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URINARY TRACT INFECTIONS AS AN URGENT NEED FOR THE CLINICAL DEPLOYMENT OF PHAGE THERAPY

A REVIEW

MARTHA REBECCA JANE CLOKIE[1],
THOMAS ERIK SICHERITZ-PONTÉN[2,3], FRANCESCA HODGES[4],
ANISHA THANKI[1], MARIE NOELLE VIEU [1],PAUL ROBSON[1],
ANDY MILLARD[1] JILL THEAKER[1] and MELISSA HAINES[1]

[1]Department of Genetics and Genome Biology, College of Life Sciences,
University of Leicester, Leicester LE1 7RH, UK
[2]Globe Institute, University of Copenhagen, 1165 Copenhagen, Denmark
[3]Center of Excellence for Omics-Driven Computational Biodiscovery (COMBio),
AIMST University, Bedong 08100, Kedah, Malaysia
[4]Innovate UK KTN, Suite 218, Business Design Centre, 52 Upper St.,
London N1 0QH, UK

SUMMARY

Bacteriophages (phages) have a long history of use in Eastern Europe and are poised for wider exploitation as novel antimicrobials in the context of antimicrobial resistance. Integrating phages into mainstream medicine requires an in-depth understanding of phages and of regulatory, economic and practical frameworks. Here we summarise insights from a UK perspective into therapeutic phage development and detail our progress towards being able to use phages for UTIs.

Phages are of interest as new medicines to target bacterial infections that are currently difficult to treat with the available therapies, and protect the medicine that protect us, by preventing the use of last line antibiotics. A pressing need has arisen for phage products to be able to treat urinary tract infections (UTIs) caused by *E. coli* and *Klebsiella pneumoniae*. Clinical trials data are needed to ensure the safety, efficacy and clinical benefits of phage treatment according to modern criteria, motivate interest from clinicians and investment from the pharmaceutical industry and thus widen access to phages. We therefore aim to conduct a human clinical trial in participants with recurrent UTIs.

We have established a UTI phage cocktail (combination) that we are optimising through a robust analysis of the phage genomes and phenotypes. On the genome front, we implement our graph-based framework to probe the genetic relationships between phages in the absence of a common marker. We describe here our repurposed ecological framework where we contextualise phage traits such as functionality in relevant physiological conditions. Ultimately, we hope to combine these approaches and correlate phage traits with therapeutic efficacy and more easily predict which phages should be developed as treatments.

Human trials can be informed by data from large scale animal trials and we show how our recent work on swine and poultry pathogens informs phage

dosage, *in vivo* dynamics and bacterial resistance. In parallel, because we will deliver phages directly to the bladder, we are currently using a murine model to collect the necessary safety and efficacy data needed for regulatory purposes.

INTRODUCTION

Antimicrobial resistance and phages

Antimicrobial resistance (AMR) is a major global problem with 2 million deaths attributed to an AMR infection in 2019 (*Antimicrobial Resistance Collaborators*, 2022) and 10 million deaths/year predicted to be caused by antibiotic resistant infections by 2050 (*O'Neill*, 2016). There is a critical need for new therapies, in light of this increasing AMR and a general lack of new antibiotic development.

To move from these startling statistics to the impact of AMR on individuals, we are often sent incredibly powerful letters and emails from patients with AMR resistant UTIs, who talk of their pain, difficulty and frustration caused by lack of access to effective treatments. We are grateful for the insights from patients who motivate us to try and find solutions. Phages are a disruptive technology and, in many ways, their lack of commonality with chemical antimicrobials along with concerns over problems of securing intellectual property and investment have flummoxed the pharmaceutical industry. We hope our work and that of others can one day direct patients' to a source of available phages within the NHS.

Bacteriophages (phages) are naturally occurring viruses that kill bacteria and thus can be used as an alternative to

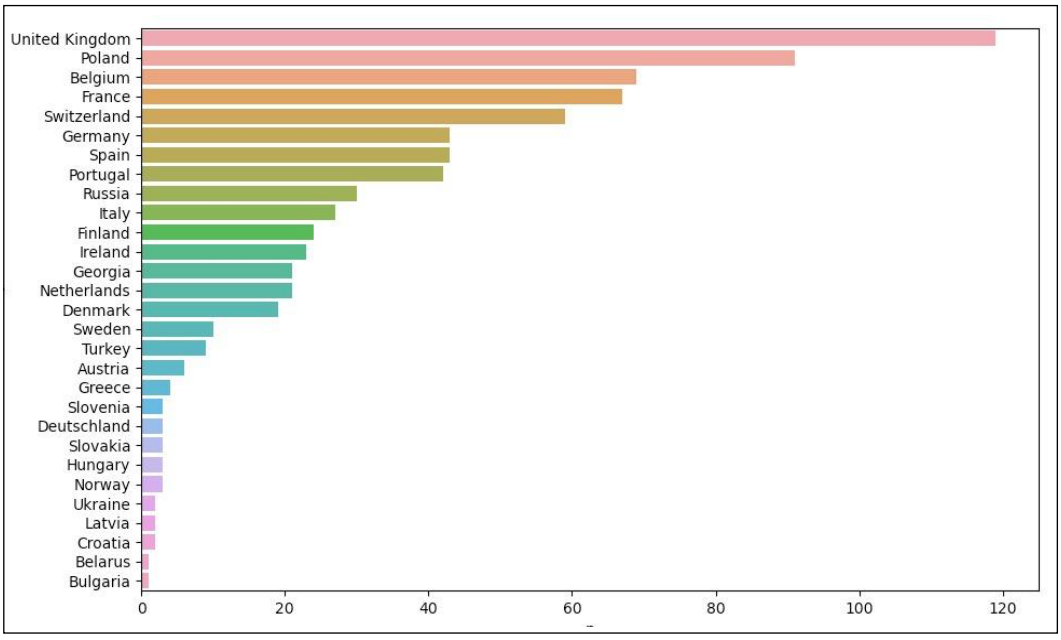

Figure 1: The number of papers on phage therapy published by country in Europe since 1941. Phage therapy papers were identified using the search terms: (PubMed entrez search for "phage therapy", or "phage cocktail", or "bacteriophage therapy", or "bacteriophage cocktail".

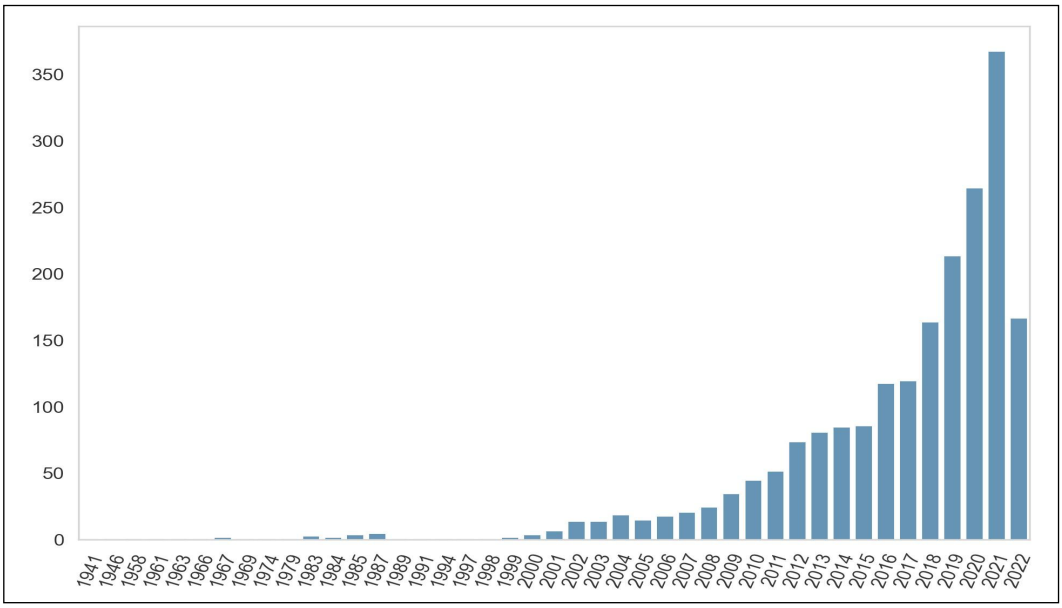

Figure 2: Number of phage papers published over time. Number of phages therapy papers published globally from 1941-2022.
Phage therapy papers were identified using the search terms: (PubMed entrez search for "phage therapy", or "phage cocktail", or "bacteriophage therapy", or "bacteriophage cocktail".

antibiotics in order to prevent or treat bacterial diseases. Although phages were discovered in 1915 and 1917 and used as antimicrobials soon after this, they were not widely used after antibiotics were discovered. Phages were seen as being problematic due to their narrow specificity towards individual bacterial species, or often a subset of strains within a species, and because of the inherent complexities associated with developing a biological entity in comparison to an antibiotic (*Gordillo* et al., 2019).The routine use of phages as antimicrobials in Georgia and Russia and for compassionate use in some European countries such as France and Poland (*Gordillo* et al., 2019) did continue (*Abedon*, et al., 2011) and currently Belgium has been increasing the amount of phage use for compassionate cases (*Djebara* et al., 2019). In the UK there are limited compassionate use studies e.g. *Dedrick* et al. (2019). This relatively small number of phage treatments largely reflects concerns regarding the safety, efficacy and consequences of using an unlicensed product, the lack of availability of phage products, and a lack of knowledge from the medical community.

Evidence for interest in the use of phages to treat bacterial diseases in Europe can be seen in Figure 1 which shows the number of papers on phage therapy published in Europe since 1941 by country. Figure 2 shows how the total number of phage papers written since 1941 has increased each year. Grant funding across the world and resources invested by biotechnology, pharmaceutical and agricultural companies have also increased.

In order for phage treatment to be accepted in the UK, it is essential to have doctors and patients' interest, awareness and understanding of how phages work and how they differ from antibiotics. The Medicines and Healthcare products Regulatory Agency

(MHRA) will also need to approve phage products.

The relative benefits of personalised and standardised phage products have been extensively reviewed elsewhere (*Pirnay* et al., 2011) and are not the subject of this paper. There is a role for personalised phage treatment in cases where emergency treatment is required (in case of multi-antibiotic resistant bacteria, or complications secondary to antibiotic use). However, there are currently no effective UK structures for phage provision. The practicability of administration in context of the English health care system needs to be assessed.

Progress towards a UK clinical trial and the scope of this review

In this review paper, we summarise the rationale for the development of clinical trials of phages developed to treat urinary tract infections (UTIs) caused by *Escherichia coli* and *Klebsiella*.

The following aspects are considered in the review below: a) burden of AMR in the context of UTIs; b) lessons learned from recent systematic reviews of phage therapy and rapid reviews of papers on phage toxicity; c) lessons learned about factors affecting phage efficacy from the on-going laboratory *in vitro*, *in situ*, *in vivo*, and bioinformatic work at University of Leicester.

A major hurdle to phage therapy is using optimally useful phages that have the correct target specificity. This requires well understood phage sets. Here we show how we have developed approaches and tools to study this. We highlight findings from our work on studying phage efficacy under different physiological settings. We provide a rationale for developing an ecological framework to identify useful phages according to their 'traits'. We also summarise how a graph-based analytical pipeline approach can provide additional information on phage relationships and be useful to probe different aspects of individual phages and inform phage cocktail composition. Ultimately we hope to be able to map key 'therapeutically' good traits within the graph-based system and the ecological framework, in order to expedite future development and use.

We then move to showcase data from our laboratory studies where we show how we have constructed phage cocktails (combinations of multiple phages) to kill *E. coli* and *Klebsiella*, and how we are building on this work to create improved cocktails with optimal physiological properties and host ranges. There is a lack of pharmacokinetic data within phage therapy on how to best deliver and formulate bacteriophages and indeed what the optimal phage dose is. We have recently carried out trials in animal settings, both in swine and poultry and present insights from these studies focusing on how they inform the design of our human clinical trial.

The regulation regarding phage products has also been the subject of much review across the globe [8] and is not a main focus here. Regulatory bodies within the UK are becoming increasingly engaged in the development of regulatory frameworks for non-traditional antimicrobials. The conclusions of the review include suggested next steps for the research groups such as ours who wish to advance phage therapy.

THE MOTIVATION FOR FOCUSING ON PHAGE THERAPY FOR UTIS

Literature studies and consultation with general practitioners, clinical microbiologists, infectious disease specialists and urologists highlighted innovative Urinary Tract Infection (UTI) treatments as a priority because of their high burden

on health care and patients, and because they can act as a driver and spreader of antimicrobial resistance.

Each year globally there are 400 million UTIs diagnosed and 236,786 UTI-related deaths (*Zeng* et al., 2022). In England UTI-related morbidity, mortality and NHS costs are high with 175,000 UTI-related hospital admissions annually at a cost of over £450 million. A third of all UTI hospital admissions have a length of stay greater than 7 days (*NHS England*, 2022).

UTIs are a major part of prescribing costs in primary care, and the most common Healthcare Associated Infection (HCAI) (*Mantle*, 2015). Furthermore, the number of deaths following *E. coli* bacteraemia has been increasing (*Abernethy* et al., 2016) (We have used the 2019-20 data to avoid confusing the story with a COVID19 confounding effect). *E. coli* bacteraemia is estimated to cost £20 million per year in England. Resistance to antibiotics increases the cost per infection by £180 - £430 depending on the resistance type (*Naylor* et al., 2019). Indeed, *E. coli* is the commonest cause of Bloodstream Infections (BSIs) and UTIs frequently lead to BSIs. In England in 2020 the number of *E. coli* BSIs was estimated at 37,823 and over a quarter of *E. coli* BSIs (29.3%) were resistant to one or more antibiotic treatment. The resistance in *E. coli* isolated from blood resistant to third-generation cephalosporins has increased significantly (*UK Health Security Agency*, 2021).

UTIs and AMR

This burden will increase further as bacteria become more resistant to antibiotics resulting in additional deaths and disability (*Cassini* et al., 2019). The key bacteria that cause both UTIs and sepsis are *E. coli* and *Klebsiella* along with Enterobacter species (*UK Health Security Agency*, 2021; *Medina* and *Castillo-Pino*, 2019). These bacteria are classified in England as 'key pathogens' because of their rapidity in developing AMR, a lack of alternative antibiotics to treat them and the fact they cause common infections such as UTIs (as well as BSIs, pneumonia and surgical wound infections).

The healthcare problems associated with *E. coli* and *Klebsiella* are compounded by antibiotic resistance, usually caused by Extended-Spectrum Beta-Lactamases (ESBL). ESBL-producing *E. coli* and *Klebsiella* are on the list of World Health Organisation 'priority pathogens' which is the highest category (*World Health Organization (WHO)*, 2017). Of major concern, both *E. coli* and *Klebsiella* are becoming more resistant to antibiotics even amongst antibiotics that are in the 'Watch' and 'Reserve' categories [18].

The inappropriate antibiotic prescribing for UTIs is a key driver of antibiotic resistance (*Pujades-Rodriguez* et al., 2019). To address this, a main target of the national AMR plan, 'Tackling antimicrobial resistance 2019 to 2024: the UK's 5-year national action plan', is to halve healthcare associated Gram-negative bloodstream infections (GNBSIs) and therefore the risk to develop sepsis. One of the priorities to achieve this target is to reduce urinary tract infections (UTIs) including catheter associated UTIs.

Therefore, as a test case to progress lytic phages towards a widespread clinical use in the UK we have focused on treating UTIs. Phage therapy is a potentially effective and safe complementary measure to the antibiotic treatment of UTI or CAUTI (Catheter Associated Urinary Tract Infections) when requiring last line antibiotics treatment. Treating UTIs and its complications could contribute to protecting antibiotics and prevent further antimicrobial resistance, which are key priorities in the UK 5

Years national AMR plan (*Pujades-Rodriguez* et al., 2019). Phages could do this by:
a) reducing the consumption of antibiotics associated with recurrent UTI, therefore antibiotic consumption for UTIs;
b) reducing the risk of recurrent UTIs becoming resistant to 'Access Antibiotics' and,
c) reducing the risk of complications of recurrent UTIs including BSIs.
Another key part of this strategy document - of key importance to our work is that 'bacteriophages' are now included as part of the new therapeutics for development.

ANALYSIS OF THE SYSTEMATIC REVIEWS OF PHAGE THERAPY

Our clinical trial work builds on a body of previous phage clinical trials and and we summarise data from this work. To synthesise all available information from previous phage therapy trials, electronic databases were systematically searched (June 2022 for all systematic reviews about phage therapy in humans) and analysed here. This identified 14 papers that had systematically reviewed clinical trials, case studies and case series of phage therapy in humans. All of these papers were published in the last five years, further demonstrating the growing interest in human phage therapy.

The following paragraphs are a preliminary analysis of these thirteen systematic reviews (*Rahimzadeh* et al., 2018; *Dąbrowska*, 2019; *El Haddad* et al., 2019; *Saperkin* et al., 2019; *Clarke* et al., 2020; *Steele* et al., 2020; *Geneviere* et al., 2021; *Kenneth*, et al., 2021; *Liu* et al., 2021; *Thomas* et al., 2021; *Aranaga* et al., 2022; *Özal* et al., 2022; *Uyttebroek* et al., 2022; *Walter* et al., 2022) that mainly identify case studies and case series of human phage therapy, and randomised control trials (RCTs). This lack of high-quality trials means that there are knowledge gaps in many aspects of phage therapy (*Saperkin* et al., 2019; *Uyttebroek* et al., 2022).
All the papers analysed conclude that phage therapy, using lytic phages, is a promising alternative antimicrobial strategy. Complex and intractable infections, due to bacterial strains resistant to available antibiotics, do respond to phage therapy (*Clarke* et al., 2020).

Phage pharmacokinetics (how the host affects the phage's absorption and distribution within the body tissues and microbiota) and pharmacodynamics (how the phage affects the host through toxicity, side effects and inhibition of bacterial growth) affect the success of phage therapy (*Dąbrowska*, 2019; *Suh* et al., 2022). Phage therapy is effective in treating bacterial infections (excluding infection caused by intracellular pathogens) provided that the following factors are taken into consideration: the use of specific phages for each bacterial strain (*Aranaga* et al., 2022), the precise characterisation of the bacteria and its concentration at the site of infection, the co-infection with other species of bacterial strains; the mode of administration (*Dąbrowska*, 2019; *Suh* et al., 2022); the phage concentration at time of administration, its dosage and frequency of administration; the patient's clinical condition; the potential development of phage resistant bacteria; the concomitant administration or not of antibiotics. When effective, phage therapy reduces bacterial concentrations and degrades biofilms thus allowing healing and improving outcomes. This occurred through the administration of either a single phage or a phage cocktail.

Systematic reviews of phage therapy

on specific infection sites have been for bone, joint and prosthetic infections, burn wounds and superficial infections; for these sites of infection, phages can be administered topically or by direct injection to the infection site. Although oral administration effectively delivers phages to the alimentary tract, it is the least effective for systemic phage penetration. The most efficient delivery was achieved by injections (intravenous - IV, intraperitoneal - IP, or intramuscular - IM) that led to effective phage dissemination within minutes, but these delivery routes are likely to generate a greater response from the immune system (*Dąbrowska*, 2019).

In most studies reviewed, phage treatment is considered to be safe because there are no adverse effects either in recent trials, case studies or case series. Most studies however do not include a deep analysis of safety and toxicity issues and indeed comprehensive and standardised reporting of potential toxicities associated with phage therapy is generally lacking in the published literature (*Liu* et al., 2021).

The heterogeneity of the studies included in the systematic reviews means that it is difficult to draw conclusions (*Walter* et al., 2022; *Suh* et al., 2022). The studies covered a wide variety of pathologies (from mild to life-threatening) and were caused by a diverse set of bacterial pathogens, infection types and locations within the body. They also differed in whether they used single phages or cocktails and in administration routes (intravenous, oral, local, or combined). Phage therapy was sometimes combined with other treatments (such as antibiotics). In some cases, phage sensitivity testing was carried out before the start of the treatment, in others it was not (empirical phage therapy with a standardised phage cocktail), or this information was lacking. For some studies, no information was provided on either the phage formulations (liquid or powder) or the concentration of the phage solutions. Outcome parameters were poorly defined and the follow-up period was short, with no information on long-term effects.

In conclusion, all of the systematic human phage therapy reviews show the potential of phage therapy but stress the need for high quality clinical trials, taking into consideration the large number of variables that can affect efficacy and safety outcomes, devising treatment guidelines, or designing clinical trials and case studies. The critical analysis of clinical information, the building of strong clinical databases, the design of consensual therapeutic guidelines, and the availability of regulatory policies are essential steps that need to be taken (*Azevedo* et al., 2022). The conduct of well-designed and sufficiently powered trials would facilitate registration and wide acceptance of phage treatments (*Saperkin* et al. 2019).

The systematic reviews show more confidence for the short-term adoption of phage therapy for topical applications, and in bio-preservation, biodecontamination and bio-sanitization (*Ssekatawa* et al., 2021). They express more confidence in phage therapy through individualised treatment with phages matched to the bacteria, but with an expectation that in the longer-term phage therapy can be used in the early stages of infection and on a larger scale, reducing the up-front use of antibiotics, helping to preserve them (*Suh* et al., 2022).

ASSESSING PHAGE SAFETY

An early step in developing a phage therapy clinical trial is to consider all potential safety issues with phage. We cover the general concerns in this area in order to show how we will address them in our work. Various mechanisms can cause drug toxicity: a) on-target (or mechanism-based) toxicity; b) immune hypersensitivity and immune response; c) off-target toxicity; d) bioactivation/covalent modification; e) idiosyncratic events (*Guengerich*, 2011).

The key phage characteristics that are critical to consider to assess the safety of phage treatment are listed below:

1) **Phage interaction with human cells.** Because phages replicate in bacterial cells, it was previously thought that they are unable to proliferate in eukaryotic cells (*Kwiatek* et al., 2020). However, some studies suggest that interactions between phages and eukaryotic cells are possible (*Podlacha* et al., 2021) and thus it is important to further understand this and from a regulatory perspective determine if they accumulate inside eukaryotic cells and the potential consequences of this.
2) **Phage target specificity and life cycle.** Phages have a narrow infection spectrum, and therefore leave the commensal microbiota unaffected. However, depending on the phage replication cycle, they may contain genes coding for factors that increase or generate pathogenicity of the bacteria for example, bacterial toxin genes (*Tiwari* et al., 2014). Thus, understanding both specificity and phage life cycles and how these impacts on the 'ecology' of an infection is critical to understanding their safety.
3) **Phage mediated immune responses.** Phages can elicit/ induce immune response either from a) the crude phage lysates; b) in response to the *in situ* 'lysates' coming from the destruction of the bacterial cell wall *in situ* (*Tsonos* et al., 2014; *Dąbrowska*, 2019). Understanding the extent of these reactions is key for successful phage deployment.
4) **Phage movement within the human body.** Phages are able to pass through bodily barriers such as organs (*Tsonos* et al., 2014; *Van Belleghem* et al., 2017).

Reviews of phage safety data highlight that the lethal impact of phage therapy is extremely rare (*van Belleghem* et al., 2017; *Liu* et al., 2021; *Aranaga* et al., 2022). Indeed, most published accounts of phage therapy report no adverse events after phage administration via oral, inhaled, or intravenous (*Tsonos* et al., 2014; *Principi* et al., 2019; *Kwiatek* et al., 2020; *Suh* et al., 2022). Adverse events reported were transient and very rarely required stopping the phage therapy. They included: fever, shortness of breath and wheezing; in another case hypotension, diarrhoea, epistaxis, oropharyngeal pain, cough, rhinalgia, and decreased blood bicarbonate (*Liu* et al., 2021; *Suh* et al., 2022).

As stated above, increased immune responses can occur, directly due to phages or due to the bacteriophage-mediated lysis from a large bacterial load that causes the release of endotoxins, resulting in cytokine release syndrome (*Van Belleghem* et al., 2017; *Kwiatek* et al., 2020; *Liu* et al., 2021; *Suh* et al., 2022).

Hypersensitivity and cytokine release syndromes can also be induced by bacterial components and toxins present in phage preparation (*Liu* et al., 2021). Phage preparations can also impact the innate and adaptive immune system directly, resulting in production of phage antibodies. The anti-bacteriophage humoral response seems to be dependent on a number of factors;

largely the route of bacteriophage administration and the dosage (*Principi* et al., 20019; *Kwiatek* et al., 2020; *Liu* et al., 2021).

Some chemical components used in the purification process of phages can have toxic effects for example intoxication from Caesium Chloride (CsCl) can result in gastrointestinal distress, hypotension, syncope, numbness, or tingling of the lips (*Liu* et al., 2021). Other biological effects include transaminases, transient septic episodes and anaphylactic related decompensation (*Liu* et al., 2021). Bacterial resistance to phages was reported in some clinical case reports (*Aranaga* et al., 2022).

Limitations of phage safety studies

Although minimal toxicity has been reported from both *in vivo* animal and human phage therapy studies, many concerns have little data associated with them and thus need to be taken into consideration. The distribution of phages within the body and their impact on tissues and physiological processes are largely unknown. This is in part because most of the studies focusing on this issue were carried out on temperate phages (*Liu* et al., 2021). Furthermore, the impact of phage therapy on the human microbiome is unclear (*Liu* et al., 2021).

Very few studies have documented the effects of the release of endotoxins or reported data on immunogenicity of phage (*Liu* et al., 2021). Similarly, data on phage preparation are under-reported in animal and human studies, including genotype information (*Liu* et al., 2021). Although phages are clearly distinct from standard API's (active pharmaceutical ingredients) very few studies have actually defined the median effective dose (ED50), lethal dose for 50% (LD50), or the therapeutic index (TI) (*Liu* et al., 2021). We discuss a dose response below in connection with our animal studies.

Another limitation is that no studies were found that assessed genetic toxicity of phages or the effects of phage therapy on pregnancy growth and development. A more general point is also that the animal trials were carried out on rodents and not large animals (e.g., pigs), this limits the generalizability to humans (*Liu* et al., 2021). Finally, there are currently no animal or human studies that present data on assessing the risk of emergence of bacterial resistance against phage (*Principi* et al., 2019).
Several recommendations for toxicity studies of phage therapy were made (*Principi* et al., 2019; *Liu* et al., 2021; *Suh* et al., 2022). We are currently addressing these in our pre-clinical work in anticipation of the clinical trial work.

1) **Factors relating to the phage formulation:**
   a) Describing the methods used to propagate and purify the phage preparations should be standard.
   b) Quantify and document the presence of bacterial components in phage preparation especially endotoxin.
   c) Ensure the removal of chemical contaminants in phage preparation.
2) **Factors relating to the characterisation of the phages used:**
   a) Include an analysis of immunogenicity of phages in both animal and human studies.
   b) Include information on the characteristics of phages used in animal studies and clinical studies, including their morphology, genetics, and protein profile, as well as the composition of the phage preparations, including the levels of bacterial contaminants and other impurities.
   c) Sequence the phage genome and ensure it does not contain genes

enabling the lysogenic lifestyle, antibiotic resistant genes (ARG), genes for phage-encoded toxins.

3) **Factors relating to data that should be collected from the patients:**
   a) Reports on the safety of phage therapy should include information on the general health of participants, adverse events, chemistry, and hematologic testing data.
   b) Information on immune responses should be evaluated prior, during, and after phage therapy.

FACTORS TO CONSIDER FOR PHAGE EFFICACY.

Developing well-characterised phage sets

Having set the scene for safety concerns we now consider aspects that relate to efficacy. To develop an effective phage therapy product requires an understanding of the phage specificity and behaviour with respect to the pathogen of interest. A knowledge of how many phage types are required to kill the most relevant circulating serotypes or micro diversity within that bacteria strain is essential. We have built a large collection of AMR *E. coli* and *Klebsiella* strains isolated from urinary samples from patients with UTIs and representing the most prominent circulating sequence and capsule types. This is supported by the UK literature for uropathogenic *E.coli* (*Doumith* et al., 2015; *Day* et al., 2019), unfortunately for *Klebsiella* the data on dominant serotypes is less well-defined (*Gorrie* et al., 2018; *Caneiras* et al., 2019).

One approach to minimise bacterial resistance towards phages and maximise target specificity within a species, is to administer a phage cocktail. To ensure that the phage cocktail is effective, phages in the cocktail should not hinder each other and preferably be synergistic. Experiments have been conducted at Leicester university to investigate the interactions of phages within a cocktail (*Haines* et al., 2020). A 'virulence index' has been used to define and quantify the phage cocktail efficacy (*Storms* et al., 2020). This method quantifies bacterial death by measuring the optical density of bacteria over time. Using this method, a score of '0' means that there is no impact on the bacteria and '1' is maximum impact with all bacteria killed. By comparing the local virulence index of individual phages, to that of phage combinations (doublets/triplets/sextuplets) positive and negative interactions between phages can be identified (*Haines* et al., 2020).

A clear example of synergy was seen between the phages UP17 & JK08 that target *E.coli*. The synergy was seen across several clinical isolates, but was particularly marked for *E. coli* KR2733 where virulence index for UP17 alone was 0.3, for JK08 alone was 0.45, but when both phages were used in combination the impact increased to 0.95.

The current study suggests that some phage adds to the efficacy of other phages while being of limited efficacy on their own. This phenomenon would not have been identified using the current phage selection methods. This highlights the need for well characterised phage sets to be generated in order to produce 'nuanced'- less obvious phage combinations.

The impact of Oxygen on phage infection

A number of the pathogens on the World Health Organisation's priority list of antibiotic resistant bacteria (*Shrivastava* et al., 2018) are facultative

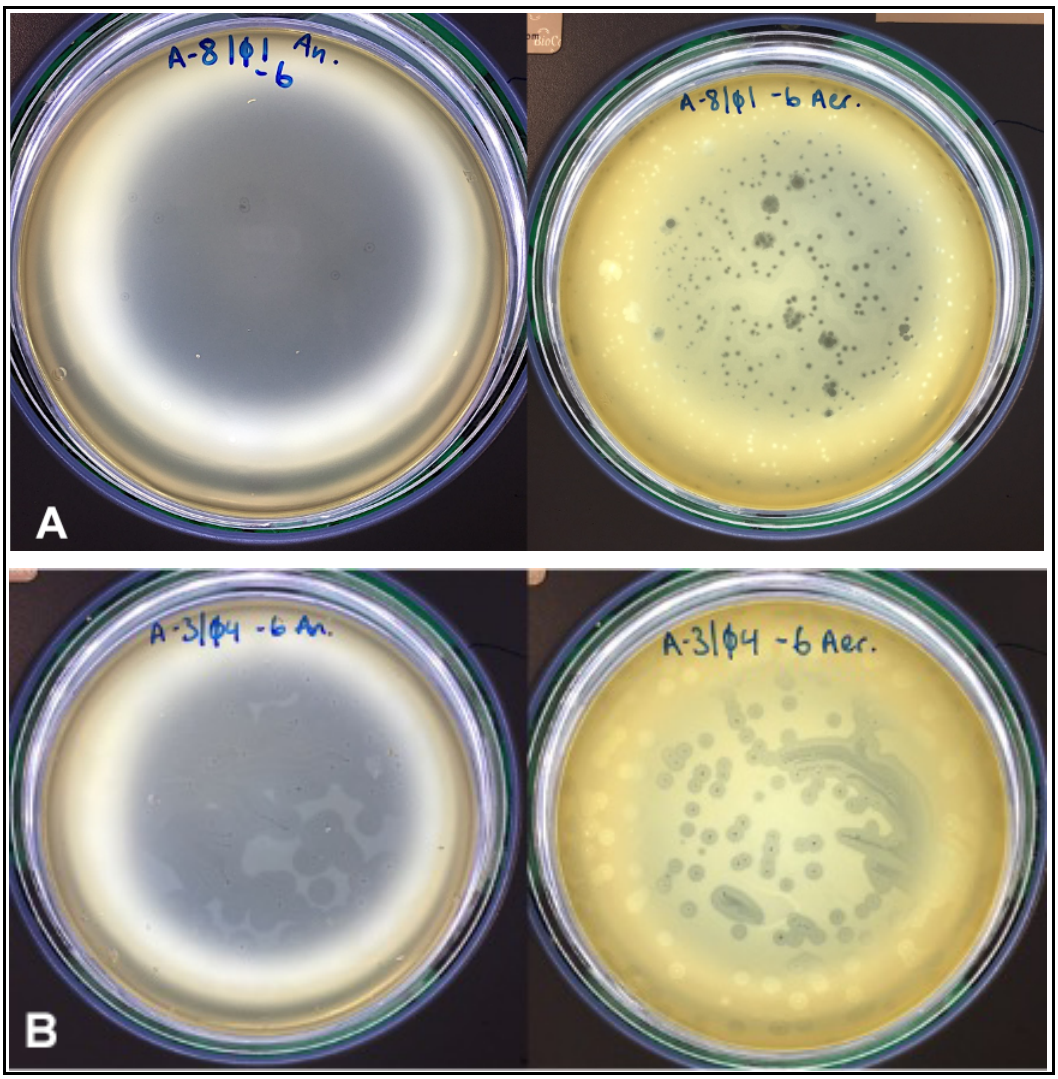

Figure 3: Specific bacteria and phage combinations respond differently to oxygen concentration.
A: The phage is unable to infect the bacterial host under standard aerobic conditions of oxygen yet when it infects the host in an anaerobic environment it grows well.
B: The opposite is true and the phage infects well under aerobic conditions

anaerobes, which means that they are able to switch between aerobic and anaerobic respiration. This allows them to survive or even thrive, in diverse environments with varying levels of oxygen available. In humans these environments include skin wounds, the urinary tract, the digestive system, and the lungs where these pathogens can cause biofilm. We showed that according to plaque morphology, some phages work infections.

It is part of phage development to assess their ability to work in conditions under which they are likely to encounter their bacterial hosts. In a bladder infection this may include the ability of phages to work under microaerophilic conditions or possibly in anaerobic conditions if the infection occurs within a better in anaerobic conditions and others in aerobic (Figure 3).

There is little in-depth knowledge of how changes in oxygen availability affects the efficiency with which a phage can infect its host. Our recent review reports on phage phenotypes under aerobic growth conditions and conditions where oxygen is limited (*Hodges* et al., 2021). Ultimately it concluded that oxygen availability can have a clear impact on phage effectiveness. This conclusion is also supported by unpublished results from our previous work which demonstrates that oxygen availability can have an effect on the burst size, latent period, adsorption rate, and efficiency of plating (EOP) measured in the characterisation of a phage (Figure 4). From this figure we can see that φA1 had a shorter latent period and faster adsorption rate under anaerobic conditions, a higher EOP ratio under aerobic conditions, and there was no significant difference measured in the burst size for this phage between aerobic and anaerobic conditions.

The review also highlights the need for standardising the measurements of phage virulence taking into consideration the bacterial environment. Measurements such as burst size, latent period, and adsorption rate are all affected by the environmental conditions under which the phage encounters its host (*Hodges* et al., 2021) and this is not usually accounted for in experimental design or virulence testing.

Data collection of phage virulence under different bacterial environments would contribute greatly to the reliability of *in vitro* phage characterisation when work is translated to *in vivo* studies and larger scale trials.

Of note, in our work we showed that different parts of a phage life cycle are 'better' or 'worse' in terms of contributing to phage reproduction, under high or low oxygen conditions. We do not currently know which of these attributes best correlates to a good therapeutic outcome, but these data suggest a lot more characterization could be done to relate these properties on a larger scale to efficacy and thus ultimately use the information to better predict which features will work well for particular infections under particular conditions.

The ability of phage to work in different pH and in biofilms

Ideally, phages should be tested in conditions that mimic the bacterial host environment. The bladder and urine are a potentially hostile, de-activating environment for phages. The pH of human urine can vary from 4.5 – 8.0, so it is important to test efficacy in this pH-range. Furthermore, a buffer could be used for the final phage cocktail for intravesical therapy within the murine model of infection and ultimately in humans. Such as salts, for example magnesium or calcium salts could also be added to improve efficacy (*Jończyk* et al., 2011).

Additional *in vitro* testing will consider the ability of phage cocktails to combat bacterial virulence factors such as biofilms (biological matrices that bacteria produce to protect themselves from the environment). Their importance in urinary tract infections has been previously shown for *E. coli* and *Klebsiella* (*Hancock* et al., 2010). Our previous work (*Haines* et al., 2020) demonstrated the efficacy of the phage cocktail against *E.coli* biofilms (11/19 isolates), but required improvement for biofilms created by *Klebsiella* (5/19 isolates). The phage cocktail has now been refined and experimental work is in progress to determine the efficacy of the improved combination.

Phage efficacy in model systems

Over the last decade we have developed several physiologically relevant models, in which to study phage efficacy (*Nale*

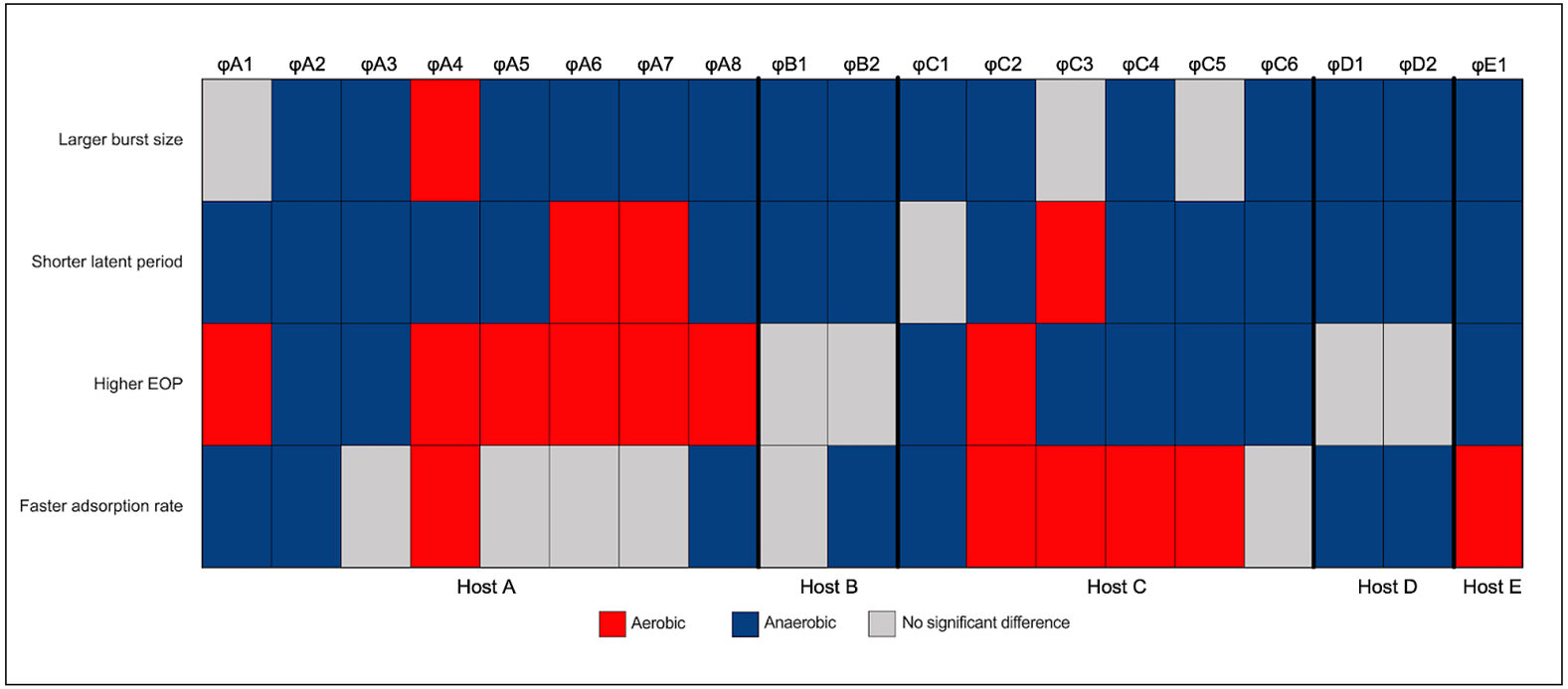

Figure 4: A heatmap (unpublished data) that indicates whether a phage infection was 'better' under aerobic or anaerobic conditions. Better is defined as potentially contributing to more phage progeny. Measurements better under aerobic conditions are labelled in red, and those better under anaerobic conditions are labelled in blue. Grey represents no significant difference.

and *Clokie*, 2021). These include a range of relevant cell lines that provide critical data on how phages interact with components of the immune system. Insect models are incredibly useful to establish dosing regimens and to look at the impact of bacteria that are resistant to phages. Artificial gut models allow the observation of how phages impact on other microbiota. For this work we have designed an artificial bladder that we will use in conjunction with catheters from patients in order to show how effective our phages are on natural UTI biofilm communities.

PHAGES MUST NOT INTERFERE WITH CURRENT AVAILABLE THERAPY

The development of phage therapy requires study of the interactions of phages with current therapy, such as antibiotics. The current work at the University of Leicester includes assessing the positive or negative interactions between common antibiotics used to treat UTIs and our phage collections. There are many examples of phage-antibiotics synergies (PAS) and an excellent review on this phenomenon (*Łusiak-Szelachowska* et al., 2022). There have been reports of instances where antibiotic resistant bacterial isolates have become sensitive after selective pressure of phage treatment.

Frameworks to recognise 'phage types' to progress therapy

Phages in their natural environments, like all viruses, have evolved to not immediately kill all of their bacterial hosts and to avoid generating resistance ie. they have a plethora of ways to ensure their survival until they can target an appropriate host. However, a subset of phages will have sets of characteristics that render them more appropriate at performing this than others. Unfortunately, it is not known in a general context which phages have optimal traits that are suitable for therapy. A major research focus within our laboratory is to do exactly that. We have repurposed an ecological framework that is based on a botanical framework (*Clokie* et al., 2020) and we are currently trying to understand what ecological features across all phage groups render particular phages effective.

The CSR concept was developed by Grimes in the 1970s to the 2000s in order to classify all UK flowering plants into a functional type with the view to better understanding individuals, communities and to be able to make predictions about what types of plant species we would expect in specific environments (*Grime*, 1979). All plants, Grimes argued, can be divided into those which are good competitors, stress tolerators or ruderals (Figure 5). Competitor plants are those that when environmental conditions are good they create a good infrastructure before reproduction, A classic example is stinging nettles. Stress tolerant plants in contrast, are highly conservative with their 'resources' such as wild thyme with tiny leaves and flowers. The final category, ruderals are good at coping with disturbance and can get to their reproductive state very quickly in order to exploit a newly formed environment. A classic ruderal is a poppy which is a symbol of war because it is one of the first plants to grow on land disturbed by trenches. Plants can also be any combination of these categories. As this scheme has stood the test of time within botany, rather than designing an entirely new set of axes from the outset we are trying to understand if this may also serve a useful purpose within phages.

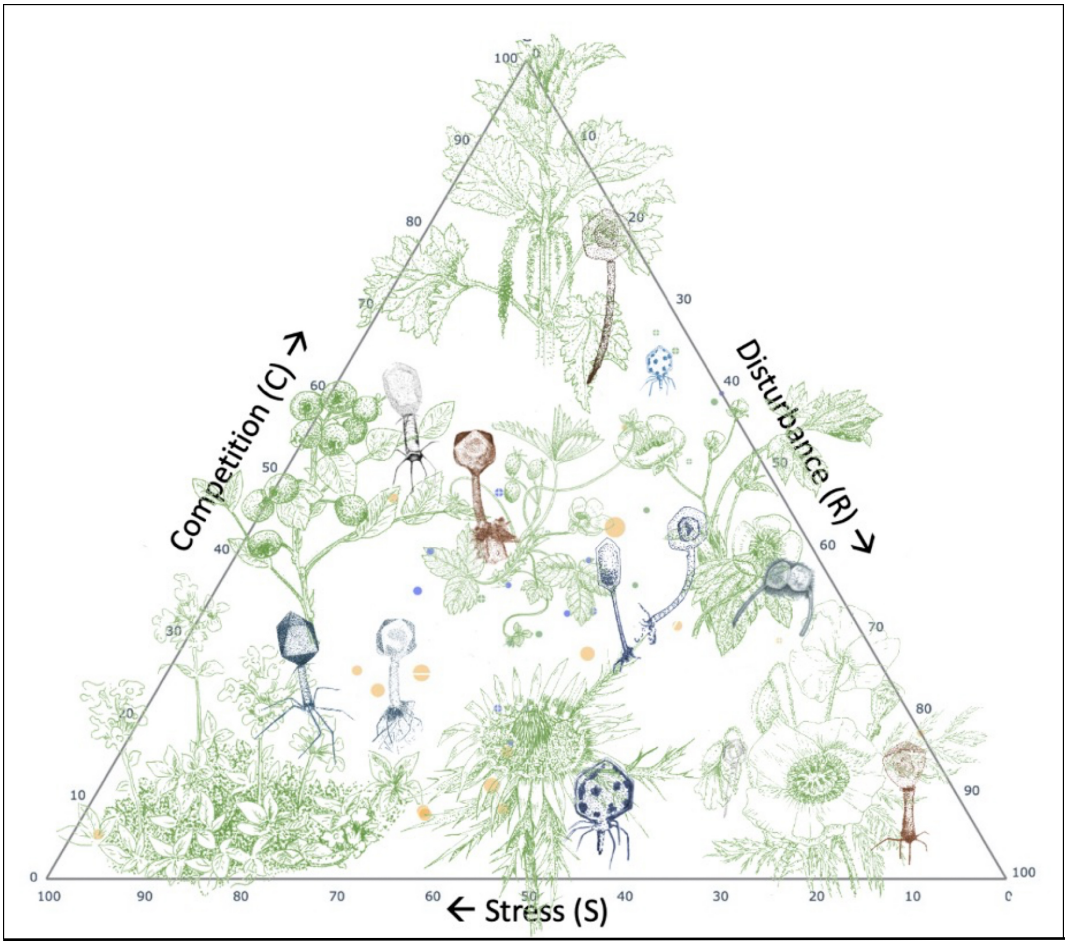

Figure 5: Plants that represent attributes according to an axis of competition (C), stress (S) or disturbance (D). The 'familiar' species we chose to depict these categories are stinging nettle (C), thyme (S) and poppy (D). CR is sea holly, CS is blueberry and CR is buttercup. Wild strawberry represents CSR. Phages have been imposed on the diagram to illustrate that they will also conform to a range of ecological strategies (*Grime*, 1979).

Phage traits are more hidden than those associated with plants, but it is likely that many traits can be predicted from phage genomes such as the genes that encode for parts of their replication and translational machinery. They are also likely to include genes that encode proteins associated with stability, or stress. As an initial proxy for this scheme, we have looked at the proportion of phage genes that are expressed at different points in the life cycle (*Clokie* et al., 2020). Based on these concepts, phages could be classified as a) competitor phage which encodes proteins that enable significant rearrangements to the bacterial cell; b) ruderal phage disturbance surviving phage) and c) stress phage which encodes proteins that facilitate the survival of the bacteria whilst replication can occur. Although we have been limited to date by the small number of phage transcriptomes that have been collected, data suggest our theory is plausible (Figure 6).

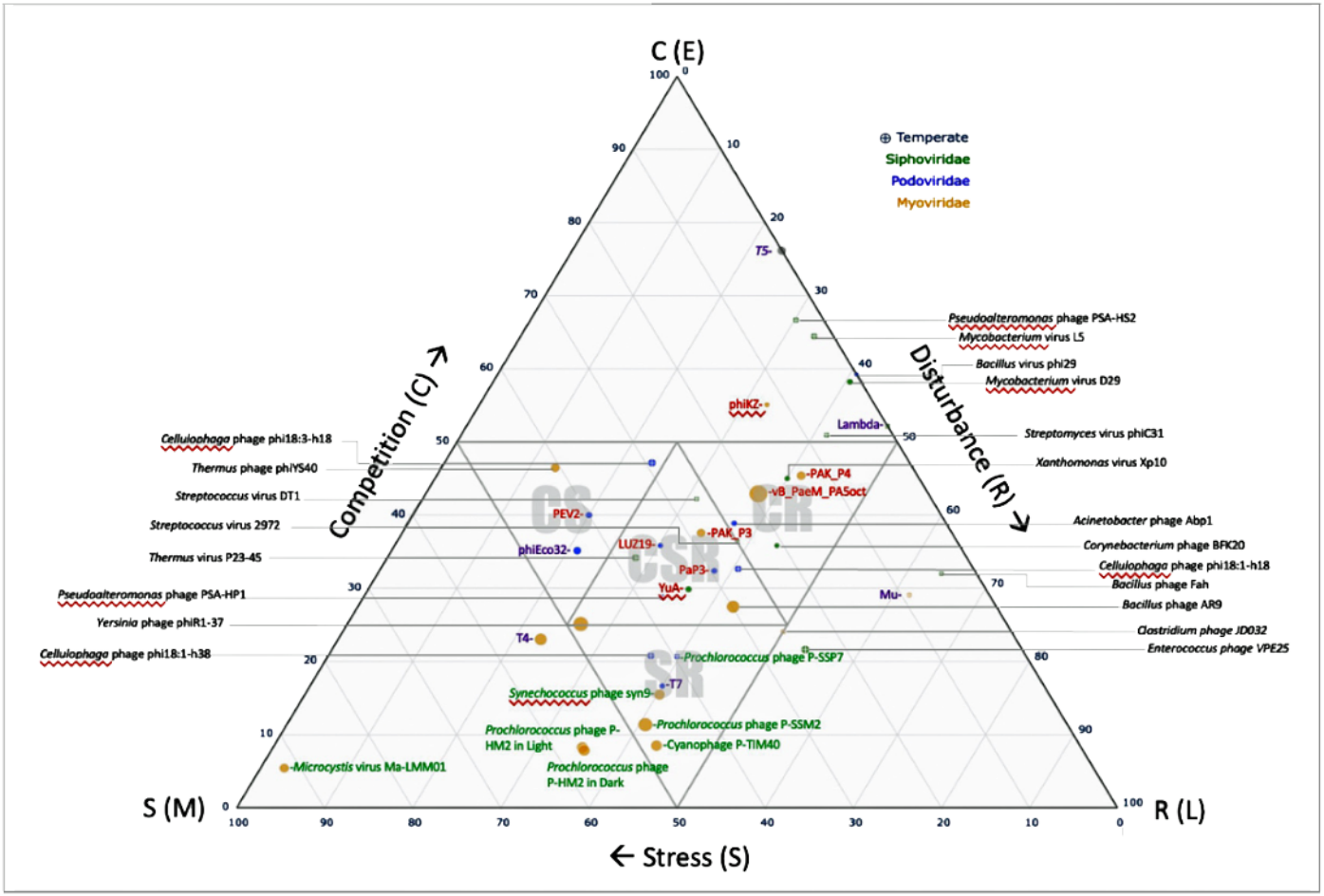

Figure 6: The position of 42 phages based on the proportions of early, middle, and late genes within the CSR triangle, which reflects their ability to tolerate competition, stress, or disturbance. The colour and size of the dots represent the phage classification into myoviruses, siphoviruses, and podoviruses and their genome size. Although the phages were all examined during their lytic cycle, those with a cross have access to the temperate cycle. Associated labels of phages that target *Pseudomonas* are shown in red, *Escherichia* in purple, and cyanobacteria in green.

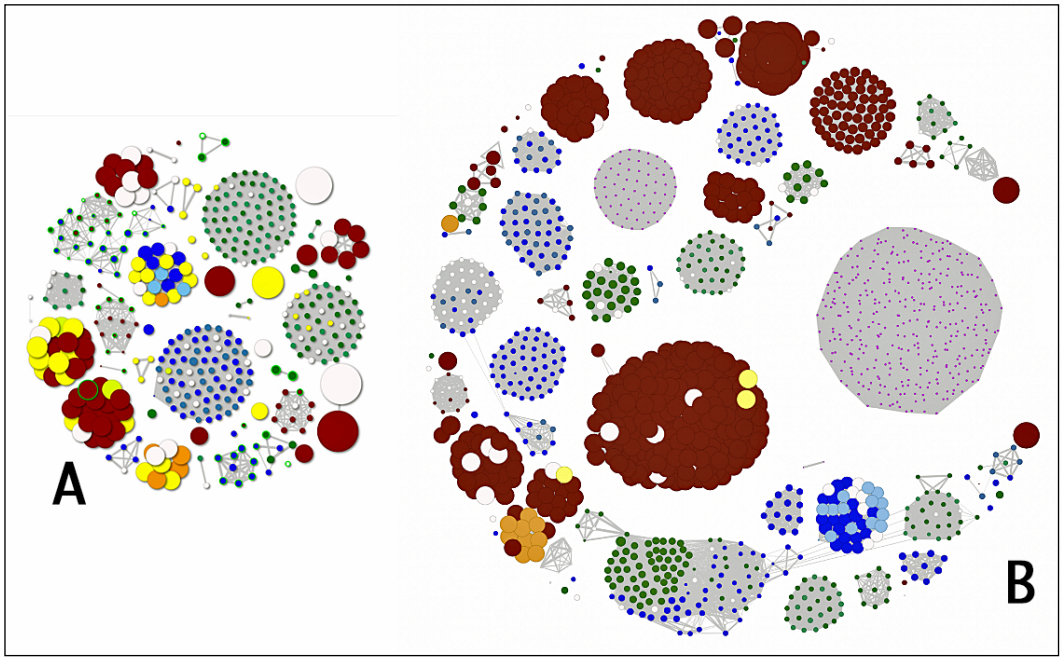

Figure 7: A schematic representing all genomes in NCBI for phages that target *Klebsiella* (A) and *E. coli* (B) respectively. The colours represent phage taxonomy.
Although recent large-scale changes to phage taxonomy have disbanded three large families, for backwards compatibility, phages from the former *Myoviridae*, *Podoviridae* and *Siphoviriade* are included alongside the recently described families. The colours represent these groupings, *Siphoviridae (cobalt blue)*, *Demerecviridae (light blue)*, *Ackermannviridae* (orange), *Microviridae* (pink) and taxonomically unclassified (white).
A) The *Klebsiella* clouds have 1270 phages within ~20 clouds.
B) The *E. coli* cloud has 1657 phages composed of ~30 clouds.
The figures are scaled so that in both cases dot size is reflective of the genome size.

Another key tool that we show data for is a graph-based method that we have developed to look at the evolutionary relationships between phage genomes. Ultimately, we hope to link these two concepts together and therefore to impose our ecological framework onto our clouds network. In this review we will showcase the clouds-based approach with respect to bacteriophages that target both *E. coli* and *Klebsiella pneumoniae*.

In the last five years, we have increasingly been working to build both experimental and computational frameworks to determine and select phages and phage combinations with maximum therapeutic suitability. To support the experimental workflow and to get a rapid overview of the phages and phage cocktails isolated and developed in our laboratory, we have developed a graph-based method to quickly characterize their genomic and evolutionary relationships (*Rangel-Pineros* et al., 2021).

To explain our graph-based method, in Figure 7A, the clouds towards 11:00 o'clock have multiple cloud connections that, from our experience, are indicative that they have a temperate lifestyle. This is consistent with the fact that they contain known prophages (*Rangel-Pineros* et al., 2021). Although there are a larger number of phages within the *E. coli* cloud (Figure 7B), it allows us to see some interesting trends. Because *E. coli* phages are comparatively well understood, we know which are temperate

and again they as expected in this figure, being within the clouds that are connected closely to each other such as those at 6:00 o'clock. It is also clear that there are clouds that contain phages with very small, and some with much larger genomes.

The phages represented by yellow dots are those that we are characterising in detail, many of which came from a collaboration with Ellie Jameson who has extensively characterised their physiological parameters and host-ranges (*Townsend* et al., 2021). We have been adding to this body of data and in general, and as expected, we notice more similar 'behaviours' in terms of physiological parameters, from phages within a cloud than between those in different clouds.

Phage traits

In addition to virulence and host-range, many other facets of phage biology are likely to be useful to inform clinical development. These are yet to be defined and referred to here as 'phage traits.' These will be the genes/proteins to render phages so effective at killing under specific conditions. They may also be traits that activate immune responses that convey less clearance from the human body - or those that can enter eukaryotic cells in order to clear infection. Conversely, it is equally important to establish which traits make phages less effective, for example what traits link to the safety questions raised above, or to the phage life phage cycle? Answering these questions would improve phage cocktail design and facilitate effective phage selection and clinical phage bank design.

One concept we are also investigating is if we can use traits/attributes from one phage taxa to inform others within the clouds described above. For example, if one cloud member carries a specific toxin gene, does the whole group have the potential ability to also do this? Similarly, if there are any temperate markers associated with the phage, or known phenotypic characteristics of transduction for example, should the whole cloud be down prioritised as therapeutic phages? Once we have a greater understanding of what traits are measurable and what they mean, we will ultimately be able to project them onto the phage clouds and also combine this with their different ecological lifestyles to help work out their therapeutic potential. For example, to be able to quickly project a phage's therapeutic suitability onto the phage clouds, we developed a machine learning based pipeline checking for presence of temperate markers, antimicrobial resistance genes, and virulence genes (*Yukgehnaish* et al., 2022).

Our efficacy studies of phage cocktails in various animal settings show that the best performing phage combinations are often those containing phages from different phage clouds with different ecological strategies. This is important within the challenge of bacterial phage resistance.

Traits that we would deem to be incredibly useful within a phage are those that allow the phage to be useful and to function in its intended location such as within a human environment. A phage ideally would also be amenable to propagation and formulation at high titre and easily separated from any potential toxins that might make infection more severe. It is also becoming increasingly clear that phages are not fully inert to human cells, some phages appear to drive an increased immune response whereas others appear to reduce the amount of cytokines and chemokines that are produced during an infection. These are likely to be traits to be taken into consideration during the selection of clinically relevant phages.

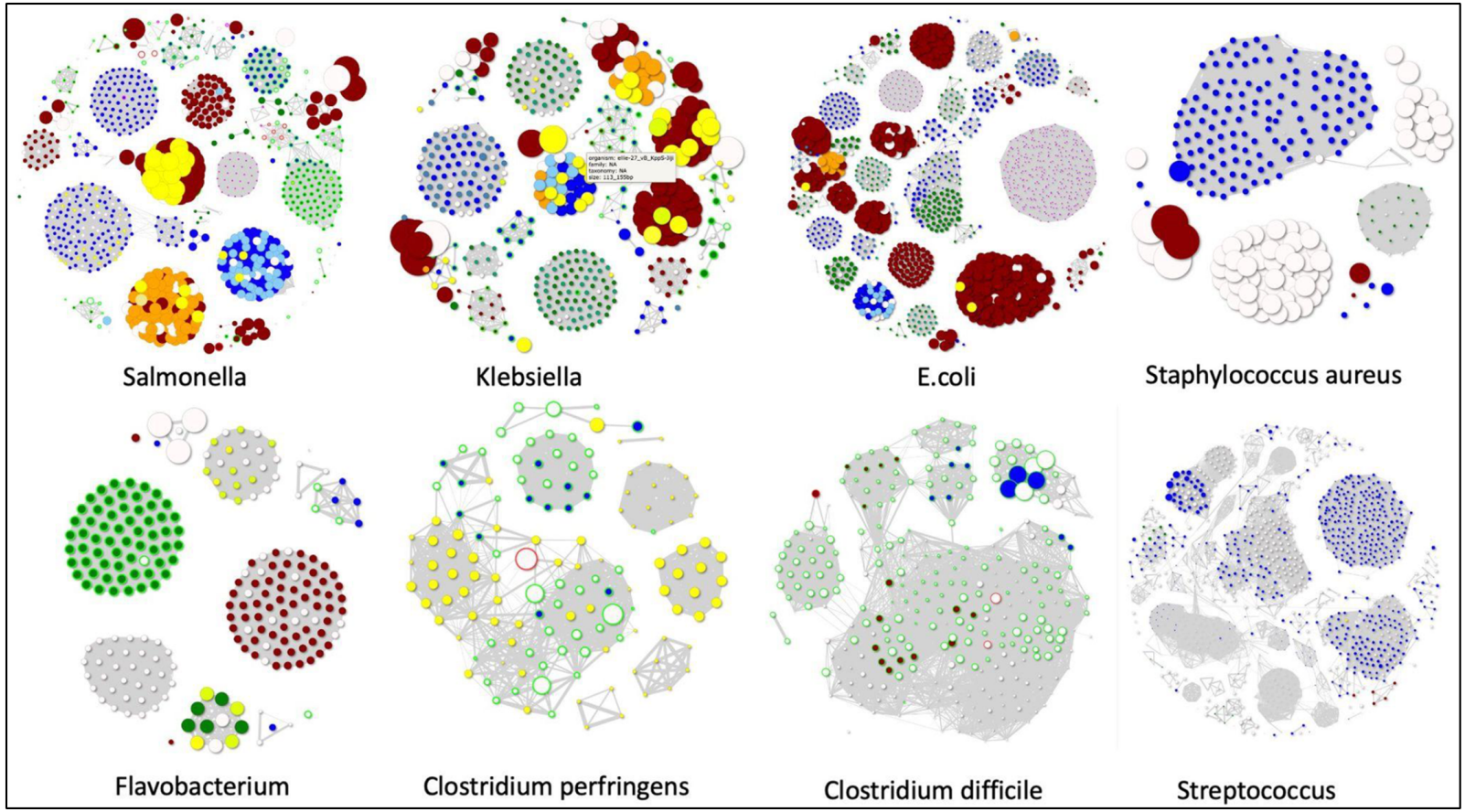

Figure 8: The *Klebsiella* and *E. coli* clouds alongside clouds for several other important pathogens. Ultimately, we hope to use this approach so see if the biology of the phages correlates with these groupings. Clearly the number of phages within each group dictates aspects of the cloud formation, but regardless of this we can see features such as connectivity, largest number of representations and number of discrete clouds all of which will form starting points to understanding dynamics specific phage groups.

Overall patterns within clouds

Schematic phage clouds for several common bacterial pathogens of humans and other animals are shown in Figure 8. Clearly phage clouds that contain a lot of species have a difference in density to those with relatively few representatives. We are currently investigating if we can identify patterns within host specific phage cloud space and apply that information to the phage cloud space from unrelated hosts.

LESSONS FROM ANIMAL STUDIES

Phages can amplify *in situ*

As stated in our Systematic Reviews of clinical phage use, there is little data available on the ability of phages to replicate *in vivo*, which impacts our ability to determine the appropriate dose to use within our human trials. Studies in animals can inform us of dose responses and show us how the dose changes the efficacy. Furthermore, as mentioned above, very little work has been carried out in large animals and thus pig trials can be particularly informative in terms of the pharmacodynamics and pharmacokinetics.

From the pig data (Figure 9A and B) it was clear that when phages were given to the animals who were then challenged with *Salmonella*, there was a reduction of bacterial numbers and an increase in phage amplification throughout their digestive tract. In contrast, when phages were given to the pigs in the absence of bacterial challenge the number of phages observed was consistently lower.

Our data show that ultimately it may be possible to add relatively small doses of the phages and demonstrate efficacy. During our study we added phages at 10^5 phage per gram within the pig feed. The selection of a low dose was dictated by the fact that we lost a significant amount of the phage titre following the industrial spray drying of the phages in order to be able to incorporate them into the animal feed. These low dosages but clear amplification are consistent with the way that phage preparations are given to patients in Georgia. In general a low titre, highly diverse phage mix is given to patients with the idea being that the correct phage within the mixture will amplify *in situ*.

Lower dosages are more effective than higher dosages

Although the pig work was a relatively small study, the data on *Salmonella* reduction and phage amplification provided us with the incentive to test the pages on a larger scale. We have subsequently carried out a large-scale chicken trial with 672 birds (Figure 9C). The main objective of this trial was to show safety and efficacy and determine the optimal phage dose. The most striking observation from our data was that the lowest phage dose was the most effective in terms of clearing *Salmonella* from the intestine of the birds. Here we have presented the data in terms of the amount of *Salmonella* bacteria that were shed from the chickens in terms of what could be recovered from sampling the pens (there were sixteen pens per treatment group). When data were analysed in terms of the number of colonies shed from individual birds, the same trend was apparent and the lowest dose (0.1x) was the most effective. The reason for this highly effective lower dose is unknown, but we hypothesise that it is related to the lower dose having a lower rate of bacterial killing which would allow higher levels of localised replication and infection prior to clearance by the host immune response.

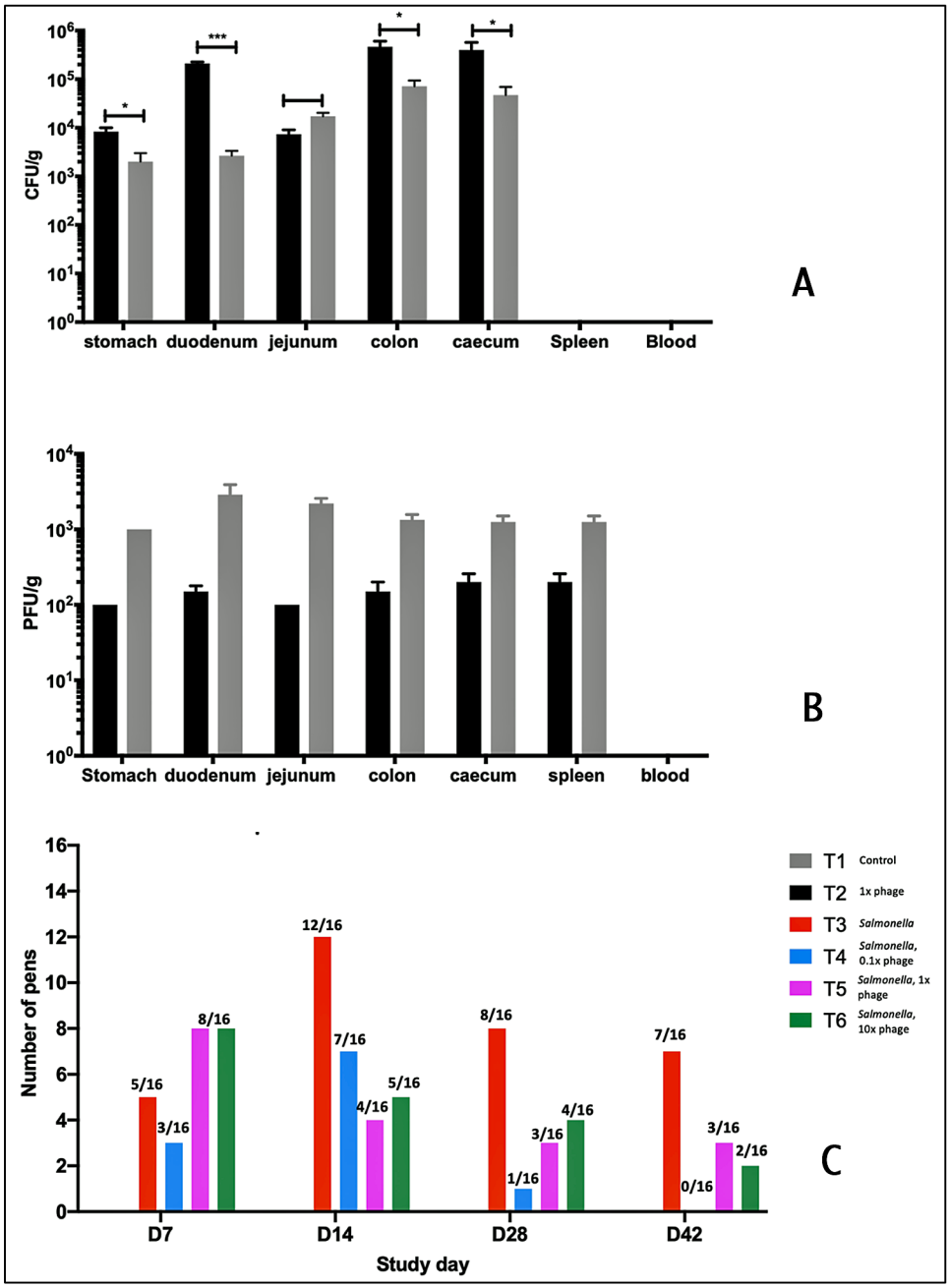

Figure 9: A) the number of bacteria isolated from the different regions of a pig digestive five days post challenge (*Thanki* et al., 2022). In the sample group that were given phages, significantly fewer bacteria were found in the stomach, duodenum, colon and caecum than were found in the control group. **B)** the number of phages in each section of the gut, with the animals given the bacterial challenge shown in grey. In all cases, the animals that received bacterial challenge had a greater number of phages in the sample. **C)** the data from a poultry study where phages were given in feed to poultry and the bacteria enumerated from the chicken pens. These data show that phages reduce the spread of infection by reducing the bacterial load shedding from the animals. At the lowest phage dose (0.1x), no bacteria were found in any pen. The other two doses (1x and 10x) also had a markedly good impact on the amount of *Salmonella* that can be recovered after 42 days.

Resistance

One of the knowledge gaps that we recognised from the systematic reviews was information on bacterial resistance. Clearly there is a lot of data on this within the literature, but most of it is from *in vitro* work. We isolated bacterial colonies from the multiple days up to 42 of the study and in no cases did we find examples of bacteria that were resistant to our phages. Forty-two days is not a particularly long time-frame but it is encouraging that we did not see resistance on this time scale. Future work will be needed in order to confirm that specific phages do not drive high resistance rates. Clearly, it is of paramount importance to ensure that wedo not repeat the mistakes of the past and breed phage resistance in these settings that would make human based treatments ineffective

CLINICAL TRIAL

The overall aim of the current pre-clinical UTI based studies within our group is to provide the necessary data that will allow us to conduct a clinical trial in humans. We plan to conduct a phase I/II multi-centre clinical trial with participants who have known recurrent *E.coli* and *Klebsiella* UTIs.

Our pre-clinical work is currently in progress and involves the use of a murine UTI model to provide preliminary efficacy and safety data of our defined phage cocktail in an *in vivo* model. The model will involve the direct intraurethral administration of phages to mimic how they would be used in humans where they would be catheterised directly into the bladder. We are using relevant clinical strains of *E. coli* and *Klebsiella* that have already been well characterised within murine models, and are using our optimised phage cocktails. In addition to the murine work, we will also assess human urinary epithelial cell toxicity and immune responses.

In the USA, there is an interesting clinical trial that is currently recruiting patients, being carried out by Adaptive Phage Therapeutics - APT (NCT04287478). There are similarities with our work, in that researchers involved with this trial will treat UTIs caused by *E.coli* and *Klebsiella*. The main difference is that they will provide 'personalised' phage therapy as opposed to the 'off-the shelf' product we will be using. To ensure our trial provides data on efficacy, our inclusion criteria will ensure only participants who have bacterial isolates that are sensitive to our defined phage cocktail. Another difference is that the APT trial will compare two routes of administration, intravesical and intravenous, while our clinical trial will assess the intravesical route only.

REGULATION

In a recent report published by the Antibacterial Resistance Leadership Group (ARLG) based in the USA, it was indicated that the compassionate use of phages is a viable regulatory pathway for the use of phages to treat patients who have few options left. The ARLG endorsed the collection of systematic data on patients who receive phage therapy through this access pathway until regulatory bodies approve a licensed phage therapy (*Suh* et al., 2022).

At present in the UK, there are no licensed phage products and no specific

regulatory frameworks for the use of phage-based products in humans, so compassionate use is the only way phage therapy can be administered to a patient. In the UK, phage therapy can be prescribed to a patient on a 'named patient basis'. This type of prescription is only carried in certain circumstances when particular criteria are met and it considers the special needs of an individual patient. Pathways established to drive innovation in medicine by the UK government and within the UK's National Health Service (NHS) such as the Promising Innovative Medicine Designation (PIM) and the Innovative Licensing and Access Pathway (ILAP) could potentially provide routes for licensing phage-based therapies for compassionate use on a wider scale, though it is not yet clear how this would work.

Within the UK, following clinical trial data to support safety and efficacy of phage products for human therapeutic use will be regulated through the Medicines and Healthcare products Regulatory Agency - MHRA. It is clear that phage products used on a large-scale, and for compassionate use will have to be produced under GMP (Good Manufacturing Practice) conditions. Although there are no facilities currently available for this to occur within the UK, there is significant demand for this resource and growing interest in setting up such a facility that UK academics, doctors and companies producing phages for clinical trials can access. There are several ongoing initiatives that are actively working towards making phage therapy more accessible for clinicians and patients in the UK as well as facilitating discussions with key stakeholders on the potential for establishing a phage production facility in the UK. Globally such facilities can be accessed although costs can be prohibitive with a significantly large proportion of the resource required for a clinical trial being directed into GMP phage production.

CONCLUSIONS AND FINAL THOUGHTS

In this paper we have outlined why UTIs are a major problem and why there is a desperate need to develop novel treatments. We also summarised the reviews on the extensive body of literature that strongly suggests that phages are inherently safe. There is a caveat to this of course, which is that many aspects of toxicity have not been rigorously assessed and such studies are needed.

From our work we show that the earlier key phage traits can be integrated into a phage selection programme, the better. Simply identifying phages on the basis of their host range and virulence is likely to result in omitting useful phages. Our current approach may lead to identifying phages/phage cocktails which are good all-rounders; on the other hand it could be that a phage needs to have the ability to cope with stress such as a low oxygen environment, which will result in higher virulence than as measured under optimal conditions.

Our work on larger animals and within large scale settings., and mouse work using a UTI model, provides a pre-clinical framework for a human clinical trial of phage therapy for UTI

Importance of the long view

It is important to remember that we are building on a large body of literature assembled by our Georgian, Russian and Polish colleagues. In addition, there is more clinical interest than ever before. It is possible to go straight from phage isolation to genome sequencing.

Bacteriophage bioinformatics

There are many bioinformatic challenges that bacteriophage genomes present us. Some of our authors have a strong background in using bioinformatics to extract information from ancient life, complex metagenomes and unique evolutionary trajectories – we have combined forces to apply this knowledge to identify phage diversity. The extreme diversity of phage genomes limits our ability to identify the function of the majority of their putative open reading frames. This means that even genes that we know must be present within the genome such as major structural genes are not always identifiable. This may even extend to whole bacteriophage genomes which may not be recognisable either from metagenomic data sets or from whole bacterial genomes. To address this, we have written Phageboost that looks to find novel phages from within bacterial genomes or metagenomes based on feature space rather than sequence similarity (*Sirén* et al., 2021).

Furthermore, the fact that they have no genes in common means that a comparative scheme such as that used to barcode or other forms of life based on shared ribosomal RNA is not available. To address this, we designed the graph-based phage cloud approach that we presented above to examine genome relationships. To identify phages suitable for therapy we exploited our knowledge of attributes that render phages unsuitable for therapy and developed Phageleads as screening tool (*Yukgehnaish* et al., 2022).

Molecular and structural biology

Another pertinent aspect of phage therapy is that unlike the case for previous generations of phage researchers, we can manipulate the genomes of an increasing number of phages. There are an increasing number of recombineering and selection approaches coming on-line, CRISPR based technologies for example are becoming more standard and synthetic biology and Gibson assemblies followed by approaches such as 'rebooting' in easier hosts means that phages can be manipulated to understand the relevance of key features and also potentially to expand and extend their properties. These approaches will be increasingly important to gain fundamental knowledge to expand phage properties.

Phage structural biology is likely to be of increasing importance to progress our understanding of therapeutically relevant phages. Again, new approaches such as Alphafold2 that predicts protein structures e.g. (*Dowah* et al., 2021; *Tunyasuvunakool* et al., 2021) will be of importance to predict key structures that can then be verified using standard approaches and direct biochemical and functional analysis. Furthermore, using cryo-electron microscopy and electron tomography to resolve phage protein and phage-host protein complexes are likely to be important in understanding how bacteriophages function.

Final thoughts inspired by patients

We have outlined the healthcare problem, the economic backdrop and the lack of availability of antimicrobials, particularly with respect to UTIs caused by *E. coli* and *Klebsiella*. We have also detailed our approaches to developing and testing phage sets. The length and multifaceted nature of this paper reflects the many aspects to phage therapy development. Whilst understanding the science and elucidating a mechanistic basis for why phages work is of paramount importance to developing phages in a sustainable and effective manner, it is important to remember that AMR infections in people is a real problem now and thus we need to expedite

phage development programmes to save lives and prevent misery.

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