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Andrea Garretto
Loyola University Chicago

Taylor Miller-Ensminger
Loyola University Chicago

Alan J. Wolfe
Loyola University Chicago

Catherine Putonti
Loyola University Chicago, cputonti@luc.edu

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Andrea Garretto[1], Taylor Miller-Ensminger[1], Alan J. Wolfe[2] and Catherine Putonti[1,2,3,4*]

[1] Bioinformatics Program, Loyola University Chicago, Chicago, IL USA

[2] Department of Microbiology and Immunology, Stritch School of Medicine, Loyola University Chicago, Maywood, IL USA

[3] Department of Biology, Loyola University Chicago, Chicago, IL USA

[4] Department of Computer Science, Loyola University Chicago, Chicago, IL USA

* Corresponding Author email: cputonti@luc.edu

Abstract | The discovery of bacteria in the female urinary bladder has fundamentally changed current dogma regarding the urinary tract and related urinary disorders. Previous research characterized many of the bacterial components of the female urinary tract, but the viral fraction of this community is largely unknown. Viruses within the human microbiota far outnumber bacterial cells, with the most abundant viruses being those that infect bacteria (bacteriophages). Similar to observations within the microbiota of the gut and oral cavity, preliminary surveys of the urinary tract and bladder microbiota indicate a rich diversity of uncharacterized bacteriophage (phage) species. Phages are vital members of the microbiota, having critical roles in shaping bacterial metabolism and community structure. Despite the fact that phages have been discovered in the urinary tract, such as phages that infect *Escherichia coli*, sampling them is challenging owing to low biomass, possible contamination when using noninvasive methods, and the invasiveness of methods that reduce the potential for contamination. Phages could influence bladder health, but an understanding of the association between phage communities, bacterial populations, and bladder health is in its infancy. However, evidence suggests that phages can defend the host against pathogenic bacteria and, therefore, modulation of the microbiome using phages has therapeutic potential for lower urinary tract symptoms. Furthermore, as natural predators of bacteria, phages have garnered renewed interest for their use as antimicrobial agents, for instance in the treatment of urinary tract infections.

- Phages are abundant members of the microbiota of the lower urinary tract.
- Active or lytic phages have been isolated from urine samples, but the majority of phages within the urinary microbiota persist through dormant infections, the lysogenic life cycle.
- Evidence suggests that phages have a role in modulating the composition of the urinary microbiota, similar to that observed in microbiota of other organs of the human body.
- Phage therapy, or the use of phages to treat pathogenic bacterial infections, is an active area of research within urology, given their potential use to treat urinary tract infections.

Introduction

Bacteriophages (phages) are ubiquitous viruses that infect bacteria; they are the most abundant biological entities, far exceeding even bacteria [1]. Surveys of the marine environment have resulted in an estimated prevalence of 10^{30} phages in the oceans alone, meaning that for each bacterial cell in the ocean there are ~10 phages [1]. In addition to their abundance within the marine environment [2], phages are prevalent within the soil [3,4] and in freshwater [5]. Phages have even been isolated from some of the most inhospitable conditions, including desert sands [6], sea ice [7], and the depths of the ocean [8]. In addition to our surroundings, phages are abundant in and on plants [9] and within the bodies of insects and mammals [10,11]. Indeed, the human gut alone is home to an estimated 2 trillion phages [12], greatly exceeding the number of eukaryotic viruses in our bodies [13]. Although phages are unable to infect eukaryotic cells, evidence has been reported of direct interactions, such as direct transcytosis of phages across cell layers of the gut, lung, liver, kidney and brain [15], between phages and human cells [14,15]. However, the full extent of direct and indirect effects of phages is unknown, as researchers have only just begun to speculate about the roles of phages in the human body, including the human immune and central nervous systems [12].

As phage genomes can be dsDNA, ssDNA, double-stranded RNA (dsRNA), or ssRNA, linear or circular, and even segmented, sequencing phage populations often is limited by the genomic nucleic acid extraction protocol and might need amplification before sequencing [16]. Amplification can introduce biases such as quantitative biases, the preferential amplification of ssDNA viruses, stochastic biases, systemic biases [17-19]. Nevertheless, whole-genome sequencing technologies have enabled researchers to identify new phage species. In contrast to cellular organisms, no universal marker exists for phages because no gene is conserved within all phages. To identify phages, researchers often target genes that encode structural proteins such as phylogenetic markers [20]. However, these signature sequences are far from comprehensive [21]. Only a small fraction of phage sequence diversity is represented in extant sequence databases, and it is heavily biased for sequences of phages with DNA genomes that infect bacterial species that are routinely studied in the laboratory, such as *E. coli*, *Pseudomonas* spp., and *Bacillus* spp. [22-24]. Metagenomics, the high-throughput sequencing of mixed, complex communities of microbes, has enabled exploration of the diversity of phages on Earth [2-5] and the human body [25-31], including in the lower urinary tract [32-36].

The Human Microbiome Project (HMP), which characterized human microbiota using 16S ribosomal RNA sequencing and metagenomic whole-genome shotgun sequencing, revolutionized our understanding of bacteria that inhabit the human body [37]. However, the bladder was not included in the HMP publications, which focused on the oral cavity, nasal cavity, skin, gastrointestinal tract, and vagina [37,38]. In the absence of a urinary tract infection (UTI), the bladder was thought to be sterile and so it was not included in the HMP [39]. This dogma resulted, in part, from the widespread use of standard clinical microbiology urine culture protocols, which are designed to detect common fast-growing pathogens with basic nutrient needs and no aversion to oxygen (especially *E. coli*). Thus, the standard protocol does not detect anaerobes, slow-growing bacteria, or bacteria with complex needs. However, diverse bacterial and fungal species have been detected in urine obtained directly from the bladder via transurethral catheterization (herein catheterization) or suprapubic aspiration that is negative using standard culture using an enhanced urine culture method called expanded quantitative urine culture (EQUC) and/or DNA sequencing methods, such as *Lactobacillus*, *Corynebacterium*, *Streptococcus*, *Actinomyces*, *Staphylococcus*, *Aerococcus*, *Gardnerella*, *Saccharomyces* and *Candida* spp. [40-49]. These and

other studies of the bladder microbiome and microbiota have revealed associations of bladder bacteria with postoperative UTIs [50,51], urgency urinary incontinence (UUI) [44,45,47], and response to overactive bladder treatment [52]. For instance, the microbiome of women with UUI had increased levels of *Gardnerella* spp. and decreased *Lactobacillus* spp. relative to the microbiome of women without UUI [44]. Some bacteria are even associated with the lack of symptoms and an abundance of *L. iners* seems to provide protection against post-instrumentation UTI [46,50,51,53,54]. These results suggest that the bladder might possess its own protective microbiota and that dysbiosis results in disorders, such as UTI and UUI [55,56]. An effort to generate a genomic catalogue of bacteria isolated from the bladder that was published in 2018 revealed that the genomes of bladder bacteria are quite distinct from bacteria isolated from the gut, but somewhat similar to those of the vagina [57]. This suggests an interlinked female urogenital microbiota, i.e. strains resident of the vaginal community could be transferred to the urinary tract and vice versa.

Although the HMP focused on characterizing the bacterial fraction of the human microbiota, sequencing of some viral genomes was unavoidable because viral DNA was present in the samples and because prophage DNA (a stage in the lysogenic cycle of temperate phages when the phage genome is either integrated into the host genome or remains in the cytoplasm as a self-replicating plasmid) was present within the bacteria [58]. Subsequent to the original initiative, bacterial and viral communities within the five niches studied in the HMP were extensively investigated [59], most notably the communities inhabiting the gastrointestinal tract as it is a high biomass niche and can be studied using stool samples as a proxy. These viral communities include both eukaryotic viruses and phages. The gut virome (the viral component of the microbiome) has been the focus of numerous studies [25-31], each leading to the same conclusion: phages are key members of the gut microbiota. A core phage community exists within the gut of healthy individuals [30]and disruption of this core phage community (dysbiosis) has been associated with certain gastrointestinal symptoms and disease, such as Crohn's disease and ulcerative colitis [27,29-31,60,61]. Within the gut, seven phage taxa were found to be associated with type 2 diabetes, establishing a type 2 diabetes-specific gut phage community [62]. Other studies have characterized the viromes of the body sites included in the HMP [63-68]; the data from these studies are publicly available (**Table 1**). Like the gut, associations between phage communities and patient symptoms and/or disease have been identified in these other body sites. For example, phages within the oral cavity have been linked to periodontitis [65]. In contrast to the sites of the HMP, investigation of the phage communities within other niches of the human body has only recently begun. These associations within the gut and oral cavity are active areas of investigation as the extent to which phages modulate the human microbiota remains an open question. Furthermore, phage communities and their putative role in disease and/or symptoms have yet to be determined in the other niches studied within the HMP. Although the investigation of the urinary microbiota was launched independent and subsequent to the HMP, considerable progress has been made in characterizing this niche. Investigations into the phages of the lower urinary tract present challenges unique to this niche, but have greatly benefited from the work of their predecessors exploring the viromes of the five HMP niches.

Studies of the bacterial communities of both the lower urinary tract in women and men have revealed a diverse community of species. Furthermore, studies of the urinary microbiome have documented clinical relevance of bladder bacteria; including associations with urinary symptom levels, treatment response, and UTI risk [42,44,45,51-54,69-72]. Phages are the most abundant biological entities in the human body, and given observations made in other organs phages are probably vital members of the lower

urinary tract microbiota with the potential contribution to urinary symptoms and/or disease. Their role in the lower urinary tract is largely unknown. In this Review, we describe the current knowledge of phages within the urinary tract and their possible contribution to urinary tract health. We provide a brief introduction to phages followed by a discussion of both culture-based and culture-independent studies of the viruses of the urinary tract. We present some of the challenges in studying the urinary microbiota. Finally, we consider the clinical relevance and applications of phages. Both historical and current applications of phage therapy for lower urinary tract infections and other disorders are discussed.

[H1] The phage life cycle

Phages have three distinct, generally well-characterized life cycles for propagation and reproduction: lytic, lysogenic and chronic [72]. All phages infect their bacterial host by binding to surface receptors, a process called adsorption (**Fig. 1**); these receptor binding proteins are often quite specific, leading to a narrow range of hosts (strains or species) that a particular phage can infect [73]. Following adsorption into the host cell, the phage injects its DNA or RNA genome into the host's cytoplasm. In the lytic cycle, the phage genome replicates and phage proteins are synthesized [74]. For double-stranded DNA (dsDNA) phages, DNA is inserted into the protein procapsid, whereas for single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA) phages, the capsid is formed around the nucleic acid (**Fig. 1**). The bacterium's cell wall breaks ('bursts'); phage proteins called holins can form holes in the cytoplasmic membrane or spanins can degrade the outer membrane [74], and the phage progeny disperse into the surrounding environment. Some phages are obligately lytic, but others, called temperate phages, can alternate between the lysogenic and lytic cycles. In the lysogenic cycle, the phage genome is either integrated into the host genome or remains in the cytoplasm as a self-replicating plasmid [75] (**Fig. 1**). The phage genome (now called a prophage) generally replicates in synchrony with the host chromosome, some phage genes are also known expressed by the bacterium. [75]. Temperate phages, such as the model phage λ, are capable of going through the lytic and lysogenic cycles. Their prophages can remain dormant for generations until, often, an environmental cue, such as host starvation, change in nutrients, temperature [75,76], triggers entry into the lytic cycle — a process known as induction. This switch between the lysogenic and lytic cycle can also be determined by the phage-produced peptide communication system (the 'arbitrium' system), in which progeny phage lysogenize when this peptide is abundant within the environment [77]. In addition to the lytic and lysogenic cycles, phages can reproduce by chronic infection; in this process phages, for example the filamentous phage M13, are shed from the bacterial cell without killing the host cell [78]. The majority of known phages can be associated with one of these three life cycles, but additional modes of infection and reproduction, such as pseudolysogeny, have been described [72,76,78].

Given these multiple mechanisms of infection and persistence, unsurprisingly phages can have profound effects on microbial communities (**Fig. 1**). Phages can transform a microbial community through predation (lysis) [79-81]. Furthermore, phages can affect bacterial diversity within a community [82-85], including adaptation in susceptible host species such as loci associated with phage resistance [86,87]. Coevolving lytic phages can increase diversity within bacterial populations by selecting for multiple modes of resistance [84]. Phages have also been shown to alter apparent competition among bacterial strains [84]. Exposure to temperate phages can increase bacterial virulence (a process referred to as lysogenic conversion) [88,89] by, for example, encoding toxins [90,91]. Case reports detail shiga toxin-

producing *Escherichia coli* strains, most commonly associated with enteric infections, found within the urine of individuals with UTIs [92-94]. In this example, the shiga toxin is carried by a phage, integrated within the *E. coli* genome. Thus, lysogeny can be beneficial for the bacterial host [95]. Some temperate phages can transfer genetic material from one cell to another (the process of transduction (Fig 2)) because they integrate their genome into their host's genome; this process can benefit the recipient host cell. Indeed, temperate phages are well known to mediate horizontal gene transfer (HGT) and have helped spread virulence and/or resistance factors through bacterial communities [96]. Similarly, lytic phages can also transfer bacterial DNA via transduction [97]. Data exist that support both frequent and infrequent phage-mediated spread of antibiotic resistance genes [98-101]. Phages also can contribute to HGT indirectly; for example a 2017 study identified two 'superspreader' phages, which are phages that promote extensive plasmid transformation [102]. In this scenario, phage lysis spreads intact host plasmids, enabling HGT via transformation. The two superspreader phages discovered were 50-times more efficient in dispersing antibiotic resistance genes [102]. Given the large genetic diversity present within phage communities [103], investigation of the complexities of phage-host dynamics is in very early stages [22,31,104,105].

[H1] **Viruses of the urinary tract**

Viruses are abundant members of the human microbiota, found throughout the body including the urinary tract. These viruses include those that infect human cells (eukaryotic viruses, Box 1) as well as both lytic and lysogenic phages.

[H2] Eukaryotic viruses

The urinary tract harbours a diverse eukaryotic viral community, including adenoviruses, anelloviruses, papillomaviruses, and polyomaviruses [32-36,106-116]. Adenoviruses can be detected in urine [106], and can range from limited, localized infections in otherwise healthy individuals to severe and potentially fatal infections in immunocompromised individuals [107]. Torque teno virus (TTV), also referred to as small anellovirus, has largely been studied in relation to immunodeficiency in renal transplant recipients [108]. Rani et al. [32] collected midstream clean-catch urine from 22 kidney transplant recipients; whole-genome sequencing was conducted for the urinary viromes of these samples and 108 different subtypes of TTV were detected. The most prevalent eukaryotic viruses in urine samples are human polyomavirus 1 (BK virus) and 2 (JC virus) [109]. Both of these polyomaviruses seem to have little effect on healthy individuals, but each can lead to nephropathy and hemorrhagic cystitis in immunocompromised populations [110,111]. Metagenomic sequencing of the bladder microbiome (both the bacterial and viral fractions) of 30 individuals enabled reconstruction of the full JC virus genome in five of the samples [33]. JC virus and other polyomaviruses have also been detected in other viromes from urine samples (obtained using an undescribed voided urine collection method) [34]. Human papillomaviruses (HPVs) also have been detected in voided urine [112] and bladder tissue [113,114]. Certain HPV genotypes have been attributed to condylomata acuminatum of the bladder [115,116], but these high-risk genotypes associated with cervical cancer are rare. In one investigation of the urinary virome, 95% of the 20 participants sampled had HPV sequences detected in their urine [35]; for eight patients, these samples were collected via intermittent catheterization and the for the others, an undescribed voided urine collection method was used. In the case of the latter, whether contamination from either the skin microbiota or the vaginal microbiota,

both of which are known to contain HPV [117], occurred is unknown. However, eukaryotic viruses are estimated to represent just a small fraction of the urinary virome [33-36].

[H2] Lytic phages

Lytic phages have been isolated directly from urine on numerous occasions. The first phage from urine was isolated by the co-discoverer of bacteriophages Félix d'Hérelle in 1917 when he observed that this invisible microbe lysed the Shiga bacillus, despite not knowing exactly what a phage was at the time [118]. A century later, two studies isolated phages capable of infecting *Pseudomonas aeruginosa* from urine samples [119,120]. Transmission electron microscopy (TEM) of the isolated phages provided information about the phages' morphology, which includes a siphophage, a tailed phage with a long, thin, often flexible tail structure, [119] and two tailless phages (Fig 3) [120]. A fourth *Pseudomonas*-infecting phage, harvested from a bacterial isolate from a urine sample collected via catheterization has been discovered (Johnson et al., in preparation). This phage is capable of lysing *P. aeruginosa* PAO1. Coliphages, or phages that infect *E. coli*, have also been isolated from voided and catheterized urine samples. Dallas and Kingsbery [121] found 100,000 colony-forming units (CFU) /ml of bacterial growth in routinely plated urine samples (collected using an unknown method) and, upon closer inspection, phage plaques. Furthermore, four coliphages were isolated from clinical urine samples and their morphologies were determined to be siphophages using TEM [119]. A further seven coliphages were isolated from the bladder of four women with UUI (in urine collected via catheterization) [122]. From the complete genomes of these seven coliphages, six (phages Greed, Sloth, Envy, Pride, Gluttony, and Lust) resemble coliphages that were isolated from cattle slurry [123]. This observation similarity suggests that the human urinary virome might include strains found within other hosts, having a regulatory role in the urinary microbiota. The seventh coliphage identified, phage Wrath, most closely resembles a lysogenic *Bacillus* phage sequence. TEM images suggested that these phages had siphophage morphology (Fig 4). Testing of the host range of the phage Greed showed that in addition to its ability to lyse the laboratory strains *E. coli* C and K-12, it is also capable of infecting and lysing some *E. coli* strains isolated from urine samples, including the uropathogen *E. coli* CFT073 [124]. Thus, within the urinary microbiota, Greed might be effective in thwarting the proliferation of uropathogenic *E. coli strains.* However, the lytic phage population is only one part of the phage community within the lower urinary tract.

[H2] Lysogenic phages

Lysogenic phage communities have been routinely under-reported in the human body, a direct result of the methods used to collect and sequence viral isolates [125]. In fact, evidence suggests that lysogenic phages are the most abundant phage type within the gut microbial community [126]. Similar observations have been made within the bladder. Several prophages have been identified within *E. coli* isolates collected from catheterized urine from the bladder [127]. Numerous prophage sequences have been identified within the genomes of four *Gardnerella* strains isolated from urine specimens obtained via catheterization from the bladders of adult women with UUI, although a lytic *Gardnerella*-infecting phage has yet to be isolated, possibly owing to the challenges of growing this phage in the laboratory, and is a currently unexplored area of phage biology [128]. Analysis of these four genomes and other publicly available *Gardnerella* genomes revealed that phage infections were pervasive within the urinary

microbiota [128]. This examination of lysogenic phages was then expanded to include 181 bacterial isolates, which was representative of the phylogenetic diversity within the bladder [129]. These samples were collected from women with or without lower urinary tract symptoms. Over 400 phage sequences were identified; the majority (86%) of these bacterial isolates harboured one or more lysogenic phages [129]. Furthermore, many (57%) of the phages identified in this study [129] exhibited no sequence similarity to any known phages, indicative of a vast unexplored phage population residing in the bladder.

To date, three published studies have employed a metagenomic approach to sequence the viral fraction of the urinary microbiota. The first study, conducted by Santiago-Rodriguez et al. [35], sought to determine whether the urinary virome was affected by urinary tract health status. The viral fraction (eukaryotic viruses and extracellular phages) of urine collected from 10 individuals with and 10 individuals without a diagnosed UTI were sequenced. For each cohort, samples were collected either via catheterization or voided urine from five men and five women. As previous research has shown, clean-catch studies of voided urine from women routinely contain bacterial taxa from vaginal contamination [41]. Furthermore, the bacterial taxa of the male and female urinary microbiota are not identical [130,131]. Only 27% of the viral sequences produced in this study were homologous to known viruses, the majority (>99%) of which represented phage genes [35], again suggesting a large unexplored phage community within the urinary tract. In a second study, urine samples were collected via the voided mid-stream clean-catch method from 14 men and eight women who received a kidney transplant [32] and the viral fraction was isolated and sequenced. Phages are present in these viromes, but this study did not mention phages and sequence data is not publicly available; instead, the authors focused solely on eukaryotic viruses. The subsequent study of Thannesberger et al. [34] also found a large phage community, but the authors concluded that the phage community primarily consisted of relatives of known species, the majority resembling Chlamydia microviruses, which infect *Chlamydia* spp. This study included two healthy individuals and four individuals with human cytomegalovirus (CMV) infections. However, information about how the urine was collected or demographics of the patients was not provided. This omission, compounded by the small sample size, limits our ability to frame the study's results with respect to other virome studies.

Viral diversity within the urinary tract also has been studied by sequencing the entire urinary microbiota. In 2018, Moustafa et al. [36] published a study in which metagenomic sequencing was performed for urine samples from 49 individuals with suspected UTIs (collected via the clean-catch method). As this study did not select for the viral fraction, most of the sequenced data corresponded to bacterial genetic material. Nevertheless, viral — primarily phage — sequences were detected [36]. Similar to the study conducted by Santiago-Rodriguez and colleagues [35], this study examined samples from individuals with UTIs and detected sequences homologous to those from phages that infect bacteria commonly found within the urinary tract and associated with UTIs, including those of the genera *Escherichia*, *Enterococcus*, *Lactobacillus* and *Pseudomonas* [36]. Abundant bacterial species harbouring prophages would result in an abundance of phages; thus, one would expect to identify phages infectious of UTI-associated bacterial taxa. In a similar approach, sequencing was undertaken of urine collected using catheterization from 10 asymptomatic women and 20 women with overactive bladder [33]. Partial and complete viral genomes were reconstructed in 12 of the 30 samples sequenced, including the complete genomes of novel phage strains [33]. Partial and complete phage genomes also exhibited sequence homology to previously characterized lytic or lysogenic phages that infect *Gardnerella*, *Lactobacillus* and *Streptococcus* species. These bacterial species are dominant members of the urinary microbiota of

healthy women as well as women with overactive bladder symptoms [44]; thus, one would expect to readily identify phage infection of these taxa. In sequencing both the bacterial and viral members of the microbiota, associations between phages and their hosts can be inferred. As both of these studies have highlighted, phages that infect dominant bacterial taxa within the urinary microbiota can be identified [33,36]. One can, therefore, postulate that novel phage sequences (phages that do not share sequence homology with any known, sequenced phage or prophage sequence) are infectious of a bacterial taxa within that same individual's urinary microbiota, whereas more prolific phage species, which are representative of more deeply sequenced viral sequences, are probably infectious of dominant bacterial taxa.

Culture-based and culture-independent studies have revealed a large, active phage population within the lower urinary tract. The diversity present has yet to be comprehensively catalogued, but the consistent finding that the majority of phage sequences detected do not resemble known, sequenced phages suggests a novel community within the urinary tract. In parallel to continued efforts to catalogue this community, future studies should conduct comparisons of the urinary virome to the viromes of other areas of the human body. In particular, comparisons to the gut virome are warranted given emerging evidence that viruses of the gut have been found elsewhere in the body [12].

[H1] Challenges of studying bladder phages

The bladder has orders of magnitude less microbial biomass than the gastrointestinal tract, oral cavity or vagina [43,132,133]. DNA concentrations are often low, a challenge faced by both those studying the bacterial and those investigating the viral constituents of the bladder [134]. Thus, two of the metagenomic studies of the urinary virome employed amplification before sequencing [34,35]. This technique is efficient for increasing viral genomic material, but these amplification methods have well documented biases [17-19]. For instance, multiple displacement amplification (MDA) can increase the DNA template concentration for sequencing, but small circular viral genomes are over-amplified. Both MDA and single-primer amplification (SISPA) methods have also been found to under-amplify viral genomes with GC contents at the extremes [135]. Perhaps of greater concern are the methods by which urine is collected and the anatomical microbiota that the collected urine represents. The method of urine collection is a frequently debated and investigated topic in the field [55,130,134], owing to the need to balance the invasiveness of procedures during collection and the purity of the sample obtained. This debate is not unique to the bladder, urinary tract, or urine; biopsies and stool samples give quantitatively and qualitatively different results for the gut [136] and methods of sampling the gut microbiota are still being refined [137,138]. Studies of voided urine have routinely observed vaginal contamination of clean-catch samples [36,48]. Virome studies by Santiago-Rodriguez and colleagues [35], Rani and colleagues [32], and Moustafa and colleagues [36] investigated voided urine samples. Thus, whether the viruses detected resided in the bladder and/or in the urethra, vagina, or skin remains unknown. Another study, in which the bacterial communities in the bladder were obtained via paired samples by catheterization and suprapubic aspiration from women were compared showed that both methods did not isolate microbial communities that resembled the skin or vaginal microbiomes and successfully avoided vulvovaginal contaminants [41]. Moreover, the communities identified by the two methods were similar [41]. Although a similar study has not been conducted comparing the virome of urines collected via catheterization and suprapubic aspiration, one

would assume that both represent the same community of the bladder microbiome. Thus, catheterized urine samples have a lower probability of contaminants than voided urine samples [127]. Study of less-invasive methods for collection is an ongoing pursuit [139]. In a 2019 study, the use of the non-invasive Peezy midstream device (Forte Medical) was tested[140]. The results showed that voided urine collected by the Peezy was less prone to contamination, having a bacterial abundance distinct from the periurethra [140]. This device is a promising step towards a sampling method that is less-invasive than catheterization, which is the current best method for sampling the bladder's microbiota.

[H1] Phages and urinary tract health

The associations between phage communities, bacterial populations, and the human host are not yet fully understood. Some evidence suggests that phages might contribute to human health [141], in particular the gut in which they have been suggest to have roles including maintaining a stable bacterial community within the gut and providing an innate defense to pathogenic species [27,29-31,60]. These studies of the gut will probably inform future studies of the urinary tract and other niches of the human body, providing a model for conducting such studies and expanding our knowledge of phage genetic diversity within the human body. Paralleling those discoveries of associations between phage communities of the gut and GI symptoms, associations have also been made within the bladder: variation was observed in the abundance of lysogenic phages in bacteria isolated from asymptomatic individuals and those with overactive bladder, in which the microbiota of women with OAB included more *Lactobacillus* phages than the microbiota of women without OAB [129]. However, notably, the *Lactobacillus* species between these two cohorts varied which might be contributing to the observed difference and, therefore, warrants further investigation [129]. Variation, determined via the β diversity statistic and principle component analysis, was not found in the extracellular phage populations of individuals with or without UTI symptoms [35]. Although the bacterial communities differ between individuals with and without UTIs, the virome does not seem to change in response, suggesting that UTI symptoms are not associated with changes in the virome [35]. Further investigation of this observation is needed as the sample size was limited. However, importantly, understanding of the diversity of phages within the urinary tract has only just begun, in contrast to the gut phage communities. Cataloguing the phage community in both asymptomatic and symptomatic individuals is a critical first step in understanding if and how phages contribute to urinary tract health. All of the aforementioned studies discovered a large collection of novel viral sequences indicative of a unique genetic diversity present within the urinary tract. Further investigation of phage–bacteria dynamics in the bladder and urinary tract could reveal indicators for early detection of symptoms.

Phages could also offer a defense to the human host against pathogenic bacteria. Studies of the gut communities have revealed unexpected ways in which phages interact with human cells, organs, and immune system [12,29]. The prevalence of phages on the mucosal surfaces of the gut might confer a direct benefit to the human host by protecting the epithelium from bacteria [142]. This study's findings suggest that phages and mucosal surfaces have coevolved such that phages bind to mucosal glycoproteins; this phage mucosal layer reduces adherence of bacterial pathogens [142]. Changes in the mucosal phage population have been associated with ulcerative colitis [61]. Evidence also suggests that phages have increased virulence to bacteria when human cells are present [143]. In this study, phages were found to reduce *Clostridium difficile* numbers more efficiently in the presence of human cells [143]. Furthermore,

phages can interact directly with human cells. Studies have found that the wild type T4 phage and its substrain HAP1 can bind to cancer cell membranes and inhibit or attenuate melanoma tumour growth [144]. Although phages cannot infect eukaryotic cells, there are several means in which they can enter eukaryotic cells. A phage could be a passenger, as a cell of an invasive bacterial species that harbours a phage could enter a eukaryotic cell [145,146]. Alternatively, eukaryotic cells can take up free phages by endocytosis [145,147]. A *Staphylococcus*-infecting phage was capable of infecting bovine mammary epithelial cells and clearing intracellular *S. aureus* [147]. One study showed that phages are capable of penetrating epithelial cell layers via endocytosis with an estimated 31 billion phage particles passing through these layers of the gut into the body daily [15]. Given this observation, in a study of the urinary virome, comparison with the gut virome should be considered in order to identify if urinary phages originated from the gut. Within the human body, phages can modulate immune responses [148]. For instance, T4 phages mediated inhibition of T-cell proliferation via the CD3 complex [149] in vitro and stimulated of humoral responses in mice in vitro and in vivo [150]. Phage-mediated immunoregulation holds promise, such as for attenuating the expression of proinflammatory cytokines during UTIs [151]. The mechanisms by which phages interact with the immune system remains an active area of investigation [148].

Appreciation is growing of the therapeutic potential of modulating the human microbiome. Induction and release of temperate phages can lyse sensitive competitor strains or lysogenize other cells [152,153]. For instance, the gut bacterium *Enterococcus faecalis*, which has also been associated with UTIs, uses its prophages to colonize when competing strains are present [152]. Alternatively, an individual's bacterial infection can be treated with obligately lytic phages, known as phage therapy. In the face of the increasing threat of antibiotic-resistant bacterial strains, phage therapy has regained interest [154]. Phage therapy was a promising area of UTI treatment in the early 20th century. For instance, in a 1928 report, phages isolated from sewage were 90% efficient in lysing *E. coli* and *P. aeruginosa* strains isolated from catheterized urine samples [155]. The USA and Western Europe abandoned phage therapy when antibiotics became commercially available (amongst other reasons) [156]; this area of research and treatment continued in Eastern European countries. Phage therapy is a publicly available treatment for individuals with UTIs in Russia, Poland, and the Democratic Republic of Georgia. In one study [157], 41 *E. coli* and 9 *Klebsiella pneumoniae* strains isolated from individuals with UTI were challenged with phages from collections from the Democratic Republic of Georgia. Only one *E. coli* isolate was resistant to the individual phages and phage cocktails tested, and one phage was capable of lysing all *K. pneumoniae* strains. Similar efficiencies have been observed for other bacterial species that cause UTI symptoms. A single patient, for whom gentamicin, ceftazidime, ciprofloxacin and meropenem were unable to clear the root cause of the UTI (*P. aeruginosa*) for > 2 years, was successfully treated with a combination of 6 phages from the Eliava Institute in Tbilisi collection [158]. Phage treatment was administered via catheterization every 12 h for 10 days, and meropenem was administered starting on day 6 through 30 and urine samples were negative 1 year later[158]. A 2-year long clinical trial of bacteriophages for treating UTI in patients undergoing transurethral resection of the prostate (NCT03140085) at the Tzulukidze National Center of Urology (Tbilisi, Georgia) concluded in 2017. Participants were treated with either an antibiotic, a phage (bacteriophage Pyo and adapted substrains of Pyo), or a placebo, the latter two were administered via catheterization for 7 days [159,160]. The study was unable to draw any statistically reliable conclusions, but it did conclude that phage treatment of UTIs might be effective and safe [161]. Phages have also been explored for their potential use in pretreating long-term catheters with phages to minimize bacterial biofilm development and catheter blockage, which can cause catheter-associated UTIs (CAUTIs) [162]. Catheters have been pretreated with phages that infect *P. aeruginosa* [163], *Proteus*

mirabilis [164], and *E. coli* [165] with varied success. The pretreatment of catheters with two phages were found to considerably reduce *P. mirabilis* biofilms for up to 168 hours post treatment [164].

Increased understanding of phage, microbiota, and human host interactions is imperative for the feasibility of phage therapy of urinary tract symptoms and infections. Phage therapy has the potential to combat antibiotic-resistant bacterial infections, and anecdotal evidence of its success certainly warrants further investigation [166]. Phage therapy has already proven effective in the treatment of bacterial infections in other areas of the human body. In the highly publicized case of a life-threatening *Acinetobacter baumannii* infection, all modern antibiotics were found to be ineffective and over a hundred phages were tested before the few phages capable of saving the patient's life were found [167]. Phage–drug cocktails are promising as well; for instance, such a cocktail was used to clear a vascular graft *P. aeruginosa* infection [168]. The *Pseudomonas* phage OMKO1, used in combination with ceftazidime, was able to completely clear the infection as bacteria resistant to the phage were more sensitive to ceftazidime and vice versa [168]. All phages infect their bacterial host by binding to surface receptors, a process called adsorption (**Fig. 1**); these receptor binding proteins are often quite specific, leading to a narrow range of hosts (strains or species) that a particular phage can infect [73]. This specificity is in direct contrast to broad-spectrum antibiotics and has the benefit of targeting the pathogen with no effect to commensal bacteria. However, this specificity means that phage therapeutics will probably have to be developed on a patient-by-patient basis. In the aforementioned *A. baumannii* case, nearly 100 *A. baumannii* phages (selected from a larger collection of phages known to infect multi-drug resistant *A. baumannii* strains) were screened against clinical isolates from the patient; the vast majority of the phages tested had no effect against the clinical isolates [167]. A phage therapy effective for a larger patient population (n>1) will, therefore, probably be a cocktail of phages, including phages capable of infecting different strains. Phage cocktails also provide the benefit of outpacing pathogen evolution, a strategy similar to that employed for the vascular graft infection [168].

Given the rise of antibiotic-resistance, phage therapy is a promising replacement or augmentation to antibiotic treatment for infections throughout the body, including infections of the urinary tract. Several clinical trials are or have been conducted, including the recent trial for UTIs [160,161], the results of which provide critical data for moving forward

[H1] Conclusions

Whole-genome sequencing and new enhanced culture methods have been of great benefit to the study of the microorganisms within the bladder and the rest of the lower urinary tract, but considerable work remains to be done. An ongoing debate is occurring surrounding the potential presence of vulvovaginal and/or skin bacterial contaminants in urine samples of the urinary microbiota, and the same conversation is relevant to the new study of the lower urinary tract virome. Most of the studies discussed herein, with a few exceptions [35,122,128,129], have used voided urine for isolation of lytic phages or sequencing of the urinary virome. To the best of our knowledge, the phage populations of adjacent anatomical locations have yet to be investigated so the rate of incidence of viral contamination is unknown. As we have just begun to explore the phage communities within the urinary tract, such considerations must be kept in mind. More samples of the urinary virome must be sequenced to

462 determine if, like in the gut [30], a core phage community exists within the bladder, the urethra, the
463 periurethral niche and adjacent urogenital niches. Only through such efforts can we fully ascertain what
464 a healthy and an unhealthy phage community consists of. Whether a shift from the lysogenic life cycle to
465 the lytic cycle is a cause or consequence of bacterial community dysbiosis or urinary symptoms in
466 currently unknown. Studies such as those by Moustafa et al. [36] and Garretto et al. [33] will be particularly
467 powerful in capturing the dynamics between phages and their hosts, increasing understanding of their
468 interactions. These studies should become increasingly attainable as the costs of sequencing continue to
469 decline

470 Knowledge of the phage communities within the lower urinary tract and their role in urinary tract health
471 is a vital first step in the development of new strategies to treat urinary symptoms and infections.
472 However, critical to effective and reliable phage therapy strategies is the understanding of the extant
473 beneficial microbiota. Phage therapies should ideally cause minimal to no disturbance of this
474 community. In contrast to broad-spectrum antibiotics, phages can be directed very narrowly toward a
475 specific pathogen within the community. Given the observed novelty of many of the phages sequenced
476 from urine and from the bladder [35,129], perhaps the genomes of the modifiers of urinary tract health
477 have already been sequenced. Our understanding of the phage population of the urinary tract is in its
478 infancy and future studies will highlight new areas of investigation.

479

480

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Competing interests

The authors declare no competing interests.

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Author contributions

A.G. and T.M.-E. researched data for the article, all authors made substantial contribution to discussion of content, wrote the article and reviewed and edited the manuscript before submission.

Tables

Factor	**Airway**	**Gastrointestinal tract**	**Oral cavity**	**Skin**	**Urogenital tract and/or vagina**

Total number of metagenomic viral contigs	268	29,107	48,904	2,461	422
Unique viral clusters*	70	3,645	6,963	210	68
Total number of genomes	107	510	886	491	101

862 **Table 1.** Current number of viral sequences from virome studies of HMP anatomical sites.

863 *Clusters correspond to genetically distinct groups. (Data retrieved from the Integrated Microbial
864 Genomes/ Virus (IMG/VR) system [90].)

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Figures

Fig. 1. Lytic and lysogenic cycles of phages and their impact on microbiota. Active phages infect a susceptible bacterial host, binding to surface receptors, and inject their DNA entering into the lytic or lysogenic life cycle. In the lytic cycle (left), the phage genome replicates, producing mature virions. The phage virons then burst the host cell and diffuse through the surrounding environment. Thus, the susceptible bacteria within the microbiota are killed, leaving resistant (or non-host) bacteria. Within the lysogenic cycle (right), the phage genome either integrates into the bacterial genome (prophage) or persists as an extrachromosomal plasmid. As the infected bacterial cell reproduces, the phage genome is also replicated. Cell divisions produce a population of bacterial cells harbouring the phage genomic material. Environmental factors can induce a lysogenic (or latent phage) to enter the lytic cycle.

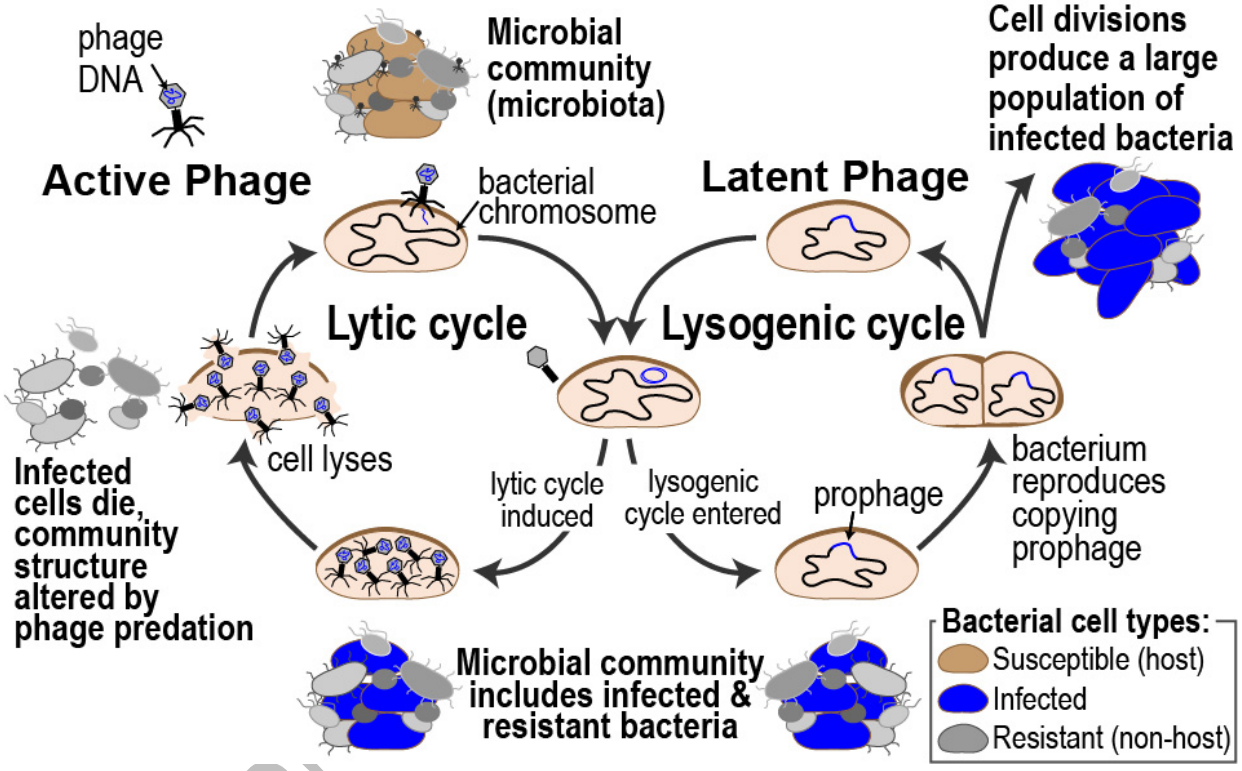

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Fig. 2 The process of transduction. In transduction, bacterial DNA is transferred from one cell to another by phages. Adapted with permission from Sirha et al. Nature Reviews Urology 15, 750–776 (2018) 169.

[Not shown here]

Fig. 3 Phage tail morphologies. Siphoviridae families have a baseplate at the distal end of the tail to which receptor-binding proteins (RBPs), such as tail fibres and tail spikes, are attached, tailless phages are just a capsid. Adapted with permission from Nobrega et al. Nature Reviews Microbiology 16, 760–773 (2018)[170].

[Not shown here]

Fig. 4. Bacteriophage Greed, isolated from catheterized urine microbiome sample. The phage's capsid (head) containing the phage genomic material can be seen, as well as the phage's tail structure. Tail fibers are not visible. The scale bar represents 50 nm. Samples were positively stained with 2% (wt/vol) uranyl acetate and observed at 80 kV using a Hitachi H-600 transmission electron microscope (TEM).