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Review

Encapsulated Phage Cocktail: The Destined Environmental Biocontrol Agent For Pathogenic *Leptospira*

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ABSTRACT

Leptospirosis is one of the often-neglected fatal zoonotic diseases endemic to most developing countries. The disease transmits mostly through contact of rodent urine contaminated with pathogenic *Leptospira* in the environment. This review discusses the need for environmental bioremediation of these pathogens and the reasons phage could very well be employed for that purpose. With a few modifications like encapsulation and cocktail formulation, the functionality and stability of phage as the natural predator could easily be heightened. Host specificity, ability to auto-dose and co-evolve along its hosts, effectiveness against biofilms and independence of its production are some of the promising features of a phage. Here we also highlight the interactions and interference among phages in a cocktail, transduction probability, and hypothetic usage of phage lysin in biocontrolling pathogenic *Leptospira*.

Key words: Bacteriophage, biocontrol, environmental bioremediation, *Leptospira*

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INTRODUCTION

Leptospirosis has been around for many centuries, especially in tropical countries. It is always overlooked for several reasons: the underdeveloped state of rural health departments in most tropical countries, the non-specific disease symptoms, and the confusing clinical diagnosis.

Rodents are the most common animal reservoir and vector of leptospirosis. These animals spread the pathogens in their urine via the environment, either among others of their own or to another species. With over 250 identified pathogenic *Leptospira* serovars, nearly all species of mammals could be infected, making eradication of the disease unlikely. Since the disease is bacterial, treatment if infected is with the use of antibiotics. This infection could damage the kidneys, liver, lungs, and brain and cause fatality if left untreated.

The only logical paths to prevention right now are through hygiene and environmental bioremediation. Bacteriophages have been one of the popular options for microbial bioremediation and biocontrol purposes since decades ago in mostly wastewater, food, agriculture, and aquaculture industries (Withey *et al*., 2005; Choińska-Pulit *et al*., 2015). Some studies also showed successful use of bacteriophages in biocontrol of pathogens like *Aeromonas* sp. in aquaculture (Le *et al*., 2018; Cao *et al*., 2020), *Salmonella* in food production (Wall *et al*., 2010; Islam *et al*., 2020; Li *et al*., 2020), and *Ralstonia solanacearum* in plantation (Fujiwara *et al*., 2011). Hence, phage-mediated biocontrol of pathogenic *Leptospira* sees a promising potential in decreasing the quantity of bacteria in the environment, thus reducing the probability of human contact with them.

The lethal pathogenic *Leptospira*

An estimated one million people worldwide are infected

by pathogenic *Leptospira* annually, along with 58,900 deaths due to severe leptospirosis (Costa *et al*., 2015). These figures might be just gross underestimations as most countries where leptospirosis is highly endemic are in developing states (Goarant, 2016), resulting in poor diagnostic capabilities and poor surveillance of the disease. Leptospirosis is a worldwide rodent-carried bacterial zoonosis affecting both humans and animals resulting in morbidity and mortality. Among all diseases that are transmitted by zoonotic agents, leptospirosis is one of the leading ones that contribute to the global disease burden, as it results in premature deaths (Torgerson *et al*., 2015).

The causative agent, pathogenic *Leptospira* comes from a genus of spirochaete bacteria, which are aerobic, right-handed helical shaped with distinctive hooked ends and endoflagella (Fang *et al*., 2023). A total of 20 species have been identified within the genus and divided into three clades: pathogens, intermediate, and saprophytes according to their 16S rRNA sequence: the "pathogens" clade comprises the 10 species responsible for severe diseases in humans including *Leptospira alexanderi, Leptospira alstoni, Leptospira borgpetersenii, Leptospira interrogans, Leptospira kirschneri, Leptospira kmetyi, Leptospira mayottensis, Leptospira noguchii, Leptospira santarosai,* and *Leptospira weilii* (Cordonin *et al*., 2020); five "intermediate" species that may cause opportunistic mild diseases in humans are categorized under the second clade; while the non-infectious environmental "saprophytes" clade comprises the remaining six environmental species which are known to consume decaying matter (Picardeau, 2012).

Direct or indirect exposure to pathogenic *Leptospira* leads to leptospirosis (Haake & Levett, 2015). Infection starts with the entry of pathogenic *Leptospira* into the bloodstream, followed by its exit from the circulation into tissues and its adhesion to exposed components of the extracellular matrix, and penetration of the endothelial cells that line the blood vessels (Picardeau, 2017). The disease usually has an incubation period of 1-2 weeks, but could be less than that or up to a month (Ko *et al*., 2009; Sivakumar & Pelly 2014). Within 4-20 days, immunologic reactions caused by the *Leptospira* antigens could be observed (Colvin *et al*., 2016). Some major effects of a person contracting the disease are mild fever, Weil's disease, and pulmonary hemorrhage syndrome, which are similar to those of malaria and dengue fever (McBride *et al*., 2005). The disease is complex and has several modes of transmission and nonspecific clinical manifestations (Mazhar *et al*., 2016).

Underlying threats in the environment

Human infection with the pathogen predominantly occurs via the contact of abraded skin or mucous membranes with water or moist soil that is contaminated with the urine of infected animals (Ko *et al*., 2009). In 1917, the role of rats as a source of human infection was first discovered (Monahan *et al*., 2009), and subsequently, some previous studies have demonstrated that other wild mammals including dogs, cows, goats, sheep, and turtles can also act as potential carriers or secondary hosts (Chadsuthi *et al*., 2017).

Although pathogenic *Leptospira* only multiplies inside the hosts (Mohammed *et al*., 2011), secretions of infected animals that flow into water bodies might be carrying the pathogen, where humans are infected following recreational or occupational exposure. Pathogenic *Leptospira* colonizes the proximal renal tubules of reservoir hosts and is excreted through urine into the external environment. Leptospirosis is then transmitted through the urine which is contagious as long as the surroundings are still moist (Guerra, 2009). The animals first contaminate mud or stagnant freshwater with the pathogens, which then would be washed into flowing water bodies with the help of rain and flood, in which they can survive in water and soil for more than 20 days up to several months (Trueba *et al*., 2004; Bierque *et al*., 2020; Davignon *et al*., 2023).

Leptospira survive best in fresh water, damp soil, and moist environments (Ridzlan *et al*., 2010). They could be in alkaline soil, mud, swamps, streams, and rivers, even tissues of dead animals (Mohammed *et al*., 2011). The pathogen favors surviving in higher temperatures and wetter seasons (Levett, 2001; Storck *et al*., 2008; Vanasco *et al*., 2008), while humans prefer outdoor activities in the natural environment during these times, thus making contact between *Leptospira* and us straightforward. Multiple developing areas, with poor sanitation practices and ineffective drainages, are prone to have a significant number of cases during severe weather events (World Health Organization., 2010). Living in densely populated areas (Levett, 2001), near water bodies and garbage accumulation contributes to the increased exposure of humans to the *Leptospira* species.

As a result of a current significant level of deforestation, increasing anthropogenic activities in the forested habitats and jungle, large and increasing population of reservoir animals, rainy seasons, and floods, humans are at a greater risk of being exposed to new serovars of *Leptospira* (Lau *et al*., 2018). In a sizeable handful of cases, flooding chiefly contributes to the expansion of leptospirosis. After the

2014 major flooding event in Kelantan, Malaysia, an epic outbreak with an incidence rate exceeding 100 per 100,000 population was recorded with an increment of total incidents from 29.0% cases occurred during preflood to 59.0% cases postflood (Firdaus *et al*., 2018). According to the study also, the incidence of leptospirosis increases 2–3 weeks after heavy rainfall and flooding, which confirms the incubation period of the disease upon exposure. An eight-fold increase in leptospirosis happenings was reported in Mumbai, India (Maskey *et al*., 2006) after a severely heavy rainfall. Other examples of the disease outbreak right after flooding events are in South America (Liverpool *et al*., 2008); New Caledonia (Goarant *et al*., 2009), Italy (Pellizzer *et al*., 2005), Indonesia (Victoriano *et al*., 2009); Lao People's Democratic Republic (Kawaguchi *et al*., 2008), and many more.

This constantly re-emerging zoonosis can have negative consequences for biodiversity, human and livestock health, animal welfare, and the economy of a country. Leptospirosis may occur anywhere worldwide but countries with humid tropical climates further encourage its happenings, due to the hot and wet conditions in favor of the bacterial lifespan (Levett, 2001). A report from Argentina reveals more than three-quarters of cases happened during seasons of high humidity and temperature (Vanasco *et al*., 2008).

The disease is hence considered endemic to several tropical countries like Malaysia (Thaya *et al*., 2013) for instance. Leptospirosis is well-known as the rat-urine-disease ["*penyakit kencing tikus*" in the national language] and nicknamed "the Great Mimicker" (Ministry of Health Malaysia, 2011), as the symptoms bear deceptive resemblances to those of other diseases, and thus was always overlooked, underdiagnosed or misdiagnosed with malaria, dengue or other illnesses. Other interchangeable names of the disease in Malaysia are "*demam pesawah*" [rice-field fever] and "*demam lumpur*" [mud fever], indicating leptospirosis often happens to those who have recently been to these areas. Surveillance to the Ministry of Health (Ministry of Health Malaysia, 2011), Malaysia recorded an exponential increase in the number of cases and deaths due to leptospirosis, from only 263 cases in year 2004 to 1,418 cases (2009), and 2,268 cases (2011) to 8,291 cases (2015). This translates to a 34 times increment within 11 years. Outbreaks of leptospirosis in Malaysia were also mostly related to contaminated water bodies. During the Eco-Challenge held in Sabah in the year 2000, 80 athletes representing 26 countries were suspected to have contracted the disease following exposure to contaminated river water (Garba *et al*., 2017).

The recurrence of leptospirosis cases in one place further signifies the importance of pathogenic *Leptospira* biocontrol in the environment. As a case in point, one month after an 18-year-old student from the Labuan Matriculation College, Malaysia died of suspected leptospirosis on October 29, 2014 (Bernama, 2014a), two new cases were reported again at that same institute (Bernama, 2014 b). In New York City, a cluster of severe leptospirosis cases (Rettner, 2017) had incessantly happened in a one-block section of a neighborhood over two months. The patients faced kidney and liver failure with one fatality. It is therefore simply cogent that bioremediation of environments should be considered where leptospirosis occurs in masses.

Current efforts and options of preventing leptospirosis and their complications

Right now, attempts to prevent the disease are by not contacting the potentially infected animals and contaminated water or soil (Zavitsanou *et al*., 2008). Protective equipment to prevent contact and general self-hygiene are encouraged. However, for these steps to be executed, people need serious awareness, which is unfortunately lacking where it is required most, like in the slums of the developing world. The use of rodenticides (Victoriano *et al*., 2009) is harmful to the environment and crop plants.

Surveillance of *Leptospira* is conducted either through direct detection of the bacteria via culturing, histopathology, and immunostaining of tissues or clinical specimens, and nucleic acid amplification tests (NAATs); or indirect methods by detecting leptospiral antibodies such as the microscopic agglutination test (MAT), the enzyme-linked immunosorbent assay (ELISA), and lateral flow methods (Sykes *et al*., 2022).

Vaccines for animals exist for certain types of *Leptospira* which may decrease the risk of spreading to humans (Slack, 2010), but unquestionably if were to be applied against wild rodents like rats is not within the bounds of possibility. To date, the only vaccines available for human use are limited and come with highly undesirable side effects (Azevedo *et al*., 2023).

The antibiotic doxycycline, when used in an effort as a chemoprophylaxis measure among travelers, is of unclear benefit (Slack, 2010). Usage of doxycycline is suggested for people who have a high risk of exposure as a short-term protection (Victoriano *et al*., 2009). As a case in point, doxycycline has been provided once a week as a prophylaxis to minimize infections during leptospirosis outbreaks in endemic regions (Pavli *et al*., 2008). Doxycycline was also used in reducing the number of leptospirosis

cases in military personnel undergoing exercises in the jungles (Pavli & Maltezou, 2008). However, this treatment is shown to not reduce the rate of infection (Bhardwaj *et al*., 2010), and there is no strong evidence that chemoprophylaxis is effective in containing outbreaks of leptospirosis (World Health Organisation, 2011). This is crucial as it is evidence of unnecessary use of the chemical which may increase the probability of its resistance.

Treatment, if infected, is also with antibiotics - ampicillin, ceftriaxone, doxycycline, and penicillin (Lim *et al*., 2011); but even with the drugs, Weil's disease and severe pulmonary hemorrhage syndrome result in death rates greater than 10% and 50%, respectively (McBride *et al*., 2005). Resistance to antibiotics by the pathogenic *Leptospira* is therefore a concern worth paying attention to. Poggi, De Giuseppe & Picardeau (Poggi *et al*., 2010) confirm the practicability of mutations in genes for the spontaneous resistance of *Leptospira* strains to streptomycin and spectinomycin in vitro. These mutated genes could then be replicated along with the bacterial division, or undergo genetic exchange among bacteria through conjugal transfer of plasmid DNA (Derbyshire & Grey, 2014), hence the spreading of antibiotic resistance. Therefore, genes responsible for the resistance will remain in existence following the bacteria that have since replicated, even without continuous exposure to the specific antibiotic.

There is a variety of chemicals that could be used to target pathogenic *Leptospira* because they are quite sensitive to acid and basic disinfectants (Faine *et al*., 1999; Richard *et al*., 2021), and so are quite easy to kill. However, *Leptospira interrogans* is shown to resist chlorination in tap water (Huang *et al*., 2014). The survival and virulence of pathogenic *Leptospira* spp. was estimated to be well preserved over 20 months even in waters with low temperatures and pH (Andre-Fontaine *et al*., 2015). There has yet to be any way that could effectively be used as a preventive step for the spreading of *Leptospira* in water bodies.

It is coherent that, since we do not have any other straightforward options, nor would be creating enough alertness for the vast majority of people all around the world shortly, phage-mediated biocontrol has to be one of the best solutions for reducing risks of human contact with water bodies contaminated with pathogenic *Leptospira*.

Phage-mediated biocontrol of pathogenic *Leptospira* in the environment

Bacteriophages, or phages in short, are natural intracellular obligate parasites to their host bacteria (Moye *et al*., 2018). They reproduce by letting the hosts transcribe and translate their genome, resulting in the formation of their progenies. Phages can be isolated from almost everywhere where bacteria exist (Weber-Dąbrowska *et al*., 2016), from wastewater to the human digestive system. They play an important role in mediating the cycle of nutrients and energy in an environmental ecosystem through bacterial lysis (Krishnan *et al*., 2017).

Phages were discovered by the French microbiologist Felix d'Herelle in 1917 (Keen, 2012). The unknown bacteria-killing agents were reported by many scientists before him, but it was d'Herelle who recognized their viral nature and coined the name "bacteriophages". He later distinguished the phages targeting a series of pathogenic bacteria and suggested using them therapeutically for prevention and treatment of infectious diseases.

Two categories namely lytic and temperate phages can be differentiated by their contrasting approaches in infecting their hosts: a lytic phage initiates immediate transcription machinery to produce progenies upon infection, and the viruses lyse the host cell once ready to be released, causing a bactericidal effect. On the other hand, a temperate phage stays latent inside its hosts, only to enter the lytic phase in the presence of environmental stresses (Jankauskaite *et al*., 2018).

As owls were used to hunt down rats, phages could be used to kill pathogenic bacteria. Exploitation of the valuable ability of lytic phages to lyse their hosts can be seen in various applications including treatment of multi-drug resistant pathogenic bacteria infections (Jamil *et al*., 2018), food contamination, and environmental bioremediation or biotreatment (Jankauskaite *et al*., 2018). With the added benefit of preventing the elimination of beneficial bacteria (Lin *et al*., 2017), the application of phage for treatment and preventing a series of bacterial infections is highly suggested. They are natural antibacterial agents safe for humans and animals (Sillankorva *et al*., 2012).

The other favorable condition is the effectiveness of a phage's host lysing mechanism. Rather than stopping bacteria from doing one specific process, a phage actively lyses the bacteria by puncturing holes inside out on the cell wall and cell membrane using lytic proteins (Salmond & Fineran, 2015). As most bacterial cells are protected by a cell wall of polysaccharides - one of the vital virulence factors for immune-evasion and protection against antibiotics (Drulis-Kawa *et al*., 2015), the ability of phage to lyse the wall is of benefit.

Current findings of phage targeting *Leptospira*

To the best of our knowledge, the only phages against the genus *Leptospira* [leptophage] that have been isolated, purified, and phenotypically characterized are vB_LbiM_LE1 [renamed6, and abbreviated LE1] (Girons *et al*., 1990; Kameni *et al*., 2002), vB_LbiM_LE3 [LE3], and vB_LbiM_LE4 [LE4] (Schiettekatte *et al*., 2018). These phages have been isolated from urban sewage and infect the saprophyte *L. biflexa*. To date, there is not yet any report on the discovery of lytic phage with the potential to lyse a *Leptospira* from the "pathogens" clade.

Yin and yang between a lytic phage and its host *Leptospira* in the environment

The concept of yin and yang dualism best describes the relationship between a parasitic phage and its bacterial host. In a normal environment where the quantity of pathogenic *Leptospira* is low, the number of phages should also be low due to insufficient host availability. However with the increase of *Leptospira* –carrying rodents in a certain area, the number of phages should also be manually heightened to neutralize the environmental influx of these pathogens. These two seemingly contrary organisms are interrelated where the survival of one would be influenced by that of the other for a balanced ecosystem. For instance, the presence of *Leptospira* could increase the number of its phage, which will in turn be reduced along with its vanishing host.

By discovering and characterizing a phage with a small host range that can lyse pathogenic *Leptospira*, the use of phage could be focused on the prevention of pathogens from spreading in the environment. Outside a host animal, it seems that these bacteria have a near nil reproductive output – although in a few cases, limited binary fission has been observed (Ritchie & Ellinghausen 1965), probably brought about by the long-term absence of any host species, but in a general sense the *Leptospira* do not multiply. Nevertheless, with sustainable conditions, the pathogens can still last for a prolonged timespan (Levett, 2001), resulting in a slow decrease in their number, even though they may not be propagating. Trueba *et al*. (2004) suggested that *Leptospira* can form biofilms to facilitate their persistence in the environment. In an environment where carrier hosts regularly add fresh urine, the pathogenic colonies can maintain an almost continuous presence.

Prominent characteristics of an obligate lytic phage suggesting its potential for *IN SITU* biocontrol application

Every aspect of a lytic phage is designed for efficient infection and killing of its host, in the meantime reproducing its progenies.

Attachment mechanism and host specificity

Having recognized its host bacterium, a lytic phage fixes to its surface, penetrates the cell wall, and injects the genetic material inside. Attachment to selective receptors on the surface of bacteria, including pili, flagella, lipopolysaccharides, proteins, or teichoic acids in gram-positive bacteria is required for phages to enter these cells (Lindberg, 1973; Hyman, 2022). This specificity of adsorption means a phage can only interact with certain microbial species or strain-bearing receptors to which it can bind, hence the phage's narrow host range.

This explains the motivation behind phage therapeutic application which is host-specificity (Duckworth & Gulig, 2002). They are selective hunters fixed to attacking only a particular species of bacteria and are therefore not likely to disturb the other beneficial microorganisms naturally present in the environment. Even the human body is populated by over a thousand species of essential microbes, including those helping us make nutrients we otherwise would not have (Morowitz *et al*., 2011).

Nevertheless, the query of whether a sudden manual influx of phages would adversely disrupt a preciously stable microbial community is worth being contemplated. This could however be well managed by using only host-specific phages, as production of progenies and reinfection only happen when that particular host is available. Experiments on the determination of a phage's host range should be conducted not only by targeting bacteria of the same species or genus but also those ubiquitous in the areas where the biocontrol was to be carried out. Broad spectrum phages, as reported by some studies (Ross *et al*., 2016; Tang *et al*., 2019; Li *et al*., 2020), should be scrutinized before application, especially in the environment as there is yet to be any method of selection eliminating viruses from the nature.

Production of progenies – phage's ultimate trump card in environmental biocontrol application

After its attachment and secretion of enzymes responsible for degrading the host's outer layers,

penetration of the phage's genetic material through its host initiates the infection process. The genome of the phage is then translocated into the host's, followed by the ceasing of bacterium products synthesis, and the commencement of phage components production (Sharma, 2013).

This feature of self-replication provides a phage with an advantage over other possible biocontrol agents. In the environment where water flows and flooding happens, a chemical would be diluted and never enough to serve its purpose of killing the host. This is where resistance easily happens as under dosage could not be prevented. For instance, it is not difficult to make microbes resistant to antibiotics in the laboratory by exposing them to concentrations not sufficient to kill them (David *et al*., 2008). The danger of using chemicals in the environment could be seen when fish farmers provide antibiotics as part of the fish food (Rodgers & Furones, 2009). The unconsumed food and fish feces will settle to the bottom of the riverbed with the antimicrobial substances dissolved into the water. This could alter the composition of the microbiota (Ding & He, 2010) by killing those susceptible and allowing the enhanced growth of those resistant.

With this, the hypothesis is that a phage with a larger burst size makes a better biocontrol agent. The higher the number of progenies reproduced within a shorter production time, the higher the phage's efficacy (Drulis-Kawa *et al*., 2012), as the chance for reinfection is heightened.

Host-killing and continuous reinfection

By way of self-assembly, mature phage particles are formed inside the bacterium. Due to the action of the phage enzymes and the growing intracellular pressure, the cell wall bursts open and the new virions get into the surrounding medium (Sharma, 2013). Released phage progenies will penetrate new uninfected hosts and the whole cycle is repeated until all the bacteria sensitive to this phage are reinfected and lysed.

This reproductive mechanism is especially useful as phage-mediated environmental remediation is dependent on a phage's fast self-amplification on site. Phage quantity will be easily diluted in situ, due to weather changes like sudden raining. The self-replicative activity of a phage bluntly translates into its auto-dosing capability during the biocontrol of bacteria. Environmental therapy is considered an active treatment as it requires active phage replication to achieve the desired outcome. The comparison between active and inundative treatments is discussed in (Abedon, 2019).

There is strength in numbers

Phages are estimated to outnumber bacteria by ten-fold in the environment (Brüssow & Hendrix, 2002). Isolating phages infecting the same host strain should be far from laborious even from one environment sample, as local phage diversity is high in many instances (Breitbart & Rohwer, 2005). A study by Flores *et al*. (2011) made it intelligible that isolation of phages infective to bacteria from different geographical locations is possible. Furthermore, it is likely that *Leptospira* be targeted by an unknown diversity of genetically distinct phages (Schiettekatte *et al*., 2018). Hence, the concern for pathogens resisting phages is excessive, as we could always find several different ones targeting the same host easily. Furthermore, using multiple phages targeting the same pathogen in a cocktail provides a built-in contingency against the development of phage resistance in bacteria (Potera, 2013).

Effectiveness against biofilms – tearing down a pathogen's shield against antibiotics

Pathogenic *Leptospira* were shown to develop biofilm (Ristow *et al*., 2008; Kumar *et al*., 2016) – a thick layer of viscous materials that protect them from antibiotic treatment (Harper *et al*., 2014). Biofilms are clusters of cells that are entrapped by an extracellular polymeric substance [EPS] matrix layer produced by the bacteria (Flemming & Wingender, 2010). Many phages are equipped with tools that can digest this common form of bacterial growth, which antibiotics and biocides can be essentially ineffective against. Studies have shown that genes encoding enzymes for breaking down the matrix were found in multiple phage genomes (Leiman, 2004; Yan *et al*., 2014). Experiments using phages to treat biofilm layer were conducted on various surfaces such as stainless steel (Ganegama Arachchi *et al*., 2013), polystyrene (Kelly *et al*., 2012), glass (Siringan *et al*., 2011), and ceramic tiles (Viazis *et al*., 2011). Phage's possible role in the reduction of biofilm and public good production is hence bigly recognized – as shown by the phage engineered to express a biofilm-degrading enzyme (Lu & Collins, 2007). In the experiment, phage $T7_{control}$ was genetically incorporated with gene dspB for the production of a polysaccharide depolymerase cloned from *Actinobacillus actinomycetemcomitans*. The phage $T7_{DspB}$ showed heightened effectiveness in biofilm-removing and reducing the bacteria within.

Independence of phage production

Moreover, in contrast to antibiotics that were mostly biosynthetically produced, a phage is not dependent on an organism for production; phages can be found wherever their hosts are available. The Discovery of new antibiotics requires new culturable species to be grown for the detection of antimicrobial activity, which in turn necessitates a suitable condition for the production of the chemical to reach a certain threshold. In other words, there is no warranty for any new antibiotic to be uncovered soon, as it is fully luck-dependent. The shortage of new-class antimicrobials itself (AlRuthia *et al*., 2017), despite the amount of work put into its discovery, clearly validates the dilemma faced by researchers. Be that as it may, given the limited options of alternative therapies faced by clinicians, our priority should be the judicious use of phages.

Ability to coevolve along its host – overcoming phage resistance

The foremost issue on using phage that many have been struggling with is the likelihood that the evolution of phage resistance echoes the spread of antibiotic resistance. Kunin *et al*. (Kunin *et al*., 2008) described the adaptation of bacteria in the environment towards phage predation pressure locally. If resistance towards phage emerges in a short time at a great rate, phage application in the environment seems redundant.

Hampered phage adsorption

Phage resistance mechanisms could vary among bacteria. Every step required for a successful phage infection could be targeted. Liu *et al*. (Liu *et al*., 2002) show a significant decrease by a factor of 10^6 in phage BPP-1 efficiency of forming plaques on Bvg-phase *Bordetella* sp. cells. This is due to the masking of cell receptor pertactin autotransporter [PRN] by the bacteria during the Bvg- stage, which is required for phage adhesion (Ge *et al*., 2020).

However, almost all virulence factors [e.g., adhesins and toxins] of *Bordetella pertussis* are activated by the BvgAS two-component system. In the course of Bvg+ mode, BvgS is active and BvgA is phosphorylated, and the bacterial virulence-activated genes are induced. Expression of virulence-repressed genes happens throughout Bvg- mode (Moon *et al*., 2017).

The ability of a phage to antagonistically coevolve with its host can be seen from its faculty for tropism switching (Liu *et al*., 2002). In the study, one of the two identified tropic variants switched tropism to favor the Bvg- phase host while the other indiscriminately lyse both Bvg+ and Bvg- phase strains. This remarkably demonstrates the competence of a phage in adapting to its host receptor alterations.

CRISPR-Cas Immune system

The popular antiphase mechanism by the host, CRISPR-Cas immune system is first reported by (Barrangou *et al*., 2007). Serving some bacteria as guards that cleave phage DNA entering the cell, the system consists of short palindromic DNA repeats interspaced by non-repetitive ones which are adapted from the phage DNA. Biogenesis of cr-RNAs, together with Cas proteins, form CRISPR-Cas complexes; these complexes interfere with and cleave matching foreign phage DNA sequences in the cell upon recognition (Hille & Charpentier, 2016). This stops phage replication by disabling the translocation of its DNA into the host's.

Several studies (Fouts *et al*., 2016; Beriwal *et al*., 2018; Fernandes *et al*., 2019; Xiao *et al*., 2019) reveal the findings of the CRISPR-Cas system in *Leptospira,* especially the pathogenic ones These further raised concern over the emergence of phage-insensitive strains when environmental pathogens are challenged. However, some phages are blessed with the ability to evade this host immune system. An example is provided by (Mendoza *et al*., 2020), where phage ΦKZ can evade the host's CRISPR destruction mechanism by encoding protective protein barriers. The building of a protective proteinaceous compartment around the phage DNA kept Cas proteins and endonucleases of *Pseudomonas aeruginosa* at bay. The study also suggests that ΦKZ developed immunity to the host's phage-resisting system in nature. Several other counteracting evasion strategies of phages are an encoding of phage's own CRISPR-Cas system (Seed *et al*., 2013), possession of anti-CRISPR/Cas genes (Bondy-Denomy *et al*., 2013), and nucleotide mutation (Deveau *et al*., 2008).

Phage resistance in exchange for virulence

About phage resistance also, several studies show the correlation between the virulence of a bacterium and its ability to not be affected by a phage. This is due to the bacteria cell receptors targeted by phages are responsible for their virulence factors, and in return for phage resistance, alteration of

the receptors is necessary, which directly causes an attenuation of virulence (Inal, 2003). The induction of virulence-repressed genes during the Bvg- stage albeit prevention of phage adsorption by *Bordetella pertussis* as discussed earlier is a case in point. A study shows phage-resistant *Yersinia pestis* bacteria strains have either a notable increase in the time taken to kill the hosts or an absolute attenuation of their virulence (Filippov *et al*., 2011). Another research confirms the happening of phage-driven complete loss of virulence in a fish pathogenic bacterium (*Flavobacterium columnare*), resulting in incapability to affect the host survival (Laanto *et al*., 2012). In addition, with the acquisition of phage resistance comes the depletion of bacterial metabolic fitness (Gómez & Buckling, 2011; Koskella *et al*., 2012). These astonishing studies proofs phage resistance can be costly, in a good way as the pathogens could either be lysed or loss their virulence in exchange for survival.

The Red Queen's hypothesis

Nevertheless, in the natural environment, phages and their respective host bacteria never outrun each other in the evolutionary arm's race, as they are both desperately adapting for survival. Immunity of a host towards phage infection brings about an evolution of the phage to overcome that resistance, which in turn leads to a counter-mutation by the host for continual survival (Buckling & Rainey, 2002; Stern & Sorek, 2011). To maximally minimize phage resistance, many researchers have suggested using a cocktail of multiple different phage species targetting a similar host at once.

Phage cocktail

Also goes by the name of multipage (Hall *et al*., 2012) and polyphage therapy (Chan & Abedon, 2012), the use of several phages targeting either one specific strain of pathogen or a few host species at once could bring various advantages if applied with discretion.

In a study, the bactericidal efficacy between sequential and simultaneous applications of different phage types is compared (Hall *et al*., 2012). The researchers first suggested there might be pathogens that could evolve and resist multiple phages simultaneously. In that case, sequential continuous exposure of the bacteria to new phages could lengthen the time taken for resistance occurrence, which coequals a more effective phage treatment. The results however concluded the better strategy is to apply all given phages in a combination.

Phage cocktail combating cross-resisting mutants

The use of several phages targeting different receptors on the hosts' surfaces could heighten the effectiveness of a phage cocktail. This could lessen the emergence of cross-resistance by the host towards multiple phages, as phage resistance mostly starts from masking or loss of certain bacterial surface receptors responsible for phage binding (Labrie *et al*., 2010). Phages in a cocktail might have different infection mechanisms, but the need to bind to similar receptors may render them useless. Ong *et al*. (Ong *et al*., 2020) show a cocktail of two phages provides longer suppression of the growth of *P. aeruginosa* compared to single phage treatments because these phages recognize different unique components in host lipopolysaccharide receptors for successful attachment. Hence the absence of cross-resistant strains, apart from one which was observed only after the 5th round of co-culture.

Besides, the multi-phage-resistant host might pay a higher fitness cost compared to their phage-susceptible counterparts (Torres-Barceló & Hochberg, 2016). These receptors (examples previously mentioned), besides binding phages, could be the virulence-determining factors of the bacteria. Attenuation of the pathogen could be the result of those receptor's alteration.

Identification of host receptors responsible for phage attachment could be done using a molecular approach by creating and testing the phages' ability to infect mutated hosts with defects on different parts of LPS, as proposed by (Filippov *et al*., 2011). In the study, they determined different receptors for the phages, postulating that the formulation of a cocktail using these phages could be favorable. Again, truncation of the pathogen's virulence is proven, resulting in a 50% increase in the lethal dose and longer survival of hosts (mice).

Interaction among phages in a cocktail

Thorough research should be done to determine the functionality of a phage cocktail. There might be interference phenomena among the viruses, due to host competition and other reasons (Hall *et al*., 2012). Those with a higher one-step growth rate (time taken for one infection cycle) could produce more progenies within a shorter time frame, thus competing with hosts from other phages. This potentially selects the growth of resisting mutants, eventually resulting in a pausing state on that particular phage's

replication, followed by a sudden increment of the others, if cross-resistance did not happen. In due course, we might as well use consecutive single phage treatments instead of providing them all at once. The interaction and its effects would differ among phage types and are yet to be well understood, hence the need for more advanced and detailed experiments before the application of new phages.

A phage's sex life

The phage's general reproduction cycle is considered asexual. From host infection to replication of progenies, there is no room for genetic exchange or recombination between two parental phages to happen. Hence, deleterious alterations in the genomic nucleotide sequence can easily happen between each generation, gradually but eventually resulting in irreversible accumulation of these mutations among progenies. This phenomenon is well explained metaphorically by Hermann Joseph Muller as an "irreversible ratchet mechanism" that occurs in non-recombining species (Muller, 1964). The existence of Muller's ratchet in viruses is proven in several laboratories (Chao *et al*., 1992; Clarke *et al*., 1993; Dobbins *et al*., 2004).

In due course, as reverse mutations hardly happen, Muller's ratchet will bring about a mutational meltdown (Gabriel *et al*., 1993), where dysfunctional individuals are produced ultimately effectuating a reduced phage population size or extinction. For phages to withstand this successive mutational assault without continuously loosing their Darwinian fitness, genetic exchange occurs now and then. The damaging impact of deleterious mutation accumulation may be less common even among these asexual phages, as they benefit from genetic recombination. When two phages co-infect a bacterium, an exchange of genes can occur among the parental phages, giving birth to new phage types with varied genomes. This resembles sex between two organisms, or even polygamy when more phages are involved. The progenies may inherit genetic materials from both or multiple phages, allowing a 50% or lesser chance for their genomes to receive the parental mutations (purifying selection).

Although sex may be naturally favored for the above reasons, genetic variability among phages is not generally preferred in phage therapeutic applications. The sole purpose of using phage here is to decrease bacterial count, while itself reduced too due to dependence on the host for survival. The mechanisms of viral recombination, independent assortment, and incomplete linkage, are detailed in (Fleischman, 1996). Both mechanisms result in new viruses with different characteristics. Therefore, it is of high priority to determine the process and rate of genetic recombination in chosen phages before use.

The selfish phage

Another conundrum faced by scientists is the occurrence of a "Prisoner's dilemma" during phage coinfection. Prisoner's dilemma is a metaphorical behavioral theory, where a law prosecutor, who lacks sufficient evidence for a full conviction (2 years imprisonment) of two individuals, gives each prisoner a choice to either betray (convict) the other and be freed or remain silent and serve a shorter period (1 year) behind bars. As both prisoners are in solitary confinement, the only rational decision is to betray the other, due to both self-interest and distrust.

The same situation happens in an environment where a host could be co-infected by multiple different viruses, as some of them may take advantage by using more and producing fewer proteins essential for a complete infection cycle. Several studies looked into this type of competition-adapting phage, named the "cheater phage" (Dennehy & Turner, 2004) or defective interfering (DI) particle (Turner *et al*., 1999). They mutate to lose the required genes such as protein-coding regions, but hold on to the sequences recognized by host replication machinery, spontaneously or not. These defective viruses therefore replicate at the expense of other normal ones (Stauffer Thompson *et al*., 2009).

One of the pioneering observations of cheater phages was during the characterization of filamentous M13 phages that target *Escherichia coli*. It was observed that truncated virions, containing circular single-stranded DNA less than half the size of standard M13 readily present in 20% of a serially sub-cultured stock (Griffith & Kornberg, 1974). These particles, with only their structural genes, such as those responsible for replication and packaging, cheat obligately during ordinary M13 coinfection and acquire capsid proteins produced by the full-length M13 (Secor & Dandekar, 2020).

This phenomenon is mostly due to the sudden increase in intracellular competition during co-infection (Turner *et al*., 1999), which indirectly indicates a multiplicity of infection too high might do more harm than good, as it induces host competition among phages. Cheater phages are detrimental to phage cocktails, as they limit the production of other phage's progenies, hence splitting the supposedly collaborated host-lysing effect. As expected, some viruses evolved counter strategies to curb co-infection (superinfection exclusion), which should prevent another phage from cheating, as seen in

bacteriophage φ6 (Dennehy & Turner, 2004). Nevertheless, combinations of phages that do not interact antagonistically should minimize undesired disadvantages in a phage-mediated bioremediation.

Selection of phages for cocktail-making

Besides all the favorable characteristics described previously, these are two basic preferences when choosing phages for therapeutic applications. When hosts are abundant, larger burst sizes, shorter eclipse (the time required for synthesizing phage proteins & genome), and latent (the time between phage attachment & bacterial lysis) periods all increase absolute virion production. In (Figure 1), hypothetical one-step growth curves of phages A, B, and C were illustrated using plaque assays to estimate the number of phages in the solution. Comparison between phages A and B shows the only difference is the number of progenies produced at the end (burst size). Logically, phages with larger burst sizes reproduce more progenies after a single infection, directly auto-dosing the quantity of phage in the surroundings, promoting further reinfection. Phage C, despite having a similar burst size as B, requires a shorter latent period, hence a faster infection activity. In a surrounding where environmental factors like weather and temperature are constantly changing, timing is of utmost importance, thus phage C possesses the best characteristic for this biocontrol application. Therefore, isolation of bacteriophages should be conducted from different sources and compared among themselves to increase the chance of finding those that best fit the descriptions of phage C.

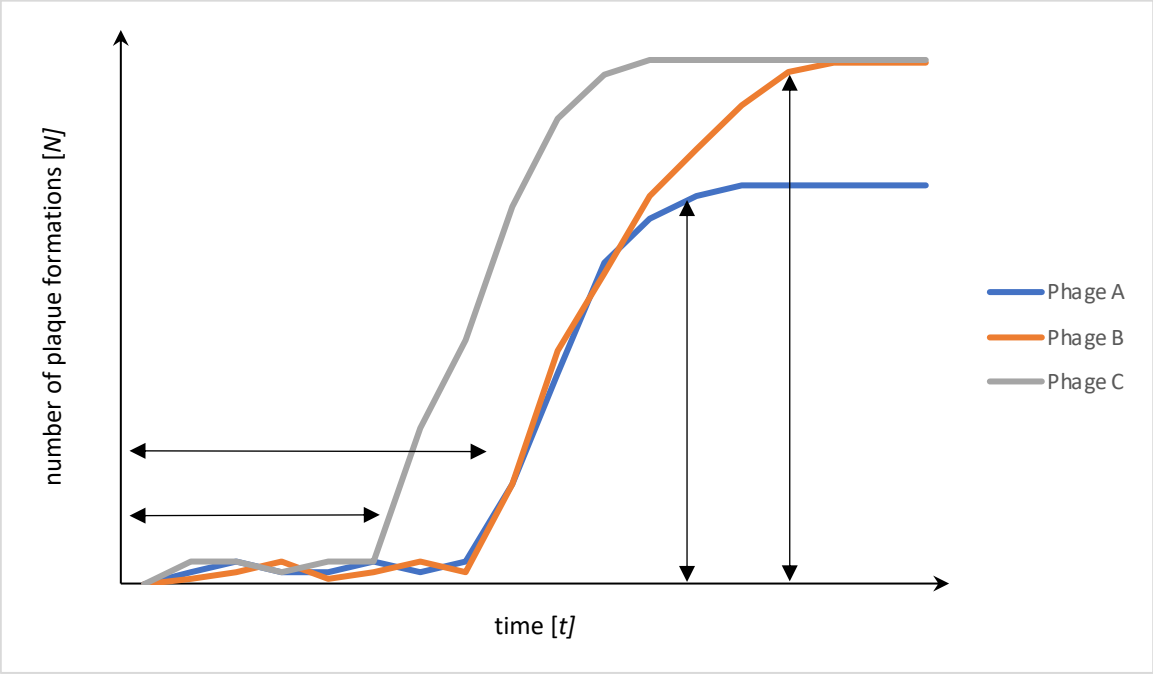

Fig. 1. Hypothetical one-step growth curves of phages A, B, and C.

Phage encapsulation for stability

A noteworthy study on the influence of environmental conditions on phages describes a result of varied stability, survival rates, and host-infectivity of different phages (Kim, 2007). For environmental antimicrobial purposes, an agent has to minimize the hosts' ability to develop resistance and reach its host targets in a sufficient load exceeding the minimum inhibitory concentration (Abedon, 2011). While the formulation of phage cocktails could enhance the former ability, encapsulation possibly helps prolong their persistence in the environment, which is of utmost importance to prevent them from being rendered non-viable before encountering their host.

The quandary of phage-mediated environmental biocontrol of pathogens is whether the agent could withstand harsh surroundings, such as high temperatures above 50°C (Gabiatti *et al*., 2018), fluctuating pH and chemicals, even metal ions like copper ions with concentration exceeding 300 mg/L (Li & Dennehy, 2011). In addition, the phages also face the challenge of being diluted. Insufficient density might indirectly impair phage activity. For a cost-effective application, phage lifespan in storage should also be prolonged. Current available phage-preserving techniques are not promising in terms of phage stability and viability. With that, the proposal of a protective 'shield' for the phage is fairly appealing.

As environmental biocontrol of pathogenic *Leptospira* requires the phage to be used in damp soil or stagnant and flowing water, encapsulation could provide phage protection from undesirable conditions, immobilization of phage preventing dilution, and a longer existence both while in use and storage. The capsule should be made up of non-toxic substances and harmless to the environment. It should also not negatively impact the phage activity.

The application of encapsulated phage in livestock farming is proven by (Wall *et al*., 2010) where multiple phages were microencapsulated together in biodegradable alginate microspheres and administered orally to pigs preinoculated with *Salmonella enterica*. Significant reductions in the pathogen counts in both the cecal and ileal samples of the pigs are observed post-treatment. Application of the alginate/CaCO3 encapsulation method to protect phages targeting *Salmonella* and *Staphylococcus* in the acidic gastrointestinal tract has also been tried out (Colom *et al*., 2017). Other well-orchestrated studies provide examples of utilizing different materials for phage encapsulation: chitosan-alginate beads (Abdelsattar *et al*., 2019), liposomes (Cinquerrui *et al*., 2018), and electrospun biopolymers (Korehei & Kadla, 2014) to name a few.

Concerns regarding phage transduction

The horizontal transfer of genes which encourages the evolution of bacteria (Arber, 2014) is facilitated through transduction. The random selection of genes when transduction occurs made the spread of antibiotic resistance genes and virulence factors possible, as Faruque & Mekalanos (Faruque & Mekalanos, 2012) show the participation of phages in horizontal gene transfer of *Vibrio cholerae*. This issue has raised the question of whether the application of phage-mediated biocontrol in the environment is suitable (Guenther *et al*., 2012).

Nonetheless, the probability of a bacterial gene carried by a phage is minimal. This could be calculated by multiplying the frequency of transduction particles occurring with the fraction size of the bacterial genome that fits within the phage particle. For example, the genome of lytic phage targeting *Leptospira biflexa*, phage LE4 composed of 47,866 base pairs (bp) of double-stranded DNA (Schiettekatte *et al*., 2018). The genome of *Leptospira biflexa* serovar Patoc strain Patoc1 (Ames strain) consists of three replicons with a total of 3,956,086 bp (Picardeau *et al*., 2008). If LE4 could infect and lyse this particular strain of bacteria, the probability of transduction could be calculated as follows:

Phage LE4, genome size = 47,866 bp

So, transduction element size = 47,866 bp

If host genome size = 3,956,086 bp, the size of a host DNA fragment that could be packaged into a phage's head is 47,866/3,956,086 × 100% = 1.2% of the host genome.

Using methods as suggested by (Raya *et al*., 1989) with necessary modifications we could identify the transduction frequency of a lytic phage. Assuming the frequency of transduction happening is 1 × 10^-5, by multiplying it by 1.2%, the probability of a bacterial gene being carried in the phage is just 1.2 × 10^-7. For a specific gene (e.g. resistance gene) to be carried, the chance is even more minute. Supposing the size of the gene makes up 1/1000 of the bacterial genome, the probability of transducing it is only 1.2 × 10^-10.

Moreover, as discussed before, the phages used should be host-specific. If there happens to be a faulty progeny that carried out transduction, it has to be of the same strain as the host, which could easily be lysed on the next phage infection.

Hybrid phages engineered for the transduction of antibiotic-sensitive genes

Phage transduction is shown to potentially be used for the transfer of DNA encoding antibiotic-sensitive genes into selected resistant bacteria (Edgar *et al*., 2011; Yosef *et al*., 2015). These astonishing studies aim to reverse the common perspective of labeling phage transduction of DNA as a risk in phage therapies. Instead of traditional lytic phage therapy, these studies suggest using temperate and transducing ones for prophylactic treatments against drug-resistant bacteria. By broadening the phage host range, for example by exchanging tail fibers (Ando *et al*., 2015) or creating mutant phages that propagate in targeted hosts, researchers use the transducing mechanism of phages to inject DNAs into bacterial genomes. Plasmids encoding tail genes of different phages, desired genes, and phage-packaging genes were designed and encapsulated into transduction-effective phages, making them hybrid particles (Yosef *et al*., 2017).

These theories indirectly explained the possibility of exploiting phage transduction for therapeutic purposes, but on the other hand, are however still in their early stages. Lytic phages with a narrow host range are preferable for phage-mediated environmental bioremediation up to the present time.

Suggestions regarding phage lysin

As previously described, after replication and formation of lytic phage progenies, the bacterial host has to burst open for the release of these new phages. With exceptions of some RNA and small DNA phages that may produce proteins that interfere with peptidoglycan biosynthesis (Fischetti, 2008), for most double-stranded DNA phages dissemination of progenies could be resolved by the release of lysin, a type of peptidoglycan hydrolase (Gilmer *et al*., 2013). As the bacterial high internal pressure is maintained by a highly cross-linked cell wall, peptidoglycan digestion by the lytic enzymes results in disruption of the wall's integrity and hypotonic lysis of the cell (Fischetti, 2008).

An attractive advantage of using lysin instead of phage itself is it avoids the unpredictability of phage infection kinetics. The benefit of using phage, which is its replication only at the expense of bacterial survival (Abedon, 2009), might present a weakness. The phage could reproduce continuously whenever and wherever the host is available, in this case complicates the calculation of the proper dosage required. Thus, the importance of minimum inhibitory concentration (MIC) (Vipra *et al*., 2013) is apparent for the application of an effective phage titer, at the same time making sure the amount introduced is not immoderate, as to steer clear of potential undesired effects.

Nevertheless, just like antibiotics were produced by fungi, phage lysin is just another chemical product, which resistance might easily occur as it is unable to counter-evolve along the host. As opposed to the idea suggesting the occurrence of lysin-resistant bacteria is unlikely (Fenton *et al*., 2010), phages were the ones that evolved naturally with their respective hosts, not their enzymes. All in all, prudentialism is key and more findings were to be reported before their application.

CONCLUSION

Humans have been using antibiotics and other chemical antimicrobials to combat pathogenic bacteria for decades. They are convenient, speed-effective, and low in production cost. Nevertheless, with chemical use being out of the question regarding environmental bioremediation of pathogens, it is time to turn to the natural prey-predator system between phages and bacterial hosts. Phages have high variability (Weinheimer *et al*., 2023) and are ubiquitous and abundant in nature, ranging from 10^4 to 10^8 particles per milliliter aquatic environments (Paul & Kellog, 2000), and 10^{8} to 10^{9} per milliliter sewage waters, ranging from (Ballesté *et al*., 2022). With current technology and knowledge, we could easily characterize and exploit the bactericidal ability of phages for the well-being of humans. For example, metagenomics has made possible the in-water detection of previously unidentified phages (Breitbart *et al*., 2002). Besides, the ability to reproduce only in the presence of targeted hosts brings about both the advantages of self-dosing and minimal environmental negative impacts. Nevertheless, only suitable phages, such as those with species-specific host ranges that infect pathogenic *Leptospira*, should be used. Besides, obvious weaknesses in the environmental application of bacteriophages like physical sensitivity and the difference in flow rate of the waterbodies need to be addressed. Novel encapsulation methods that could enhance the delivery, for example, through the periodic release of these viruses into the contaminated environment should be created. Pathogenic *Leptospira* bestowed diseases and deaths to innocent ones, hence the utilization of the pathogen's predator to kill them is in all ways ideal. There are of course risks in every attempt to introduce new treatments but with more research and understanding, the application of phages could very well bring betterment to our current world.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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