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The enigmatic roles of *Anelloviridae* and *Redondoviridae* in humans

Louis J Taylor[1,*], Emma L Keeler[2,*], Frederic D Bushman[2] and Ronald G Collman[2,3]

Anelloviridae and *Redondoviridae* are virus families with small, circular, single-stranded DNA genomes that are common components of the human virome. Despite their small genome size of less than 5000 bases, they are remarkably successful — anelloviruses colonize over 90% of adult humans, while the recently discovered redondoviruses have been found at up to 80% prevalence in some populations. Anelloviruses are present in blood and many organs, while redondoviruses are found mainly in the ororespiratory tract. Despite their high prevalence, little is known about their biology or pathogenic potential. In this review, we discuss anelloviruses and redondoviruses and explore their enigmatic roles in human health and disease.

Addresses
[1] Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, MD, USA
[2] Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA
[3] Department of Medicine, Pulmonary, Allergy and Critical Care Division, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

Corresponding Author:
Ronald G Collman (collmanr@pennmedicine.upenn.edu)
[*] Contributed equally.

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Introduction

The advent of culture-independent sequencing technologies has revealed remarkable depth and complexity of the human virome, the community of human-associated viruses that infect human cells or human-associated cellular microorganisms. Beyond discrete diseases caused by viral infection, current understanding suggests that the virome can be a key regulator of human health and disease, with studies associating virome composition with chronic inflammation and immunosenescence [1,2], and disease states, including ulcerative colitis [3,4], inflammatory bowel disease [5–7], diabetes [8,9], graft-versus-host disease [10], and cancer [11]. Numerous viruses are present in healthy individuals [12–14] and the virome may even play a beneficial health role in some contexts [15,16].

Two members of the human virome, *Anelloviridae* and the recently described *Redondoviridae*, are small circular DNA virus families of particular interest because they are both widely distributed or ubiquitous in humans, yet poorly understood. Despite their similarities, they differ in localization in the body, and only anelloviruses have been experimentally demonstrated to infect human cells [17–21]. Here, we will review these two virus families, including key questions outstanding for the field.

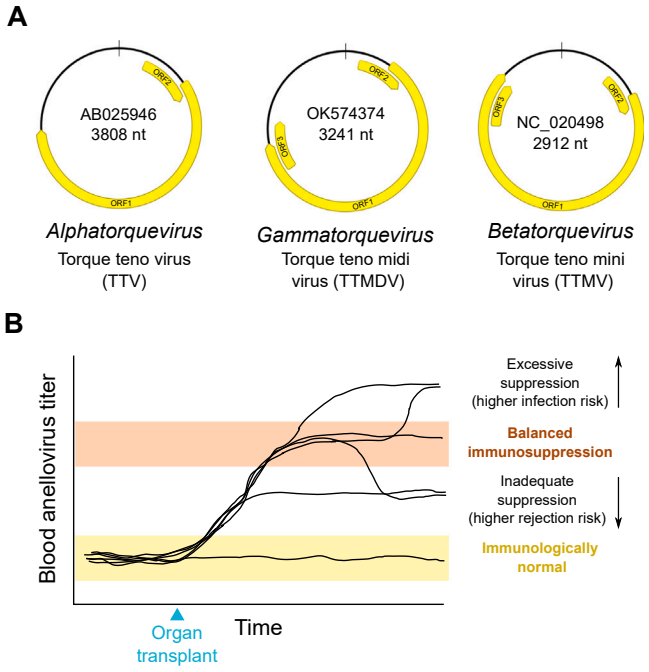
Anelloviridae: ubiquitous members of the human virome

Anelloviruses, members of the *Anelloviridae* family, are present in adult humans at rates approaching 90% in some cohorts and sample types [22–31]. Human anelloviruses were initially identified in 1997 in transfusion-associated hepatitis samples [32–34]. Subsequent studies revealed high prevalence globally, presence in multiple body sites, and persistence over time [28,29,35,36,37]. Knowledge about anelloviruses has been limited in part by its unclear role in human disease and the lack of a robust cell-culture system, although a recent report of anellovirus launched *in vitro* from a synthetic gene system should facilitate future progress [21].

Taxonomy and virology

Anelloviruses have circular, single-stranded DNA (ssDNA) genomes ranging from 2 to 3.9 kb in length. The *Anelloviridae* family is exceedingly diverse and includes multiple genera that are defined based on genome size and organization, and host species [38,39]. Multiple anellovirus genera infect humans and non-human primates (Figure 1a). The *Alphatorquevirus* genus was the first group discovered in humans and includes the torque teno viruses (TTVs) with genomes ranging from 3.7 to 3.9 kb in length, the *Gammatorquevirus* genus contains the torque teno midi viruses with genomes of approximately 3.2 kb, and *Betatorquevirus* includes the torque teno mini viruses that have the smallest genomes of approximately 2.9 kb [39,40]. Anellovirus genera and

Figure 1



Anelloviridae genome organization and potential utility as an indicator of immune status. **(a)** Map showing representative genomes for members of the three anellovirus genera that infect humans. Positions of open-reading frames are indicated by yellow arrows. **(b)** Model showing potential utility of anelloviruses as a biomarker of immune status. The black lines show different hypothetical trajectories of blood anellovirus titer during immunosuppressive treatment after organ transplantation. Shaded boxes indicate plasma anellovirus titer setpoints of normal immune function (yellow) and balanced, 'ideal' immunosuppression (orange), with regions carrying risk of inadequate or excessive immunosuppression as indicated.

species are demarcated by the ORF1 nucleotide sequence, with 44% and 69% identity separating anellovirus genera and species, respectively [39,41]. Currently, 33, 16, and 15 species are recognized in the *Alphatorquevirus*, *Betatorquevirus*, and *Gammatorquevirus* genera, respectively (NCBI Taxonomy, Apr 2021). Anelloviruses are also widely distributed in other animal species, including examples such as Gyrovirus, which includes chicken anemia virus, a fowl pathogen [42]; *Kappatorquevirus* and *Iotatorquevirus* that infect swine and may cause or exacerbate porcine disease [39,43]; and *Lambdatorquevirus* that infects sea lions [44], among others.

The cell type in which human anelloviruses replicate is likely an immune cell, but the host-cell identity is unclear with several cell types implicated in *ex vivo* samples [18,20,28]. *In vitro* replication of anellovirus has been observed in stimulated primary peripheral blood mononuclear cells [17,19,45]. Anellovirus genome replication and recombination has also been detected in cell lines [46,47], although no robust cell-culture system or reverse genetic system exists. Since other small circular DNA viruses can be rescued from cloned DNA [48–50], including porcine anelloviruses [51], a reverse genetic system for human anelloviruses is potentially achievable.

Anellovirus genomes encode multiple proteins in overlapping reading frames. The largest, ORF1, is conserved across anelloviruses. Although specific functions of ORF1 have not been tested *in vitro* or in culture, the presence of an arginine-rich region reminiscent of those in some small DNA virus capsid proteins suggests that ORF1 may have a role in capsid structure [52]. Spliced mRNA products have been identified in studies of an anellovirus isolate, TTV HEL32, a member of the *Alphatorquevirus* genus [53]. Anelloviruses encode two or three additional ORFs that overlap with either the 5′ or 3′ end of ORF1. The presence, length, and sequence of these ORFs often differ between different genera. In *Alphatorquevirus*, ORF2 may promote viral infection by counteracting host immune responses [54]. Other nonstructural proteins have been implicated in the induction of apoptosis in infected cells [55,56]. A detailed overview of anellovirus proteins, splice forms, and potential functions is available in [57,58].

Replication mechanisms are not well-defined for anelloviruses. By analogy to other small circular ssDNA viruses whose replication cycle is better understood, anelloviruses contain genomic structural elements similar to those involved in initiation of virus replication [52,59]. However, in circular Rep-encoding single-stranded (CRESS) DNA viruses, those structures are nearly universally stem loops [52,60–62], distinct from the T-shaped structure identified in anellovirus genomes [59]. The DNA stem loops in CRESS viruses are recognized by the cognate replication-associated (Rep) protein, which cleaves the stem loop to initiate a process known as rolling-circle replication (RCR). Anelloviruses do not encode a classical Rep protein. While RCR motifs similar to those in CRESS virus Rep proteins have been identified in anellovirus ORF1 proteins [63,64], the anellovirus ORF1 does not contain a helicase domain, which is required for replication in CRESS viruses [65,66]. Additionally, the N-terminus of anellovirus ORF1 is arginine-rich and positively charged, properties common in CRESS virus capsid proteins [61,62]. However, direct analogy between anellovirus ORF1 and viral proteins from other ssDNA viruses is not possible and more work is required to understand the function(s) of ORF1, as well as the mechanisms of anellovirus replication.

Interactions between anelloviruses and their human hosts

Human anelloviruses were first identified in blood samples and transfusion recipients [32–34]. Subsequent studies illuminated the ubiquity of anelloviruses in diverse contexts, both geographically across human populations [24–26,28,31] and spatially across human body sites ranging from saliva, to blood, and organ-tissue samples [22,23,27,30]. Anellovirus prevalence in adult populations often exceeds 90% [24,26,28,29]. In contrast, anelloviruses are much less common in infants and young children under two years of age [14,23,67], and prevalence has been shown to increase with age [37,68]. In early life, acquisition of anelloviruses likely occurs through peripartum, oral/fecal, and breastfeeding routes [69–71]. Increased anellovirus abundance and diversity was observed in infants delivered by standard vaginal delivery compared with C-section, suggesting that vertical transmission may occur during vaginal delivery [72]. On the other hand, a recent study of infants found that the earliest positive blood sample was at approximately 1 month of age and increased to nearly all infants by 12–18 months [73]. Anelloviruses are also transmitted parenterally (including organ transplantation) and may also be sexually transmitted [74–79].

Anelloviruses exhibit extreme genetic diversity, both between and within individuals, and people can be colonized contemporaneously by multiple different anellovirus strains [31,78,80]. Anellovirus communities are highly individualized; strains are rarely shared between unrelated individuals [31,78,81], though family members show greater overlap of anellovirus strains [82]. Studies of patients undergoing organ transplants and blood transfusions show transmission of donor strains that intermix with recipient strains [31,78,79]. Anellovirus lineages can be quite stable within individuals over time, persisting for months or years [31,78,83]. The persistence of individual lineages, together with evidence of anellovirus transmission and colonization via transfusion and other routes, suggests that the swarms of anellovirus present within adult humans are likely the product of both evolution of existing lineages and introduction of new strains over time.

One hypothesis for the existence of diverse anellovirus communities within individuals is that it may enable anelloviruses to evade eradication by the immune system. Increased blood anellovirus levels following immunosuppressive treatment and in states of immune deficiency such as AIDS indicate that anelloviruses are under tonic immune control [30,83–86]. The relationship between anelloviruses and the human immune system is complicated by the fact that anelloviruses likely replicate in an immune-cell type [17,45], as evidenced by acutely decreased anellovirus titers with myeloablative chemotherapy [87,88]. Antibody responses have been detected against anellovirus proteins, though with variable prevalence compared with the ubiquity of viral DNA [89–92], but part of this variability may be technical due to the challenge of designing reagents that capture the diversity of anellovirus-protein sequences. In contrast, despite the evidence for immune control, little is known about T-cell-mediated or other immune responses to anelloviruses.

Anelloviruses in humans: commensal or pathogen?

Anelloviruses are common in healthy people, so definitively linking them to disease is challenging. Although first identified in cases of transfusion-transmitted hepatitis [32–34], subsequent studies failed to confirm a pathogenic role in hepatitis [93,94]. Elevated anellovirus levels are reported in blood or tissue in multiple diseases, including pulmonary fibrosis, asthma, COVID-19, and others [95–97], but this finding may simply reflect inflammation or other drivers of viral activation, and current evidence does not suggest a causative role by themselves in human disease [58,98,99].

As a likely commensal that establishes persistent colonization of humans at very high prevalence, anelloviruses are resoundingly successful viruses and could be a useful model for persistent viral infection. Conversely, what effect lifelong infection with these diverse variants has on shaping the immune system (which clearly recognizes and exerts some control) has not been explored. Although anellovirus strains are generally very individual-specific, Arze et al. [31] identified lineages (defined as > 97.5% identity of the ORF1 nucleotide sequence) in blood-donor samples that were highly similar to those of the transfusion recipient, and found no difference in transmission likelihood based on similarity or divergence from strains already present in the recipient. This finding suggests that there is little immunity to superinfection or superinfection exclusion. Combined with the observation of anellovirus persistence, these observations raise the possibility that anelloviruses might be useful as gene-therapy vectors.

Anelloviruses as a potential clinical biomarker of immune function

One of the most interesting areas of anellovirus study is the strong link between blood anellovirus levels and immune competence, whether due to disease or to medical treatments. This observation has led to the notion that monitoring blood anellovirus levels could be used to titrate therapeutic immune suppression in organ-transplant patients, where it is challenging to achieve a balance between suppressing organ rejection and excessive susceptibility to opportunistic infection [30,84,100–102]. Multiple studies have demonstrated that anellovirus blood levels increase with therapeutic

immunosuppression, and show a relative drop before episodes of acute organ rejection, implying increased immune competence that may drive rejection. Thus, it is plausible that anellovirus levels could provide a measure of 'integrated' immune function (despite lack of knowledge on the specific mechanisms of immune control) and serve as a clinically useful biomarker with which to titrate immunosuppressive therapy in organ transplantation [103–106] (Figure 1b).

Redondoviridae: a new virus family found in the human ororespiratory tract

Redondoviridae, named for its circular genome structure ('redondo' for round in Spanish), is a newly described viral family that recently was identified in metagenomic data [62,107] (Figure 2a). It is taxonomically located within the *Cressdnaviricota* phylum, which was recently created to unify seven families of CRESS DNA viral genomes [108]. *Redondoviridae* is the lone family in the *Recrevirales* order, and contains a single genus, *Torbevirus* with two species, *Brisavirus* and *Vientovirus*. First reported in 2017 in throat and saliva metagenomic sequences from a single patient with respiratory symptoms [107], redondoviruses have since been detected in both healthy and disease specimens across distinct populations, locales, and ororespiratory sample types, with prevalence rates ranging from 2% to 82%, depending on sample type, method of detection, and population studied [62,109–111] (Figure 2b). The known global distribution of redondoviruses includes the United States, Spain, Italy, the United Kingdom, Vietnam, China, Cameroon, Botswana, Tanzania, and Ethiopia [62,109–115].

Genome structure

Redondoviridae have circular DNA genomes of approximately 3.0 kb [62,116] and by analogy with other CRESS DNA viruses are inferred to be ssDNA. The genome contains three ambisense ORFs separated by intergenic regions (Figure 2c). The ORFs encode a 449–531 amino acid putative capsid protein (Cp), a 334–363 amino acid replication-associated protein (Rep), and a third ORF (*ORF3*) that encodes a 200 amino acid protein with no identifiable homology to any other protein sequences in the NCBI database or conserved predicted functional domains. The *Cp* and *Rep* ORFs are oppositely oriented, with *ORF3* overlapping the 5′ end of the capsid gene in a separate reading frame [116].

The redondovirus genome contains a stem-loop structure with a conserved nonanucleotide motif (5′-TATT ATTAT-3′) located at the beginning of the *Rep* coding sequence. The inverted repeat that forms the stem varies in length, whereas the nonanucleotide motif is conserved [62]. The stem-loop structure is a candidate for the origin of replication (ori), as has been reported in the related porcine circovirus [117]. Knowledge of the genome expression and virion structure remains limited, mainly owing to its very recent identification and lack of a cell-culture propagation system.

Redondovirus replication

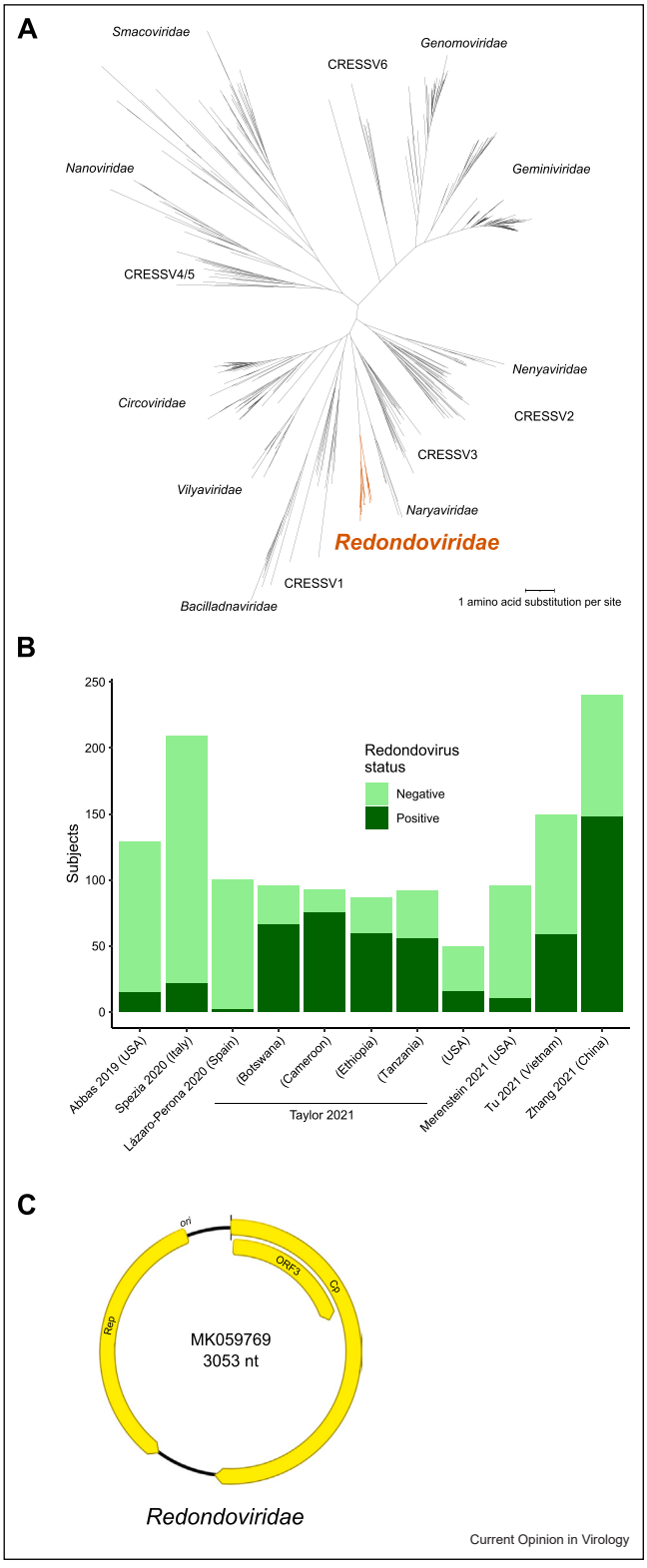
The redondovirus Rep protein contains two conserved domains, an RCR endonuclease and a superfamily-3 helicase domain [118,119]. The N-terminal RCR domain possesses three conserved motifs. Motif I is thought to recognize the ori, Motif II contains histidine residues required for coordination of divalent metal ions, and Motif III contains the catalytic tyrosine residues crucial in Rep function [61,118]. The Rep protein was recently shown to catalyze multiple reactions *in vitro*, which is consistent with its presumed role in mediating redondoviral replication and recombination [111].

Redondoviruses are predicted to replicate via a rolling-circle mechanism, which is characteristic of related circular ssDNA viruses such as porcine circoviruses [60,119]. RCR is a process of unidirectional replication of a circular nucleic acid molecule. According to the standard RCR model, upon viral entry and uncoating, the ssDNA is converted to dsDNA by the host polymerases. The viral Rep protein binds to the stem-loop structure, nicking the dsDNA and generating a free 3′-hydroxyl end from which DNA synthesis can begin. This initial component of the RCR model was validated for redondovirus Rep *in vitro* [111]. Next, the Rep protein remains covalently bonded to the 5′-phosphate end; after the first round of DNA synthesis, the Rep protein releases one ssDNA genome, and the dsDNA is available for additional rounds of RCR [120]. This Rep-mediated cleavage rejoining has been hypothesized to enable recombination events, evidence for which is observed in longitudinal redondovirus sequences generated by single-genome amplification from human subjects [111].

Redondoviruses in humans

Redondoviruses were discovered through metagenomic sequencing and subsequently identified more broadly by PCR-based surveys. Redondoviruses are found commonly in the oral cavity and respiratory tract of humans, and infrequently in the gut, but are absent in blood, vaginal, skin, and other metagenomic datasets [62,97,110,111]. While CRESS DNA virus sequences are common environmental or laboratory contaminants [121,122], the lack of redondovirus detections in environmental samples, contamination controls, and laboratory reagents suggests that they are authentically present in human samples [62]. Redondoviruses lack features of bacteriophages such as Shine–Dalgarno sequences [62] or representation in CRISPR spacer databases [123], implying a eukaryotic host cell. However,

Figure 2



Redondoviridae phylogeny, genome organization, and prevalence across studies. **(a)** Phylogeny of Rep protein sequences from members of the Cressdnaviricota order. Rep protein sequences were aligned with MUSCLE (v3.8) [133], followed by tree construction using RaxML (v8.2) [134] and visualization using iTOL (v6) [135]. The *Redondoviridae* branches are colored orange. A FASTA file of the Rep amino acid sequences used to build this tree was adapted from prior publications [122,136] and is available on GitHub: https://github.com/louiejtaylor/cressdnaviricota_reps. **(b)** Redondovirus prevalence by qPCR in ororespiratory samples across studies. Specimens varied among studies, including saliva, gingival tissue, naso- and oropharyngeal swabs, sputum, and endotracheal aspirates. Numbers are aggregated from all conditions tested in the cited studies, including healthy and a diversity of disease conditions. **(c)** Redondovirus genome map. Positions of open-reading frames are indicated by yellow arrows. The stem-loop putative origin of replication initiation is labeled (ori).

presently no *in vitro* replication model exists and their definitive host cell within the ororespiratory tract remains unknown. Even whether they grow in human cells is unclear.

Redondovirus appears to establish a chronic colonization state, with persistence of lineages showing > 99% nucleotide identity (proposed as the cutoff for strains [111]) over periods of 2–3 weeks, 5 months, and even up to two years [111,113]. At the same time, longitudinal sampling showed that distinct redondovirus species (> 25% nucleotide divergence) can be present in individual subjects contemporaneously, that different strains and species can be present at different times, and that recombination may occur between them [111,113]. Thus, the human ororespiratory tracts exhibit both stable and dynamic redondovirus colonization over time.

A wide range of nonhuman species have been surveyed metagenomically without consistent detection of redondovirus sequences, including domestic animals, nonhuman primates, urban and other wildlife, aquatic animals, insects, and plants [62,113,114]. A single redondovirus genome was reported from honeybees [124], although two metagenomic datasets comprising 39 honeybee samples have since been screened for redondoviruses (BioProject IDs: PRJNA599270, PRJNA407112) without detections of redondoviral sequences (data not shown). Similarly, a single redondovirus genome was identified in a porcine serum sample [125], although another study screening respiratory samples from 27 pigs by PCR did not detect Redondovirus [113]. Further surveys of nonhuman samples for redondoviruses are needed to understand the distribution of redondoviruses across the biosphere.

Redondovirus-disease associations

Redondoviruses have been detected in ororespiratory specimens both in healthy people and patients with various illnesses, with elevated levels seen in multiple conditions, including organ-transplant donors, lung-transplant recipients, febrile patients, and subjects with rheumatoid arthritis, inflammatory bowel disease, HIV infection, critical illness, respiratory and

oropharyngeal diseases, COVID-19, and periodontitis [62,97,109,110,115]. For example, both mechanically ventilated organ donors and critically ill patients harbor markedly higher levels of respiratory-tract redondoviruses compared with healthy counterparts [62,97]. However, any causal links between redondoviruses and human disease have not been established.

A particularly striking redondovirus association is with periodontal disease. Abbas et al. [62] analyzed shotgun sequencing data from three studies that queried gingival or oral samples from patients with or recovering from periodontal disease [126–128]. In all three studies, redondovirus sequence reads were high before periodontitis treatment and decreased dramatically in those who improved after treatment. Zhang et al. used nested PCR and found significantly higher prevalence of redondovirus in chronic periodontitis patients compared with healthy people [115]. Several human viruses have been implicated in contributing to progression of periodontal disease [129-132], and thus, the relationship between redondoviruses and periodontitis warrants further investigation.

Conclusions

Metagenomic sequencing has brought to light unappreciated complexity in the human virome, including highlighting previously unrecognized but highly prevalent new viruses. It is also increasingly recognized that there exists a large universe of small circular DNA viruses, including some associated with humans. *Anelloviridae* and *Redondoviridae* are two small circular DNA viruses that are highly prevalent and widely distributed in humans, exhibit extensive genetic diversity, and establish chronic colonization with both stable and dynamic features. However, there are significant differences between these families. Anelloviruses have been subject to considerable study, are widely distributed in body compartments and tissues, and have complex interactions with the human immune system, but have not yet been directly implicated in any pathogenic process. Whether and how they might influence healthy physiology is unknown, but their levels are closely linked to immune competence, raising the possibility that they might have clinical utility as biomarkers of immune function. Considerably less is known about redondoviruses, which are mainly localized to the oropespiratory tract. While redondoviruses are elevated in several disease states, most notably periodontitis and critical illness, it remains unknown whether they are recognized by the human immune system, contribute to disease, or even what cell(s) serve as their host at these mucosal surfaces. Future studies will be needed to further elucidate the enigmatic role of these ubiquitous viruses in human health and disease, as well as address the possibility of additional virus families lurking in the human virome.

Conflict of interest statement

The authors declare no conflict of interest.

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