MDR, XDR, and hv clones, e.g., ST11, 14, 15, 20, 23, 34, 37, 45, 101, 107, 147, 258, 322, 336, 340, 394, 485, and 512.

However, although EKp148 was added to a five-phage cocktail KPM1 as the 6th phage to make it KPM1-1, it might be replaced with other phages in Table 2.

Table 2. Other eleven *K. pneumoniae* phages isolated on fresh isolates of high-risk lineages.

Hunt ID	Final ID	Genome size, bp	Family	Subfamily	Genus
101301	EKp150	44,226	Autographiviridae	Slopekvirinae	Drulisvirus
101683	EKp151	39,743	Autographiviridae	Studiervirinae	Przondovirus
102031	EKp152	50,459	Drexlerviridae	NA	Webervirus
103245	EKp153	166,709	Straboviridae	Tevenvirinae	Jiaodavirus
105044	EKp154	39,616	Autographiviridae	Studiervirinae	Teetrevirus
106667	EKp155	40,515	Autographiviridae	Studiervirinae	Przondovirus
106795	EKp156	51,448	Drexlerviridae	NA	Webervirus
107365	EKp157	40,423	Autographiviridae	Studiervirinae	Przondovirus
113898-1	EKp158	50,555	Drexlerviridae	NA	Webervirus
113898-2	EKp159	43,127	Autographiviridae	Slopekvirinae	Drulisvirus
113898-3	EKp160	43,151	Autographiviridae	Slopekvirinae	Drulisvirus

Example 5. KPM2 and KPM3 phage cocktails

Another phage KEN18-2-1 was added to KPM1 in place of Ekp148 to make a new 6-phage cocktail KPM2, and this addition increased the activity of the phage cocktail KPM1 from 50% to 69%.

In addition, one more phage 15882-3, which is a recombinant phage of KEN22, KEN25, KEN37, and KEN39, obtained *in vitro* evolution method, was added to KPM2 to make a new 7-phage cocktail KPM3, and this addition increased the cocktail activity to 81%.

Example 6. Sequences

10

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CLAIMS

What is claimed is:

1. A *Klebsiella pneumoniae* phage cocktail comprising

AFR4 phage comprising a nucleotide sequence of SEQ ID NO:1, a variant thereof having at least 97% sequence identity, or a fragment thereof;

KEN39 phage comprising a nucleotide sequence of SEQ ID NO:2, a variant thereof having at least 97% sequence identity, or a fragment thereof;

KEN42 phage comprising a nucleotide sequence of SEQ ID NO:3, a variant thereof having at least 97% sequence identity, or a fragment thereof;

EKq1 phage comprising a nucleotide sequence of SEQ ID NO:4, a variant thereof having at least 97% sequence identity, or a fragment thereof; and

EKq2 phage comprising a nucleotide sequence of SEQ ID NO:5, a variant thereof having at least 97% sequence identity, or a fragment thereof.

2. The phage cocktail of claim 1, wherein the phage cocktail further comprises EKp148 phage comprising a nucleotide sequence of SEQ ID NO:6, a variant thereof having at least 97% sequence identity, or a fragment thereof.

3. The phage cocktail of claim 1, wherein the phage cocktail further comprises phage KEN18-2-1 comprising a nucleotide sequence of SEQ ID NO:7, a variant thereof having at least 97% sequence identity, or a fragment thereof.

4. The phage cocktail of claim 1, wherein the phage cocktail further comprises phage 15882-3 comprising a nucleotide sequence of SEQ ID NO:8, a variant thereof having at least 97% sequence identity, or a fragment thereof.

5. The phage cocktail of claim 1, wherein the phage cocktail further comprises phage KEN18-2-1 of claim 3 and phage 15882-3 of claim 4.

6. A pharmaceutical composition comprising any of the phage cocktails of claims 1-5, wherein the composition is formulated into a solution for parenteral administration.
7. A pharmaceutical composition comprising any of the phage cocktails of claims 1-5, wherein the composition is formulated into a solution for nebulizer.
- 5 8. A pharmaceutical composition comprising any of the phage cocktails of claims 1-5, wherein composition is formulated into a solution or aerosol for nasal spray.
9. A pharmaceutical composition comprising any of the phage cocktails of claims 1-5, wherein the composition is formulated into aerosol or dry powder for inhalation.
- 10 10. A pharmaceutical composition comprising any of the phage cocktails of claims 1-5, wherein the composition is formulated into a lotion, cream, an emulsion, ointment, or dry powder for topical administration.
11. The pharmaceutical composition of any of claims 6-10, wherein the composition comprises pharmaceutically acceptable carriers or excipients for nasal, parenteral, or topical administration.
- 15 12. A spray container containing a pharmaceutical composition of claim 8 or 9 for dispensing any of the phage cocktails of claims 1-5.
13. A method of treating a subject having *K. pneumoniae* infection, the method comprising administering an effective amount of the pharmaceutical composition of any of claims 6-11, through nasal, parenteral, or topical route.
- 20 14. The method of claim 13, wherein the subject has *K. pneumoniae* infection in the lung, urinary tract, skin, and/or blood stream.
15. The method of claim 14, wherein the subject has *K. pneumoniae* infection after lung transplant or is suffering from pneumonia, cystic fibrosis, bronchiectasis, bladder infection (cystitis), kidney infection (pyelonephritis), skin infection (cellulitis, burn wounds), and/or sepsis.
- 25 16. The method of claim 13, 14, or 15, wherein the pharmaceutical composition is optionally co-administered with an antibiotic or antibiotics, and wherein the antibiotic or antibiotics are

administered through nasal, parenteral, topical or oral route, along with the composition or separately before, after, or at the same time with the administration of the composition

17. The spray container of claim 12, wherein the spray container optionally comprises an antibiotic or antibiotics.
- 5 18. A method of disinfecting a surface, comprising applying to the surface the composition comprising any of the phage cocktails of claims 1-5, wherein the composition is formulated into a solution, lotion, aerosol, or powder form.
19. A method of disinfecting a fish tank or aquafarm, comprising applying to the water the composition comprising any of the phage cocktails of claims 1-5, wherein the composition is
10 formulated into a solution or powder form.
20. EKq1 phage comprising a nucleotide sequence of SEQ ID NO: 4, a variant thereof having at least 97% sequence identity, or a fragment thereof.
21. EKp148 phage comprising a nucleotide sequence of SEQ ID NO: 6, a variant thereof having at least 97% sequence identity, or a fragment thereof.
- 15 22. KEN18-2-1 phage comprising a nucleotide sequence of SEQ ID NO: 7, a variant thereof having at least 97% sequence identity, or a fragment thereof.
23. Phage 15882-3, a recombinant phage of KEN22, KEN25, KEN37, and KEN39, comprising a sequence of SEQ ID NO: 8, a variant thereof having at least 97% sequence identity, or a fragment thereof.
- 20 24. A method isolating a new *K. pneumoniae* phage to obtain a *K. pneumoniae* phage having broad antibacterial activity against MDR, XDR, or PDR *K. pneumoniae* subspecies, wherein the method comprises steps of collecting phages from the environment water or human blood, isolating the phage by performing plaque assay with other *Klebsiella* species than *K. pneumoniae*, and testing lytic activity on *K. pneumoniae*.
- 25 25. The method of claim 25, wherein the *Klebsiella* species other than *K. pneumoniae* is *Klebsiella quasipneumoniae*, and optionally MRSN 829456.

26. A *Klebsiella pneumoniae* phage cocktail comprising 3 or more, 4 or more, 5 or more or 6 or more of the following:

AFR4 phage comprising a nucleotide sequence of SEQ ID NO:1, a variant thereof having at least 97% sequence identity, or a fragment thereof;

5 KEN39 phage comprising a nucleotide sequence of SEQ ID NO:2, a variant thereof having at least 97% sequence identity, or a fragment thereof;

KEN42 phage comprising a nucleotide sequence of SEQ ID NO:3, a variant thereof having at least 97% sequence identity, or a fragment thereof;

10 EKq1 phage comprising a nucleotide sequence of SEQ ID NO:4, a variant thereof having at least 97% sequence identity, or a fragment thereof; and

EKq2 phage comprising a nucleotide sequence of SEQ ID NO:5, a variant thereof having at least 97% sequence identity, or a fragment thereof.

EKp148 phage comprising a nucleotide sequence of SEQ ID NO:6, a variant thereof having at least 97% sequence identity, or a fragment thereof.

15 KEN18-2-1 phage comprising a nucleotide sequence of SEQ ID NO:7, a variant thereof having at least 97% sequence identity, or a fragment thereof.

15882-3 phage comprising a nucleotide sequence of SEQ ID NO:8, a variant thereof having at least 97% sequence identity, or a fragment thereof.

20

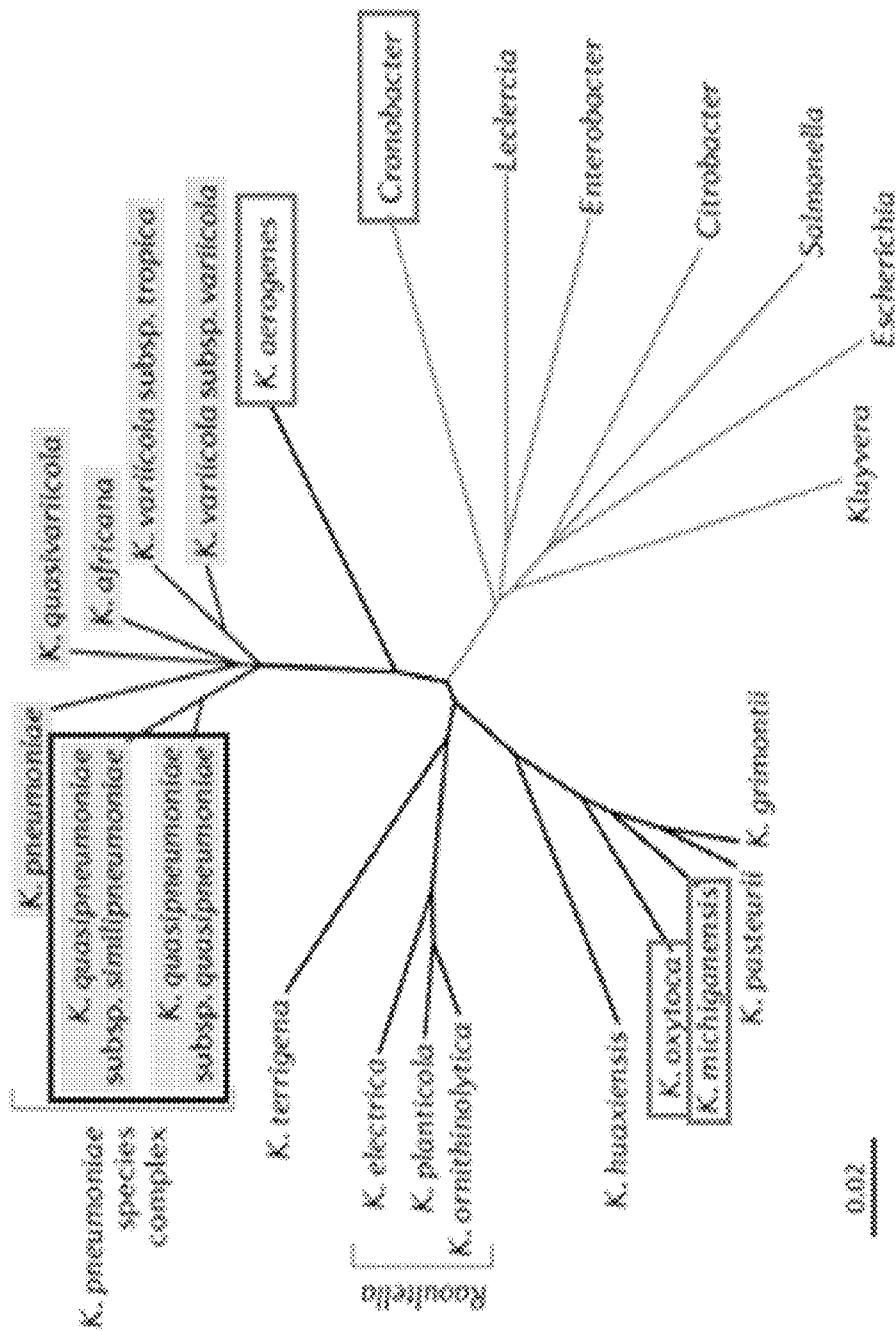
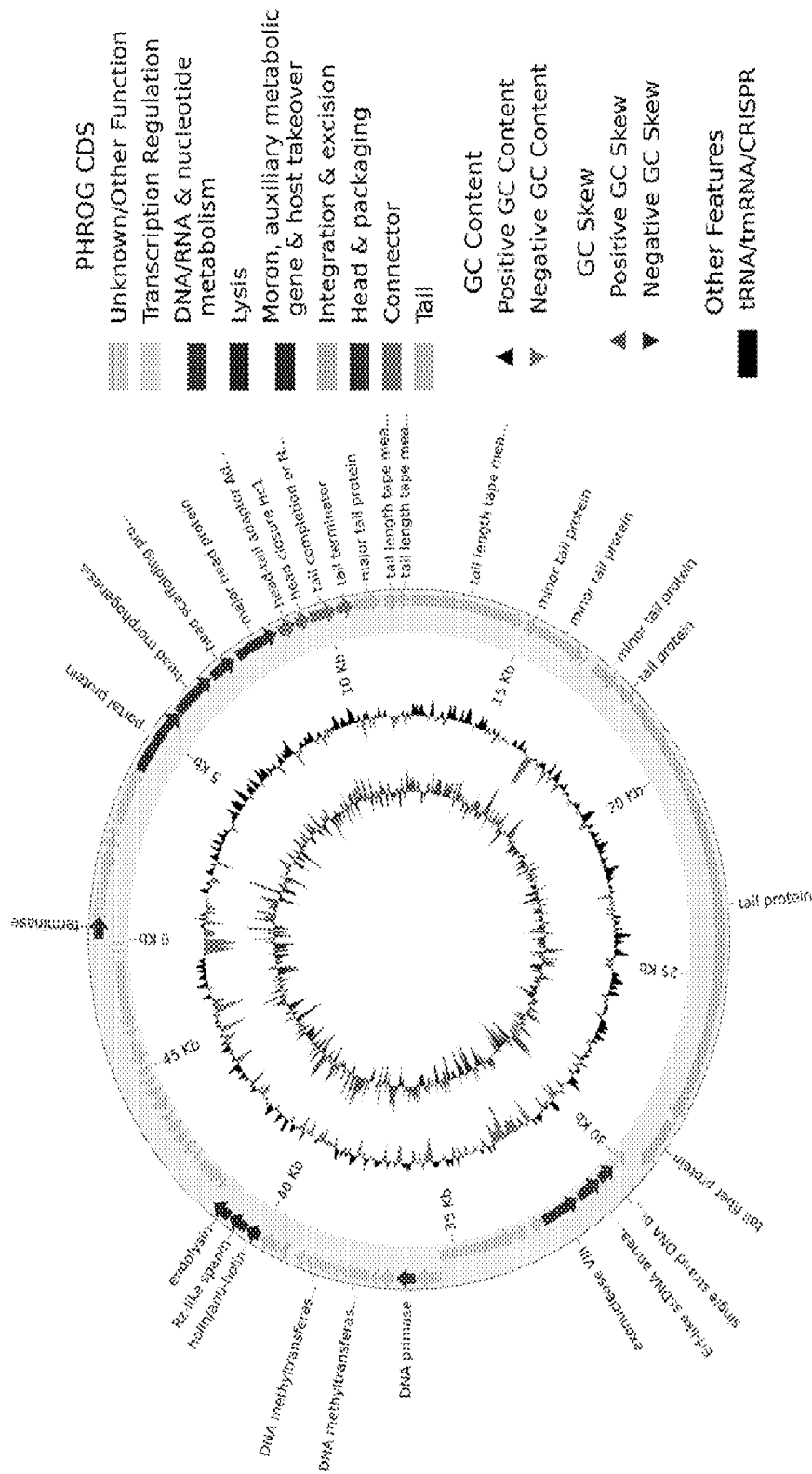


Figure 1



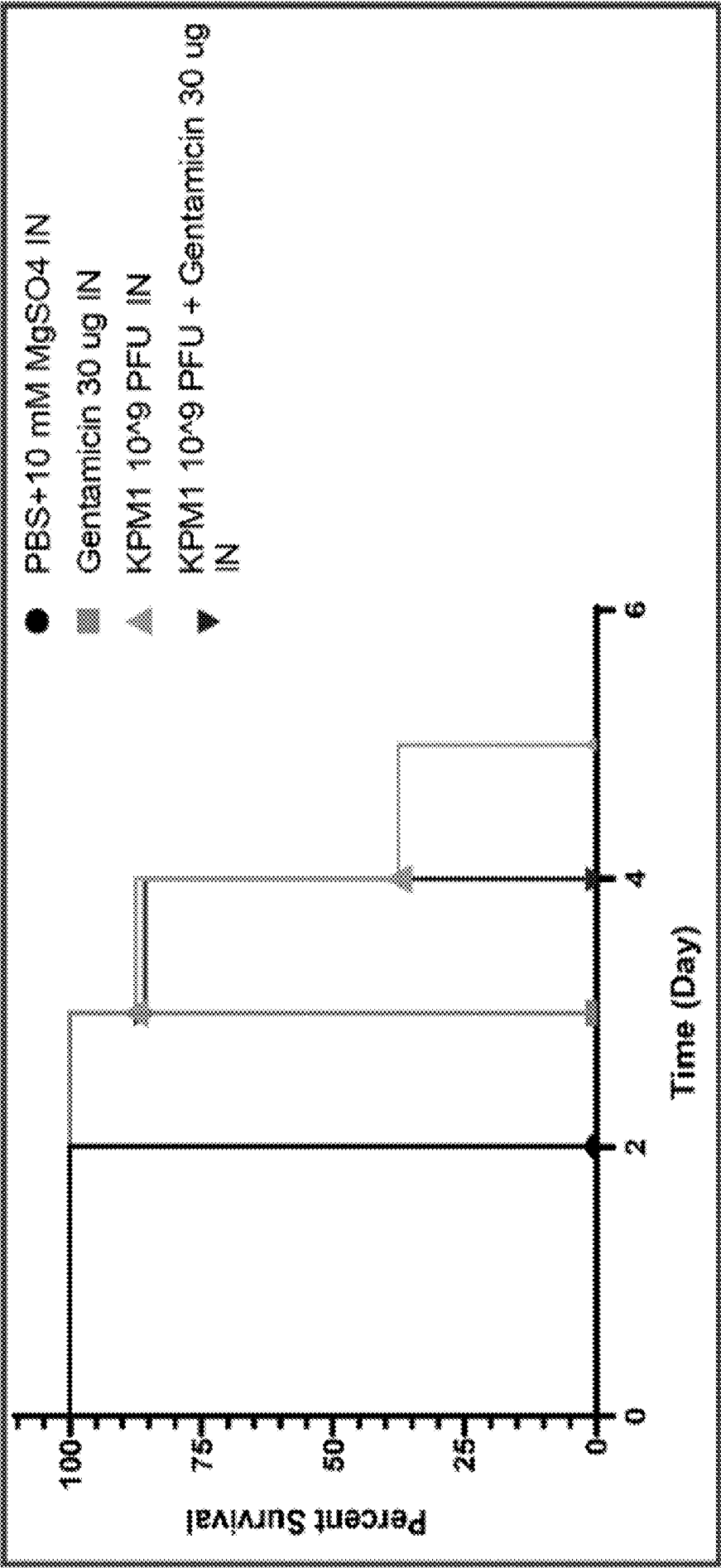


Figure 3

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/051399

A. CLASSIFICATION OF SUBJECT MATTERIPC: **A01N 63/40** (2025.01); **A61K 35/76** (2025.01); **A61P 31/04** (2025.01); *A01P 1/00* (2025.01); *C12N 7/00* (2025.01); *C12R 1/92* (2025.01)CPC: **A01N 63/40**; **A61K 35/76**; **A61P 31/04**; *C12N 2795/10321*; *C12N 2795/10331*; *C12N 2795/10332*; *C12N 7/00*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2019/0290709 A1 (TECHNOPHAGE, INVESTIGACAO E DESENVOLVIMENTO EM BIOTECNOLOGIA, SA) 26 September 2019 (26.09.2019) para [0019]-[0021]; para [0064]	1-2, 6-10, 26
A	US 2014/0295528 A1 (Wang et al.) 10 February 2014 (10.02.2014) para [0411]; SEQ ID NO: 97	21
X	OR555718, European Nucleotide Archive Accession No: OR555718, Klebsiella phage EKq1, complete genome, 09 October 2023 [online]. [Retrieved on 02 December 2024]. Retrieved from the Internet [URL: https://www.ebi.ac.uk/ena/browser/view/OR555718] Entire document	20
A	OL539448, European Nucleotide Archive Accession No: OL539448, Klebsiella phage vB_KpnD_PeteCarol, complete genome, 20 December 2021 [online]. [Retrieved on 02 December 2024]. Retrieved from the Internet [URL: https://www.ebi.ac.uk/ena/browser/view/OL539448] Entire document	1-2, 6-10, 26



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“D” document cited by the applicant in the international application

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

02 December 2024 (02.12.2024)

Date of mailing of the international search report

04 February 2025 (04.02.2025)

Name and mailing address of the ISA/US

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/051399

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	OX335428, European Nucleotide Archive Accession No: OX335428, Klebsiella phage mtp6 genome assembly, chromosome: 1, 03 October 2022 [online]. [Retrieved on 02 December 2024]. Retrieved from the Internet [URL: https://www.ebi.ac.uk/ena/browser/view/OX335428] Entire document	21

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Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13ter.1(a)),
☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: **11-17**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I+, claims 1-10, 20-23, 26, directed to a *Klebsiella pneumoniae* phage, or a phage cocktail, or a pharmaceutical composition comprising a phage cocktail. The phage, phage cocktail, or composition will be searched to the extent that the phage (or phages of the cocktail) encompass: AFR4 phage comprising a nucleotide sequence of SEQ ID NO:1, KEN39 phage comprising a nucleotide sequence of SEQ ID NO:2, KEN42 phage comprising a nucleotide sequence of SEQ ID NO:3, EKq1 phage comprising a nucleotide sequence of SEQ ID NO:4, and EKq2 phage comprising a nucleotide sequence of SEQ ID NO:5, EKp148 phage comprising a nucleotide sequence of SEQ ID NO:6. The first named invention was determined based on this being the first listed set of phages, and corresponding nucleotide sequences (claims 1 and 2). This first named invention has been selected based on the guidance set forth in section 10.54 of the PCT International Search and Preliminary Examination Guidelines. It is believed that claims 1-2, 6-10(in part), 20-21, 26 encompass this first named invention, and thus these claims will be searched without fee to the extent that the phages encompass AFR4 phage comprising a nucleotide sequence of SEQ ID NO:1, KEN39 phage comprising a nucleotide sequence of SEQ ID NO:2, KEN42 phage comprising a nucleotide sequence of SEQ ID NO:3, EKq1 phage comprising a nucleotide sequence of SEQ ID NO:4, and EKq2 phage comprising a nucleotide sequence of SEQ ID NO:5, and/or EKp148 phage comprising a nucleotide sequence of SEQ ID NO:6. Additional phage(s), and corresponding nucleotide sequence(s) will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected phage(s), and corresponding nucleotide sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be phages: AFR4 phage comprising a nucleotide sequence of SEQ ID NO:1, KEN39 phage comprising a nucleotide sequence of SEQ ID NO:2, KEN42 phage comprising a nucleotide sequence of SEQ ID NO:3, EKq1 phage comprising a nucleotide sequence of SEQ ID NO:4, and EKq2 phage comprising a nucleotide sequence of SEQ ID NO:5, KEN18-2-1 phage comprising a nucleotide sequence of SEQ ID NO:7 (claims 1, 3, 6-10(in part), 20, 22, 26).

Group II, claims 18-19, directed to a method of disinfecting a surface, comprising applying to the surface a phage cocktail composition.

Group III, claims 24-25, directed to a method of isolating a new *K. pneumoniae* phage to obtain a *K. pneumoniae* phage having broad antibacterial activity against MDR, XDR, or PDR *K. pneumoniae* subspecies.

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

The inventions listed as Groups I+, II and III do not relate to a single special technical feature under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I+ includes the special technical feature of a composition comprising or consisting of a *Klebsiella pneumoniae* phage, that is not required by Group II or III.

Group II includes the special technical feature of a method of disinfecting a surface, comprising applying to the surface a phage cocktail composition, that is not required by Group I+ or III.

Group III includes the special technical feature of a method of isolating a new *K. pneumoniae* phage to obtain a *K. pneumoniae* phage having broad antibacterial activity against MDR, XDR, or PDR *K. pneumoniae* subspecies, that is not required by Group I+ or II.

The inventions of Group I+ each include the special technical feature of different phage(s), and corresponding nucleotide sequence(s), and is considered a distinct technical feature.

Common Technical Features

Group I+, Group II, and Group III share the common technical features of a *Klebsiella pneumoniae* phage cocktail comprising 3 or more *K. pneumoniae* phages.

No technical features are shared between the different phage nucleotide sequence of Group I+ and accordingly these groups lack unity a priori.

Additionally, even if the inventions listed as Group I+ were considered to share the technical features of a pharmaceutical composition comprising any of the phage cocktails, wherein the composition is formulated into aerosol or dry powder for inhalation, a solution for parenteral administration or nebulizer, or a lotion, cream, an emulsion, ointment, or dry powder for topical administration, these shared technical features are previously disclosed by the prior art, as further discussed below.

The feature shared by Groups I+, II, and III and the feature shared by the inventions listed as Group I+ are taught by US 2019/0290709 A1 to Technophage, Investigacao e Desenvolvimento em Biotecnologia, SA (hereinafter "Technophage").

Technophage teaches a pharmaceutical composition comprising a *Klebsiella pneumoniae* phage cocktail comprising 3 or more different *K. pneumoniae* phages (para [0019]-[0021] "a purified bacteriophage comprising a nucleic acid having a nucleotide sequence with at least 90% sequence identity to SEQ ID NO:7 (corresponding to *K. pneumoniae* phage F92/15), or at least 99% sequence identity to SEQ ID NO:8 (corresponding to *K. pneumoniae* phage F105/15), at least 98% sequence identity to SEQ ID NO:9 (corresponding to *K. pneumoniae* phage F134/15), or at least 95% sequence identity to SEQ ID NO:10 (corresponding to *K. pneumoniae* phage F141/15), and having antibacterial activity against *K. pneumoniae*...a pharmaceutical composition comprising at least two different phage"; para [0064] - "Another aspect of the invention relates to cocktail compositions of one or more bacteriophage and/or phage products of the invention, as well as combinations with other phage, including F391/08 (previously disclosed in PCT/PT2011/000031). Still another aspect relates to pharmaceutical compositions of the phage(s) and/or phage product(s), as well as methods of using same in the treatment and prevention of bacterial infections, in particular, respiratory infections caused by *P. aeruginosa* and/or *K. pneumoniae*"), wherein the composition is formulated into aerosol or dry powder for inhalation (para [0020] "the composition is formulated for administration as an aerosol"; para [0145] "administration by inhalation").

As the technical features were known in the art, they cannot be considered special technical features that would otherwise unify the groups.

Therefore, Groups I+, II and III inventions lack unity under PCT Rule 13 because they do not share the same or corresponding special technical feature.

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Box No. III **Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-2, 6-10(in part), 20-21, 26, limited to AFR4, KEN39, KEN42, EKq1, EKq2, EKp148, and SEQ ID NOs: 1-6**

- Remark on Protest**
- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.