Centro de Investigación en Alimentación y Desarrollo, A.C.

CARACTERIZACIÓN DE BACTERIÓFAGOS LÍTICOS DE CEPAS CLÍNICAS DE *Pseudomonas aeruginosa* MDR

Por:

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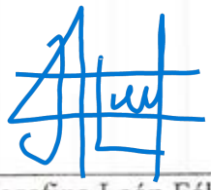

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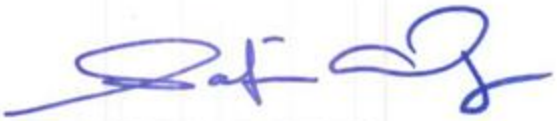

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RESUMEN

Pseudomonas aeruginosa (PA) es una bacteria que causa infecciones nosocomiales y el tratamiento de pacientes con estas infecciones se complica por la capacidad de esta bacteria para desarrollar resistencia a antibióticos y formar biopelículas. Por lo anterior, se buscan alternativas para controlar dichas infecciones, como el uso de bacteriófagos (fagos) líticos. Por lo que el objetivo del proyecto fue evaluar la actividad *in vitro* de fagos líticos aislados de cepas clínicas de PA multidrogo resistentes (MDR). En la primera parte del proyecto, a 17 cepas PA de pacientes con infecciones nosocomiales se les realizó prueba de susceptibilidad a carbapenémicos (aztreonam, imipenem, meropenem), identificación molecular de genes de resistencia a antibióticos y formación de biopelículas (*mex*A, *mex*B, *opr*M, *psl*A, *psl*D), se analizó el pangenoma, y se evaluó su capacidad de formar biopelículas. Siete de las 17 cepas fueron resistentes a los carbapenémicos probados. Las 17 cepas presentaron amplificación para los genes de resistencia a antibióticos y formación de biopelículas. En el análisis del pangenoma, se detectaron 12,273 pangenes, siendo 4,813 genes el genoma central. El porcentaje de genoma accesorio varió entre 19-29%. Se encontraron genes de resistencia a aminoglucósidos y betalactámicos principalmente y entre 230-240 factores de virulencia. La secuencia tipo (ST) más predominante fue la 233 descrita como de alto riesgo. Todas las cepas produjeron biopelícula. De acuerdo con estas características, las cepas en estudio se consideran epidemiológicamente relevantes. En la segunda parte del proyecto, se lograron aislar cinco fagos (AR1 ATCC, AR2 ATCC C, AR1 S, AR2 I, AR1 R1), los cuales se caracterizaron morfológica, biológica y genómicamente. La prueba de rango de hospedero mostró que los fagos pudieron lisar a 54 de las 80 cepas utilizadas para este ensayo. Los fagos con mayor actividad lítica son el AR2 I y el AR1 S, por lo que se puede considerar que los fagos tienen un amplio rango de hospedero. Los cinco fagos son genéticamente diferentes. Tuvieron tamaños de genoma entre 46,379 y 213,521 pb, de 72 a 209 ORFs, no se encontraron ARNt, ni genes de virulencia ni de resistencia a antibióticos. Más del 70% de los ORFs codifican para proteínas hipotéticas. El análisis bioinformático sugiere que los genomas de los fagos presentan una organización funcional por módulos típica en fagos con tallo. Las caracterizaciones indican que los fagos son seguros para utilizarse en terapia, sin embargo, se debe realizar una caracterización completa para garantizar su eficacia y seguridad.

Palabras clave: *Pseudomonas aeruginosa*, bacteriófagos, biopelículas.

ABSTRACT

Pseudomonas aeruginosa (PA) is a bacterium that causes nosocomial infections, and the treatment of patients with these infections is complicated by the ability of this bacterium to develop resistance to antibiotics and form biofilms. Hence, alternatives are sought to control these infections, such as the use of lytic bacteriophages (phages). Therefore, the objective of the project was to evaluate the *in vitro* activity of lytic phages isolated from clinical strains of PA multidrug resistant (MDR). In the first part of the project, 17 PA strains from patients with nosocomial infections underwent carbapenem susceptibility tests (aztreonam, imipenem, meropenem), molecular identification of antibiotic resistance genes, and biofilm formation (*mex*A, *mex*B, *opr*M, pslA, *psl*D), the pangenome was analyzed, and its ability to form biofilms was evaluated. Seven of the 17 strains were resistant to the carbapenems tested. The 17 strains showed amplification for antibiotic resistance genes and biofilm formation. In the pangenome analysis, 12,273 pangenes were detected, with 4,813 genes being the core genome. The percentage of accessory genome varied between 19-29%. Genes of resistance to aminoglycosides and beta-lactams were found, mainly and between 230-240 virulence factors. The most predominant sequence type (ST) was 233 described as high risk. All strains produce biofilm. Based on these characteristics, the strains under study are considered epidemiologically relevant. In the second part of the project, it was possible to isolate five phages (AR1 ATCC, AR2 ATCC C, AR1 S, AR2 I, AR1 R1), which were characterized morphologically, biologically, and genomically. The host range test showed that phages can lyse 54 of the 80 strains used for this assay. The phages with the highest lytic activity are AR2 I and AR1 S, so it can be considered that the phages have a wide host range. The five phages are genetically different. They had genome sizes between 46,379 and 213,521 bp, from 72 to 209 ORFs, no tRNA, virulence genes, or antibiotic resistance were found. More than 70% of the ORFs coded for hypothetical proteins. Bioinformatic analysis suggests that phage genomes exhibit a modular functional organization typical of stem phages. Characterizations indicate that the phages are safe for use in therapy, however full characterization must be performed to ensure efficacy and safety.

Key words: *Pseudomonas aeruginosa*, bacteriophages, biofilms.

1. SINOPSIS

Pseudomonas aeruginosa (PA) es una bacteria gramnegativa establecida en el agua, el suelo y varios organismos huéspedes, tiene la capacidad excepcional de generar resistencia a los antibióticos por su capacidad de mutación y adquirir genes de resistencia horizontalmente. Esta bacteria puede prosperar en una amplia variedad de nichos ecológicos y causar daño en diferentes huéspedes, ya que es una bacteria versátil y de fácil adaptabilidad debido al mantenimiento de genes de virulencia y metabólicos en su genoma. La importancia de esta bacteria radica en que provoca una importante morbimortalidad entre los pacientes inmunocomprometidos y los que requieren ventilación mecánica, como los pacientes con quemaduras y los que padecen fibrosis quística (FQ).

A lo largo de los años, han surgido cepas PA multirresistentes debido al uso indiscriminado y generalizado de antibióticos ya sea por una administración excesiva, la automedicación, la prescripción aleatoria de medicamentos inadecuados y el uso prolongado de antibióticos, por lo que es imperativo el desarrollo de nuevos métodos alternativos para controlar a esta bacteria. En este contexto, existe un renovado interés por las terapias basadas en bacteriófagos (fagos), que son virus que infectan bacterias y son parásitos intracelulares obligados que utilizan la maquinaria del huésped para replicarse. Se ha reportado que existen fagos con una especificidad de huésped donde infectan cepas específicas de una sola especie de bacteria; no obstante, existen fagos que pueden infectar múltiples especies. Adicionalmente a esta especificidad de huésped, los costos de producción y purificación son mucho más económicos que los de los antibióticos, lo cual hace atractiva la propuesta de utilizar fagos para tratar infección por bacterias MDR. Sin embargo, en la actualidad existen muy pocos datos clínicos relacionados con estudios de fagos contra esta bacteria en México.

Por lo tanto, resulta necesario realizar estudios sobre el aislamiento y caracterización morfológica, biológica y genómica de fagos contra *Pseudomonas aeruginosa* para que posteriormente puedan ser seleccionados y evaluados en modelos *in vivo*, lo que proporcionará información confiable para diseñar cócteles efectivos, ya sea utilizando fagos mixtos o en combinación con antibióticos,

logrando un gran avance en la investigación clínica.

1.1. Justificación

Las “superbacterias” se han convertido en uno de los problemas de salud pública más importantes en el mundo. En Europa se ha estimado que los microorganismos multidrogo resistentes (MDR) causan alrededor de 25,000 muertes por año, mientras que en los Estados Unidos el reporte es de 23,000 muertes anuales. En México, la vigilancia epidemiológica de las infecciones nosocomiales está a cargo de la Red de Vigilancia Epidemiológica Hospitalaria. Adicionalmente, el Instituto de Seguridad y Servicios Sociales para los Trabajadores del Estado (ISSSTE) realiza su control y vigilancia de las infecciones nosocomiales a través del Comité para la Detección y Control de Infecciones Nosocomiales, encontrando a *P. aeruginosa* entre los primeros agentes causales.

La Organización Mundial de la Salud (OMS) reporta que este problema se encuentra en todo el mundo donde *Escherichia coli, Klebsiella pneumoniae, Staphylococcus aureus* y *P. aeruginosa* constituyen el 50% de las bacterias resistentes a antibióticos como la cefalosporina, un antibiótico de tercera generación. Ante esta perspectiva, la OMS ha sugerido la generación de métodos alternativos para el tratamiento de dichas infecciones en vez de antibióticos.

Diversos estudios se han iniciado para estandarizar métodos de tratamientos alternativos que utilizan nuevas formas de acción para obtener una actividad antimicrobiana. Estos métodos incluyen terapia por bacteriófagos, terapia de quelación de hierro, péptidos antimicrobianos, vacunación profiláctica, terapia fotodinámica y terapias basadas en óxido nítrico. Uno de estos métodos que está recobrando interés es el del desarrollo de terapias basados en fagos respaldado por varios estudios que sugieren que la terapia basada en fagos líticos y degradadores de biopelículas puede ser prometedora para el tratamiento de infecciones nosocomiales.

Por lo que esta propuesta se establece para dar un primer paso en la valoración de la terapia con fagos para tratar infecciones causadas por *P. aeruginosa* como una alternativa inicial para atender

un problema de salud que afecta a todos los niveles de atención en el ISSSTE, por lo que los beneficios impactarán en bienestar del paciente y en un ahorro de costos a largo plazo.

1.2 Antecedentes

1.2.1 Bacterias Multirresistentes a Antibióticos

El uso persistente de antibióticos, la automedicación y la exposición a infecciones en los hospitales, ha provocado la aparición de bacterias resistentes a múltiples fármacos (MDR) responsables del 15.5% de las infecciones hospitalarias (IHA) en el mundo. El término "ESKAPE" abarca seis de estos patógenos con creciente resistencia a múltiples fármacos y virulencia: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* y *Enterobacter* spp. Los patógenos ESKAPE son responsables de la mayoría de las infecciones nosocomiales y son capaces de "escapar" de la acción biocida de los agentes antimicrobianos (Mulani *et al*., 2019).

A. baumannii y *P. aeruginosa* resistentes a carbapenémicos junto con β-lactamasas de espectro extendido (BLEE) están en la lista de prioridad crítica de patógenos (Mulani *et al*., 2019).

1.2.2 *Pseudomonas aeruginosa* e Infecciones Asociadas a la Atención a la Salud (IAAS)

P. aeruginosa MDR se definen como cepas resistentes a varias clases de antibióticos: penicilinas, cefalosporinas, carbapenémicos, aminoglucósidos y fluoroquinolonas. El antibiótico de elección administrado a pacientes con infecciones contra *P. aeruginosa* MDR era la colistina (polimixina E). Sin embargo, el aislamiento reciente de cepas resistentes a la colistina puede crear un riesgo de brotes epidémicos graves en condiciones hospitalarias (Krylov, 2014).

P. aeruginosa es un patógeno común en hospitales y particularmente en unidades de cuidados intensivos debido a la resistencia innata que posee a muchos antibióticos y antisépticos, la capacidad de adquirir genes que participan en mecanismos de resistencia a múltiples clases de antibióticos y la capacidad de sobrevivir en ambientes húmedos. Esta bacteria se ha asociado a varias infecciones potencialmente mortales como endocarditis y septicemia, infecciones del tracto urinario, cistitis, neumonía e infecciones de heridas quirúrgicas. Es el cuarto patógeno nosocomial más comúnmente aislado que representa el 10% de todas las infecciones adquiridas en el hospital y la segunda causa más común de neumonía y la tercera causa más común de infección del torrente sanguíneo por microorganismos gramnegativos (Pachori *et al*., 2019).

P. aeruginosa es una bacteria común en hospitales y particularmente en unidades de cuidados intensivos, ocasionando infecciones asociadas a la atención de la salud (IAAS), las cuales son un problema de salud pública importante debido a la frecuencia con que se producen, la morbilidad y mortalidad que provocan y la carga que imponen a los pacientes, al personal sanitario y a los sistemas de salud (Pachori *et al*., 2019).

La OMS define a las IAAS, también conocidas como infecciones nosocomiales o intrahospitalarias, como “infecciones contraídas por un paciente durante su tratamiento en un hospital u otro centro sanitario y que dicho paciente no tenía ni estaba incubando en el momento de su ingreso. Dichas infecciones pueden aparecer después del alta y representan el evento adverso más frecuente asociado al cuidado del paciente” (IMSS, 2017).

Según encuestas nacionales de prevalencia de IAAS recientes y datos de los programas de seguimiento de la bacteriemia hospitalaria de varios países europeos, se estima que estas infecciones afectan, en promedio, a 1 de cada 20 pacientes hospitalizados, lo que corresponde a un total anual de 4.1 millones de pacientes; de estos, se estima que alrededor de 37,000 pacientes fallecen cada año en la Unión Europea por esta causa (OPS, 2012).

Una característica de *P. aeruginosa* que le permite subsistir en ambientes adversos y causar infecciones nosocomiales, es su capacidad para formar biopelículas, las cuales son notoriamente difíciles de manejar debido a la baja permeabilidad de la membrana externa a los antibióticos y a

los mecanismos de resistencia a los antibióticos que permiten la resistencia cruzada a múltiples clases y tipos de antibióticos (Chan *et al*., 2016).

1.2.3 Microbiología de *P. aeruginosa*

P. aeruginosa es un miembro del género *Pseudomonas*, aerobio gram negativo/anaerobio facultativo que se encuentra ubicuamente en el suelo y en los ambientes acuáticos. Debido a la producción de pigmentos solubles en agua como la pioverdina, que es un pigmento fluorescente de color verde amarillo, y la piocianina que es un pigmento azul-verde, es fácilmente detectable en agar (Pachori *et al*., 2019).

Metabólicamente, *P. aeruginosa* es oxidasa positiva (es decir, prefiere crecer en ambientes aeróbicos o microaerobios) y fermentación sin lactosa, pero también es capaz de usar nitrito o nitrato como un receptor terminal de electrones en condiciones anóxicas (incluso dentro del pulmón con fibrosis quística (FQ)). Se han secuenciado los genomas de múltiples cepas de *P. aeruginosa*, y su análisis indica que el tamaño total del genoma de este patógeno oscila entre 5.5 y 7 Mbp y es rico en GC (es decir, >65% de contenido de GC), con un genoma accesorio de hasta 200 kbp, lo cual indica la capacidad de *P. aeruginosa* para adquirir elementos genéticos mediante transferencia horizontal de genes, como transformación, conjugación y transducción, demostrando la versatilidad y adaptabilidad de este organismo (Malhotra *et al*., 2019).

Como grupo, los miembros del género *Pseudomonas* tienen requisitos nutricionales mínimos para su crecimiento y pueden utilizar una amplia variedad de fuentes ambientales para la nutrición; *P. aeruginosa* a menudo solo necesita acetato y amoníaco como fuente de carbono y nitrógeno, respectivamente. Este requerimiento nutricional mínimo le permite crecer en ambientes marginales, como superficies secas de quirófanos, salas de hospitales, clínicas y equipos médicos, así como lavabos, duchas, incluso contaminando el agua destilada, demostrando ser una fuente importante de infección nosocomial (Pachori *et al*., 2019).

1.2.4 Mecanismos de Resistencia y Biopelículas de *P. aeruginosa*

El mecanismo de resistencia a los antibióticos en *P. aeruginosa* se da por resistencia intrínseca (resistencia que de manera natural se encuentra presente en la bacteria) a una variedad de agentes antibacterianos debido a la baja permeabilidad de su membrana externa y a la expresión de múltiples enzimas modificadoras de antibióticos, como las enzimas modificadoras de aminoglucósidos, betalactamasas que incluyen betalactamasas de espectro extendido (ESBLs) y metalo-beta-lactamasas (MLBs); bombas de eflujo de antibióticos como MexAB-OprM, MexEF-OprN, MexCD-OprJ y MexXY-OprM y resistencia adquirida (adquisición de genes de resistencia a antibióticos codificados por cromosomas o plásmidos por transferencia genética horizontal) (Chatterjee *et al*., 2016).

Las betalactamasas notificadas con mayor frecuencia en *P. aeuginosa* son las BLEE (p. ej., enzimas PER, GES, VEB), las betalactamasas de tipo OXA y las MBL, destacando los tipos IMP, VIM, NDM. Asimismo, se ha informado de un fenotipo infrecuente de resistencia a carbapenémicos en *P. aeruginosa*, que consiste en resistencia a carbapenémicos con susceptibilidad mantenida a cefalosporinas. Las enzimas modificadoras de aminoglucósidos también se detectan comúnmente en *P. aeruginosa*, como aacA4, aadA7, aph(3´)-IIb. La resistencia a la colistina se desarrolla en *P. aeruginosa* ya sea por alteraciones en los sistemas reguladores de dos componentes (PhoPQ y PmrAB) o mediante la adquisición de elementos genéticos móviles (mcr). La resistencia a las fluoroquinolonas se desarrolla por acumulación de mutaciones en los genes de girasa y topoisomerasa como en *gyr*A, *gyr*B, *par*C y *par*E. Sin embargo, la transferencia de elementos genéticos móviles, como integrones, elementos conjugativos integradores (ICE), transposones y plásmidos, desempeñan un papel en la diseminación de marcadores de resistencia a las fluoroquinolonas crpP, aac(6´)-Ib-cr o qnrVC1 (Kocsis *et al*., 2021).

Las bombas de eflujo MexAB-OprM, MexEF-OprN y MexCD-OprJ confieren resistencia a los antibióticos betalactámicos. Además, la regulación al alza de MexEF-OprN y MexCD-OprJ confieren resistencia a las fluoroquinolonas; y la regulación ascendente de MexXY-OprM también afecta la resistencia a los aminoglucósidos. La pérdida de OprD, una porina que forma canales

transmembrana estrechos se asocia con resistencia al imipenem y menor susceptibilidad al meropenem. Las cepas de *P. aeruginosa* que regulan positivamente MexEF-OprN y exhiben una expresión reducida de OprD, muestran resistencia tanto a las fluoroquinolonas como al imipenem, y una susceptibilidad reducida al meropenem (Chatterjee *et al*., 2016).

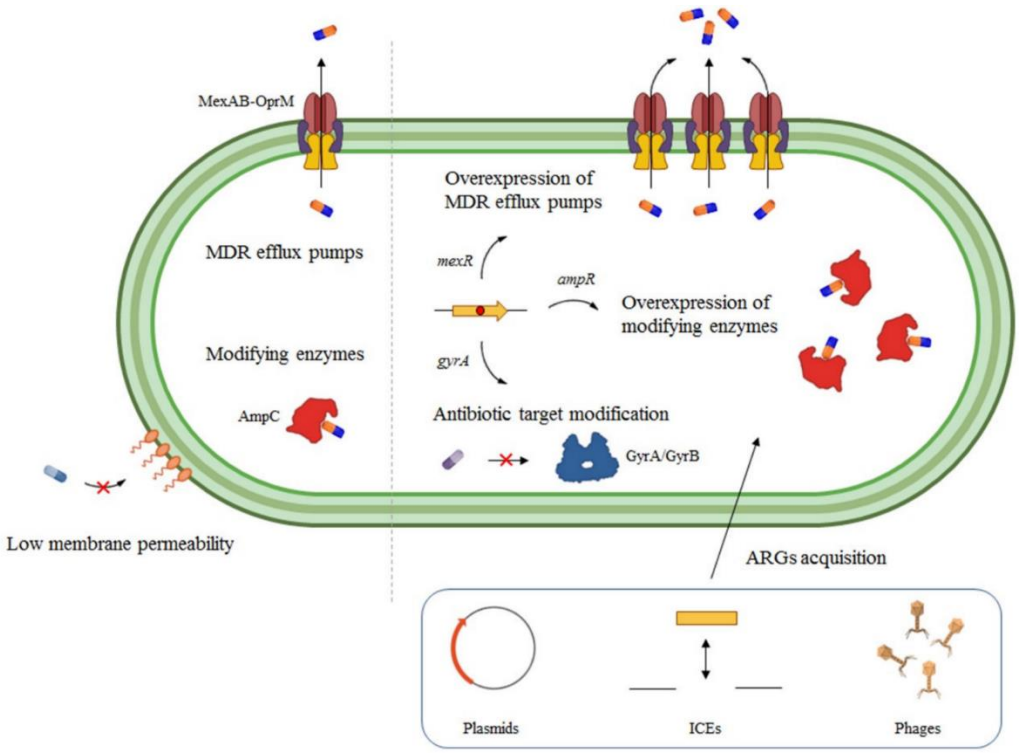

Figura 1. Representación esquemática de los principales elementos implicados en la resistencia antibiótica intrínseca y adquirida en *P. aeruginosa*. Esta bacteria posee una notable resistencia intrínseca a los antibióticos causada, entre otros factores, por la producción de enzimas modificadoras de antibióticos (por ejemplo, β-lactamasa AmpC), baja permeabilidad de la membrana externa y una gran cantidad de bombas de salida de resistencia a múltiples fármacos (MDR) como MexAB-OprM (Tomado de Sanz *et al*., 2021).

Otra estrategia de supervivencia altamente exitosa implica la producción de biopelículas. Las biopelículas se definen como comunidades multicelulares altamente organizadas de bacterias embebidas en sustancias poliméricas extracelulares (EPS) que protegen a los microorganismos del ataque del sistema inmunitario del huésped. Las bacterias en las biopelículas también aumentan su resistencia a los antimicrobianos. Aunque las composiciones de EPS son diversas según las bacterias que inician su formación, la mayoría consisten en proteínas, polisacáridos y ADN

extracelular. Los exopolisacáridos producidos por *P. aeruginosa* incluyen al menos tres polímeros distintos (Psl, Pel y alginato). La producción de alginato confiere a *P. aeruginosa* un fenotipo mucoide, y Psl y Pel están comúnmente presentes en la superficie de cepas de *P. aeruginosa* no mucoides. Aunque el mecanismo de las biopelículas que median la inefectividad inmune del huésped y la resistencia a los antibióticos no está bien definido, generalmente se reconoce que destruir las biopelículas o los exopolisacáridos secretados por las bacterias es útil para controlar la infección mediada por dichos patógenos (Mi *et al*., 2019).

El desarrollo de biopelículas en etapa temprana se compone de eventos, por ejemplo, hay una variedad de estructuras bacterianas como adhesinas, pili tipo IV y lipopolisacárido (LPS) que están involucradas en la unión a la superficie y entre bacterias, y estas estructuras bacterianas están específicamente reguladas por señales ambientales. Estudios recientes demostraron que el inicio de la formación de biopelículas se produce con un aumento de c-di-GMP, un segundo mensajero intracelular, que activa la producción de adhesinas y diversos productos de EPS. Por ejemplo, el contacto de *P. aeruginosa* a una superficie es reconocida por la proteína WspA, una proteína receptora unida a la membrana, que crea una señal para producir c-di-GMP y a su vez regula positivamente la producción de adhesina *CdrA, Psl, Pel* y alginato en *P. aeruginosa* (Lee y Yoon, 2017).

Después de que las bacterias se adhieren a las superficies o entre sí, se someten a una serie de cambios para adaptarse al nuevo modo de vida. A medida que *P. aeruginosa* unida a la superficie crece y forma microcolonias, comienzan a producir EPS y construir estructuras, y conforme la biopelícula madura, las bacterias experimentan cambios fisiológicos y se vuelven mucho más resistentes al estrés del ambiente o antibióticos. Este desarrollo y maduración de biopelículas están estrechamente relacionados con un sistema de señalización llamado quorum sensing (QS) (Lee y Yoon, 2017).

Este proceso comprende redes de genes y reguladores capaces de modular toda la vida del microorganismo. Cuando se activan estos sistemas QS, la bacteria puede producir moléculas como las acil homoserina lactonas que se difunden libremente dentro y fuera de la membrana bacteriana. Como resultado de esta difusibilidad libre, la concentración dentro del organismo refleja la

concentración afuera, lo que permite que una vez que se ha logrado una masa crítica, las moléculas QS inducen la expresión de los genes responsables de la adhesión y la producción de biopelículas. En este estado, las microcolonias de bacterias están rodeadas por una matriz densa, que las protege contra la fagocitosis y evita la penetración de antibióticos (Stefani *et al*., 2017).

La etapa final del desarrollo de la biopelícula es el desprendimiento y existen varios tipos de mecanismos como son: desprendimiento, erosión y dispersión de semillas. Estos mecanismos de separación son esenciales para crear nuevas biopelículas en nuevos nichos. Los mecanismos de desprendimiento y erosión durante la etapa de desprendimiento de biopelículas se denominan desprendimientos pasivos. El primer mecanismo es de desprendimiento, el cual consiste en que se pierde una gran parte de una biopelícula de la masa original, y la erosión es un lavado de una pequeña porción de biomasa o bacterias de la superficie exterior. La dispersión de semillas es el mecanismo de desprendimiento activo de las biopelículas de *P. aeruginosa*. En este proceso, las biopelículas de *P. aeruginosa* liberan células planctónicas individuales o microcolonias desde el centro de la biopelícula, dejando una cavidad vacía (Lee y Yoon, 2017).

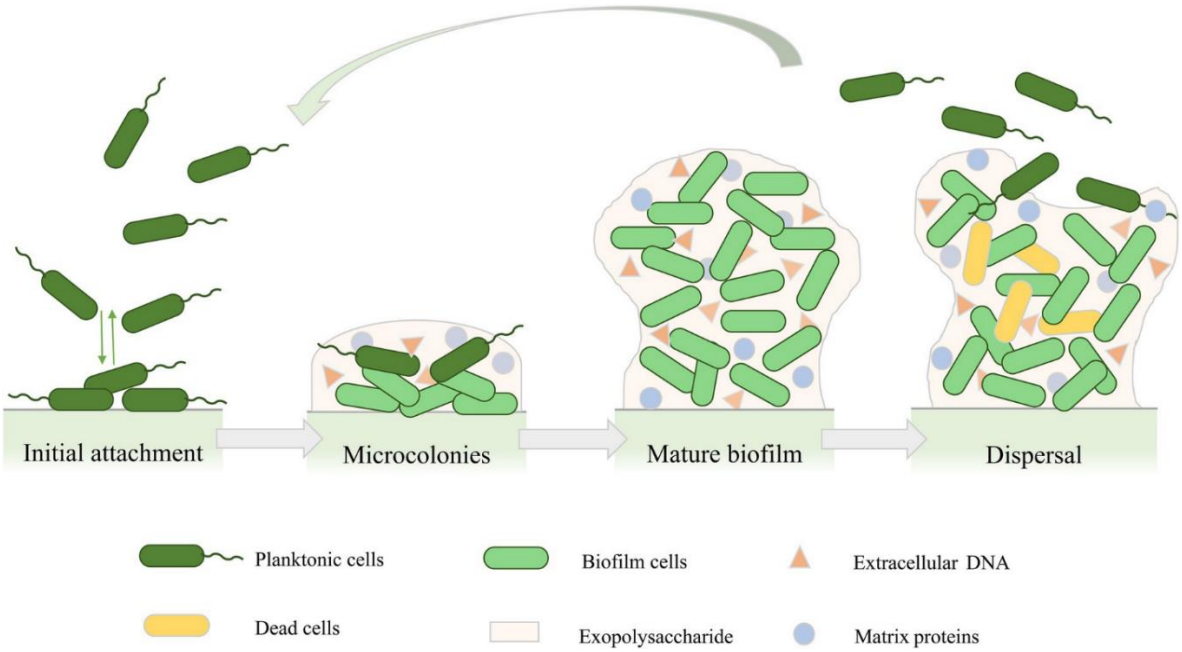

Figura 2. Pasos del desarrollo de una biopelícula de *P. aeruginosa*. La formación de biopelícula incluye cuatro etapas: unión inicial, microcolonias, biopelícula madura y dispersión de biopelícula (Tomado de Yin *et al*., 2022).

Hay tres EPS identificadas en *P. aeruginosa*: Psl, Pel y alginato. Psl es un componente importante para el inicio y mantenimiento de las biopelículas de *P. aeruginosa* al proporcionar unión a la superficie celular e interacciones intercelulares. En la etapa tardía de la maduración de la biopelícula, se ha demostrado que Psl se acumula en el exterior de las biopelículas estructuradas. Esta acumulación proporciona soporte estructural y permite la dispersión posterior de la biopelícula. El polisacárido Pel es un componente esencial para que *P. aeruginosa* forme películas en la interfaz aire-líquido y las biopelículas asociadas a la superficie sólida. Las otras funciones son actuar como una plataforma para la estructura de la biopelícula y proporcionar protección contra los antibióticos aminoglucósidos. Sin embargo, la mayoría de estos roles dependen de las cepas de *P. aeruginosa*.

El alginato es el EPS más estudiado de las biopelículas de *P. aeruginosa*, y es producido principalmente por cepas aisladas de pacientes con FQ. El alginato se conoce como un factor utilizado para distinguir las biopelículas mucosas o no mucosas, aunque se descubrió que Psl también contribuye al fenotipo mucoide de las biopelículas. El alginato juega muchos papeles importantes para las biopelículas, por ejemplo, retiene agua y nutrientes, y proporciona resistencia a los antibióticos y evasión inmune (Lee y Yoon, 2017).

P. aeruginosa es capaz de formar biopelículas en las que las bacterias están encerradas en exopolisacáridos (EPS) y son metabólicamente menos activas. La matriz extracelular en la biopelícula impide la acción de los antibióticos ya que actúan como una barrera de difusión. La baja actividad metabólica de las bacterias en la biopelícula también limita la eficacia de muchas clases de antibióticos que se dirigen a diversas vías metabólicas. Interesantemente, se ha reportado en la literatura que existen algunos bacteriófagos que tienen la capacidad de degradar las biopelículas e infectan las bacterias que residen en ellas (Malik *et al*., 2017).

Debido a su naturaleza adaptable y su alta capacidad de supervivencia, la bacteria puede sobrevivir en superficies inanimadas secas del ambiente hospitalario de 6 h a 6 meses y frecuentemente contaminando el equipo y las superficies de atención médica, como monitores, botones de ventilación, barandas, equipo respiratorio, tubos de diálisis (Pachori *et al*., 2019).

La tolerancia a los antimicrobianos por biopelículas resulta de una combinación de mecanismos, incluida la penetración restringida de antimicrobianos a través de la matriz de exopolisacáridos, la actividad fisiológica diferencial causada por la penetración limitada de oxígeno y nutrientes a través de la biopelícula como resultado del consumo bacteriano y la expresión diferencial de genes específicos. El modo de crecimiento de la biopelícula conduce al estrés oxidativo, lo que provoca una mayor mutabilidad en las bacterias asociadas a las biopelículas. Es probable que estos cambios fenotípicos jueguen un papel importante en la persistencia de la infección por *P. aeruginosa* en la mayoría de los pacientes con infecciones nosocomiales a pesar de los mejores intentos médicos de erradicación (Stefani *et al*., 2017).

Por todas las características antes mencionadas sobre *P. aeruginosa* y para fines de este proyecto, resulta prioritario determinar el potencial que tienen las cepas aisladas en estudio mediante una caracterización que permita seleccionar aquellas cepas que sean de relevancia epidemiológica, en este caso serán las cepas de relevancia clínica que presenten resistencia a dos o más antimicrobianos (multirresistencia) mediante la caracterización fenotípica, y mediante la caracterización genotípica mediante secuenciación masiva, que presenten genes de virulencia y genes que participan en la formación de biopelículas, lo cual permitirá realizar ensayos posteriores para el aislamiento de fagos, ya que se ha reportado que para combatir los mecanismos de resistencia y producción de biopelículas en *P. aeruginosa*, la terapia con fagos puede considerarse en la actualidad como un procedimiento extremadamente importante para curar las infecciones (Krylov, 2014).

1.2.5 Bacteriófagos

Los bacteriófagos (fagos) se consideran la entidad biológica más abundante en la biósfera con un número aproximado de 10^{31}, ya que se estima que el número de células procariotas es de alrededor de 10^{30} y se calcula que los fagos son al menos 10 veces mayores que esta cantidad. Los fagos son virus específicos de bacterias que aprovechan el metabolismo de sus huéspedes bacterianos para replicarse (Buttimer *et al*., 2017).

Frederick Twort (1915) y Felix d'Herelle (1917) son considerados los descubridores de los fagos. Ya se habían descrito agentes antibacterianos que insinuaron la existencia de fagos antes que los trabajos de Twort y d'Herelle, sin embargo, fueron los pioneros en atribuir este fenómeno a un origen viral. De manera rápida se reconoció el potencial de los fagos como agentes antibacterianos, con D'Herelle en 1919 que demostró que sus preparaciones de fagos eran eficaces en el tratamiento de pacientes con disentería en el Hôpital des Enfants-Malades en París. Después de esta investigación, se realizaron una gran cantidad de estudios utilizando fagos para tratar infecciones estafilocócicas, placa bubónica y cólera en humanos (Buttimer *et al*., 2017).

Las partículas fágicas (virión) contienen genoma de ácido nucleico (ADN o ARN) dentro de una envoltura proteica o lipoproteica, llamada cápside (Summers, 2005). La estructura más común en la naturaleza es la de los bacteriófagos con tallo, los cuales tienen una estructura que consiste principalmente de cabeza, tallo y fibras del tallo, sin embargo, existen otras familias de fagos con distintas estructuras. En la cabeza se encuentra encapsulado el ácido nucleico y por el tallo pasa el ácido nucleico para inyectar el material genético, mientras que las fibras del tallo ayudan al fago en su adhesión a la membrana bacteriana (Patel *et al*., 2015).

1.2.5.1 Taxonomía. Los genomas de fagos están compuestos de ADN o ARN, que pueden ser de doble cadena o de cadena sencilla. Este material genético está empaquetado en una cápside que puede ser poliédrica (*Microviridae*, *Corticoviridae*, *Tectiviridae*, *Leviviridae* y *Cystoviridae*), filamentosa (*Inoviridae*), pleomórfica (*Plasmaviridae*) o conectada a una cola (*Caudovirales*) (Dion *et al*., 2020).

La asignación de fagos en grupos taxonómicos es un paso fundamental después de su aislamiento. La taxonomía oficial de fagos fue establecida por el Comité Internacional de Taxonomía de Virus (ICTV), que organiza a los virus en niveles taxonómicos, que incluyen clase, orden, familia, subfamilia, género. Dentro del ICTV, el Subcomité de Virus Bacterianos y Arqueológicos (BAVS) es responsable de los taxones de fagos, clasificándolos en función de algunas de sus propiedades como la composición molecular del genoma (ss/ds, ADN o ARN), la morfología, la estructura de la cápside y el rango de hospederos. Recientemente, con la creciente disponibilidad de genomas

virales, el uso de genomas para la clasificación taxonómica se ha vuelto más ampliamente aceptada. Debido a los extensos esfuerzos de secuenciación para el descubrimiento de virus, el ICTV no puede ponerse al día con la gran cantidad de fagos recientemente identificados y, por lo tanto, muchos de estos virus aún no están clasificados. Un desafío detrás de este retraso es la falta de herramientas de clasificación taxonómica estándar, precisa y completa para los fagos. El estándar taxonómico en el ICTV cambia constantemente a medida que se descubren nuevos fagos. El ICTV actualizó el sistema de clasificación de fagos en agosto de 2022, en el que se eliminan varias familias importantes del sistema ICTV anterior, en donde estos cambios pueden afectar significativamente el desempeño de la clasificación familiar (Zhu *et al*., 2022).

La mayoría de los fagos aislados a la fecha tienen cola y tienen genomas de dsDNA. Por ejemplo, el informe ICTV de 1999 clasificó a los fagos con cola en tres familias, 16 géneros y 30 especies, mientras que el informe de 2018 los agrupó en cinco familias, 26 subfamilias, 363 géneros y 1,320 especies. Se han actualizado las pautas integrales para la clasificación de fagos y se espera que la lista de taxones de virus aumente en los próximos años. La gran mayoría de los fagos descritos hasta la fecha tienen una morfología con cola con un genoma dsDNA y pertenecen a la clase Caudoviricetes. Esta clase viral, aunque en proceso de reclasificación, comprende actualmente diferentes familias como por ejemplo, *Myoviridae, Siphoviridae, Podoviridae*, *Ackermannviridae* y *Herelleviridae*. Las dos últimas familias se crearon recientemente porque los enfoques basados en redes y los metanálisis indicaron que representaban grupos distintos dentro de la familia *Myoviridae* (Dion *et al*., 2020).

1.2.5.2 Ciclos de Replicación. Los fagos se pueden dividir en varios grupos de acuerdo con su ciclo de replicación; pueden infectar de manera productiva a la bacteria huésped, lo que resulta en más virus (fagos líticos) o pueden entrar en un estado latente cuando sus genomas se integran en el ADN de la célula huésped (fagos lisogénicos). Los fagos líticos/virulentos pueden matar a las células diana mientras que los fagos lisogénicos/temperados se vuelven parte del genoma de las células hospedadoras y permanecen en forma de profago por un tiempo (Doffkay *et al*., 2015).

Los fagos pueden tener ciclos de vida lítico o lisogénico como se observa en la Figura 1. Una vez

que el fago reconoce el receptor (pueden ser proteínas, lipopolisacáridos, ácidos teicoicos y cápsulas) en la célula hospedera, se adhiere e inyecta su material genético. La siguiente estrategia de replicación dependerá de si el fago es virulento o temperado. Los fagos virulentos, son capaces de replicarse a través del ciclo lítico, un proceso que implica la producción de nueva progenie viral, en donde el virus aprovecha la maquinaria enzimática de su hospedero para producir sus ácidos nucleicos y proteínas, los cuales se ensamblan y convierten en nuevas partículas virales que son liberados de la célula infectada y van a infectar nuevas células (Salmond y Fineran, 2015).

Por otro lado, los fagos temperados entran o bien al ciclo lítico o forman una asociación estable con el hospedero, llamado lisogenia, donde integra su material en cromosoma bacteriano de la célula hospedera manteniéndose como un profago. Bajo condiciones de estrés, el profago puede salir del estado lisogénico y producir viriones que posteriormente son liberados de la bacteria. Generalmente, la salida de la progenie del fago resulta de la muerte celular (Salmond y Fineran, 2015). También existe otro estado llamado pseudolisogenia que se define como una etapa de estancamiento del desarrollo de un fago en una célula huésped sin la multiplicación del genoma del fago (ciclo lítico) o su replicación sincronizada con el ciclo celular y el mantenimiento estable en la línea celular (ciclo lisogénico), que procede sin degradación del genoma viral, lo que permite el posterior reinicio del desarrollo del virus. Este fenómeno generalmente es causado por condiciones de crecimiento desfavorables para la célula hospedera (como la inanición) (Łoś y Węgrzyn, 2012). Algunos fagos de ADN muestran un estilo de vida productivo de infección crónica en el que la célula huésped no se lisa tras la liberación de partículas de fagos de progenie; en cambio, las partículas se excretan continuamente al exterior a través de la membrana. Dependiendo del fago, el genoma puede integrarse en el genoma del huésped o permanecer en el citoplasma. Los ejemplos mejor estudiados de infección crónica son los de los fagos filamentosos (Mäntynen *et al*., 2021).

Para la eliminación de patógenos en terapia de fagos, los virus más importantes son los fagos líticos. La lisogenia debe evitarse ya que puede conducir a una transferencia genética no deseada (Doffkay *et al*., 2015).

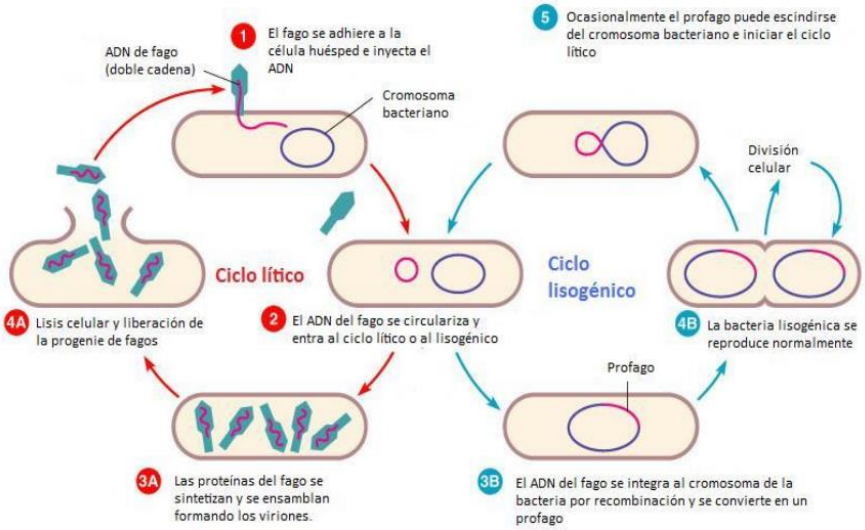

Figura 3. Esquema de los ciclos de replicación lítico y lisogénico (correspondiente al fago λ) explicados en el diagrama (Tomado de Tortora *et al*., 2010).

1.2.5.3 Uso de Fagos en Infecciones por *P. aeruginosa*. Se ha descubierto que el 94.2% de los fagos conocidos que se dirigen al género *Pseudomonas* pertenecen a la clase Caudoviricetes, que comprende tres familias de fagos de ADN bicatenario (ADNds) que difieren en las características de la cola del fago. Se han secuenciado 8 especies de fagos *Pseudomonas* sin cola: 2 pertenecientes a la familia *Inoviridae* (fagos de ADN monocatenario [ssDNA]), 2 pertenecientes a la familia *Leviviridae* (fagos ssRNA) y 4 pertenecientes a la familia *Cystoviridae* (fagos dsRNA). El 85% de los fagos del género *Pseudomonas* secuenciados pertenecientes al orden Caudoviricetes son específicos para las especies de *P. aeruginosa* y la mayoría (aproximadamente el 60%) son fagos líticos, mientras que el 21.8% son templados y el 18.2% no fueron clasificados. Entre los fagos líticos de *P. aeruginosa*, el 41% pertenece a la familia *Myoviridae* y el 38% a la familia *Podoviridae*, y la familia menos representativa es la *Siphoviridae*. Solo el 1% de los fagos líticos de *P. aeruginosa* no están clasificados. La distribución del tamaño del genoma de fagos líticos de *P. aeruginosa* por familia son bastante divergentes, con fagos *Myoviridae* que comprenden tamaños de genoma que varían de 64.1 kb a 309.2 kb, mientras que los genomas de fagos *Podoviridae* y *Siphoviridae* son mucho más pequeños y están en los rangos de 41.6 a 74.9 kb y 34.5 a 61.1 kb, respectivamente. Los fagos líticos de *P. aeruginosa* se han aislado de diferentes fuentes en todo el mundo; sin embargo, parece que las aguas residuales, incluidas las aguas residuales de hospitales y plantas de tratamiento de aguas residuales, son la mejor opción para su aislamiento (Pires *et al*., 2015).

En ausencia de antibióticos activos contra cepas de *P. aeruginosa* MDR, la terapia con fagos se convierte en la principal forma de tratar las infecciones por *P. aeruginosa*. Diferentes estudios confirman que el uso de fagos en condiciones clínicas puede ayudar a disminuir la frecuencia de infecciones nosocomiales locales. Por ejemplo, aunque una circulación espontánea de fagos ligeramente virulentos no puede garantizar la eliminación de cepas de hospital de *P. aeruginosa* en clínicas, el uso de fagos altamente virulentos adaptados a *P. aeruginosa* local ayudó a disminuir la frecuencia de infecciones hospitalarias hasta 40.8% (Krylov, 2014).

En cuanto al uso de bacteriófagos se tiene al fago PADP4 contra *P. aeruginosa* MDR que se aisló de infecciones de heridas y que pertenece a la familia *Podoviridae,* el cual se cocultivó con *P. aeruginosa* MDR y mostró una reducción significativa del crecimiento a las 12 h de incubación y a la multiplicidad de infección de 1 (Rani *et al*., 2019). También se aisló un bacteriófago MA-1 de aguas residuales que pertenece a la familia *Myoviridae,* el cual redujo significativamente la fase de crecimiento logarítmico de *P. aeruginosa*-2949 (2.5×10^3 UFC/ml) en comparación con el control (sin fago); asimismo, este fago también mostró reducciones significativas en biopelículas de 24, 48 y 74 h de edad de 2.0, 2.5 y 3.2 veces después de 6 h de tratamiento con el fago, respectivamente, en comparación con el control (Adnan *et al*., 2019).

En otro estudio, se examinó la efectividad de los fagos en el tratamiento de la infección por *P. aeruginosa* resistente a imipenem en un modelo murino experimental. Se eligió un fago con alta actividad lítica entre 29 fagos aislados de las aguas residuales del hospital local. El fago causó una disminución en la mortalidad de los ratones infectados por vía intraperitoneal con *P. aeruginosa* resistente a los antibióticos, y se concluyó que la terapia con fagos puede usarse como una terapia para pacientes con infecciones resistentes a los antibióticos. Las cepas de *P. aeruginosa* aisladas de pacientes con infecciones postoperatorias demuestran una alta sensibilidad a las preparaciones comerciales de fagos (Krylov, 2014).

También se encuentran disponibles fagos para el tratamiento de infecciones respiratorias causadas por *P. aeruginosa, B. cenocepacia, E. coli* y *K. pneumonia*. Se ha demostrado la eficacia de la actividad del fago contra cepas clínicas obtenidas del esputo con FQ y en biopelículas en etapas relativamente tempranas. Se ha demostrado que los fagos con actividad lítica poseen hidrolasas

que degradan los exopolisacáridos bacterianos. Los estudios en animales han demostrado que varias rutas de entrega de fagos son efectivas para tratar infecciones pulmonares agudas. Sin embargo, se necesita hacer más trabajo para mostrar la eficacia de la terapia con fagos en infecciones crónicas contra biopelículas más maduras que contienen comunidades polimicrobianas bien establecidas tanto en sistemas *in vitro* como *in vivo* (Malik *et al*., 2017).

Se encontró que la dosis de fago, la concentración de bacterias y el momento de la terapia con fagos tienen un impacto significativo en los resultados de la terapia con fagos. En la mayoría de los casos, administrar altas dosis de fago inmediatamente o poco después de la inoculación de los pulmones con bacterias dio mejores resultados. Es posible que se necesiten cócteles de fagos que cubran múltiples especies y que tengan el rango de huéspedes correcto para diferentes cepas y así faciliten la eliminación de infecciones crónicas. Habiendo diagnosticado adecuadamente la constitución polimicrobiana de la infección se podría formular y administrar las mezclas de fagos a medida. Con dicho propósito, se requerirían de bancos de fagos bien caracterizados con una larga vida útil para preparar tales cócteles de fagos formulados y suministrados para uso clínico. El desafío aquí es la entrega de concentraciones precisas de fagos formulados en el sitio de la infección, y en el caso de la fibrosis quística, un reto adicional es que se puede requerir que éstos penetren en las biopelículas para acceder a la bacteria (Malik *et al*., 2017).

Por lo tanto, actualmente es importante que la terapia con fagos se convierta en un procedimiento médico de rutina en oposición a lo que es ahora: demostraciones ocasionales de éxito en aplicaciones relativamente raras. Es evidente que tal transición requerirá una acumulación de grandes colecciones de fagos específicos para *P. aeruginosa* y estudios profundos de éstos. Los propósitos de tales estudios son los siguientes: (1) clasificación, utilizando procedimientos tales como microscopía electrónica (EM) y análisis RFLP, evaluación de homología de ADN con fagos previamente estudiados, secuenciación y anotación de genomas de fagos, y un estudio de características fenotípicas y (2) demostración de que los fagos elegidos para la terapia son virulentos (matan bacterias en todas las condiciones); sus genomas no codifican toxinas y otros factores de patogenicidad y virulencia ni tienen la capacidad de participar en procesos de transferencia genética horizontal, lo que lleva a la modificación de las islas de patogenicidad. Eso significa que los fagos seleccionados para la terapia deben estudiarse cuidadosamente en diferentes

condiciones. Solo dichos estudios pueden garantizar el uso seguro de los fagos en la terapia a largo plazo (Krylov, 2014).

1.2.5.4 Caracterización de Bacteriófagos.

1.2.5.4.1 Caracterización morfológica de bacteriófagos. Debido a que existen una gran cantidad de bacteriófagos en la naturaleza, es indispensable que éstos se identifiquen y se clasifiquen taxonómicamente en grupos de acuerdo con características que compartan entre sí. Para ello, se realiza una clasificación taxonómica (Adriaenssens *et al*., 2012).

La clasificación taxonómica es importante ya que tiene un valor predictivo para direccionar los experimentos que pueden demostrar tal clasificación y resultados, ya que la elección de algunos métodos depende de la estructura y clasificación taxonómica del virus. El estudio de los virus isométricos, filamentosos y pleomórficos requiere investigaciones más detalladas que la de las especies con tallo (Ackermann, 2011).

1.2.5.4.2 Caracterización biológica de bacteriófagos. Se pueden determinar parámetros como estabilidad del bacteriófago ante factores bióticos y abióticos, pero el de mayor importancia es el rango de hospedero (Gill y Abedon, 2003).

Una de las características más importantes que poseen los fagos es la especificidad por un hospedero en particular. Esta especificidad es generalmente encontrada a nivel de cepa, a nivel de especie, o más raramente a nivel de género. El rango depende principalmente de la interacción del fago con los receptores celulares y posteriormente, por los sistemas de restricción-modificación bacteriana (Jorquera *et al*., 2015).

El rango de hospedero permite determinar las cepas bacterianas sobre las que tiene actividad lítica el bacteriófago, por lo cual se considera una de las características biológicas más importantes

(Ohno *et al*., 2012). Esto a través de la capacidad del fago para introducir su material genético en el citoplasma de las células bacterianas, replicar su genoma, ensamblar nuevas partículas virales y liberar la progenie (Thiem y Cheng, 2009).

Otra característica, es la cinética de replicación, que nos permite identificar dos parámetros importantes; el periodo de latencia y el tamaño de explosión. El periodo de latencia es el tiempo mínimo que comprende desde la adsorción hasta la liberación extracelular de fagos recién formados. La replicación de ácido nucleico y la formación de proteínas se producen durante este periodo. Posteriormente, las partículas de fago experimentan maduración, es decir, el ácido nucleico y las proteínas son ensamblados, posteriormente los virus son liberados por la degradación enzimática de la pared celular bacteriana. El tamaño de explosión se define como el número de partículas de fago que se liberan por célula bacteriana que es lisada. Tanto el periodo de latencia como el tamaño de explosión son característicos de cada uno de los fagos, pero pueden variar de acuerdo con el hospedero, medio y temperatura de propagación utilizados (Raya *et al*., 2003).

1.2.5.4.3 Caracterización genómica de bacteriófagos. Este proceso se realiza mediante los perfiles de restricción utilizando diferentes endonucleasas de restricción, para obtener un patrón de digestión del material genético que pueda ser comparado con el de otros fagos y permita determinar la variabilidad genética (Gill y Hyman, 2010).

Por otra parte, la secuenciación nucleotídica del genoma es otra herramienta, que permite estructurar el genoma del fago así como identificar aquellos genes asociados con factores de virulencia, resistencia a antibióticos o de lisogenia, así como también genes con funciones estructurales, empaquetamiento de ADN, lisis y procesamiento que permite confirmar la estructura del fago, así como su mecanismo de replicación (Skurnik *et al*., 2007); además se pueden identificar productos génicos con potencial biotecnológico.

Los genomas de virus bacterianos pueden ser ADN o ARN y su material genético varía ampliamente: la longitud del genoma va de 3,405 pb a 497,513 pb, la densidad de genes tiene un rango de 0.29 a 1.36 y el número de proteínas codificadas van desde 1 a 675 (Pardini *et al*., 2017). Los genes de fagos están organizados en módulos funcionales, los genes que codifican proteínas

con funciones relacionadas se agrupan entre sí y están regulados por promotores comunes (Brüssow *et al*., 2001). El orden de los genes en estos módulos suele ser conservado, aunque sus posiciones pueden variar en diferentes grupos de virus (Hendrix, 2002).

A medida que los genomas de fagos son más grandes (>100 kb) se ha encontrado una marcada división de genes en dos categorías con diferentes comportamientos evolutivos (Comeau *et al*., 2007). El primer grupo de genes, conocido como genes esenciales, se encuentra en todos los miembros de un grupo dado de fagos. Se incluyen los genes involucrados en la estructura del fago, como la cápside y la cola, y genes del metabolismo del ADN. El resto de los genes, los genes no esenciales, generalmente no se encuentran en todos los genomas de un grupo, y son más propensos a carecer de funciones identificadas. Sin embargo, cuando se conocen las funciones de estos genes, por lo general proporcionan funciones accesorias que ayudan adaptarse al fago a su nicho ecológico (Sullivan *et al*., 2010).

Existen tecnologías que permiten la secuenciación de cientos de genomas virales y se espera que los nuevos avances permitan detectar el genoma completo de rutina de cada patógeno que sea encontrado (Margulies *et al*., 2005). La secuenciación del genoma de fagos destinados a aplicación médica y de control biológico se ha convertido en obligatorio para la aprobación reglamentaria (Hagens y Loessner, 2010).

La plataforma Illumina utiliza secuenciación en síntesis por amplificación en puente. Los oligonucleótidos para la amplificación (uno con un sitio escindible), complementario a las secuencias adaptadoras introducidas durante los pasos de preparación de la biblioteca inversa, se unen a toda la superficie de la celda de flujo. El primer paso para la carga de la biblioteca a la celda de flujo es la desnaturalización de los fragmentos de ADN de doble cadena (ADNdc) en moléculas de ADN de cadena sencilla (ADNcs) (Peña-Castro *et al*., 2013). Estas hebras son hibridadas con los oligonucleótidos en la superficie y utilizadas como cebadores para la posterior amplificación. Durante estos pasos el extremo 3' de las moléculas de la biblioteca copiados puede hibridar con los oligonucleótidos complementarios sobre la celda de flujo, formando así una estructura de puente. El paso final es eliminar una de las cadenas de los fragmentos de ADN de doble cadena usando el sitio escindible en el oligonucleótido de la superficie (Buermans y Dunnen, 2014). Después de la generación de clúster, los amplicones y un oligonucleótido de secuenciación se hibrida con una

secuencia universal, que delimitan la región de interés. Cada ciclo de la secuenciación consiste en la extensión de una sola base con una polimerasa de ADN y una mezcla de cuatro nucleótidos modificados. Estos nucleótidos se encuentran modificados de tal manera que sean químicamente escindibles en la posición hidroxilo 3' permitiendo sólo la incorporación de una sola base en cada ciclo y sean marcados con fluorescencia con cuatro marcadores los cuales permite conocer la identidad de cada nucleótido. Después de la extensión de una base y la obtención de la imagen, se realiza una escisión química para el siguiente ciclo (Shendure *et al*., 2008).

1.3 Hipótesis

1. Las cepas de *Pseudomonas aeruginosa* aisladas de pacientes del Centro Médico Nacional "20 de Noviembre" presentarán multirresistencia a antibióticos, genes de virulencia, resistencia a antibióticos y formación de biopelículas.
2. Al menos un bacteriófago aislado cumplirá con las características para ser seleccionado con potencial para el control de *Pseudomonas aeruginosa* MDR.

1.4 Objetivo General

Evaluar la actividad antimicrobiana de bacteriófagos aislados de aguas residuales del Centro Médico Nacional "20 de Noviembre" sobre cepas de *Pseudomonas aeruginosa* MDR.

1.5 Objetivos Específicos

1. Caracterizar fenotípica y genotípicamente cepas de *Pseudomonas aeruginosa* aisladas de pacientes del Centro Médico Nacional "20 de Noviembre".

2. Aislar y caracterizar biológica, morfológica y genómicamente bacteriófagos líticos obtenidos de aguas residuales de hospital con actividad antimicrobiana sobre *Pseudomonas aeruginosa* MDR.

3. Seleccionar aquellos bacteriófagos que cumplan con las características deseables para ser utilizados con potencial lítico sobre *Pseudomonas aeruginosa* MDR.

1.6. Sección Integradora del Trabajo

La información presente en este manuscrito está dividida en secciones denominadas capítulos y se presentan de la siguiente manera:

En la primera etapa de este proyecto de investigación se provee información sobre el potencial que tienen las cepas de *P. aeruginosa* estudiadas mediante una caracterización que permita seleccionar aquellas cepas que sean de relevancia epidemiológica y como segunda etapa se presenta la caracterización de los fagos aislados para garantizar que sean aptos para utilizarse como fagoterapia.

En el primer artículo se realizó una reseña sobre los hallazgos más relevantes y recientes sobre la actividad de los fagos líticos contra cepas de *P. aeruginosa* aisladas de pacientes con fibrosis quística y entornos hospitalarios, y se analizaron las perspectivas sobre el uso de la terapia con fagos en el tratamiento de *P. aeruginosa* en pacientes con fibrosis quística encontrando que es necesario el uso de enfoques terapéuticos personalizados que demuestren y garanticen la efectividad y seguridad de su uso para destruir biopelículas de *P. aeruginosa*. Además, se analizaron los desafíos de la fagoterapia tales como resistencia bacteriana, gama limitada de hospederos, falta de marco regulatorio, gran escala fabricación, estudio de biología, farmacología de fagos, e interacciones bacteria-fago que siguen dejando lagunas en el conocimiento básico. Lo anterior nos permitió concluir que los avances en biotecnología y la biología molecular pueden ayudar a superar estos problemas y esfuerzos de investigación que van acompañados de prácticas rigurosas y enfoques comerciales y regulatorios para lograr una terapia de fagos avanzada en

entornos clínicos. Este artículo se encuentra publicado en la revista Folia Microbiologica.

El segundo artículo consistió en un análisis genómico y filogenético del genoma de 17 cepas de *P. aeruginosa* aisladas de pacientes con infecciones asociadas a la atención de la salud en el hospital 20 de Noviembre, además que se detectaron genes de resistencia a antibióticos, virulencia y formación de biopelículas encontrando que la mayoría de las cepas en estudio fueron resistentes a los carbapenémicos probados. Los genes de resistencia a antibióticos (*mex*A, *mex*B y *opr*M) y de formación de biopelículas (*psl*A y *psl*D) se detectaron en todas las cepas, además se encontraron diferencias en el tamaño del genoma accesorio entre las cepas, es importante mencionar que algunas cepas aisladas en este trabajo resultaron asociadas con clones epidémicos globales de alto riesgo. Todas las cepas estaban representadas en dos grupos entre las cepas globales de *P. aeruginosa*. Se encontraron 45 genes de resistencia a antibióticos adquiridos horizontalmente relacionados con resistencia a aminoglucósidos y betalactámicos, principalmente, y entre 230-240 genes asociados a factores de virulencia. La caracterización de las cepas clínicamente relevantes de *P. aeruginosa* aisladas en este trabajo nos ilustra acerca de su diversidad genómica y múltiples mecanismos de patogenicidad. Este artículo se encuentra enviado a la revista Molecular Genetics and Genomics.

El tercer artículo se encuentra como borrador, en preparación para su envío a revista internacional indizada y arbitrada, en el cual se incluyen los resultados relacionados con el aislamiento y caracterización morfológica, genómica y biológica (en proceso) de cinco fagos (AR1 ATCC, AR2 ATCC C, AR1 S, AR2 I y AR1 R1) que infectan aislados clínicos de *P. aeruginosa*. La prueba de rango de hospedero mostró que los fagos pueden lisar a 54 de 80 cepas (71 cepas clínicas, 9 ambientales). Los fagos con mayor actividad lítica son el AR2I (47/80) y el AR1S (43/80). Todas las cepas ambientales fueron lisadas por al menos dos fagos. Por lo anterior, se puede considerar que los fagos tienen un amplio rango de hospedero. Los cinco fagos son genéticamente diferentes con tamaños de genoma entre 46,379 y 213,521 pb, de 72 a 209 ORFs en los cuales no se encontraron ARNt, genes de virulencia ni de resistencia a antibióticos y más del 70% de estos ORFs codifican para proteínas hipotéticas. El análisis bioinformático sugiere que los genomas de los fagos presentan una organización funcional por módulos típica de fagos con tallo. Los resultados de la caracterización genómica que hemos obtenido nos indican que los fagos son

seguros para utilizarse en terapia, sin embargo, se debe realizar una caracterización completa para garantizar su eficacia y seguridad.

2. CURRENT KNOWLEDGE IN THE USE OF BACTERIOPHAGES TO COMBAT INFECTIONS CAUSED BY *Pseudomonas aeruginosa* IN CYSTIC FIBROSIS

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REVIEW

Current knowledge in the use of bacteriophages to combat infections caused by *Pseudomonas aeruginosa* in cystic fibrosis

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Abstract

Pseudomonas aeruginosa (PA) is considered the first causal agent of morbidity and mortality in people with cystic fibrosis (CF) disease. Multi-resistant strains have emerged due to prolonged treatment with specific antibiotics, so new alternatives have been sought for their control. In this context, there is a renewed interest in therapies based on bacteriophages (phages) supported by several studies suggesting that therapy based on lytic phages and biofilm degraders may be promising for the treatment of lung infections in CF patients. However, there is little clinical data about phage studies in CF and the effectiveness and safety in patients with this disease has not been clear. Therefore, studies regarding on phage characterization, selection, and evaluation in vitro and in vivo models will provide reliable information for designing effective cocktails, either using mixed phages or in combination with antibiotics, making a great progress in clinical research. Hence, this review focuses on the most relevant and recent findings on the activity of lytic phages against PA strains isolated from CF patients and hospital environments, and discusses perspectives on the use of phage therapy on the treatment of PA in CF patients.

Keywords *Pseudomonas aeruginosa* · Bacteriophage · Cystic fibrosis · Multi-resistant strains · Phage therapy

Introduction

Multi-drug-resistant pathogens (MDR) have spread around the world being the common cause of nosocomial and healthcare-associated infections in hospitalized patients, resulting in ineffective treatments and increased costs (Hurley et al. 2012). The European Center for Disease Prevention and Control (ECDC) has highlighted PA as a high priority pathogen due to its ubiquitous distribution and its resistance to diverse antibiotics, mainly associated with having a thick outer membrane that prevents antibiotics from penetrating, as well as the production of antibiotic-inactivating enzymes, efflux pumps, and biofilms (ECDC 2011). Also, the bacteria have a large accessory genome that can occupy up to 20% of the entire genome and it is composed of horizontally transferable elements that include prophages, transposons, insertion sequences (IS), genomic islands (GI), and plasmids that are important for carrying antibiotic resistance genes (Subedi et al. 2018).

In CF disease, the water is not distributed in the lung due to a defective chloride channel CFTR, causing water depletion on the surface of the respiratory tract and the inability of mucociliary clearance giving rise a thick and viscous mucus that deteriorates the lungs causing chronic infections in patients with CF, who are susceptible to opportunistic bacteria mainly like PA (Nafee et al. 2014). Hence, chronic respiratory tract infection caused mainly by PA is considered a severe complication in patients with CF, and antibiotic treatment has led to the emergence of MDR strains (Cafora et al. 2019).

Currently, novel antibiotics against PA are uncommon, since pharmaceutical companies have less interest in developing and producing them, because it has evolved into an expensive, time-consuming, and bureaucratic process involving multiple interest groups, being less profitable than others

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(Projan 2003). For these reasons, new treatments against PA infections should be sought, such as phage therapy, which has regained interest, since its study has shown it as a promising alternative in the fight against this important pathogen (Knezevic et al. 2013). The use of phages in clinical PA infections, in both in vitro and in vivo studies, has been carried out for years, and results are encouraging.

Therefore, the aim of this review is to summarize and discuss recent studies describing the activity of lytic phages on both in vitro and in vivo experiments on PA strains from patients with CF and hospital environments, the clinical trials that are in process of being conducted as well as the perspectives to improve phage therapy in the treatment of PA in CF patients.

Search strategy

Searches were carried out in PubMed, Scopus, and Web of Science databases, to obtain the most relevant and recent literature on the subject. Searching in databases was done using all possible combinations of the following keywords: bacteriophage, phage, *Pseudomonas aeruginosa*, *Pseudomonas aeruginosa* MDR, therapy, infection, isolation, cystic fibrosis.

Inclusion criteria were articles published in English and Spanish, from any country, every published article about isolation, in vitro and in vivo studies made in the last 10 years and any type of scientific article (research, review, minireview, opinion). The exclusion criteria were articles describing mechanisms of phage infection, PA phenotypes associated to prophages, PA detection, use of PA phages in animal diseases, non-lytic phages, and non-CF infections.

After reviewing, 1432 articles were obtained and examined by title and abstract, out of 1179 publications were excluded after applying all the above-mentioned criteria. The remaining 253 full texts were retrieved and evaluated to determine their eligibility; finally, 103 publications were included due to their recent and relevant data on the activity of lytic phages evaluated in PA strains isolated from CF patients and hospital environment.

Pseudomonas aeruginosa and cystic fibrosis

Pseudomonas aeruginosa is considered the most common cultured respiratory pathogen in CF, and associated with a faster decline in lung function in children (Crull et al. 2016). Studies have reported that 80% of 18-year-old persons with CF are infected with PA (Cystic Fibrosis Foundation Patient Registry 2012). It has been estimated that PA infects 51,000 patients in the USA each year, of which 6700 are MDR causing 2,700 deaths only in 2017 according to the CDC (CDC 2019). According to reports from the Red Hospital for Epidemiological Surveillance (RHOVE) of the Ministry of Health in Mexico, the most frequent nosocomial infections are bacteremia (24%), pneumonia (20.7%), urinary tract infection (15.7%), surgical wound infection (15%), and others (24.6%), being PA the most frequent etiological agent in the case of pneumonia (RHOVE 2015).

Pseudomonas aeruginosa is a non-fermentative gram-negative bacterium that can grow aerobically or anaerobically at temperatures up to 42 °C. This opportunistic pathogen is ubiquitously distributed in several habitats including soil, water, wastewater, and sanitary environments (Saussereau and Debarbieux 2012). Moreover, PA is capable of forming biofilms, a thick mucus layer formed by polymeric extracellular substances that protect the bacteria from environment stresses promoting colonization and long-term survival (Drenkard 2003).

The formation of mature biofilms and the development of a mucoid phenotype of PA when infection is chronic, are considered a survival strategy (Sadikot et al. 2005). The permanent establishment of bacteria in the CF respiratory tract is the result of the rapid mutation-driven adaptation that take place in the host in response to increased competition between bacteria embedded on biofilms, where no significant genetic exchange occurred between strains but mutation selection enhances phenotypic variation. In a chronic infection, the characteristic phenotypic changes of PA are low expression of virulence factors, mucoidy, loss of motility, and stress resistance. During the process of an infection, pathogenic adaptive changes are presented by genetic changes from mutations in genes that participate in adaptive roles (for example, resistance to antibiotics and persistence) due to evolution within the host (Dettman and Kassen 2020).

Pseudomonas aeruginosa has different strategies to reduce the permeability of its outer membrane so then antibiotics cannot penetrate and exert their action. For example, efflux systems pump the antibiotics out of the cell keeping low intracellular concentrations of the antibiotic, and alterations in porin OprD trigger a reduction in membrane permeability so the antibiotic cannot penetrate, both resulting in antibiotic resistance (Chatterjee et al. 2016). Other bacterial species like *S. aureus*, *B. cepacia*, and *A. baumannii* can colonize the lungs of CF patients (Krylov 2014), but PA is the most severe because biofilm formation increases antibiotic resistance up to 1000 times in adverse conditions (Garbe et al. 2010).

Currently, there is no cure for CF, and long-term treatment against bacterial infections is given with inhaled antibiotics and antibiotic combinations such as aminoglycosides, carbapenems, cephalosporins, fluoroquinolones, and extended spectrum lactamases (Nafee et al. 2014; Chirgwin et al. 2019), which has led to the appearance of MDR bacteria (Larché et al. 2012; Hraiech et al. 2015). For CF patients, the antibiotic

colistin (polymyxin E) is one of the front-line inhaled agents used against bacterial infections, although this antibiotic exhibits a high nephrotoxicity and neurotoxicity. However, colistin-resistant strains have already been described in Australia, Denmark, the UK, and other countries (Denton et al. 2002; Johansen et al. 2008), prompting the urgent need for the development of new alternative treatments.

Taking into account all of the above, therapeutic approaches to combat resistant strains are limited and consequently, new alternatives against PA strains should be explored, highlighting the growing and renewed interest in phage studies that have been carried out throughout the world (Bush et al. 2011).

Phage therapy in CF

Clinical phage therapy refers to the administration of lytic phages to patients with the aim of eliminating the bacterial pathogens responsible of the infection (Altamirano and Barr 2019). Phage therapy has mainly been studied in Eastern Europe and the Soviet Union (Gill and Hyman 2010) and there are few clinical developments, especially for PA. In this context, the Eliava Institute has developed a pharmaceutical product called Pyo bacteriophage which is a combination of virulent phages against *S. aureus*, several species of *Streptococcus*, *E. coli* (different serotypes), *P. aeruginosa* and *Proteus*, used in the treatment and prophylaxis of purulent inflammatory and enteric infectious diseases. The clinical use of phage preparations from Ludwik Hirszfeld Institute are allowed when an effective therapy is not available against different bacterial genera including *Pseudomonas* and under rules of a therapeutic experiment (based on the Polish regulations).

The use of phage therapy to combat lung infections in humans was reported in scientific articles from Eastern European countries when they started such treatments in children with CF in 2007 in Tbilisi. Phage preparations against secondary infections in eight patients with CF were administrated by nebulization achieving a significant reduction in bacterial cells concentration in all sputum samples from patients (Kutateladze and Adamia 2010). The use of phage therapy as experimental human treatment is officially allowed in some countries such as Russia, Poland, Georgia, USA, and Canada (Górski et al. 2018).

The most important criterion for selecting a phage, or set of phages for clinical use, is host specificity (epidemiologically relevant strains), because lysis of non-targeted bacteria and adverse effects on normal flora after treatment is prevented (Thiel 2004). A problem to be faced in phage therapy is that bacteria may develop resistance mechanisms against the phages after continuous applications, leading to the joint evolution of bacteria and phages. To overcome phage resistance, different strategies have been implemented to improve the efficiency of phages, including the use of phages and their lytic enzymes, phage cocktails, synergistic phage combinations with antimicrobials, phage pre-adaptation and genetic engineering of phages genomes (Essoh et al. 2013; Pires et al. 2017).

The application of phages has advantages such as they can be easily propagated because they are a self-replicating product; their production is fast and cheap compared to antibiotics; phages have specificity for their hosts; they do not produce allergenicity reactions, although it has been described that antibodies can interact with phages, a small amount can neutralize them; and they can be applied to patients by different routes and they can be used in combination therapies, including other phages or antibiotics (Doffkay et al. 2015).

Isolation and use of PA bacteriophages

PA phages have been isolated from wastewater, water reserves, lake water, bay water, mammalian serum and compost (Table 1). For therapeutic purposes, wastewater is considered the richest source of bacteria and phages due to the presence of high levels of nutrients and because bacteria can form biofilms more easily on different water surfaces. Wastewater from hospital environment has been reported as the main source of phage isolation for clinical bacteria such as PA (Essoh et al. 2015; Kakasis and Panitsa 2019).

By December 2021, about 3500 complete phage genomes were available in the GenBank database, of which 244 correspond to phages against *Pseudomonas* species and 142 were from specific phages that infect PA strains (https://www.ncbi.nlm.nih.gov/genomes/GenomesGroup.cgi?taxid=10239&host=bacteria). Most PA phages belong to the order Caudovirales (Sepúlveda et al. 2012) and the majority of PA lytic phages belong to the *Myoviridae* family, followed by *Podoviridae* and *Siphoviridae*. There is a large variation in the phage genome sizes between families. The *Myoviridae* family phage genome size varies from 64.1 to 309.2 kb, in *Podoviridae* and *Siphoviridae* families varies between 41.6–74.9 and 34.5–61.1 kb, respectively (Tang et al. 2018). Although most phages contain double-stranded DNA, Yang et al. (2016) isolated and characterized the first phage (phiYY) of PA (strain PAO38) having a dsRNA-segmented genome, it was isolated from hospital wastewater (Yang et al. 2016). According to PA phage genome reports from 2010 onwards, 12 studies described phages that were mainly isolated from hospital wastewater and most of them belong to the *Myoviridae* family with genome sizes between 34,553 and 258,139 bp, showing variability in phage isolation and sequencing characteristics (Table 1).

Table 1 Characteristics of isolated and sequenced *P. aeruginosa* phages

Number	Phage name	Family	Isolation source	Genome size (bp)	%GC	Number ORFs	Reference
1	MP1412	*Siphoviridae*	Sewage water	61,167	–	78	(Bae et al. 2012)
2	(YMC01/01/P52 PAE BP)	*Podoviridae*	Hospital wastewater	49,381	62.16	72	(Jeon et al. 2012)
3	PA26	*Myoviridae*	Water reservoir	72,321	54.82	88	(Kim et al. 2012a)
4	PA1Ø	*Siphoviridae*	Farm wastewater	34,553	64.8	51	(Kim et al. 2012b)
5	MPK7	*Podoviridae*	Sewage water	42,874	52.14	54	(Bae and Cho 2013)
6	phiIBB-PAA2	*Podoviridae*	Hospital wastewater	42,344	52	66	(Pires et al. 2014)
7	PaBG	*Myoviridae*	Lake water	258,139	55.82	308	(Sykilinda et al. 2014)
8	KPP23	*Siphoviridae*	Bay water	62,774	56	95	(Yamaguchi et al. 2014)
9	vB_PaeM_CEB_DP1	*Myoviridae*	Hospital wastewater	66,158	55.6	89	(Pires et al. 2015)
10	AAT-1	*Siphoviridae*	Mammal serum	57,599	65.84	76	(Andrade and Kolter 2016)
11	vB_PaeP_PcyII-10_P3P1	*Podoviridae*	Compost	72,778	56	94	(Pourcel et al. 2016)
12	vB_PaeM_PcyII-10_PII10A	*Myoviridae*	Sewage wáter	65,712	55.5	93	(Pourcel et al. 2016)
13	YS35	*Myoviridae*	Hospital wastewater	93,296	49.4	172	(Yu et al. 2018)

The antimicrobial efficacy of phages in PA infections has been studied under various in vitro conditions, in different in vivo models, and also in few clinical trials (12).

The use of phage cocktails

Among new promises, the use of phage banks to supply phage cocktails for treating clinical bacterial infections is proposed to reduce bacterial resistance. This is because using a phage cocktail, the probability of two independent resistance mutations occurring within the same organism generally is lower than that of only a single mutation. At least one drug of a combination, that is, should retain activity against newly drug-arising pathogens, blocking further growth and therefore resistance evolution (Chan and Abedon 2012). The latter resistance occurs because cocktails represent the phage equivalent of a multi-drug approach to treatment, being considered as a more effective alternative (Gill and Hyman 2010; Jamal et al. 2017). A phage bank is a collection of phages that have been isolated, characterized, and are available either as phage preparations (cocktails) for matching to a particular isolated target bacterium (Gill and Hyman 2010). Phages with different affinity for the host receptor should be chosen so that they do not compete to bind in the same bacterial surface and due to selective pressures (Rossitto et al. 2018). For example, a cocktail of five phages that includes three phages of PA with a wide range of hosts generated by training (PaP1, PaoP5, PaP8), PaoP5-m1, a derivative of the phage PaoP5, and phiYY, these last two which are capable of infecting mutants with truncated O antigen; the results showed that the cocktail was effective against PA clinical isolates and limited the emergence of phage-resistant mutants in a short term (Yang et al. 2020).

The use of PA phages in combination with antibiotics

Combined therapies like phages with antibiotics may be more effective as they have a synergistic effect, helping to avoid the development of antibiotic resistance. In this regard, combined therapy has revealed a synergistic effect on planktonic cultures of PA and biofilms resulting in higher rates of bacterial lysis in biofilms in comparison to phage or antibiotic treatment separately (Chaudhry et al. 2017). The time of application is a factor that influences the efficacy of combination therapies, where phage replication and the effect of antibiotics are both density-dependent. If phages are applied over low concentrations of bacteria or bacteria non-amenable physiologically, there will be less phage density and more frequent phage application will be required (Torres et al. 2014).

Accordingly, several authors have reported that the combination of sub-inhibitory concentrations of antibiotics such as gentamicin, ciprofloxacin, ceftriaxone, and polymyxin B with specific phages of PA has antimicrobial efficacy, finding reductions in bacterial growth, demonstrating phage-antibiotic synergism and results in avoidance of antibiotic side effects occurring after administration of high doses (Knezevic et al. 2013). The phageciprofloxacin synergism on the control of PA biofilms (Sagar et al. 2016; Henriksen et al. 2019; Issa et al. 2019) was also evaluated and confirmed, the combination of two phages and bactericidal antibiotics of five classes (Chaudhry et al. 2017), the PEV20

phage with five antibiotics to destroy three PA strains isolated from sputum of CF patients (Larché et al. 2012) and a cocktail of phages with ciprofloxacin and meropenem (Ong et al. 2020).

Although most studies show a synergistic effect between the phage-antibiotic combination, studies on negative or neutral effects between systems have also been published (Tagliaferri et al. 2018). For example, in the study by Wlodarczyk et al. (2016), anti-biofilm activity is activated but no synergistic effect when phage KTN4 (*Myoviridae*) was combined with colistin against strain PAO1 cultured for 24, 48, and 72 h in vitro. The two separately antimicrobials significantly reduced the biofilm. Colistin is reported to destabilize the cell membrane and therefore can limit phage propagation, whereas KTN4 phage recognizes type IV pili as receptor and does not interfere with colistin activity. In another study, Knezevic et al. (2013) used three unrelated phages (δ, δ-1 and 001A) against their individual hosts (PA-4U, ATCC 9027 and PA-M2, respectively) in combination with sub-inhibitory concentrations of ciprofloxacin, ceftriaxone, gentamicin and polymyxin B. A significant reduction was observed only with ceftriaxone in planktonic cultures and only with the combination of phages a synergistic effect was observed, so it is concluded that not all combinations of antibiotics and phages synergistically reduce bacteria. The mode of action of antibiotics and the molecular basis of phage/host interactions plays an important role in efficacy (Knezevic et al. 2013).

The use of phage pre-adaptation and genetic engineering of phages genomes

Bacteria and phages evolve together, where bacteria acquire different mechanisms to become resistant to phage infection, and phages evolve to overcome that bacterial immunity. The emergence of phage-resistant bacteria is a problem to overcome in phage therapy (Uchiyama et al. 2016), but currently it seems to be less of a problem because a great advantage of phages is that they can be "trained," to adapt them to their target bacteria both in vitro or in vivo (Chatterjee et al. 2016). Phage pre-adaptation is a phage trained in advance in an in vitro antagonistic evolution, in a study, all phage strains were pre-adapted against all bacterial strains and then compared the efficacy of pre-adapted and non-adapted phages against ancestral bacterial strains. The results showed that, compared to ancestral phages, evolved phages have a better capacity to decrease bacterial density, which benefits by limiting the evolution of resistance in pulmonary isolates of PA from chronic CF (Friman et al. 2016). The use of pre-adapted phages is also proposed to examine the coevolution of phages and bacteria using phage-resistant PA strains (Uchiyama et al. 2016).

Bacteria have mechanisms that prevent them from being infected by phages, such as inhibition of phage absorption, targeted cleavage of injected nucleic acids via restriction-modification (R-M) system, suicide of phage-infected cells via abortive-infection (Abi) system and CRISPR/Cas systems (Li et al. 2018). For example, in Li's study, a phage-resistant mutant of PA strain PA1(PA1RG) under the infection of lytic phage PaP1 was selected. The PA1RG, due to a transition mutation in gene *wzy*, resulted in the premature formation of O antigen polymerase, which is involved in long-chain O antigen biosynthesis related to phage PaP1 recognition and binding. While *wzy* mutation did not affect bacterial growth, mutant PA1RG exhibited decreased biofilm production, suggesting a fitness cost of PA1 associated with resistance of phage PaP1 predation (Li et al. 2018). However, in another study, with the aim of knowing the role of CRISPR/Cas system in phage resistance, it was found that some PA strains cannot support the growth of any of the tested phages belonging to 5 different genera, suggesting that the CRISPR-Cas system is not a major defense mechanism against the lytic phages used (Essoh et al. 2013).

Another option is through genetically engineered phage that produces a lactonase that inhibits biofilm production in PA by reducing bacterial concentration and quorum sensing detection, this could be a therapeutic option against PA infections (Hraiech et al. 2015).

Due to the diversity of lytic phages active against PA and the difficulties in implementing phage therapy in medical practice, new phages with improved antibacterial properties that allow studying the bacterium-phage interaction must be characterized and classified (Tang et al. 2018).

Efficacy of PA phages on CF in vitro models and effects in cell models

In vitro models that simulate lung infection have been used for the study of CF caused by PA. To study phage infection, artificial sputum medium has been used (Garbe et al. 2010) and also sputum from CF patients (Saussereau et al. 2014), and to understand the effect of phages on innate immune systems, cell lines or models of epithelial cells has been studied (Alemayehu et al. 2012).

The articles considered in this review report that the isolated phages come mainly from wastewater from hospitals and water treatment plants, but also from rivers and ponds. The phage infection process has been investigated under aerobic and anaerobic conditions, both in planktonic cells and biofilms, using different multiplicities of infection (MOI), where significant logarithmic reductions are reported in treatments of 2 to 24 and 74 h. Reports indicate that the efficiency may vary according interaction time between phage and bacteria that can lead to the emergence

of phage-resistant bacteria (Pires et al. 2011; Wlodarczyk et al. 2015). Also, since it was found that the lysis by the phage against the bacteria depends on the phage-host system and not on the MOI tested, in vitro experiments are necessary to determine phage efficacy in each phage-host system (Knezevic et al. 2011). The viability of PA phages contained in particles when nebulized and administered to respiratory tract has been evaluated in two different nebulizer classes, it was found that the number of viable phages administered during nebulization depended on both the nebulizer and the nebulized phages, so the viability of phages in the inhaled particles in the lower respiratory tract should be evaluated using nebulizers for clinical use (Sahota et al. 2015).

In the work by Garbe et al. (2010), infection of the host PA by phage JG024 was even possible under challenging conditions like the artificial sputum medium which mimics the CF lung. High viscosity and microcolony growth of the host were only small obstacles for JG024 to infect and multiply under these conditions. Saussereau et al. (2014) found that CF patients colonized by a single clonal population of PA showed several phenotypes, whereby separate colonies from a single sputum sample were not equally infected by all ten bacteriophages tested (except those containing only resistant colonies). This confirms that several phenotypes can arise from a single genotype in a patient's sputum, which can affect bacterial susceptibility to bacteriophages. Therefore, testing a set of bacteriophages against a large panel of isolated colonies for each patient would be the best approach to formulate bacteriophage cocktails, supporting the use of a personalized approach to achieve optimal treatment. Wlodarczyk et al. (2015) reported a significant reduction (70–90%) in biofilm cultures from 24 to 72 h of age for two phages (KTN6 and KT28), suggesting that phage particles can penetrate the biofilm matrix. However, the treatment of these phages caused the emergence of phage-resistant variants of PA from the biofilms persistent cells turned out to be cross resistant to both phages, probably by the loss or changes in O antigen structure. This feature was relatively stable and persistent bacteria had small colony variant morphology. It may put the applicability of the phages in study into question, although such enveloped modification may also decrease the fitness of the microbe and made them more susceptible to immune system defense. In addition, the amounts of the most important dyes secreted by the pathogen (pyocyanin and pyoverdin) significantly increased, which may prove the potency of the isolated phages to be applied as efficient antibacterial in the treatment of *Pseudomonas* biofilms. The increased rate of diffusion through the biofilm matrix after phage application highlights the degradation of biofilm structure associated with loss of biofilm matrix elements at the membrane surface and reversion to the hydrophobic properties of the membrane, or partial degradation of the matrix/biofilm structure.

By mixing one member of each genus of phages in the collection by Essoh et al. (2015), they were capable of lysing all PA isolates except four phages that appear to be also resistant to ϕKZ-like viruses. Host range depends on interactions between the phage tail fibers and the bacterial receptor, but other mechanisms are involved in bacterial susceptibility to phages. Phage infection failure may be due to exclusion of superinfection, as most CF isolates possess one or more prophages, and the presence of prophages on a bacterial chromosome immunizes bacteria against infection by phages of the same nature. Also, during lysogeny, D3-like phages modify LPS receptors, making bacteria resistant to LPS-dependent phages, including virulent phages. Another mechanism of virulent phage specificity could be related to the inhibition of the general phage defense mechanism developed by the CRISPR-Cas system, since studies have shown that the CRISPR-Cas system can be an actor in bacterial resistance to virulent phages, so it would be important to use as many phages from different genus to counteract these mechanisms (Snyder 1995; Newton et al. 2001; Essoh et al. 2013).

Besides, the effect of phages on innate immune systems in cell lines or epithelial cell models has not been thoroughly studied. A mixture of two phages has efficacy in destroying non-mucoid and mucoid PA strains, when developing as biofilm in a CFBE41o cell line of the cystic fibrosis bronchial epithelium where the phage concentration increased almost 100 fold in 24 h (Alemayehu et al. 2012). An important point is to validate that phage therapies do not cause adverse reactions within the host using assays of phage exposure in surrounding mammalian systems (Trend et al. 2018). For example, Shiley et al. (2017) using an in vitro model, characterized the exposure to phages (DMS3 and PEV2) against PA, finding a PEV2-dependent increase in IL-6 and TNF-α production but no change associated with exposure to DMS3. Additionally, it was found that DMS3 protects mammalian lung cells from PA in an infection model, therefore, DMS3 phage is a good candidate for phage therapy. Extended immune responses can lead to several health concerns. However, as phage therapy is being explored to fight bacterial infections, an increase in cytokine levels may augment antibacterial efforts (Shiley et al. 2017).

Currently, research has been aimed at studying bioavailability, pharmacokinetics and feasibility of phage therapy, as well as the cost compared to antibiotics, but these studies do not comply with regulations of countries that use evidence-based medicine (Lu and Koeris 2011).

Antimicrobial efficacy of PA phages by in vivo studies

Of fifteen articles found that describe the efficiency of lytic phages against PA in terms of in vivo studies, five studies used larval models of non-pulmonary hosts (*Galleria*

mellonella) (Hall et al. 2012; Beeton et al. 2015; Olszak et al. 2015; Forti et al. 2018; Jeon and Yong 2019), one used *Drosophila melanogaster* (Lindberg et al. 2014), and one of zebrafish with CF (Cafora et al. 2019), and nine lung models in mice (models of pulmonary hosts) (Debarbieux et al. 2010; Morello et al. 2011; Henry et al. 2013; Pabary et al. 2016; Waters et al. 2017; Forti et al. 2018; Jeon and Yong 2019; Alvi et al. 2020; Lin et al. 2020) (Table 2).

Debarbieux et al. (2010) reported the first lung infection model in mice utilizing a bioluminescent PA strain finding that phage treatment, in addition to curing the animals of a lethal infection, managed to prevent a pulmonary bacterial infection when administered 24 h before infection. The most common route of phage administration was intranasal, followed by intraperitoneal, and only in a recent work by Lin et al. (2020) was intravenously applied in mouse models. No in vivo studies have been done comparing phage administration by different routes in the treatment of PA associated with CF.

Some studies using a phage or a phage cocktail concluded that a cocktail is more effective as a treatment (Hall et al. 2012; Henry et al. 2013; Forti et al. 2018; Jeon and Yong 2019). Different concentrations of phage and bacteria were used in each investigation, but which ones are adequate are not defined. Regarding this, Beeton et al. (2015) research reports that the administration of phage against PAO1 extends the survival of *G. mellonella* (as PA infection model) depending on the dose. At 24 h, 100% of infected and untreated *G. mellonella* died, whereas when they were infected using 10 cells at a MOI of 10, 40% survived compared to those infected using 100 cells with the same MOI where 20% survived. Another example is Hall et al. (2012) study where phage therapy increased life time of PA PAO1-infected wax moth larvae, when the bacterial inoculum was reduced to 5000 cells and phage dose was increased by 10 times (to 5×10^6 phage particles).

Different time points are also used for the application and evaluation of both bacteria and phages. For example, the study by Morello et al. (2011) in a mouse lung infection model, the two used doses of phage P3-CHA increased survival when administered after infection and the higher dose (3×10^8 PFU) provided a higher survival rate throughout the experiment. Preventive treatment in this work seems not to be relevant since efficacy is sought in curative treatment, however, the result must be taken into account since a single dose-maintained efficacy for up to four days. In comparison, in the work of Beeton et al. (2015) the preventive treatment was just 2 h before infection and showed a better survival compared to the treatment model, by which PA was established within the cells before a dose of phage was applied in *G. mellonella*. The variations in larval survival rates as PA infection models in presence of different phages like in the study by Olszak et al. (2015) of KT28 phage, compared to the giant PA5oct, could suggest the importance of phage size and generation time rate (KT28 < PA5oct) have a significant influence on therapeutic efficacy. Other parameters that influenced significantly were slow growth rate of bacteria reduced contraction motility, low capacity for biofilm formation, and great diversity in the used strains.

In another study, the infectious load and inflammation in bronchoalveolar lavage fluid (BALF) were evaluated at different time points, using two PA strains in an acute murine infection model with a phage administered before, simultaneously, or after infection. When low infectious doses were applied, both control and phage co-treated mice cleared PA infection at 48 h, but there were fewer neutrophils in BALF than in phage-treated mice. With high infectious doses of PAO1, all phage-treated mice cleared PA infection at 24 h, while infection persisted in all control mice. Phages also reduced CFU/ml in BALF when administered 24 h after infection and both CFU/ml and inflammatory cells when administered prophylactically. There was also a reduction in the levels of soluble cytoinflammatory BALF under different conditions, concluding that phages were effective in reducing both bacterial load and inflammation in the model under study (Pabary et al. 2016).

Other works studied the correlation between in vitro and in vivo results. In this respect, Henry et al. (2013) using MOI between 0.05 and 0.2 of phages (LBL3, PhiKZ, LUZ19, and PAK_P2 to PAK_P5) found that PAK_Px phages showed the highest survival percentage, up to 100%. It was also studied whether there was a correlation on in vitro and in vivo efficacy for PAK_Px phages tested, finding that the variations on in vivo efficacy reflected genetic kinship (PAK_P3 and PAK_P5 are closely related genetically to each other, as are PAK_P1, PAK_P2, and PAK_P4). Another example is the study by Linberg et al. (2014) who tested phages efficacy in *D. melanogaster* adult females to determine the in vitro traits correlated with that shown in vivo, finding that adsorption rate, lysis time or size of burst, did not correspond with the observed mean survival time (MST). But there was a significant correlation between phage growth rate (replication) tested in vitro and MST, concluding that the faster the phage grows in vitro, the more effective it will be in fighting bacterial infection.

Jeon and Yong (2019) studied the infection model in *Galleria mellonella* and that of acute pneumonia in mice. In both organisms, phages Bϕ-R656 and Bϕ-R1836 increased larval survival by 50% and 60%, respectively, 72 h after infection and in mice by 66% and 83%, respectively, 12 days after infection. Treatment with both phages significantly reduced the bacterial concentration in mouse lungs (> 6 $\log_{10}$CFU and > 4 $\log_{10}$ CFU, respectively) on day 5. In the first model, the efficacy of the two phages against clinical MDR-PA strains was evaluated and to test the safety and

Table 2 Experimental phage therapy on pulmonary and systemic infections in animal models

P. aeruginosa strain	Phage name	Phage family	Efficient dosage	Treatment schedule	Optimal protection	Study model	Liberation route	Reference
PAK lumi	PAK-P1	*Myoviridae*	10:1 Phage-bacteria	Single dose 2 h after infection, monitored by 12 days	100% survival	Lung infection in mice	Intranasal	(Debarbieux et al. 2010)
CHA	PAK-P3 and P3-CHA	*Myoviridae*	3×10^8 PFU Preventive Tx 3×10^8 PFU 4 days before	Single dose 2 h after infection, monitored by 16 days	>95% survival 100% survival	Lung infection in mice	Intranasal	(Morello et al. 2011)
PAO1	14/1, φKZ, PNM, PT7	*Myoviridae Podoviridae*	5×10^6 PFU	Start of infection assays and reapplied phages every 12 h	100% survival	*Galleria mellonella*	Injection in abdomen	(Hall et al. 2012)
PAK, PAK-lumi and CHA	PAK_P1, PAK_P2, PAK_P3, PAK_P4, PAK_P5, CHA_P1, LBL3, PhiKZ and LUZ19	*Myoviridae Podoviridae*	MOI 0.1	Single dose 2 h after infection, monitored by 13 days	75–100% survival	Lung infection in mice	Intranasal	(Henry et al. 2013)
MPAO1 and PW8621	HWPB-1, HWPB-2, HWNPB-2 and HWNPB-3, HWNPB-1 and HWPB-3	*Myoviridae Siphoviridae*	MOI 10	Single dose 6 h after infection, monitored every 6 h for 72 h	45.7 ± 1.19 h mean survival time	*Drosophila melanogaster*	Chest injection	(Lindberg et al. 2014)
PAO1, PA45291 and BC09007	DL52, DL54, DL60, DL62, DL64 and DL68	*Myoviridae Podoviridae*	MOI 10	Single dose 2 h after infection	85% survival	*Galleria mellonella*	Injection behind the pseudopath	(Beeton et al. 2015)
PAO1, non-CF0038, CF217, CF708, CF532, CF832	KT28 and PA5oct	*Myoviridae*	MOI 100	Single dose 1 h after infection monitored at 8, 24, 48, 72, and 96 h after infection	100% survival	*Galleria mellonella*	Injection on the ventral side of the last pair of pseudopods	(Olszak et al. 2015)

Table 2 (continued)

P. aeruginosa strain	Phage name	Phage family	Efficient dosage	Treatment schedule	Optimal protection	Study model	Liberation route	Reference
PAO1 and PA12B-4973	Cocktail 1 (*P. aeruginosa* 24, *P. aeruginosa* 25, and *P. aeruginosa* 7), cocktail 2 (*P. aeruginosa* 39, *P. aeruginosa* 67, *P. aeruginosa* 77, and *P. aeruginosa* 119) and cocktail 3 (*P. aeruginosa* 3, *P. aeruginosa* 6, *P. aeruginosa* 10, *P. aeruginosa* 32, and *P. aeruginosa* 37)	*Myoviridae* *Siphoviridae* *Podoviridae*	MOI 100	At the time of the infection and 24 after infection	86% survival	Acute lung infection in mice	Intranasal	(Pabary et al. 2016)
LESB65 wild type and adapted strain NP22_2	PELP20	*Myoviridae*	2×10^7 PFU/ml	24, 36, 48, and 60 h, 6 and 7 days after infection	100% survival	Chronic lung infection in mice	Intranasal	(Waters et al. 2017)
PAO1 and PAK-lumi	PYO2, DEV, E215, E217, PAK_P1, and PAK_P4	*Myoviridae* *Siphoviridae* *Podoviridae*	MOI 0.05 MOI 25	Single dose 2 h after infection Single dose 1 h after infection	100% survival 63% survival	Respiratory infection in mice Systemic infection *Galleria mellonella*	Intranasal Injection behind the pseudopath	(Forti et al. 2018)
PAO1-GFP	vB_PaeP_PYO2, MF490236 y vB_PaeP_DEV, MF490238, vB_PaeM_E215, MF490241 and vB_PaeM_E217, MF490240	*Myoviridae* *Podoviridae*	5×10^8 PFU/ml	At the time of the infection, 30 min and 7 h after infection	83 ± 9% to 52 ± 7% survival	Zebrafish model with CF	Microinjection into the yolk	(Cafora et al. 2019)
YMC11/02/R656 and YMC11/11/R1836	BΦ-R656 and BΦ-R1836	*Siphoviridae*	1×10^7 PFU/ml, MOI 100 MOI 10	Single dose 1 h after infection, monitored every 8 h by 72 h Single dose 4 h after infection, monitored by 12 days	50% survival 66 y 83% survival	*Galleria mellonella* Pneumonia in mouse	Injection behind the pseudopath Intranasal	(Jeon and Yong 2019)

Table 2 (continued)

P. aeruginosa strain	Phage name	Phage family	Efficient dosage	Treatment schedule	Optimal protection	Study model	Liberation route	Reference
PA-1	RLP	*Podoviridae*	1×10^9 PFU/ml MOI 100	Single dose at the time of infection, monitored 1, 6, 12, 24, 48 and 96 h after infection	92% survival	Bacteremia in mice	Intraperitoneal	(Alvi et al. 2020)
P. aeruginosa 1121	øPEV20	*Podoviridae*	$>10^4$ PFU	Single dose 1 h after infection	>8-$\log_{10}$ CFU/ml	Bacteremia in mice	Intravenous	(Lin et al. 2020)

therapeutic potential of the phages they used the acute pneumonia model in mice.

Recently, Lin et al. (2020) developed a pharmacodynamic model to determine the efficacy of the øPEV20 phage as well as the phage arrangement in healthy rats. As results, when the phage was administered intravenously at a concentration of $>10^4$ PFU, there was rapid bacterial death, and the bacterial concentration was reduced 2.5 h after administering the treatment. Phage accumulation was evident in the spleen and liver at 48 and 72 h, given that both organs are important for the mononuclear phagocytic system. Interestingly, phage penetration into bladders depends on the dose administered, and 10^9 PFU/mouse was the minimum dose required to allow for phage detection in urine following intravenous administration. Phage binding to immune cells most likely affects their disposition in vivo, so future studies are needed to characterize the affinity of phages to key immune cells.

Regarding in vivo works that use a CF model, this is the one from Cafora et al. (2019) reporting it as the first time that phage therapy has been used to cure PA infections in a CF animal model where they used the PAO1 strain and the CF zebrafish model, since the structure of the CFTR channel is conserved evolutionarily between fish and mammals and cftr loss-of-function zebrafish embryos show a phenotype that mimics human disease. They found that phage treatment decreased lethality, bacterial load, and the proinflammatory response caused by PAO1 infection. In addition, phage delivery alleviated the constitutive inflammatory state of CF embryos. As can be seen in the studies considered in this review, phage therapy to destroy different PA strains in different in vivo models is successful (summarized in Table 2).

However, studies conclude that efficacy of phage-based therapy using different animal or phage models depends on time and dose. Studies describe that the efficacy depends on the time in which the treatment was administered (mostly between the first 2 h after the infection), where there is greater efficacy when phages are administered in the first hours at a high concentration and several studies in animals propose to give a curative or preventive treatment with phages to combat pulmonary infection by PA. But the appropriate dose, treatment duration, the ideal route of administration, or how to choose a host-adapted phage or cocktail has not yet been established (Hraiech et al. 2015). In addition, more studies are needed to determine how often phage should be administered and what allergic or immune reactions they can cause in the host in a short and long term. In most studies a single dose is given either at one or two hours after the establishment of the infection, but it is suggested as an attractive alternative to reduce the capacity of reinfection and the problems associated with chronic infection, administering phage cocktails, selected based on the strains that colonize the patients' lungs (Pabary et al. 2016). Because there is no standardization, comparing studies is

difficult and experiments in animals have little observation time compared to chronic lung infections in humans. As experimental treatment, there have been few case reports of experimental studies of phage treatment against PA infections in the respiratory tract in patients with CF (Hraiech et al. 2015).

As perspectives for advance of in vivo in PA infections in CF, it is proposed that more work be carried out with zebrafish model and to overcome the limitations of the murine model (without hypersecretion of mucus in mice and that the expression of the CFTR gene is in the proximal trachea), the use of other animals such as pigs and ferrets is proposed because like humans, they have a high expression of CFTR in the serous cells of the submucosal glands in cartilaginous airways, which simulates lungs with CF (Lavelle et al. 2016). The use of these in vivo models, in addition to providing active phage titers high enough to target PA MDR in lungs, could also address phage pharmacokinetics. Likewise, to address the efficacy against PA MDR in CF and adverse effects using various phage delivery routes, phage pharmacodynamics could also be investigated (Abedon 2017).

Clinical studies, challenges and promises of PA phage therapy in CF.

In order to find projects under development in the US about phage therapy for CF, a search was conducted on Clinicaltrial.gov in December 2021 and we found four registered studies. Regarding the first one, although it has been completed, the results have not been published yet. The aim of the study was to evaluate a cocktail of ten phages on PA strains in sputum samples from CF patients at the University Hospital in Montpellier, France (NCT01818206). Other two studies are currently in progress. The first one is a prospective, randomized, placebo-controlled, double-blinded, single-site study of Yale Phage Therapy (YPT) in CF subjects with chronic PA airway infections. The study has 3 parallel arms of "standard" phage dose, "high" phage dose, and placebo, with all study materials GMP-manufactured, to demonstrate efficacy and safety of nebulized phage therapy (NCT04684641). The second is a phase 1b/2a, double-blind, placebo-controlled, randomized, single-dose study conducted to assess the safety and tolerability of the inhaled AP-PA02 phage cocktail in patients with chronic pulmonary PA infection and CF (NCT04596319). No patients have yet been recruited in the latest study. This is a phase 1b/2a study with the main objective of determining if BX004-A phage is safe and tolerable. Exploratory objectives include whether BX004-A reduces PA bacterial load in sputum in CF subjects with chronic PA lung infection (NCT05010577).

Currently, the German consortium Phage4Cure is working on studying the efficacy and safety of a cocktail of inhaled phages against chronic pulmonary infection by PA. The aim of the project is the clinical application of phages in Western Europe and Germany using good manufacturing practice standards throughout the production chain and obtaining approval for its use by regulatory authorities (Wienhold et al. 2019).

Therefore, the paucity of literature on clinical studies involving strains and patients with CF shortens the conclusions that can be described from the use of phage therapy. Few clinical data are available as most studies are based on clinical case reports as well as in vitro and in vivo studies (Hraiech et al. 2015). Deficiencies include the lack of adequate controls or the small number of patients in clinical trials, which could be considered as an important reason why phage therapy has not been used and approved worldwide. Controlled and regulated clinical research is required for phage therapy to be used as an approved medical treatment. For formal testing of safety or efficacy, only fully sequenced phages that are shown to be non-virulent by genome and proteome analysis, that their genomes do not encode virulence or toxicity genes, and that are not involved in horizontal gene transfer, should be used. Moreover, quality control is of utmost importance, it should include stability (shelf life) and cytotoxicity (Karumidze et al. 2012; Doffkay et al. 2015).

Failures in phage use by early researchers have been associated with factors, such as inappropriate phage choice, for example having narrow host range, poor phage preparation, and insufficient purity which favors poor stability or viability leading to phage disintegration before application, lack of understanding about the mechanism of action and the diversity of phages (Gill and Hyman 2010; Kim et al. 2012c). The main failure causes of phage therapy in the airway setting in CF are the development of bacterial resistance to phage, the difficulty of the phage to interact with the bacteria, and phage binding to dead bacterial cells, which are abundant in chronic infection (Trend et al. 2017).

For phage therapy, phage administration directly into the respiratory tract can promote contact between phage and target bacteria, accelerating host lysis. Inhaled phage formulations can include materials such as buffers and excipients that support the lung environment and must perform experiments to ensure that the aerosol achieves the delivery of viable phages within the lungs (Bodier et al. 2017). Therefore, there are still aspects to be addressed so that phage therapy on PA in CF can be approved and used in a safe and effective way.

Factors influencing the large-scale application of phages that have limited the conclusions of the most recent trials include the production of phage cocktails, the context of the current legal framework, as well as the limited number of patients included in clinic studies (Sybesma et al. 2018).

The use of phage-coded lytic enzymes

Today, the use of phage-encoded products such as lytic enzymes, like endolysins, depolymerases, and virion-associated peptidoglycan hydrolases (VAPGH) for therapeutic application, is on clinical development. Lytic enzymes such as lysins and holins are produced intracellularly by the host in order to induce cell wall degradation for facilitating the release of newly formed virions and leading to bacterial death (Guo et al. 2017; Ghose and Euler 2020). On the other hand, depolymerases are enzymes that facilitate phage adsorption by degrading capsular polysaccharides and cleaving EPS in the biofilm matrix and phages use VAPGH to degrade peptidoglycan (PG) and inject the phage genetic material into the cell (Pires et al. 2017; Yang et al. 2018).

Interestingly, it has been reported that some PA lytic phages produce depolymerases which have features such as increased absorption and removal of biofilms from the target bacteria. Depolymerases are commonly identified by a halo surrounding phage plaques. Most of phage-derived depolymerases were identified by prediction through genomic annotation and the diversity and biological roles of them are not yet fully studied. Few PA bacteriophage-encoded depolymerase genes have been reported. Therefore, the combination of phage-derived depolymerases and other antimicrobial agents such as antibiotics might be helpful to control antibiotic-resistant bacterial infections (Mi et al. 2019). Synthesis of depolymerases in some phages is useful for biofilm destruction. However, this is not a common trait; through genetic engineering, genes producing enzymes that degrade EPS have been inserted into some phages but its degradative activity is diminished compared with the unmodified phages (Parasion et al. 2014).

Recently, research has increased in the use of all these lytic enzymes for their therapeutic value against PA (Yang et al. 2018; Mi et al. 2019). A lytic phage of PA encodes a depolymerase-like protein that degrades the EPS of PA and increases bactericidal activity, further disrupting host bacterial biofilms (Mi et al. 2019). In another study, the phiYY-encoded lysin, called Ply17, showed great antibacterial capacity against the outer membrane of PA treated with permeabilizer (Yang et al. 2018). The endolysin LysPA26, purified from the phage JD010, contains a lysozyme-like domain that has activity against PA MDR (and other Gram-negative bacteria) without using an external membrane permeabilizer. Additionally, this endolysin has, with the ability to remove biofilms and reduce up to 4 log units of PA for 30 min (Guo et al. 2017).

Lytic phage enzymes have as advantages that there is little risk of generating resistance to lysins because they target specific molecules and can lyse active or resting cells, even bacteria within biofilms (Antonova et al. 2018).

Current status of the legal framework of clinical phage therapy

The current European regulatory framework has not considered the value of decades of historical clinical data on phage therapy because it has not been ratified with currently accepted regulatory standards (Verbeken et al. 2012).

Several investigators have described that a new regulatory framework must be developed for phage therapy. Good manufacturing practices that rule the production of medicines for human use generate high costs and are sometimes not essential. Instead, important parameters to control are the quality and safety of phage preparations (Verbeken et al. 2014).

Working with phages for a clinical treatment approach differs from industrial approaches to produce phage-based products, the industry seeks to commercialize phage cocktails for economic causes, differing from phage therapy, which is based on a personalized approach to the patient. Weak intellectual property makes it difficult to attract capitalists, which means that developers of phage therapies have difficulty following regulatory frameworks and the high costs (Verbeken et al. 2012).

Even though humans are in contact with phages daily, their immunogenicity and safety have not yet been fully studied, presenting a problem for the introduction of phage therapy. It has been described that some phage proteins can cause adverse reactions when stimulating the immune system. For that reason, phage therapies and their derived products must be manufactured with current pharmacopoeia and good manufacturing practices (Cooper et al. 2016).

Therefore, in clinical human studies, the efficacy, toxicity, and adverse effects of new drugs must be established, and the health of the patients must be prioritized over the generation of results (Cooper et al. 2016).

Conclusions

Phage activity against PA biofilms in chronic CF infections has been studied and tested, and although in vitro and in vivo efficacy has been improved using advanced techniques to formulate cocktails, training as well as its use in combination with antibiotics, it is necessary to use personalized therapeutic approaches that demonstrate and guarantee the effectiveness and safety of its use to destroy PA biofilms in CF. In addition, regulated and approved clinical studies have not yet been conducted to investigate how phages safely and persistently degrade and lyse the PA biofilm matrix in patients with CF, so in future clinical investigations against these infections, isolated phages and their lytic enzymes characterized and selected, tested in vitro in cocktails or in

combination with antibiotics, and in vivo in models of PA MDR-infected pulmonary and non-pulmonary hosts should be used.

Furthermore, phage therapy continues presenting challenges to have consistent and efficient results such as bacterial resistance, limited host range, regulatory framework, large-scale manufacturing, study of biology, phage pharmacology, and bacteria-phage interactions that continue to leave gaps in basic knowledge. Therefore, advances in biotechnology and molecular biology can help overcome these problems and research efforts that are accompanied by practical and rigorous commercial and regulatory approaches to successfully advance phage therapy in clinical environments are needed.

Author contribution All authors contributed to the conception and design of the study. Josefina León-Félix had the idea for the article and María José Martínez-Gallardo performed the literature search. Data analyzes were carried out by María José Martínez-Gallardo and Claudia Villicaña. The first draft of the manuscript was written by María José Martínez-Gallardo and Josefina León-Félix. Martha Yocupicio-Monroy and Sofía Lizeth Alcaraz-Estrada critically revised the work. All authors read and approved the final manuscript.

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Declarations

Competing interests The authors declare no competing interests.

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3. COMPARATIVE GENOMIC ANALYSIS OF *Pseudomonas aeruginosa* STRAINS ISOLATED FROM PATIENTS WITH HEALTH CARE-ASSOCIATED INFECTIONS IN A MEXICAN HOSPITAL

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Comparative genomic analysis of Pseudomonas aeruginosa strains resistant to carbapenems isolated from patients with health care-associated infections in a Mexican hospital

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Comparative genomic analysis of *Pseudomonas aeruginosa* strains resistant to carbapenems isolated from patients with health care-associated infections in a Mexican hospital

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Abstract

Pseudomonas aeruginosa (PA) is an important opportunistic pathogen that causes different infections on immunocompromised patients. Within PA accessory genome, differences in virulence, antibiotic resistance, and biofilm formation have been described between strains, helping to the emergence of multidrug-resistant strains. The genome sequences of 17 strains isolated from patients with healthcare-associated infections in a Mexican hospital were genomically and phylogenetically analyzed and were detected antibiotic resistance, virulence, and biofilm formation genes. Most of the 17 strains were resistant to the carbapenems meropenem, imipenem, and aztreonam. The antibiotic resistance (*mexA*, *mexB*, and *oprM*) and the biofilm formation (*pslA* and *pslD*) genes were detected in all strains. Differences were found between strains in accessory genome size. The strains had different sequence types, and some were like that of global high risk epidemic PA clones. All strains were represented in two groups among PA global strains. Horizontally acquired resistance genes related with resistance to aminoglycosides and beta-lactams were found, mainly, and between 230-240 genes associated with virulence factors. The strains under study were variable in terms of their accessory genome, antibiotic resistance, and virulence genes. With these characteristics, we provide information about the genomic diversity of clinically relevant PA strains.

Keywords

Pseudomonas aeruginosa; nosocomial infections; multidrug resistant; pangenome

1. Introduction

Currently, most antibiotics have become ineffective for the control of bacterial infections since bacteria have generated multi-resistance to these kinds of drugs due to the indiscriminate and widespread use of antibiotics [1]. These multidrug-resistant (MDR) pathogens have been called "superbugs" and have become one of the most critical public health problems worldwide since cause more than one million deaths a year worldwide and around 35,000 deaths per year in the United States [2, 3].

The World Health Organization (WHO) reports that 50% of infections caused by *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (PA), show resistance to antibiotics such as cephalosporin, a third-generation antibiotic. This group of bacteria induces healthcare-associated infections (HAIs) or nosocomial infections, which are defined as infections contracted by a patient during treatment in a hospital that patient does not have at the time of admission [4]. HAIs affect one in 20 hospitalized patients, and in Mexico the yearly death toll is 37,000 by this kind of infections [5].

In Mexico, the epidemiological surveillance of HAIs is in charge of the Hospital Epidemiological Surveillance Network. Additionally, the Institute for Social Security and Services for State Workers (ISSSTE) carries out its control and surveillance through the Committee for the Detection and Control of Nosocomial Infections, finding PA among the first microorganisms [6].

Pseudomonas aeruginosa is a gram-negative bacterium established in water, soil, and various host organisms. In humans, it is considered the fourth most isolated nosocomial pathogen, accounting for 10% of all HAIs, and the second most common cause of pneumonia [5].

PA has the exceptional ability to generate resistance to antibiotics, for its ability to mutate genes and transfer horizontally acquired resistance genes [7]. Both clinical and environmental strains can thrive in a wide variety of ecological niches and cause damage in different hosts, since it is a versatile bacterium with easy adaptability due to the maintenance of virulence and metabolic genes in its genome. The importance of this bacterium lies in the fact that it causes significant morbidity and mortality between immunocompromised patients and those requiring mechanical ventilation, such as burn patients and those with cystic fibrosis. Some sequence types (ST) referred to as high-risk clones are extensively issued and frequently found in PA [8].

The increased availability of genomes has made it possible to perform comparative genomic analyzes of PA strains in distinct parts of the world [7, 9, 10]. These studies have defined the pangenome of strains with a "core genome" which are the genes present among all members of the species and represent up to 90% of the total PA genome, which is highly conserved, and been defined an "accessory" genome conformed by genes that are present in but missing in other PA strains [7]. PA genome size varies between 5.5 and 7 Mbp and such variability is owing to it has a great accessory genome that are specific DNA blocks of each strain and can occupy up to 20% of the entire genome. This accessory genome is integrated of horizontally transferable elements including prophages, plasmids, insertion sequences (IS), genomic islands (GI), and transposons. The accessory genome is significant for bearing acquired antibiotic resistance and virulence genes, with lateral transfer of these genes among strains contributing to the developing of more virulent strains. Additionally, mutational changes may promote antibiotic resistance and virulence. Genome characterization studies of PA isolates such as Aggarwal et al. 2015 [11] and Subedi et al., 2018 [10] have shown that the pangenome contains large heterogeneity in accessory genome size among 22 strains which was correlated

93 with the quantity of prophages, insertion sequences, and genomic islands. The strains from these
94 studies had different sequence types and were distinct from the worldwide PA epidemic clones.
95 The isolates previously characterized have genes that confer resistance to aminoglycosides, beta-
96 lactams, quaternary ammonium compounds, sulfonamides, chloramphenicols and tetracyclines.
97 Therefore, knowing the PA genome aids to comprehend the genetic modifications that are related
98 with more resistant and pathogenic strains and until the time of writing this article, no published
99 articles were found about comparative genomic analysis between PA strains isolated in Mexico.
100 Hence, the objective of this study is to compare the genomic variability within PA strains isolated
101 of hospitalized patients with different HAIs from the Centro Medico Nacional 20 de Noviembre
102 in Mexico City, for which the complete genomes of 17 strains were sequenced and genomic
103 analysis was performed by comparing evolutionary relationships, genomic divergence, antibiotic
104 resistance, and virulence factors.
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2. Materials and methods

2.1 Bacterial strains and antibiotic susceptibility testing

We studied 17 clinical strains of *Pseudomonas aeruginosa* provided by the Central Epidemiological Surveillance Laboratory, Centro Medico Nacional 20 de Noviembre ISSSTE of Mexico City isolated from different samples of patients with HAIs between the years 2019, 2020, and 2021, as are shown in Table S1. The disk diffusion method was performed based on the guidelines recommended by the Clinical and Laboratory Standards Institute (CLSI) to determine antibiotic susceptibility patterns, using PA ATCC 27853 as control strain. The antibiotic disks (BD BBL, USA) used in this study included meropenem (10 µg), imipenem (10 µg), aztreonam (30 µg). The isolates with resistance to 2 antibiotic classes were considered as PA resistant to carbapenems [12].

2.2 DNA extraction and PCR detection of resistance and biofilm formation genes

The strains were grown in 5 ml of TSB liquid medium at 37°C for 24 h and genomic DNA was extracted following the method described by Chen and Kuo (1993) [13]. PCR reactions for detection of efflux pump (*mexA*, *mexB*, and *oprM*) and biofilm-forming (*pslA* and *pslD*) genes were done using specific primers listed in Table S2. Reactions were carried out in 0.2 mL microcentrifuge tubes in a reaction volume of 25 µL containing 5X buffer, 25 mM $MgCl_2$, 10 mM nucleotide triphosphates (dNTPs), 0.1 µM primers and1-unit Taq DNA polymerase (GoTaq Promega). PCR conditions consisted of one cycle at 95 °C for 5 min; 30 cycles of 30 s at 95 °C, 40 s at Tm specific for each primer, 1 min at 72 °C and a final elongation at 72 °C for 10

135 min. The PCR products were separated on a 1% ethidium bromide agarose gel with 1X TAE (70
136 v, 300 mA, 1 h) and visualized using UV light transilluminator [14].

137 *2.3 Whole Genome Sequencing*

138 Once a 3-µg sample of genomic DNA with a purity of 1.8 (absorbance ratio of 260/280
139 nm) was obtained and its integrity verified by agarose gel electrophoresis, the samples were
140 sequenced by service at the Laboratorio Nacional de Genómica para la Biodiversidad del Centro
141 de Investigación y de Estudios Avanzados del IPN (LANGEBIO, CINVESTAV-IPN, Irapuato
142 Unit). The sequencing of the genetic material was carried out by the synthesis method with the
143 Illumina Miseq system in 2x150 format.

144

145 *2.4 Genomes assembly and analysis*

146 MiSeq sequencing resulted in a range of 653,270 to 2,199,951 reads per sample. With
147 FastQC version 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) was
148 evaluated the quality of the raw reads, and Trimmomatic version 0.36 [15] to remove adapters
149 with default parameters (SLIDINGWINDOW:4:30 MINLEN:36). A *de novo* genome assembly
150 was done using SPAdes version 3.13.1 [16] using the default settings. Prokka version 1.14.5 was
151 then used for annotations of the assembled genomes [17]. The PA PAO1 genome (RefSeq
152 accession number NC_002516.2) was used as a reference in the present work. The preliminary
153 genome contigs were rearranged and aligned to the reference genome with the MAUVE multiple
154 genome alignment software [18, 19]. Genomic islands were determined using IslandViewer [20],
155 and PHASTER to identify and annotate prophages [21]. To know the type of sequence (ST) of
156 each strain, the pubMLST database was used [22].

157 *2.5 Pangenome and phylogenetics*

158 Pangenome analysis was done with Roary version 3.13.0 [23]. Common genes in at least
159 99% of the strains were considered central genes. The accessory genome was obtained by
160 subtracting the core genes from the total genes of the genome of each strain. Two hundred fifty-
161 two genomes of PA strains (235 previously reported complete genomes and 17 genomes from
162 this work) were aligned with Parsnp version 1.2 having the PAO1 strain sequence as a reference,
163 and then it was used to make a maximum likelihood tree taking in account the core genome
164 single nucleotide polymorphisms (SNPs).

165

166 *2.6 Screening of antibiotic resistance and virulence genes*

167 The genomes of PA isolates were screened to identify acquired antibiotic resistance genes
168 by using Resfinder 3.0 (Centre for Genomic Epidemiology, DTU, Denmark) [24]. Virulence
169 genes associated with adherence, quorum sensing, type IV secretion system, alginate production,
170 toxins and protease production were searched in the genomes using the virulence factor database
171 (VFDB) [25].

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178 **3. Results and discussion**

179 *3.1 Antibiotic susceptibility profile*

180 A total of 17 strains of PA isolated from different samples of hospitalized patients with
181 HAIs were obtained. Seven of the 17 strains had antibiotic resistance, eight strains had
182 intermediate resistance, while two strains were susceptible to the three antibiotics tested. The
183 strains showed greater resistance to imipenem and meropenem (carbapenems), compared to
184 aztreonam (monobactam), irrespective of the year of collection; however, it would be appropriate
185 to use a greater number of strains from 2020 and 2021 to make a fair comparison (Table 1). Also,
186 it is described that carbapenems are the most active drugs against PA [26], so it is important to
187 highlight that most of the strains in this study were resistant to this class of antibiotics, which
188 stands out the need to find new alternatives to control this pathogen. Jeukens et al. (2017) [27]
189 reported that seven strains from CF patients were mostly susceptible to imipenem and
190 meropenem but resistant to ampicillin and amoxicillin, however, these strains were isolated in
191 1993, so it can be inferred that the bacterium have become more resistant over the years. The
192 emergence of *Pseudomonas* species resistant to carbapenems and monobactams has been
193 reported. In several studies such as the work of Bajpai et al. (2019) [26] where 126 isolates were
194 resistant to multiple kinds of antibiotics showing greater resistance to meropenem, 92 (72.5%);
195 aztreonam, 87 (69%) and imipenem, 78 (62.6%). In another work, Subedi et al. (2018) [10]
196 reported 22 susceptible and intermediate resistance strains to imipenem and aztreonam
197 (carbapenems) but resistant to gentamicin (aminoglycoside) and ciprofloxacin (quinolone), so
198 susceptibility to antibiotics may vary according to the classes of antibiotics.

199

200 *3.2 Detection of antibiotic resistance and biofilm formation genes*

201 Given the high levels of resistance present in the strains under study, the detection of
202 some genes involved in antibiotic resistance and biofilm formation was sought. For example, the
203 mexAB-oprM efflux pump system has been reported to be very common and aids PA intrinsic
204 resistance to β-lactams (including carbapenems), chloramphenicol, macrolides, quinolones,
205 tetracycline, and novobiocin, thus, the genes (*mex*A, *mex*B and *opr*M) related to this antibiotic
206 resistance system were searched for in the 17 strains, as were the biofilm formation genes (*psl*A
207 and *psl*D) and the extracellular obtaining of the polysaccharide synthesis locus mediated by
208 polysaccharides (*psl*) that have been reported to enhance the formation of biofilms that promote
209 resistance to antibiotics and protect the pathogen from the host's immune system [14], finding
210 that all genes were detected in the 17 strains as shown in Fig. 1. Our results coincide with the
211 previous work of Ugwuanyi et al. (2021) [14] where they describe that the *mex*A, *psl*A and *psl*D
212 genes were detected in 39 (100 %) isolates, but not the *mex*B and *opr*M genes, which were
213 identified in 34-36 isolates (88-92 %), respectively. However, Abbas et al. (2018) [28] informed
214 the detection of *mex*AB-R in 100% of PA isolates from urinary tract infections in Egypt [14].

215

216 *3.3 General characteristics of genomes*

217 The contigs generated by *de novo* assembly of the 17 PA strain genomes ranged from 96
218 in strain 19256 to 754 in strain 20019. Like other published PA genomes [9, 10, 27, 29], the
219 genomes in this study had a size of 6.6 to 7.3 Mbp and a mean C+G content of 65.95%. Genome
220 size oscillated extensively between strains, with up to 1.0 Mbp more DNA than PAO1 strain. The
221 number of coding sequences (CDS) oscillate from 5,969 (strain 19029) to 6,844 (strain 19133).

222 The five PCR amplified genes presented above (*mex*A, *mex*B, *opr*M, *psl*A and *psl*D) were
223 manually searched and found to be present in all the genomes of the strains, which was confirmed
224 by the PCR results. The general characteristics of the genomes are shown in Table 2.

225 A total of 12,273 orthologs were obtained from analyzed strains in this study. The
226 genomes of the 17 strains isolated in this work contain more ortholog genes than the study of
227 Subedi et al. (2018) [10] where incorporated 22 PA strains from various origins and they found
228 9786 orthologs in the pangenome. The larger pangenome found in the present study may be due
229 to the diverse type samples of patients that were used to isolate the strains since the size of the
230 pangenome can increase depending on the diversity of the strains in study, and it depicts the
231 accumulated genetic information between a group of bacterial genomes. Additionally, of 12,273
232 pangenes, this study described 4,813 genes that formed the core genome which was common in
233 at least 99% of the strains, no soft-core genes were found (common in 95-99% of strains), there
234 were 2938 shell genes (common in15-95% of strains) and 4521 cloud genes (common in 0-15%
235 of strains) (Fig. 2). Previous reports have informed core genomes of 5316 (6406 pangenes) [7],
236 5233 (9344 pangenes) [8], and 4910 (9786 pangenes) [10] in different strains of PA. Even though
237 these studies used less (less than 17) genomes, the results can be compared. Finding the smallest
238 core genomes in this study may be due to the use of more genomes for the alignment, as well as
239 the use of incomplete draft genomes and definition of the core genome (≥99% similarity in each
240 strain) [10]. Within the core genome were genes related to resistance to most antibiotics,
241 respiratory, metabolic and virulence genes, and the presence in all strains of metabolic and
242 respiratory genes gives the bacterium the ability to survive on a variety of carbon sources for
243 energy [8].

244 Besides the core genome, PA has an accessory genome that varies across strains, where
245 most of these genes are acquired horizontally. In this work, accessory genes were calculated by
246 removing the central genes (4813) from the number of CDS. The accessory genome ranged from
247 19% to 29% of the complete genome, which is larger than previously reported, which may be
248 attributed to the use of draft genomes that may overrate the number of accessory genes giving a
249 seemingly larger genome [10]. In these accessory genomes were found genes associated with
250 antibiotic resistance and virulence, as well as genes that aid strains to survive in hostile
251 environments like strains cultured from sites containing toxic organic compounds and heavy
252 metals which are unsuited for PA habitation. Therefore, the increase in the number of accessory
253 genes found in the present work may be because the strains have acquired many genes to be able
254 to grow and survive in the hospital environment [10]. According to previously reported accessory
255 genomes, a large number of undetermined genes were also found in this study, either because
256 their function is unknown, or they are pseudogenes. It is important to highlight that in these
257 accessory genomes there were many genes that participate in replication, recombination, and
258 repair, mainly integrases, recombinases and transposases, whose function is DNA mobility [8].
259 As important elements of accessory genome, genomic islands (GI) and prophages were also
260 identified in the genomes [30]. The results indicate that the predicted number of GI was ranged
261 from 27 to 90 and of prophages from one to five in the 17 genomes (Table S3), but only with
262 complete genomes can the total quantity from these genes in the accessory genome be known.

263 According to the Multi-Locus Sequence Typing (MLST) analysis, 10 different sequence
264 types (ST) were found in the 17 genomes, only one constituted a new type. The ST was allocated
265 to every strain based on seven integers that are the alleles at seven house-keeping loci that gives a
266 matching number in the MLST database, therefore if a strain did not coincide with the existent

267 database, it was taken as a new ST. The results indicate that five isolates belong to ST 233, three
268 isolates belonged to ST 309 and two strains to ST 3045 (MLST profiles are listed in Table 4), the
269 remaining strains correspond to seven different STs. These results indicate that the strains in
270 study belong to a wide variety of STs and coincide with anteriorly reported clinical epidemic
271 isolates [31, 32, 33, 34]. Most frequently reported international high-risk MDR PA clones, ST
272 111, 175, 233, 235, 277, 357, 654, and 773 [34, 35, 36]. ST 233 has been described as a high-risk
273 clone in Mexico, the United States, South Africa, and Japan. An ST 111 strain resistant to
274 carbapenems and aztreonam [37], which coincides with the 19133 strain that had the same ST
275 and presented resistance to the three antibiotics. The ST 233 was the most common ST found in
276 this work, which has been reported in MDR hospital strains [37].

277 *3.4 Phylogenetics*

278 All strains did not follow a specific pattern and were well represented in two groups, nine
279 strains in group 1 and eight in group 2 (Fig. 3). This coincide with previous studies where it has
280 been described that PA strains from different sources fall into two main groups, where group 1 is
281 the largest, and includes the most investigate worldwide strains DK2, PAO1, LESB58 and PAK,
282 while group 2 is slightly smaller and contains the studied virulent strain PA14 [38].

283 Interestingly, three groups of strains that were grouped by clade had the same ST in each
284 clade. Strains 19121, 19241, 19256, 19299 and 21049 were grouped within group 1 and had ST
285 233 reported as high-risk clones, also within the group. Group 1 strains 20061 and 21068 had ST
286 3045 and within group 2 strains 20019, 19071 and 19291 with ST 309 were grouped in the same
287 clade.

288 *3.5 Genetic profiles of antibiotic resistance*

289 A total of 45 different acquired antibiotic resistance genes were found in the present work
290 using ResFinder database (Fig. 4). Most resistance genes associated to aminoglycosides and beta-
291 lactams were observed in the strains from this study. As in the 22 PA strains reported in the work
292 by Subedi et al. (2018) [10], the genes *bla*PAO (resistance to b-lactams), *fos*A (resistance to
293 fosfomycin), *aph(3')-IIb* (resistance to aminoglycosides) and *catB7* (resistance to
294 chloramphenicol) were also detected in the 17 strains analyzed. One strain (19291) has five
295 resistance genes that were not detected in other strains; *aph(6)-Id*, *aph(3')-VIa*, *aph(3")-Ib*
296 aminoglycoside, *bla*IMP-15 and *cat*A1 that confer resistance to aminoglycosides, beta-lactams
297 and phenicols.

298 *3.6 Virulence genes*

299 Virulence genes related with adherence, quorum sensing, type IV secretion system,
300 alginate production, toxins and protease production were searched in our 17 PA genomes using
301 the Virulence Factor Database (VFDB). Differences in virulence genes were found for genes
302 involved in adherence, iron uptake, and secretion systems (Table 3). This was more evident for
303 genes with the type III secretion system (*exoS*, *exoU*, *exoY* and *exoT*). The exoT and exoS toxins
304 were the first to be identified in PA, participating in related functions such as altering the host
305 cell actin cytoskeleton, inducing host cell rounding, inhibiting phagocytosis, and even causing
306 cell death [39]. ExoY rifts the intracellular cAMP in eukaryotic cells and causes cell rounding
307 [39]. ExoU is considered the most virulent effector as it disrupts eukaryotic membranes in
308 different cell types. Based on these characteristics, PA strains can be classified into two groups;
309 strains expressing ExoT and exoU internalize poorly and cause rapid host cell death, while strains
310 expressing exoS and exoT internalize efficiently and cause slow cell death and in the strains in
311 this study the presence of exoS and exoU is important, as the 10 strains with exoS can evade the

312 action of antibiotics due to their ability to invade mammalian cells and that they can remain
313 inside the cells, whereas the presence of exoU in seven strains gives them a survival advantage by
314 increasing resistance [39]. The *exoS* and *exoU* genes are mutually exclusive which was verified in
315 the strains under study since one of the two genes was found in each strain [40]. The *exoU* gene
316 is part of genomic islands, so the strains studied that possess *exoU* showed larger accessory
317 genomes. The *exoY* and *exoT* genes have been described as the most prevalent secretory toxins in
318 strains, being found in all and 15 of the strains, respectively (Table 4) [10].

319 Regarding the type VI secretion system in PA, it has been reported that the phospholipase
320 D gene (*pldA*) promotes chronic infections [10]; however, *pldA* was absent in eight of the 17
321 isolates in this study (Table 4). Adhesion associated genes such as type IV pili (*pilA*) are involved
322 in adherence to and invasion of mucosal surfaces, consequently, *pilA* mutants exhibit less
323 adherence and invasion of airway epithelial cells [41]. In this study, *pilA* gene was found in seven
324 of the 17 strains (Table 4), including some associated with respiratory tract infections.

325 Conclusion

326 In this study, a comparative genomic analysis was performed between PA isolates from
327 patients with HAIs. The strains under study were different in terms of their accessory genome,
328 antibiotic resistance, and virulence genes, and phylogenetically, the 17 strains were distributed
329 throughout the two PA groups described. However, all strains have all the characteristics to
330 become resistant to antibiotics used in hospitals, as well as possessing the necessary
331 pathogenicity to cause infections in humans. Knowledge of the genes shared by most PA isolates
332 makes it possible to search for, propose, and approve new therapies to combat the great diversity
333 of human infections caused by this bacterium. Therefore, this information can be used to

334 elucidate mechanisms or search for alternatives that help combat this virulent and multiresistant
335 bacterium in the hospital environment.

336

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458 Statements & Declarations

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461 Competing interests

462 The authors declare no competing interests.

463 Author contributions

464 All authors contributed to the study conception and design. Juana Salazar-Salinas, Omar
465 Fernando Mendoza-Vázquez and Gabriel Damazo-Hernández provided clinical isolates. Josefina
466 León-Félix and María José Martínez-Gallardo design the experiments. Experiment execution was
467 performed by María José Martínez-Gallardo. Data analysis was performed by Josefina León-
468 Félix, María José Martínez-Gallardo, and Claudia-Villicaña. The first draft of the manuscript was
469 written by María José Martínez-Gallardo. Martha Yocupicio-Monroy and Sofía Lizeth Alcaraz-
470 Estrada critically revised the work. All authors read and approved the final manuscript.

471 Data availability

472 The datasets generated during and/or analyzed during the current study are available from the
473 corresponding author on reasonable request.
474

Tables

Table 1. Antibiotic susceptibility profile of *Pseudomonas aeruginosa* strains.

Strain	Antibiotics (Carbapenem class)					
	Halo size (mm)	Interpretation Aztreonam	Halo size (mm)	Interpretation Imipinem	Halo size (mm)	Interpretation Meropenem
19021	6	R	6	R	6	R
19029	18.62	I	10.46	R	18.62	I
19071	6	R	6	R	6	R
19121	23.82	S	6	R	6	R
19133	16.77	R	6	R	6	R
19182	21.73	S	23.71	S	27.4	S
19199	14	R	11.5	R	10	R
19205	25	S	24.05	S	24.25	S
19241	21.5	I	6	R	6	R
19256	23.83	S	6	R	6	R
19291	20.43	I	6	R	6	R
19299	15.32	I	6	R	6	R
20019	0	R	0	R	0	R
20061	20	I	14	R	18	R
20238	0	R	0	R	0	R
21049	21	I	0	R	0	R
21058	15	R	8	R	7	R

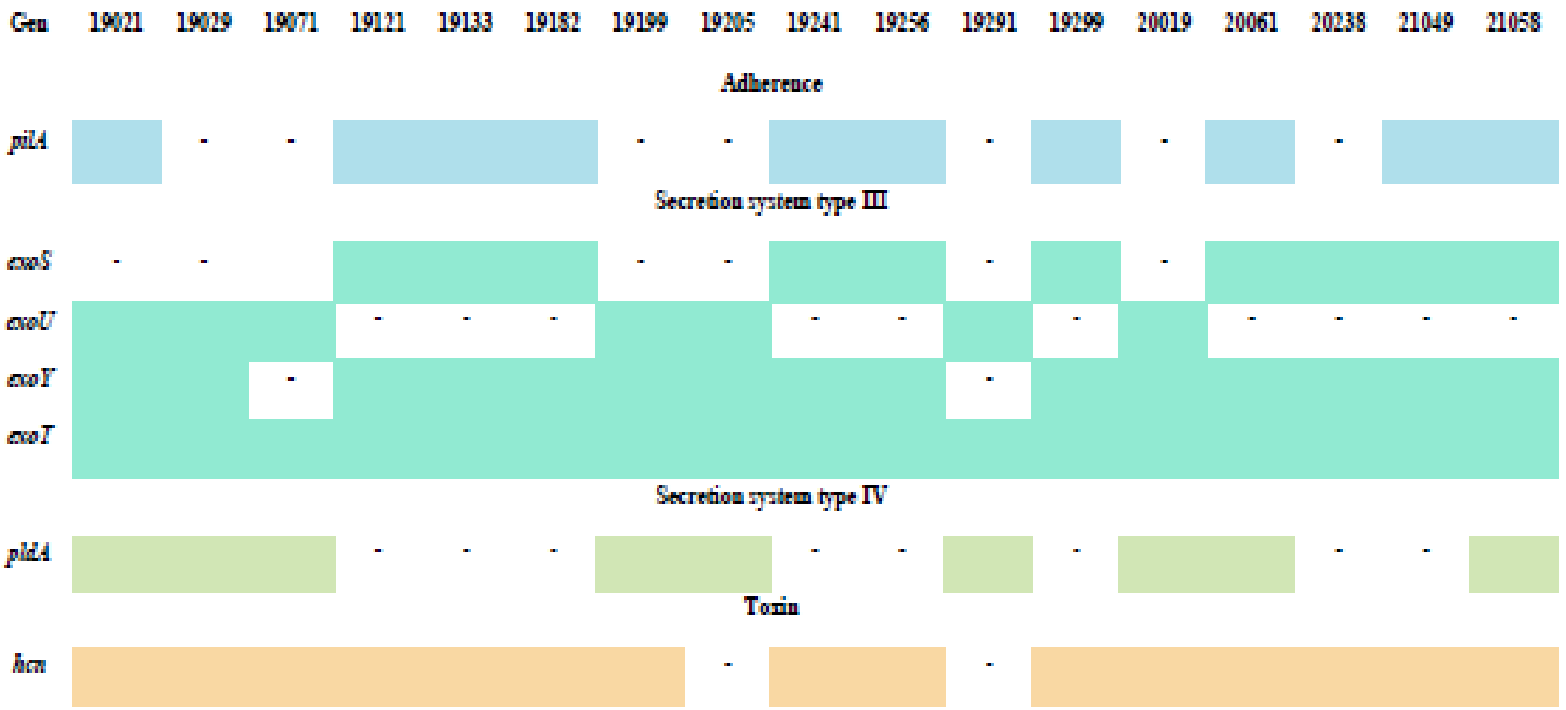
Table 2. General characteristics of the *Pseudomonas aeruginosa* strains genomes. *Sequence types were determined by the Multilocus Sequence Typing Database. Sequence types not listed in the MLST database have been considered as new.

Strain	Sequence type*	No. of contigs	Lenght (bp)	%GC	CDS	tRNA	Accesory genes
19241	233	552	6893744	65.93	6297	67	1484
19299	233	727	6840867	65.9	6240	65	1427
19256	233	96	6898377	65.99	6281	68	1468
20019	309	754	7094083	65.87	6539	71	1726
20061	3045	532	7153863	65.69	6607	70	1794
20238	17	301	7002407	65.85	6439	72	1626
21049	233	251	6966072	65.98	6360	68	1547
21058	3045	686	7349074	65.3	6800	70	1987
19205	532	659	7003379	65.78	6427	73	1614
19182	27	218	6651343	66.13	6104	68	1291
19029	235	217	6600218	66.31	5969	69	1156
19071	309	546	7073259	65.88	6496	64	1683
19199	308	317	7162628	65.79	6617	67	1804
19133	111	486	7324582	65.68	6844	69	2031
19021	New	224	7020763	65.81	6474	65	1661
19291	309	222	7018393	65.91	6418	67	1605
19121	233	101	6933644	65.99	6302	68	1489

Table 3. Virulence factors detected in the 17 PA strains analyzed in the VFDB.

	19021	19029	19071	19121	19133	19182	19199	19205	19241	19256	19291	19299	20019	20061	20238	21049	21058
Adherence	85	85	87	92	96	88	85	83	92	92	85	92	86	87	87	92	90
Antimicrobial activity	10	12	10	12	10	10	10	8	12	12	10	11	10	10	8	9	9
Antiphagocytosis	25	25	25	25	24	25	25	25	23	25	25	23	24	24	25	25	23
Biosurfactant	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Enzyme	4	4	4	3	3	3	4	4	3	3	4	3	4	4	3	3	4
Iron absorption	28	28	25	30	24	27	27	28	28	30	27	27	25	27	27	29	26
Protease	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
Quorum sensing	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Regulation	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3
Secretion systems	65	63	60	64	63	62	60	61	60	64	62	60	60	62	62	64	59
Toxins	4	4	4	4	4	4	4	1	4	4	1	4	4	4	4	4	4
Total genes	235	235	229	244	240	233	229	224	236	244	229	234	227	232	230	240	232

Table 4. Heterogeneity in virulence genes in PA genomes found for adherence genes, iron uptake, and secretion systems.



Figures

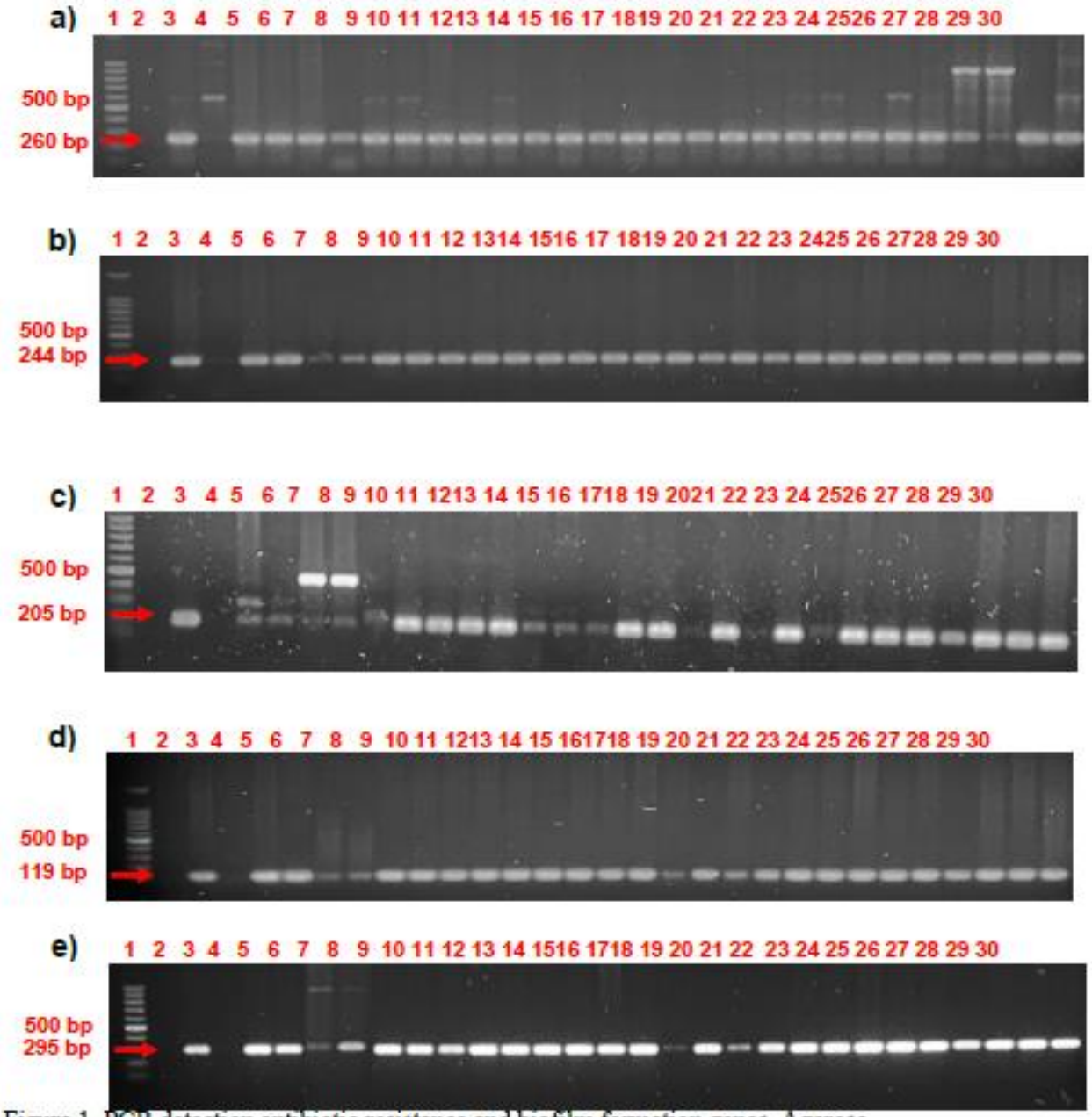


Figure 1. PCR detection antibiotic resistance and biofilm formation genes. Agarose electrophoresis of amplicons using primers for PA genes as follows: a) *mex*A, b) *mex*B, c) *opr*M, d) *psl*A, e) *psl*D. Lane 1: 100 bp DNA ladder, 2: blank, 3: positive control, 4: negative control, lane 5-30 *Pseudomonas aeruginosa* strains. The amplicon size for each primer set is indicated at the left.

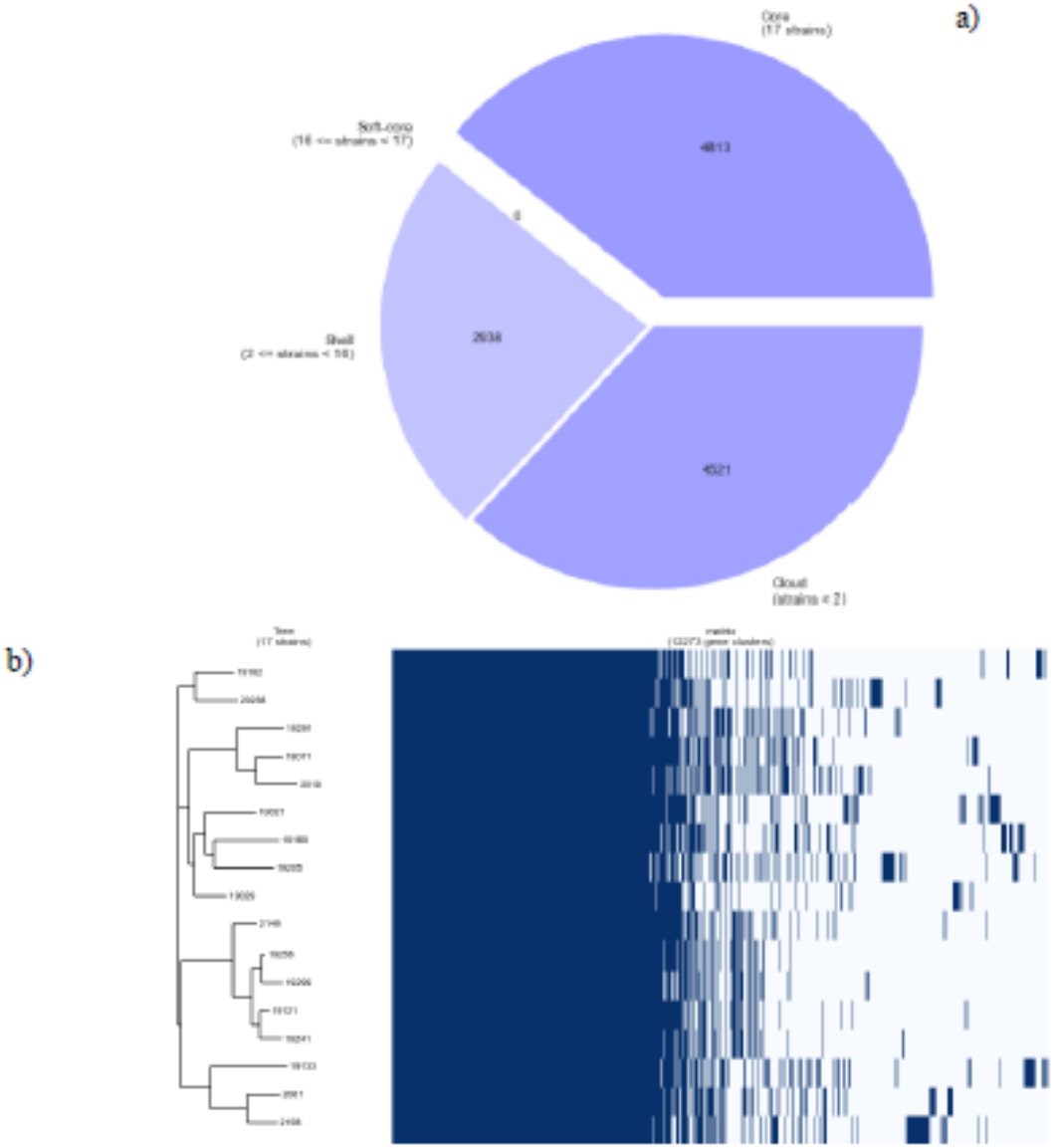


Figure 2. Pangenome of the 17 PA strains studied. a) Pie chart of the breakdown of genes. The pangenome is subdivided into core and accessory genes. The accessory genome is further subdivided into cloud and shell genomes, which are based on the number of genomes that contain the gene(s). The number of genes present in each category are shown. The number of strains necessary for a gene to meet the defined category is shown in parentheses; b) A tree compared to a matrix that represents the gene content, in terms of presence/absence of core and accessory genes of the pangenome inferred from the comparison of the 17 PA complete genomes.

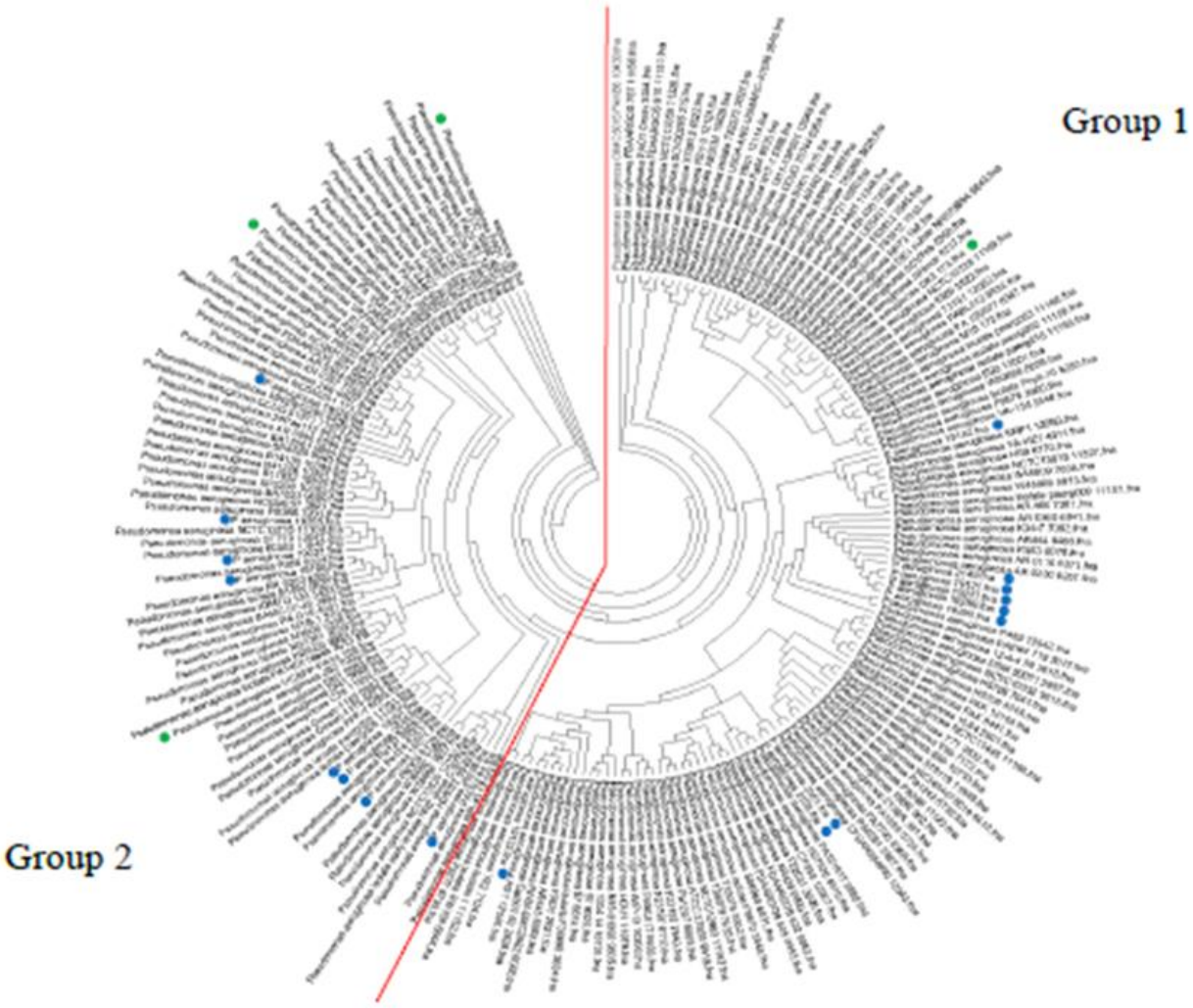


Figure 3. Phylogenetic analysis of *Pseudomonas aeruginosa* isolates. Strains used in this study are indicated by a blue circle. The positions of some reference strains are shown in green circles, such as *P. aeruginosa* VRFPA04, *P. aeruginosa* UCBPP-PA14, *P. aeruginosa* PAO1 and *P. aeruginosa* DK2.

	19021	19029	19071	19121	19133	19182	19199	19205	19241	19256	19291	19299	20019	20061	20238	21049	21058	Resistence
aac(3)-Id				■					■			■				■		Aminoglucoside
aac(6')-29a					■										■			Aminoglucoside
aac(6')-29b	■				■			■							■			Aminoglucoside
aac(6')-33			■							■			■					Aminoglucoside
aac(6')-Ib3	■		■				■	■		■			■	■				Aminoglucoside
aac(6')-Ib-cr	■		■				■	■		■			■	■				Aminoglucoside
aac(6')-Il									■			■				■		Aminoglucoside
aac(6')-Il				■			■									■		Aminoglucoside
aadA1b	■		■				■	■		■			■	■	■			Aminoglucoside
aadA2																■		Aminoglucoside
aadA6	■						■											Aminoglucoside
ant(2'')-Ia	■						■	■							■			Aminoglucoside
aph(3'')-Ib											■							Aminoglucoside
aph(3'')-IIb	■	■	■	■	■	■	■	■	■		■	■	■	■	■	■	■	Aminoglucoside
aph(3'')-VIa											■							Aminoglucoside
aph(6)-Id											■							Aminoglucoside
blaCTX-M-15	■																	Betalactams
blaGES-1	■						■								■			Betalactams
blaGES-14			■															Betalactams
blaGES-19			■															Betalactams
blaGES-2																	■	Betalactams
blaGES-20			■							■			■					Betalactams
blaGES-9	■																	Betalactams
blaIMP-15											■							Betalactams
blaOXA-2	■		■				■	■		■			■	■	■			Betalactams
blaOXA-395					■			■										Betalactams
blaOXA-396						■												Betalactams
blaOXA-4																■		Betalactams
blaOXA-486				■					■			■		■		■	■	Betalactams
blaOXA-488	■	■					■											Betalactams
blaOXA-494						■												Betalactams
blaOXA-50			■							■	■		■	■	■			Betalactams
blaPAO	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	Betalactams
blaVIM-2				■	■				■			■		■		■		Betalactams
catA1											■							Phenicol
catB2	■																	Phenicol
catB7	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	Phenicol
cmlA1																■		Phenicol

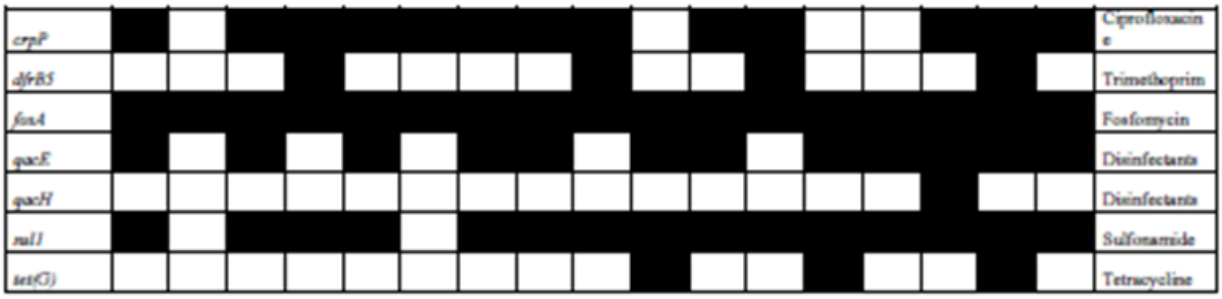


Figure 4. Antibiotic resistance genes profile detected by Resfinder database. Associated resistance: Beta lactams: - *bla*OXA-50, *bla*PAO, *bla*NPS-1 Aminoglycosides: *aph3'-IIb*, *aph(6)-Id*, *aph(3'')-Ib*, *aac(3)-IId* Fosfomycin: *fosA* Sulphonamide: *sul1* Chroramphenicol: *cmlA1*, *catB7* Tetracycline: *tet*(G). Quaternary ammonium compounds: *qacEdelta1*.

Supplementary material

Table S1. *Pseudomonas aeruginosa* strains isolated from clinical samples of patients with different HAIs. Abbreviations. UTI: Urinary tract infection, RTI: Respiratory tract infection, STI: Bloodstream infection, SSI: Surgical site infection, IIA: Intra-abdominal infection.

Code	Isolation year	Type of sample	Associated HAI
19021	2019	Urine	UTI
19029	2019	Sputum	RTI
19071	2019	Blood culture	STI
19121	2019	Blood	STI
19133	2019	Bronchial secretion	RTI
19182	2019	No data	No data
19199	2019	No data	No data
19205	2019	Wound secretion	SSI
19241	2019	Blood	STI
19256	2019	Wound secretion	SSI
19291	2019	Catheter site discharge	Catheter site
19299	2019	Abdominal secretion	SSI
20019	2020	Bronchial aspirate	RTI
20061	2020	Blood	STI
20238	2020	Urine	UTI
21049	2021	Bronchial secretion	RTI
21058	2021	Peritoneal dialysis fluid	IIA

Table S2. List of primers for efflux pumps and biofilm-forming genes used in this study.

Primer	Sequence	Amplicon size (bp)	Tm (°C)	Gene function
mexA-F *mexA*-R	5′-ACC TAC GAG GCC GAC TAC CAGA-3′ 5′-GTT GGT CAC CAG GGC GCC TTC-3′	260	57	Antibiotic Resistance (Efflux pump)
mexB-F *mexB*-R	5′-GTG TTC GGC TCG CAG TAC TC-3′ 5′-AAC CGT CGG GAT TGA CCT TG-3	244	56	Antibiotic Resistance (Efflux pump)
oprM-F *oprM*-R	5′-CCA TGA GCC GCC AAC TGT C-3′ 5′-CCT GGA ACG CCG TCT GGA T-3′	205	57	Antibiotic Resistance (Efflux pump)
pslA-F *pslA*-R	5′-TGG GTC TTC AAG TTC CGC TC-3′	119	55	Biofilm formation

	5′-ATG CTG GTC TTG CGG ATG AA-3′			
pslD-F	5′-CTC ATG AAA CGC ACC CTC CT-3′	295	52	Biofilm formation
pslD-R	5′-TGC GAC CGA TGA ACG GAT AG-3′			

Table S3. Number of predicted genomic islands and prophages in each of the 17 genomes.

Strain	Genomic islands	Prophages
19241	58	3
19299	56	2
19256	54	2
20019	77	4
20061	73	2
20238	71	1
21049	57	3
21058	90	2
19205	78	5
19182	45	3
19029	27	2
19071	56	4
19199	73	5
19133	86	2
19021	69	1
19291	71	2
19121	54	2

4. ISOLATION AND CHARACTERIZATION OF LYTIC BACTERIOPHAGES OBTAINED FROM HOSPITAL WASTEWATER WITH ANTIMICROBIAL ACTIVITY AGAINST *Pseudomonas aeruginosa* MDR

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Estado: Revisión interna

1. INTRODUCTION

Pseudomonas aeruginosa (PA) is a Gram-negative, non-fermentative aerobic bacterium extensively founded in soil, domestic water, and hospital wastewater (Jiang et al. 2020) and is one of the most frequent pathogens producing nosocomial infections in patients with cystic fibrosis, patients with burns or other wounds, and also can adhere as a biofilm on the surface of different materials (Yuan et al. 2019). PA presents a great antimicrobial resistance both by innate and acquired mechanisms, so antibiotic resistance in bacteria is an emerging threat worldwide and the development of new antibiotics is less common, which calls for new strategies to control bacterial infections in humans (Alvi et al. 2021).

Among these, as bacteriophages (phages) can cause the death of bacteria (Kwon et al. 2021), phage therapy is a promising alternative in the treatment of infections with multidrug resistant (MDR) pathogens (Alvi et al. 2020). Phages are the most abundant viruses that lyse bacteria in nature, being able to follow two replication cycles, phages that follow the lytic cycle and phages that follow the lysogenic cycle; however, lytic phages are desirable to treat bacterial infection because they cause the death of host bacteria (Jiang et al. 2020).

Phages have advantages over antibiotics, such as their specificity at the genus and even species level, replication at the infection site, they are innocuous and have a low production cost (Alvi et al. 2020). Hence, the isolation and characterization of new phages, as well as the evaluation of their ability to lyse bacteria or destroy biofilms, is a priority to strengthen the development of antibacterial. *In vitro* and *in vivo* studies of phage therapy have been performed in recent years to determine its ability to control PA infections (Yuan et al. 2019; Alvi et al. 2020; Jiang et al. 2019; Alvi et al. 2021; Kwon et al. 2021). These studies, have demonstrated the potential therapeutic effect of the phages used in terms of a significant reduction in morbidity and mortality. Nevertheless, in addition to these effects, phage-host interactions need to be studied in detail, in terms of phage dynamics in natural settings, phage resistance in clinical environments and biosafety (Yuan et al. 2019).

In previous works on phage therapy, it has been determined that a key factor for a successful phage therapy is a correct selection of phages, for which some biological parameters must be considered, such as phage specificity, efficacy and following a lytic cycle (Kwon et al. 2021). In this study, we isolated and characterized five phages (AR1 ATCC, AR2 ATCC C, AR1 S, AR2 I and AR1 R1) which were selected by its ability to infect PA clinical isolates. The morphology, host range, and genomic features of the phages were examined and described.

2. OBJECTIVE

Isolate and characterize phages selected by its ability to infect PA clinical isolates. The morphology, host range, and genomic features of the phages were examined and described.

3. MATERIALS AND METHODS

3.1 Bacterial Strains and Growth Conditions

The PA clinical strains were provided by the National Medical Center “20 de Noviembre” in Mexico City. The bacterial strains of PA (ATCC 9721, 19029, 19121, 19133, 19182, 19205, and 19291) were used as host strains for phage isolation and propagation, while the remaining strains were used in the host range phage test. For subsequent assays, the strains were reactivated in trypticasein soy broth (TSB, Difco, USA), seeded by streaking on plates, and incubated at 37°C for 18 to 24 h.

3.2 Phage Isolation, Purification, Propagation and Concentration

For isolation, water samples were collected from samples of wastewater obtained from the wastewater treatment plant of the National Medical Center "20 de Noviembre” and were filtered through a filter with a 0.45 µm pore size (Corning, Alemania) and mixed with an overnight culture of PA in TSB (Green y Sambrook, 2012). Mixtures of PA strains were made to carry out the isolation of different phages, having five groups based on their susceptibility profile to carbapenems: one of sensitive strains (S) (19182 and 19205), one of intermediate strain (I) (19029), and three resistant groups (R1) (19121, 19133 and 19291), (R2) (19071, 19084 and 19199) and (R3) (19021, 19065 and 19066). After 24 h of incubation at 37 °C of the phage-bacteria mixture, a conventional spot assay was conducted for phage plaque detection (Gasic et al. 2011). A lysis plaque was selected and streaked for subculture, and this was repeated three times to obtain pure phages (Gasic et al. 2011). The phages were designated as AR1 ATCC, AR2 ATCC C, AR1 S, AR2 I and AR1 R1.

Bacteriophages were propagated via the double layer agar technique established by Jamalludeen et al. (2007) with some modifications. The mixtures of PA bacteria were grown in 50 ml of TSB, overnight at 37 °C. Subsequently, 1 ml of the bacterial culture and 100 µl of the phage were mixed

in 3 ml of TSB-Agarose (0.4%). This mixture was emptied into a Petri dish with TSA, waited for it to solidify, and incubated at 37 °C for 24 h. Phage lysates were centrifuged in a Sorvall Lynx 6000 centrifuge (Thermo-Scientific, Germany) at 8,500 × g for 15 min at 4°C. The supernatant was taken in a new PPCO tube, centrifuged at 10,000 × g for 15 min at 4°C. The supernatant was recovered in a new PPCO tube, and a final centrifugation was performed at 40,000 × g for 2 h at 4°C. The supernatant was discarded, and the pellet was resuspended in 10 ml of nanopure water. This suspension was filtered through a cellulose acetate acrodisc with a 0.2 µm pore size and the filtrate was stored at 4°C as a stock for later uses.

3.3 Electron Microscopy

Phage morphology was determined by transmission electron microscopy (TEM). The purified viral particles were negatively stained with phosphotungstic acid (2% w/v, pH 7.2) to be subsequently observed with a JEOL model JEM-1200EX TEM at 100 kV. Phage measurements were determined taking micrographs into account.

3.4 Host Range of the Phages

The host range of the phages was studied with the spot test assay as follows (Gasic et al. 2011). Briefly, ten-fold serial dilutions (102-108 PFU/ml) of phage lysate were placed on TSA lawns of agar with a PA strain. Plates were incubated overnight at 37 °C and observed for plaque formation. Clear plaque formation were determined as lysis, whereas no plaque formation as no lysis.

3.5 Bacteriophage Genome Extraction

Standard molecular biology techniques were followed for DNA isolation, according to Sambrook and Russel (2001). One ml of the purified phage suspension (1x108 PFU/ml) was taken and 1 U of DNAse I (Sigma-Aldrich, USA) and 1 U of RNAse A (Sigma-Aldrich, USA) were added, and

incubated in a thermomixer (Thermomixer, Eppendorf, Germany) at 37°C, for 30 min. After incubation, 40 µl 0.5 M EDTA (pH 8.0) (Sigma-Aldrich, USA), 2.5 µl proteinase K (20 mg/ml) (Sigma-Aldrich, USA) and 50 µl sodium dodecyl sulfate was added. SDS 10% (Sigma-Aldrich, USA), was mixed by inversion 5-10 times. Then, the tubes with the mixture were incubated at 56 °C for 2 h. At the end of the incubation, a volume of phenol (Sigma-Aldrich, USA) was added and mixed by inversion until a completely emulsified mixture was observed. Subsequently, sample was centrifuged at 3,000 × g for 5 min at 25 °C. Once centrifuged, the aqueous phase was transferred to a 1.5 ml tube and one volume of phenol-chloroform (Sigma-Aldrich, USA) was added and centrifuged at 3,000 × g for 5 min at 25 °C. The aqueous phase was collected in a 1.5 ml tube and 200 µl of 3 M sodium acetate pH 5.2 (J.T. Bater, USA) and one volume of absolute ethyl alcohol (Sigma-Aldrich, USA) were added. Afterwards, it was incubated at -20 °C overnight. After the incubation was completed, the sample was centrifuged at 15,000 × g for 30 min and the supernatant was decanted. The pellet was washed with one volume of 70% ethyl alcohol and centrifuged at 15,000 × g for 15 min. The supernatant was decanted, and the pellet was allowed to dry at room temperature. Then, 100 µl of nanopure water was added.

3.6 Analysis of Restriction Patterns with Endonucleases

The restriction of 1 µg of phage DNA was performed independently with each restriction enzyme (*Eco*RI, *Pst*I, *Bam*HI and *HinP*1I), following the manufacturer's specifications (Promega, USA). The resulting DNA fragments were separated by 0.8% agarose gel electrophoresis with ethidium bromide.

3.7 Sequencing and Bioinformatic Analysis

Genomic DNA was sent to LANGEBIO (México) for sequencing using Illumina Miseq. To assess the quality of the raw reads, FastQC version 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc) was used, and then Trimmomatic version 0.36 (Bolger et al., 2014) was used to remove adapter sequences with the minimum read

length setting of 36 and minimum coverage of 15. A de novo assembly was done with SPAdes version 3.13.1 (Bankevich et al., 2012) with the default settings. Potential open reading frames (ORFs) were predicted by GeneMark (http://exon.gatech.edu/GeneMark/) and the putative genes were analyzed by BLAST at the National Center for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Potential genes for tRNA in the genome sequence were predicted by tRNAscan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/). In addition, all identified genes were compared with the virulence factor database (http://www.mgc.ac.cn/VFs/), resistance gene identification database (https://card.mcmaster.ca/analyze/rgi) and the Allergen Protein Identification Database (http://www.allergenonline.org/) using nucleotide sequence. To classify phage lifestyle, the Computationally Approached Phage Sorting Toolkit (PHACTS) online prediction program (http://www.phantome.org/PHACTS/upload.php) was used.

4. RESULTS AND DISCUSSION

4.1 Isolation and Morphology of Bacteriophages

After enrichment with the groups of PA strains and each of the wastewater samples, lysis zones were obtained in five samples. These lysis zones were recovered to later carry out dilutions, where lysis plaques of different morphology were obtained in the five samples, for which five different phages were isolated and purified (Table 1) (Figure 1). The five phages isolated obtained from the hospital sewage were designated as AR1 ATCC, AR2 ATCC C, AR1 S, AR2 I and AR1 RI, which produced clear plaques with different sizes (Figure 1).

The isolated phages were characterized at the morphological level using transmission electron microscopy. The ultrastructural characteristics of the phages showed that the virions have an icosahedral capsid between 70-110 nm in diameter and a non-contractile tail about 120-190 nm long. The images did not show neck, base plate, spikes, or fiber in the mature phage (Table 2) (Figure 2).

Table 1. Morphology and plaque size of the isolated bacteriophages.

Phage name	**Size plaque**	**Morphology plaque**
AR1 ATCC	1.5-2 mm	Circular, irregular edges
AR2 ATCC C	0.5-0.8 mm	Circular
AR1 S	0.5-1 mm	Circular, irregular edges
AR2 I	0.1-0.6 mm	Circular, irregular edges
AR1 R1	0.5-1 mm	Circular, irregular edges

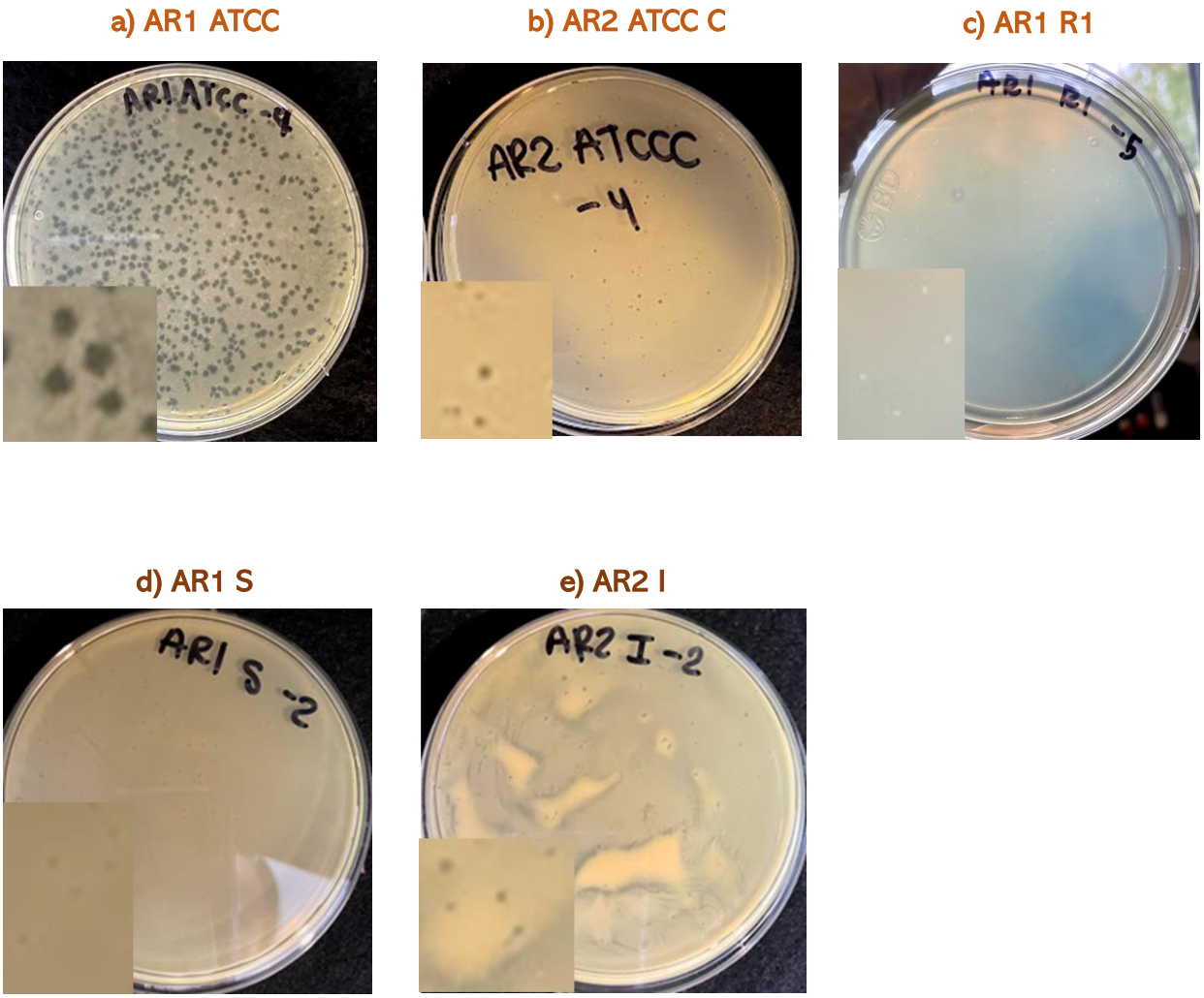

Figure 1. Plaque assays using the isolated bacteriophages on *P. aeruginosa*. a) Bacteriophage AR1 ATCC. b) Bacteriophage AR2 ATCC C. c) Bacteriophage AR1 R1. d) Bacteriophage AR1 S. e) Bacteriophage AR2 I.

Table 2. Phage head and tail size of the isolated bacteriophages.

Phage name	Capsid size	Tail size
AR1 ATCC	90 nm	150 nm
AR2 ATCC C	70 nm	140 nm
AR1 S	100 nm	180 nm
AR2 I	110 nm	190 nm
AR1 R1	80 nm	120 nm

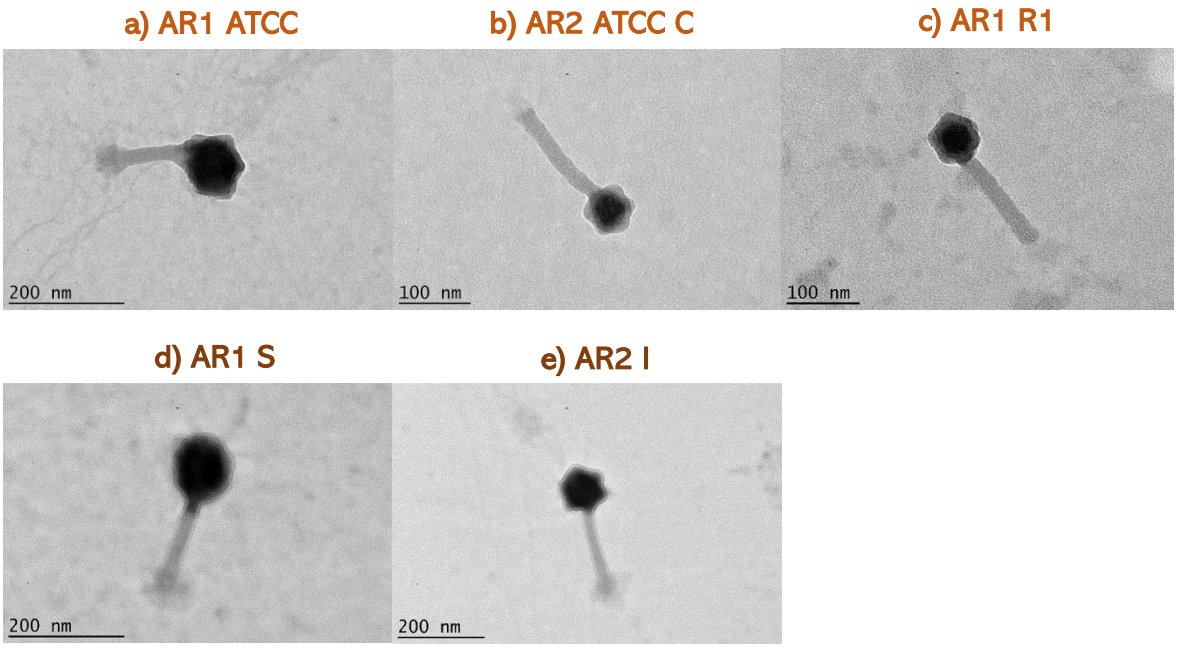

Figure 2. Transmission electron microscopy images of bacteriophages negatively stained using 2% uranyl acetate. Scale bar is equal to 100 and 200 nm.

4.2 Host Range of the Bacteriophages

The host range assay showed that the five isolated phages exhibited antibacterial activity against PA (Table 3). The host range test showed that phages can lyse 54 of 80 PA isolates (74%) (71 clinical, 9 environmental isolates). The highest lytic activity was exhibited by AR2I (47/80) and AR1S (43/80) phages. All environmental strains were lysed by at least two phages. Therefore, it can be considered that phages have a wide host range.

Table 3. Bacterial strains used for the host range spectrum of the bacteriophages.

			Phage				
Origin	Resistance profile	Strain	AR1 ATCC	AR2 ATCC	C AR1 S	AR2 I	AR1 R1
Clinic	R	19017	+	+	-	-	-
Clinic	R	19021	+	+	+	+	-
Clinic	I	19029	+	-	+	+	-
Clinic	R	19065	-	+	-	-	-
Clinic	-	19066	-	-	-	-	-
Clinic	R	19071	+	-	+	-	-
Clinic	R	19073	+	-	+	+	-
Clinic	R	19076	-	-	-	-	-
Clinic	R	19077	-	-	-	-	-
Clinic	R	19081	-	-	-	-	-
Clinic	R	19084	-	-	-	-	-
Clinic	R	19089	-	-	-	-	-
Clinic	R	19090	-	+	-	+	-
Clinic	R	19101	+	+	-	+	+
Clinic	R	19121	-	-	+	+	-
Clinic	R	19124	-	-	-	-	-
Clinic	R	19125	-	-	-	-	-
Clinic	R	19133	-	-	-	-	+
Clinic	R	19136	+	+	+	+	+
Clinic	R	19148	-	-	+	+	+
Clinic	R	19171	-	+	+	+	+
Clinic	R	19172	-	-	-	-	-
Clinic	R	19176	+	+	+	+	+
Clinic	S	19182	+	+	-	-	-
Clinic	R	19199	-	-	+	+	-
Clinic	R	19201	-	-	+	+	-
Clinic	R	19203	+	-	-	-	-
Clinic	S	19205	-	-	+	+	-
Clinic	R	19239	-	-	-	-	-
Clinic	R	19240	-	-	-	-	-
Clinic	R	19241	-	-	-	+	-
Clinic	R	19242	-	+	-	+	-
Clinic	R	19243	+	-	-	-	+
Clinic	R	19244	+	-	+	+	-
Clinic	R	19245	+	-	+	+	-
Clinic	R	19256	-	+	-	+	-
Clinic	R	19257	+	-	-	-	-
Clinic	R	19291	-	-	+	+	+

Clinic	R	19299	-	-	-	-	+
Clinic	-	20005	-	-	-	-	-
Clinic	-	20006	+	-	+	+	-
Clinic	-	20008	-	-	-	-	-
Clinic	-	20018	-	-	+	+	-
Clinic	-	20019	+	-	+	+	+
Clinic	-	20020	-	-	+	+	+
Clinic	-	20028	-	-	-	-	-
Clinic	-	20032	-	+	+	+	-
Clinic	-	20045	-	-	-	-	-
Clinic	-	20046	-	+	-	-	+
Clinic	-	20057	-	-	+	+	-
Clinic	-	20059	+	-	+	+	-
Clinic	-	20060	-	-	+	+	+
Clinic	-	20061	+	+	+	+	-
Clinic	-	20063	-	-	+	+	-
Clinic	-	20102	-	-	+	-	-
Clinic	-	20115	+	-	-	-	+
Clinic	-	20117	-	-	-	-	-
Clinic	-	20164	-	-	+	+	-
Clinic	-	20166	+	+	+	+	+
Clinic	-	20167	-	-	+	+	-
Clinic	-	20169	-	+	+	+	+
Clinic	-	20170	-	-	+	+	+
Clinic	-	20171	-	-	+	-	-
Clinic	-	20186	-	-	-	-	-
Clinic	-	20191	+	+	+	+	+
Clinic	-	20207	-	-	+	+	+
Clinic	-	20223	-	-	-	+	-
Clinic	-	20238	+	-	+	+	-
Clinic	-	21006	+	-	+	+	-
Clinic	-	21007	+	-	+	+	+
Clinic	-	21011	-	-	+	+	-
Clinic	-	21044	-	-	-	-	-
Clinic	-	21049	-	-	-	+	-
Clinic	-	21058	-	-	-	-	-
Clinic	-	21065	-	-	-	-	-
Clinic	-	21081	-	-	-	-	-
Clinic	-	21105	-	-	+	+	|-
			22/71 (31%)	16/71 (26%)	34/71 (48%)	38/71 (54%)	17/71 (24%)

Environmental (CDMX)	-	SAV-1	+	+	+	+	-
Environmental (CDMX)	-	SAV-2	+	+	+	+	-
Environmental (CDMX)	-	SAV-3	+	-	+	+	-
Environmental (CDMX)	-	SAV-4	+	-	+	+	-
Environmental (Sinaloa)	S	PA1	+	-	+	+	-
Environmental (Sinaloa)	S	PA2	+	+	+	+	+
Environmental (Sinaloa)	S	PA3	-	-	+	+	-
Environmental (Sinaloa)	S	PA5	+	-	+	+	+
Environmental (Sinaloa)	S	PA7	+	+	+	+	+

*R: Resistant, I: Intermediate, S: Susceptible.

4.3 Phage Restriction Profile

The genetic material of the phages was cut with two (*Eco*RI, *Hin*P1I) of the four enzymes used, while *Pst*I failed to cut any genetic material. *Bam*HI cut AR1 ATCC, AR2 ATCC C, AR1 S and AR2 I (Figure 3). The restriction profile suggest that the five phages are genetically different. Likewise, the restriction endonucleases used (*Eco*RI, *Bam*HI, *Pst*I, *Hin*P1I) are specific for cutting double-stranded DNA, therefore, since the phage DNA has been restricted by some of the enzymes, it indicates that the genetic material of phages is composed of double-stranded DNA.

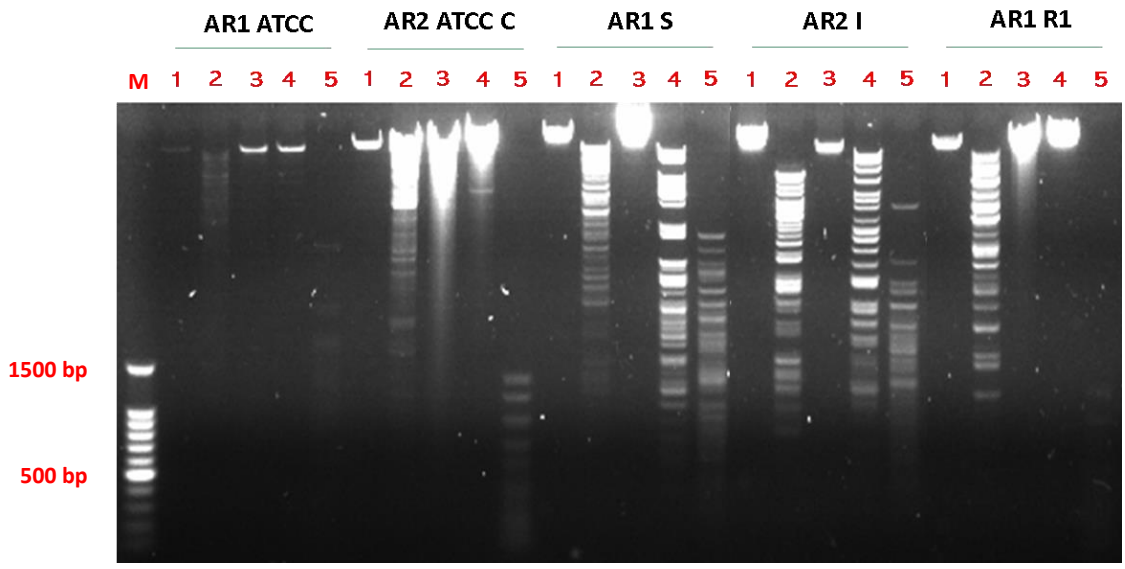

Figure 3. Ethidium bromide stained 0.8% agarose gel electrophoresis of restriction endonuclease digestion products of DNA bacteriophages AR1 ATCC, AR2 ATCC C, AR1 S, AR2 I, and AR1 R1. M: Marker 100 bp. 1, DNA without enzyme; 2, *Eco*RI; 3, *Pst*I; 4, *Bam*HI; 5, *HinP1I*.

4.4 Features of the Bacteriophages Genomes

The genome of phages AR1 ATCC, AR2 ATCC C, AR1 S, AR2 I, and AR1 R1 consists of double-stranded DNA with genome sizes between 46,379 and 213,521 bp, from 72-209 open reading frames (ORFs), and no tRNAs were found. The %GC varied from 49.31 to 55.04. Analysis of the genome reveals a lack of ORFs encoding virulence factors, antibiotic resistance genes, and allergenicity. The genomes features are summarized in Table 4. Regarding the phage life cycle, the PHACTS prediction suggested that the phages are lytic, which agrees with the absence of lysogenic genes and with our results of clear, non-turbid plaques that are characteristic of strictly lytic phages.

Table 4. General characteristics of the genomes of the AR1 ATCC, AR2 ATCC C, AR2 I, AR1 S and AR1 R1 bacteriophages.

Phage	Contigs	Length (bp)	ORFs	%CG	tRNA	Virulence genes (VFDB)	Resistance genes (RGI)
AR1 ATCC	2	46379	72	52.49	0	-	-
AR1 S	1	210420	208	49.34	0	-	-
AR2 I	1	213521	209	49.31	0	-	-
AR2 ATCC C	1	65436	94	55.04	0	-	-
AR1 R1	1	65777	93	55	0	-	-

Bioinformatic analysis suggests that phage genomes exhibit a modular functional organization typical of stem phages. Functional ORFs were categorized in various functional modules, like structure protein, DNA replication and regulation, and lysis modules. More than 75% of the ORFs correspond to hypothetical proteins with no defined function, which may translate into a lack of knowledge about the functionality of the proteins encoded by the phage genomes in the databases. In Figures 4-8 the transcriptional maps of the bacteriophage genomes are shown.

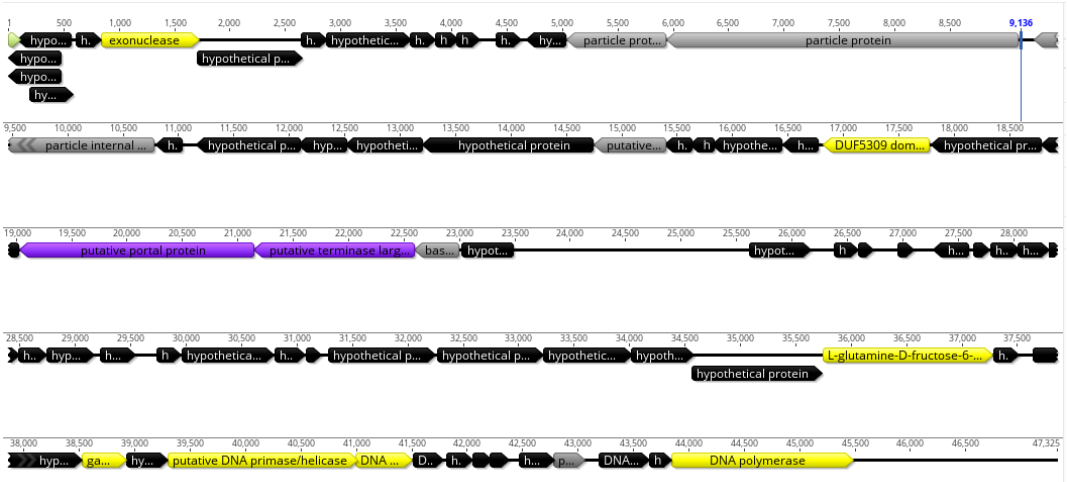

Figure 4. Graphic representation of the AR1 ATCC bacteriophage genome. Genes are indicated by arrows, the direction of which illustrates the direction of transcription. The different colors represent the function of each of the genes encoded in the bacteriophage genome: Metabolism (yellow), packaging of genetic material (purple), structural proteins (grey), proteins to induce bacterial lysis (blue), genes with unknown function (black) and genes without significance (green).

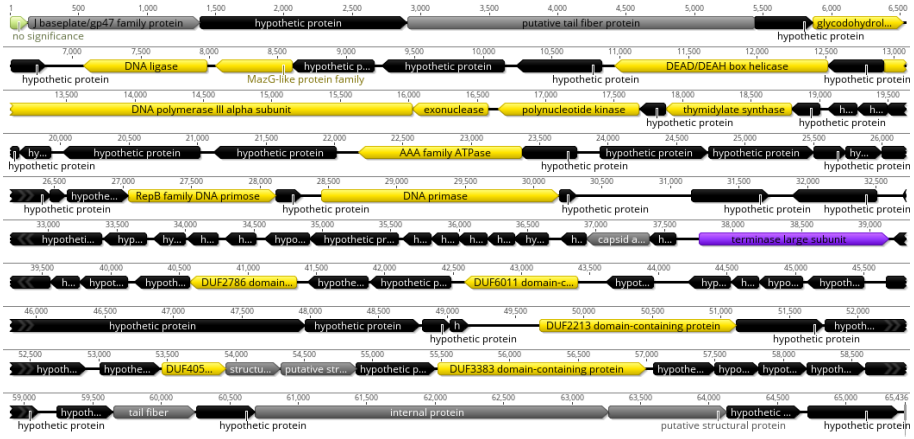

Figure 5. Graphic representation of the AR2 ATCC C bacteriophage genome. Genes are indicated by arrows, the direction of which illustrates the direction of transcription. The different colors represent the function of each of the genes encoded in the bacteriophage genome: Metabolism (yellow), packaging of genetic material (purple), structural proteins (grey), proteins to induce bacterial lysis (blue), genes with unknown function (black) and genes without significance (green).

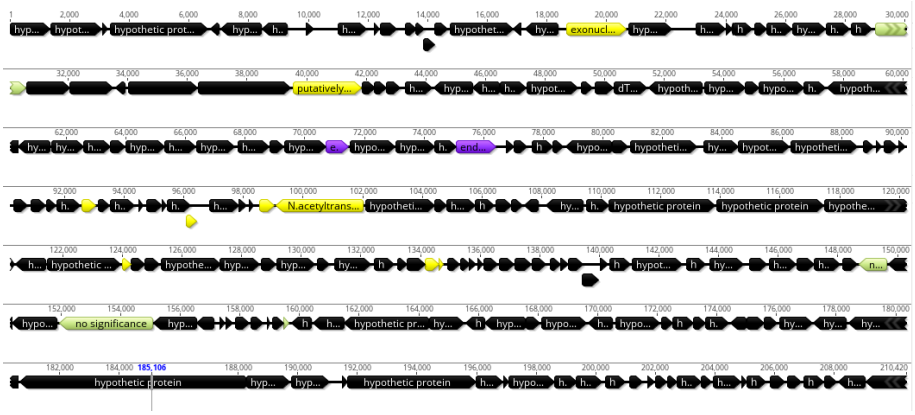

Figure 6. Graphic representation of the AR1 S bacteriophage genome. Genes are indicated by arrows, the direction of which illustrates the direction of transcription. The different colors represent the function of each of the genes encoded in the bacteriophage genome: Metabolism (yellow), packaging of genetic material (purple), structural proteins (grey), proteins to induce bacterial lysis (blue), genes with unknown function (black) and genes without significance (green).

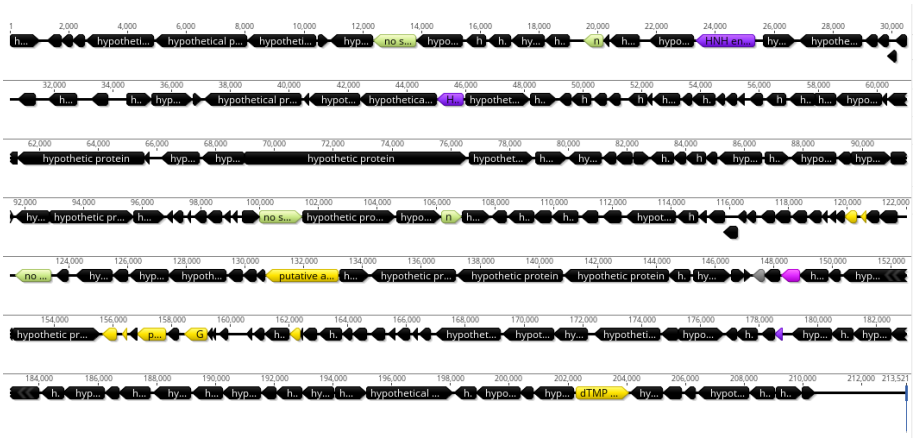

Figure 7. Graphic representation of the AR2 I bacteriophage genome. Genes are indicated by arrows, the direction of which illustrates the direction of transcription. The different colors represent the function of each of the genes encoded in the bacteriophage genome: Metabolism (yellow), packaging of genetic material (purple), structural proteins (grey), proteins to induce bacterial lysis (blue), genes with unknown function (black) and genes without significance (green).

Figure 8. Graphic representation of the AR1 R1 bacteriophage genome. Genes are indicated by arrows, the direction of which illustrates the direction of transcription. The different colors represent the function of each of the genes encoded in the bacteriophage genome: Metabolism (yellow), packaging of genetic material (purple), structural proteins (grey), proteins to induce bacterial lysis (blue), genes with unknown function (black) and genes without significance (green).

Proteins involved in receptor binding were found in the genomes of bacteriophages: the minor capsid protein (phage AR1 R1), the tail fiber subunit (phage AR1 ATCC, AR2 ATCC C), center of the base of the plate (AR1 ATCC phage). Regarding proteins that participate in replication, transcription and translation, DNA polymerase, DNA primase, DNA ligase, 3'-5' exonuclease and DNA binding protein were found in the different phages. For the assembly and packaging stage, proteins such as HNH endonuclease (AR1 S, AR2 I phage) and the long terminase subunit (AR1

ATCC, AR2 ATCC C, AR1 R1 phage) were found. For the lysis module, only a holin that controls the degradation of the host bacterial cell wall at the end of the lytic cycle of viral infection was found in the AR1 ATCC phage. Only in one phage, AR2 I, has one undesirable gene for phage therapy was found, a transposase which participates in the transfer of mobile genetic elements.

4.5 Discussion

In the present study, five new phages of PA were isolated from wastewater samples of the National Medical Center "20 de Noviembre" and for therapeutic purposes, wastewater is the richest source of bacteria and phages due to high levels of nutrients and the increased ability of bacteria to form biofilms on water surfaces and also wastewater from hospital environment has been reported as the main source of phage isolation for clinical bacteria such as PA (Essoh et al. 2015; Kakasis and Panitsa 2019). The criteria for the purification of the phage were based on the shape and size of the plaque that it develops, the selection of turbidity of the plate which is indicative of the states of lysogenization while those clear plates and defines are characteristic of lytic phages (Hyman et al, 2019).

The application of phages in clinical phases requires a thorough characterization and selection of the phages (Jiang et al. 2019). Host range is variable among phages, with some lysing all strains of a given species (broad) while others can only lyse limited strains of a given species (narrow). All five phages were able to efficiently lyse clinically isolated PA MDR (74% of the isolates) and are therefore considered to have a broad host range. Phages with broad host range have been reported in other works, where the phages tested were able to lyse at least 50% of the clinical isolates of PA (Alvi et al. 2020; Jiang et al. 2020).

The phages genomes characteristics such as length, GC content, and ORFs, are similar to that of other PA phages (Yuan et al. 2019; Alvi et al. 2020; Kwon et al. 2021). Presence of the genes encoding DNA replication enzymes like DNA polymerases, DNA ligase, DNA primase suggests that the replication of the phages is independent of the host replication machinery.

A problem with phage therapy is the possibility transduction of bacterial virulence genes between bacteria by phages, resulting in the development of a new pathogen or a multi-resistant bacterium (Yuan et al. 2019). The results of the genome annotation showed a transpose in the AR2 I phage, for which this phage would be ruled out for use at the moment, however if the phage has the potential to be used, through genetic engineering in subsequent studies this gene could be removed. In addition, a phage virulence factor analysis genome did not reveal the presence of human virulence genes, suggesting that all five phages may be suitable for use as phage therapy.

5. CONCLUSION

Based on the results obtained in the present work, most of the PA MDR strains tested were susceptible to the five isolated phages, therefore, four of the five phages can be considered as potential candidates to be used in phage therapy against infections by PA MDR.

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5. CONCLUSIONES GENERALES

Las 17 cepas clínicas de *P. aeruginosa* analizadas en este estudio mostraron multiresistencia a carbapenémicos y tienen todas las características para volverse resistentes a otras clases de antibióticos utilizados en los hospitales, además de poseer la patogenicidad necesaria para causar infecciones graves en humanos. El conocimiento de los genes compartidos por la mayoría de estas cepas permite buscar, proponer y aprobar nuevas terapias para combatir la gran diversidad de infecciones humanas causadas por esta bacteria. Por tanto, esta información puede ser utilizada para dilucidar mecanismos o buscar alternativas que ayuden a combatir esta bacteria virulenta y multirresistente en el ámbito hospitalario.

También se lograron aislar a partir de muestras de aguas residuales de hospital cinco bacteriófagos y se demostró la capacidad de éstos para infectar cepas clínicas de *P. aeruginosa*. La caracterización biológica y genómica indicaron que los fagos son seguros para utilizarse en terapia, sin embargo, se deben realizar ensayos *in vivo* para garantizar su eficacia y seguridad. Sólo en el fago AR2 I se encontró un gen que codifica para una transposasa, el cual es un gen no deseable y con lo cual quedaría descartado para ser utilizado en fagoterapia, sin embargo, mediante ingeniería genética se podría deletar este gen para utilizare y evaluar su actividad contra cepas PA.

La aplicación de bacteriófagos como agentes terapéuticos ha abierto nuevas vías para tratar infecciones que de otro modo serían difíciles de tratar. La terapia con fagos es muy eficaz para el tratamiento de microorganismos MDR, y los cuatro fagos mostraron actividad lítica eficiente contra cepas de *P. aeruginosa* MDR, por lo que los convierte en candidatos para tratar infecciones nosocomiales causadas por *P. aeruginosa*.

6. RECOMENDACIONES

Derivado de los resultados y hallazgos obtenidos de este proyecto de tesis doctoral, se emiten las siguientes recomendaciones, las cuales podrían dar oportunidad a desarrollar nuevos proyectos de investigación.

Evaluar la eficacia de los cinco bacteriófagos como una mezcla (cóctel) para determinar si se logra ampliar el rango de hospedero al lisar aquellas cepas de *P. aeruginosa* que no fueron lisadas por ningún fago de manera individual.

Evaluar la acción combinada de antibióticos con los fagos (terapia combinada) para demostrar si existe alguna sinergia en el uso de ambas terapias para eliminar de manera más eficiente a la bacteria.

Realizar ensayos *in vivo* en modelos animales, como el murino, que demuestren la seguridad del uso de estos fagos como fagoterapia antes de ser utilizados en pacientes.

Aislar una mayor cantidad de fagos para tener un banco de fagos con el que se puedan lisar la mayor cantidad de cepas distintas de *P. aeruginosa* MDR.

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