

**Understanding the Enigmatic Anelloviridae**

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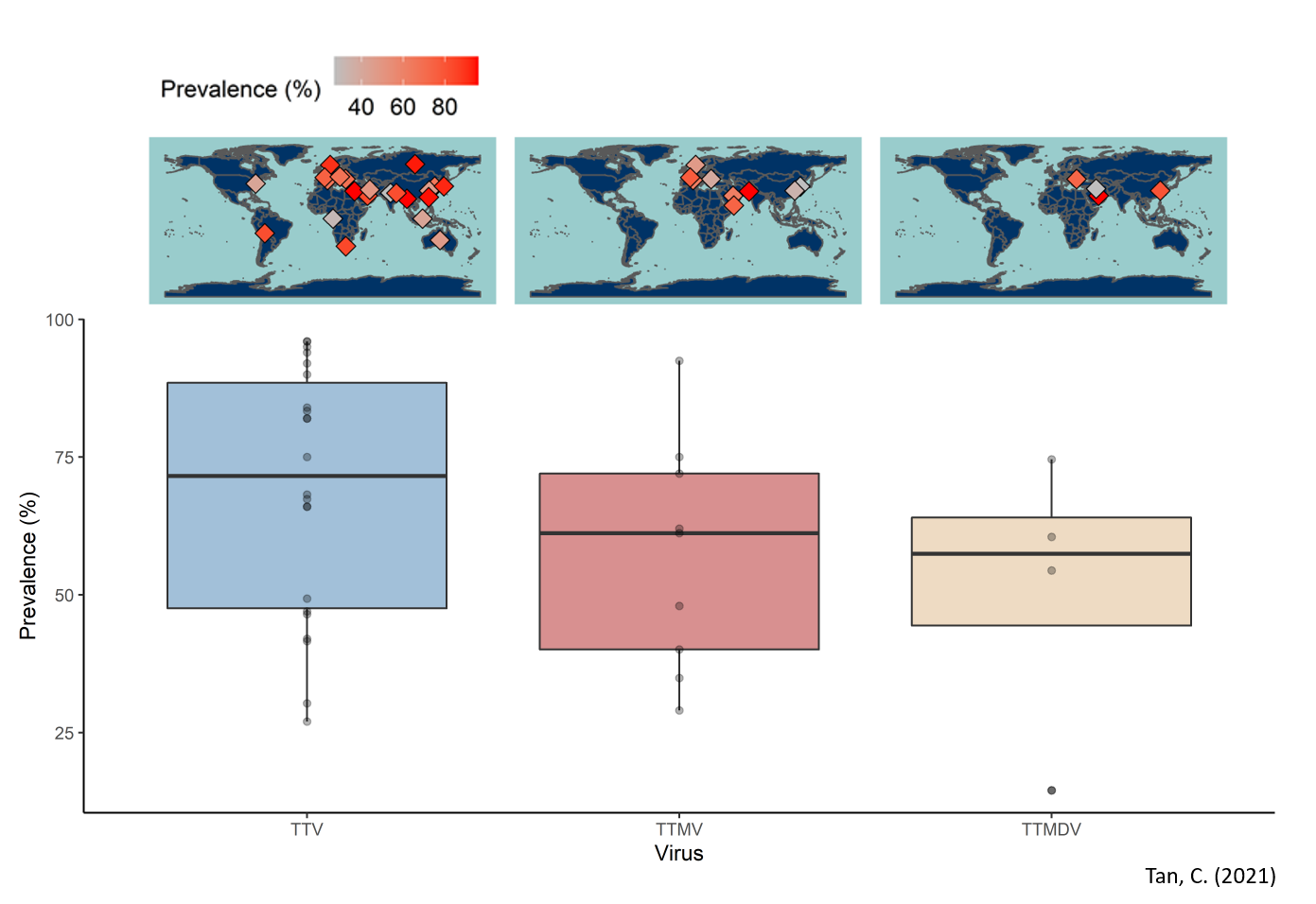
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# Abstract (200 words)

# Introduction (430 words)

The Anelloviridae is a diverse family of small, non-enveloped viruses comprising 14 genera and 76 species as of the 2019 release of the International Committee on Taxonomy of Viruses (ICTV) virus taxonomy [1]. The word ‘anello’ is Italian for ‘ring’, which refers to their circular, negative-sense, single-stranded DNA (ssDNA) genomes. As of 20 Oct 2020, there were 1143 complete anelloviruses genomes of length ranging from 1.5-4 kilobases deposited in the *NCBI Virus* database.

Anelloviruses infect a diverse range of hosts, including humans [2], rodents [3], bats [4], and livestock [5,6]. The first and most well-studied anellovirus is the human torque teno virus (TTV), which was discovered in the plasma of a hepatitis patient in 1997 [7]. The torque teno mini virus (TTMV) and torque teno midi virus (TTMDV) are also known to infect humans. Human anelloviruses are highly prevalent in the human population. Indeed, consolidation of the results from 26 studies found that the prevalence of TTV, TTMV and TTMDV were in the range of 27-96%, 29-92.5%, 14.5-74.6%, respectively (**Figure 1**). Despite this, there has been no convincing evidence thus far implicating human anellovirus infection in disease. The torque teno sus virus (TTSuV) in pigs and chicken anemia virus (CAV) in chickens are also anelloviruses in widespread circulation globally. The former has been associated with postweaning multisystemic wasting syndrome in pigs [8] while the latter has been found to cause immunosuppression and anemia [5].



**Figure 1. Global distribution of human anelloviruses**. Prevalence estimates of human anelloviruses in healthy individuals were obtained from 26 independent studies and used to generate this figure. The world maps were generated using the *ne\_countries* function from the *rnaturalearth* package in *R*.

The high prevalence and transmissibility of anelloviruses around the world warrants further surveillance. Indeed, it is possible that an animal anellovirus may acquire the ability to infect and transmit efficiently amongst humans, resulting in the emergence of a new human infectious disease that significantly affects public health. Even though global health efforts mainly involve post-emergence outbreak control [9], it is prudent to pre-empt and mitigate the emergence of the next infectious disease. However, our understanding of anelloviruses is limited relative to other more prominent pathogens. In fact, there are currently no stable cell culture systems or animal models available [10–12]. This review will draw insights from other viral families to explore the limits of our current knowledge about the *Anelloviridae*. Additionally, possible approaches to expand this knowledge will be outlined. Finally, the potential for anelloviruses to cause the next human infectious diseases will be discussed.

# **The genome, its proteins, and their interactions with the host**

## Genome structure and replication (915 words)

Anelloviruses possess two main open reading frames (ORFs), ORF1 and ORF2, but can have up to four ORFs in their genome [10]. These ORFs are found within a coding region demarcated by a TATA-box and poly-A tail sequence. Additionally, there is an untranslated region (UTR) containing a GC-rich region [13]. Transfection experiments of human TTV found that at least six proteins are generated by alternative translation initiation [14] on three mRNA species, which were produced by alternative splicing [15]. Based on the TTV genome structure and gene nomenclature (**Figure 2a**), ORF1 putatively encodes the capsid protein that contains an arginine-rich N-terminus [12], and a Rep-like protein. Additionally, ORF2 encodes a dual-specificity phosphatase [16]. Both ORFs is believed to be conserved across all anelloviruses [10]. ORF3 encodes the TTV-derived apoptosis-inducing protein (TAIP) [17,18], which is homologous to apoptin in CAV…...

Currently, the genome replication mechanism of anelloviruses is poorly understood relative to other families of circular ssDNA viruses such as *Geminiviridae* [19] or *Circoviridae* [20]. Fundamentally, anelloviruses are dependent on their hosts replication machinery since TTV replication is inhibited by administering the DNA polymerase inhibitor, aphidicolin [21]. It is believed that anelloviruses replicate via a rolling circle replication (RCR) mechanism. This is because anelloviral genomes contain a conserved motif that is part of a stem-loop structure, and a Rep-like sequence, both of which are found conserved in other circular ssDNA replicons including plasmids and viruses. In a hypothethical mechanism for anelloviral replication (**Figure 2b**), the negative-sense ssDNA anellovirus genome into double-stranded DNA (dsDNA) intermediate by host enzymes (A). Subsequently, a Rep-like protein nicks one of the two DNA strands at the origin of replication (ori) to generate a 3’OH, which acts as a primer for host DNA polymerase to incorporate new nucleotides using the other DNA strand as a template (B). As the former DNA strand is elongated, the parental strand is displaced (C). After a round of replication (D), the displaced parental strand is cleaved at the ori, the resultant 5’ and 3’ ends covalently linked, and the molecule released as circular DNA (E). The newly synthesised strand can then proceed for another of replication.



**Figure 2. Genome structure and replication.** (a) The complete genome of TTV retrieved from NCBI GenBank (NC\_002076.2) was visualised and annotated using UGene [22]. (b) Hypothetical model of RCR for anelloviruses. Annotations: host DNA polymerase (DNAP); Rep-like proteins (Rep); origin of replication (ori).

Anelloviruses have conserved motifs within a stem-loop structure, reminiscent of other circular ssDNA replicons like plasmids or viruses that replicate via RCR. For example, CAVs and TTVs contain the conserved nona- and octanucleotide motifs, 5‘-TACTATTCC-3’ and 5’-AGTTTAAA-3’, respectively. The latter motif was proposed to contain the TTV origin of replication at the +5 position [23]. These motifs resemble the origin-containing nonanucleotide motifs in multiple other circular ssDNA replicons [23–25] (**Figure 3a**). Additionally, the nonanucleotide motifs in circular ssDNA replicons tend to be found within stem-loops, which are a type of DNA secondary structure. The proposed stem-loop structure for porcine circoviruses (PCVs; family *Circoviridae*) [25] is shown in **Figure 3b**. These stem-loop structures have been shown to be essential for RCR. For instance, mutations in PCVs [26] and tomato golden mosaic virus (family *Geminiviridae*) [27] that significantly affected these stem-loops disrupted RCR. Conversely, mutations that did not affect the stem-loop structures allowed RCR, suggesting that structural conservation is more important than sequence conservation. RNAStructure [28], a tool for predicting nucleic acid secondary structure, predicts that both the CAV nonanucleotide and TTV octanucleotide motifs are within similar stem-loop structures (**Figure 3c** and **d**), which further supports an RCR mechanism for anelloviruses.



**Figure 3.** (a) Manual alignment of the octanucleotide and nonanucleotide motifs conserved in anelloviruses and other ssDNA families, respectively (right). Abbreviations: chicken anemia virus (CAV); torque teno virus (TTV); porcine circovirus (PCV); coconut foliar decay virus (CDFV); subterranean clover stunt virus (SCSV); banana bunchy top virus (BBTV). (b) Proposed stem-loop structure containing the nonanucleotide motif in PCV (U49186.1). Predicted secondary structures of the region surrounding the octanucleotide and nonanucleotide motif in TTV (c; NC\_002076.2) and CAV (d; AY843527.2), respectively. Structure prediction for TTV and CAV was performed using default parameters and all structures were renderred using RNAStructure [28]. Lower case nucleotide letters in (b) indicate the nucleotides that were forced to be single-stranded during rendering. (e) Mechanism underlying HUH endonuclease catalytic activity. M2+ indicates a divalent metal cation.

Anelloviral genomes also encode proteins [29–33] that resemble the replication (Rep) proteins crucial for RCR in other circular DNA replicons [34]. In particular, anelloviruses possess the conserved FTL and FXXK motifs [35] found in *Circoviridae* and *Geminiviridae* Rep proteins [36]. Of the nine known families of eukaryotic DNA viruses other than the *Anelloviridae[[1]](#footnote-1)*, all except *Bidnaviridae* are believed to possess Rep proteins of the HUH endonuclease superfamily [37]. As such, it is tempting to speculate that anelloviral Rep-like proteins are also of this superfamily. If true, a potential catalytic mechanism for these proteins can be modelled of that for HUH endonucleases [38]. During RCR, HUH endonucleases recognise dsDNA via a dsDNA-binding domain, and catalyse the breaking or joining of ssDNA via a series of reversible steps **(Figure 3e)**. To nick an ssDNA substrate, a catalytic tyrosine performs nucleophilic attack on the phosphorus of the substrate, resulting in the formation of a negatively charged intermediate. This intermediate is stabilised by a divalent metal cation (*e.g.* Mg2+, Mn2+) coordinated by the HUH motif, which contains an invariant histidine, an invariably polar residue (denoted U), and a second histidine or a glutamine [38]. Given the essential role of Rep proteins in RCR, a possible first step to elucidating the replication mechanism of anelloviruses could involve the identification of domains in anelloviral Rep-like proteins that share structurally homology with HUH endonucleases. This requires a protein structure solution that has not yet been described.

## Host-virus interactions

* No evidence for pathogenicity to humans; studies have only shown that TTV is marker of immune status
* Synergistic co-infection causing fatal postweaning multisystemic wasting syndrome in pigs, does not cause disease by itself
* Chicken anemia virus causes atrophy of thymus and bone marrow
* All proteins are antigenic [39] but infections are persistent 🡪 immune evasion
* Mechanisms for regulation of innate immunity
* Mechanisms for regulation of cell-mediated immunity
* ORF2 found in all anelloviruses [10]
* CAV VP2, TTMV ORF2 are dual specificity phosphatases [16]
* TTV miRNA decreases interferon production; TTV miRNA promotes cell proliferation in response to interferon [40]
* ORF3 is TAIP/ apoptin
* Transfection of ORF1 into swine macrophage line induced early anti-viral response as part of host innate immunity
* ORF1 upregulates SOCS-1, PD-1, IL-10, which are suppressors of T-cell mediated immunity
* ORF2 downregulates IFN-β and IL-13, regulators of antiviral activity and inflammation, respectively [41]
* TTV isolate and look for potential binding site targets for human mRNA transcription factors using the online PROMO program, which recognizes transcription regulatory element binding sites from DNA sequences
* 44 dsDNA sequences, homologous to human DNA transcription regulatory protein target binding sites, were identified [42]. Moreover, each homologous target sequence appeared multiple times (4 to 11 copies per target). Among these homologous targets on TTV-TA278 DNA were genetic elements for binding factors regulating synthesis of CREB (which plays an essential role in embryogenesis, cell differentiation, apoptosis, and proliferation), c-Jun (a proto-oncogene product with antiapoptotic activity), c-Myc (a proto-oncogene product that also regulates B cell proliferation), IRF2 (which competitively inhibits the IRF1-mediated transcriptional activation of IFNα and IFNβ), p53 (which has anticancer function and plays a role in apoptosis and genomic stability), STAT4 (which is required for the development of Th1 cells from naive CD4+ T cells and for IFNγ production in response to IL-12), FOXP3 (a lineage specification factor of Treg cells), and ATF3 (which represses the genes for IL-6, IL-12, and other cytokines downstream of TLR4, thereby providing a negative feedback to contain excessive inflammatory responses).
* TTV DNA used can effectively activate proinflammatory gene transcription via TLR9 in immune cells
* Lower CpG motifs in TTV than expected than based on random base usage. TLR9 binds unmethylated CpG motifs on DNA and triggers proinflammatory cytokines like IFNγ. Reduce stimulatory effect of CpGs on genome [43]
* However, we cannot rule out that human anelloviruses can alter disease progression or severity

**Figure 4.** Host-viral interactions.

# **Accumulation of genomic diversity**

## Genomic diversity (255)

Even though anelloviruses have similar genome structures, there is great sequence divergence within the family. Indeed, the amino acid sequence diversity within human TTVs is approximately 7.6, 2.2 and 2.1 fold greater than that for hepatitis B virus, human immunodeficient virus-1 and hepatitis C virus, respectively [11]. This diversity poses a major challenge for full-genome alignments during the reconstruction of evolutionary relationships, which are visualised in the form of a phylogeny. Typically, a phylogeny is reconstructed by first generating an alignment of the sequences in question. This alignment is then used as input in tree-building tools such as RAxML [44] or IQ-TREE [45] to reconstruct the evolutionary relationships of the sequences in the form of a phylogeny (**Figure 4a**). Due to the vast genomic diversity in the *Anelloviridae*, the ICTV recommends restricting family-wide phylogenetic reconstruction analyses to ORF1 and a 35% nucleotide sequence identity threshold to demarcate viral species [1]. Several studies have presented a family-wide phylogeny [10,46–49] in accordance with this. However, doing so does not consider the full-length genomic diversity of the family. Additionally, sequence alignment algorithms are known to have limited accuracy when used for aligning highly divergent sequences [50]. Moreover, as will be discussed below, there is evidence for recombination in anelloviruses. This has significant implications for phylogenetic inference since recombination results in portions of genomes representing different evolutionary histories. As such, recombinant genomes cannot be expressed by a single phylogeny (**Figure 6b**) [51]. In such cases, the resultant phylogeny topologies obtained often differs from the true phylogeny [52], especially when recombination occurs between divergent sequences [51], as in the case of anelloviruses. Given these challenges, alignment-free approaches may be more suitable for phylogenetic reconstruction. As the name suggests, alignment-free approaches quantify the similarity between sequences without the use of an alignment [50]. One common type of alignment-free approaches involves the splitting of each full-length sequence into short sequence blocks called *k*-mers (**Figure 4b**). A summary statistic representing the sequence dissimilarity (*i.e.* distance) between a pair of sequences can derived from how many of these *k*-mers are shared between said sequences. This distance is calculated for every possible pairwise combination of sequences and subsequently used to reconstruct a phylogeny via algorithms such as the neighbour-joining method [53]. Since alignment-free approaches are relatively robust to high sequence diversity and are minimally affected by recombination [50], they may be more appropriate for future studies investigating the family-wide diversity of the *Anelloviridae*.

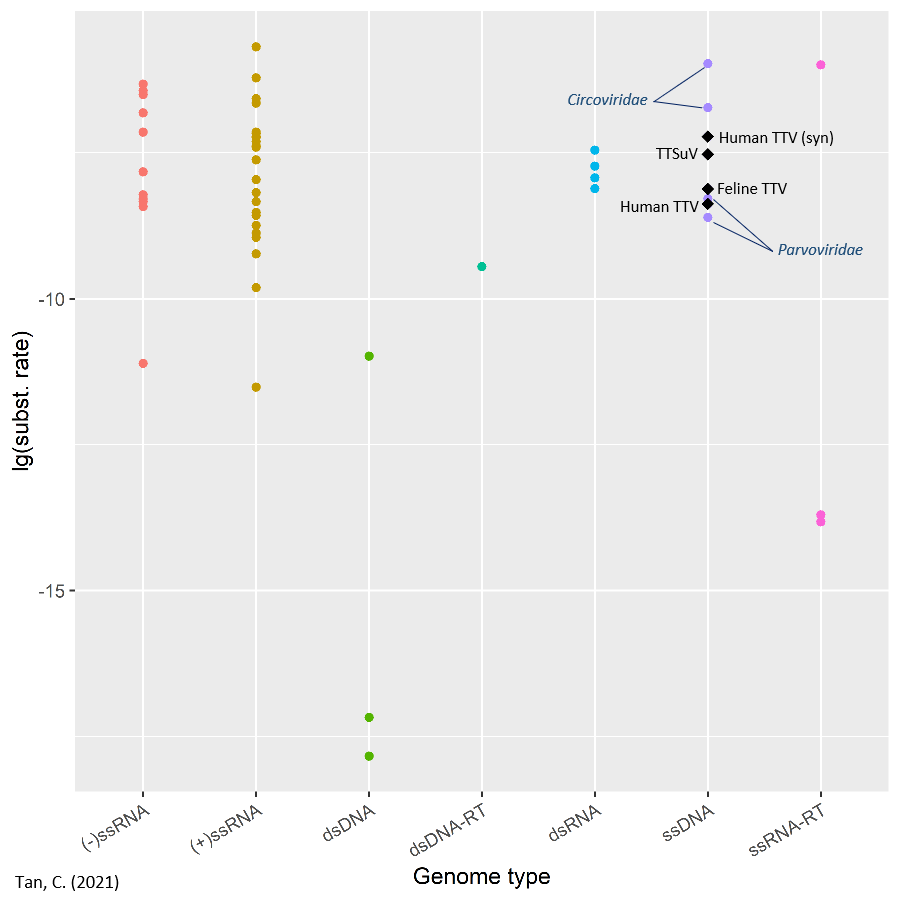
Notably, many sophisticated methods in population genetics such as those for phylogenetic dating [54] or inferring past population dynamics [55,56] are alignment-dependent. As such, future studies seeking to employ such methods could consider extracting conserved ‘core’ genes or genomic regions from the ‘un-alignable’ viral sequences (**Figure 4c**) using gene annotation tools such as *Prokka* [57], *Roary* [58]or *Mauve* [59]. These ‘core’ genes can then be combined to produce a concatenated genome alignment. Such an alignment would capture a larger proportion of the viral genomes and hence their genomic diversity while allowing downstream alignment-based analyses. Such approaches have been previously used on highly divergent members of microbial communities [60] and recently for the family *Coronaviridae* [61]. This can be done and would allow the construction of a sequence alignment that retains a larger proportion of the viral genomes. A potential ‘core’ gene alignment for anelloviruses may include regions within ORF1 and ORF2, which are found in all anelloviruses [10]. Additionally, to minimise the impact of recombination, mutations that occur independently and repeatedly in different evolutionary lineages (*i.e.* homoplasies), or genomic sites identified by recombination detection tools such as *PhiPack* [62], can be removed.



**Figure 4. Phylogenetic analyses.** Simplified workflows of (a) typical alignment-based and (b) alignment-free approaches. (c) Extraction of ‘core’ genomic regions to obtain alignable conserved genomic regions for phylogenetic reconstruction.

## Mutation rates (325)

Understanding how viruses mutate and accumulate genomic diversity has profound practical implications. For example, the emergence of SARS-CoV-2 mutations that are associated with immune escape has been of great concern with regards to vaccine efficacy and development [63] in the present COVID-19 pandemic. Additionally, elevating short-term viral mutation rates via mutagen treatment, termed lethal mutagenesis, has been shown to enhance the effectiveness of antiviral treatments [64]. Only a few studies to date have provided estimates of the mutation rate of anelloviruses [65–67], which ranges from 2.3-7.3 × 10-4 substitutions/site/year (s/s/y). The estimated substitution rate for the TTSuV genomic region containing the UTR, ORF2 and 5’ end of ORF1 sequences sampled from wild boars over six years was 5.4 × 10-4 s/s/y [65]. Additionally, the substitution rates of anellovirus sequences obtained from human sera and cat saliva reported over 16 and 6.5 years, respectively, were approximately 2 × 10-4 s/s/y [67]. Lastly, a synonymous substitution rate of 7.3 × 10-4 s/s/y was estimated from TTV-positive human sera over 12 years [66]. These rates were compared to that of other vertebrate viruses, which were collated from ten other studies [68–77] (**Figure 5**). Within ssDNA viruses, anelloviruses have mutation rates that are considerably lower than that of circoviruses (family *Circoviridae*), but in the same order of magnitude as that of parvoviruses (family *Parvoviridae*). Additionally, the mutation rates of anelloviruses are similar to that of RNA viruses. Lastly, anelloviruses mutate much faster than that of double-stranded DNA (dsDNA) viruses, which echoes previous findings that single-stranded viruses mutate faster than their double-stranded counterparts [64]. This phenomenon has been suggested to be due to single-stranded viruses being more prone to chemical damage such as oxidative deamination [78].



**Figure 5. Mutation rates of Anelloviridae in the context of other viruses**. The substitution rates of all genomic sites, obtained from the literature and expressed as substitutions/site/year, was visualised in log10 scale for the different viruses, stratified by their genome types. Anelloviruses are indicated by black diamonds and annotated with their corresponding names. The substitution rate where only synonymous mutations were considered is denoted by “syn”.

## Host-editing and recombination (530)

The mechanisms underlying the accumulation of genomic diversity in anelloviruses has not been explored. However, knowledge of these mechanisms may inform our understanding of the selective pressures that shape the genomic diversity of the *Anelloviridae*. Since genome replication is performed by host polymerases [21], which are highly accurate, nucleotide misincorporation may play only a minor role in the accumulation of mutations.

Host innate antiviral responses may contribute to the genomic diversity. For example, host APOBEC3 proteins are cytidine deaminases that have been found to cause C->T and G🡪A mutations in other DNA viruses like human papillomavirus [79,80]. Cytidine deaminases converts cytidine in a DNA template to uradine, which is then recognised and base-paired with adenosine on the complementary strand during DNA replication (**Figure 6a**). While such host-editing by APOBEC3 has not been demonstrated for anelloviruses, *in vitro* experiments found that these proteins preferentially bind ssDNA over dsDNA and may account for higher C->T mutations on the negative-sense strand of HIV reverse transcripts [81]. This suggests that negative-sense ssDNA anelloviruses are likely to be subject to host-editing. Investigations of how anelloviruses evade or overcome APOBEC3-mediated editing may prove to be invaluable in our understanding of how viruses evolve.



**Figure 6. Potential mechanisms of accumulating genomic diversity**. (a) Reaction equation catalysed by host cytidine deaminases. Cytidines on ssDNA is deaminated to form uradine, which is subsequently base-paired with adenosine during DNA replication, resulting in C 🡪 T and G 🡪 A mutations. (b) Schematic illustrating how recombination produces new combinations of point mutations and confounding phylogenetic inference.

Recombination plays a substantial role in shaping the extant genomic diversity of virusesby generating new genetic combinations that could not have been attained via point mutations alone [82]. Indeed, a single recombination event between two viral genomes could generate new combinations of their existing mutations, introducing several nucleotide polymorphisms at a time (**Figure 6b**), which could prevent the accumulation of deleterious mutations [83–85]. Moreover, recombination has even suggested to result in viral lineages gaining the ability to infect new host species. For example, human adenoviruses may have emerged from the recombination of chimpanzee and bonobo adenoviruses [86], while turkey coronaviruses may have emerged from chicken infectious bronchitis virus and another unknown coronavirus [87].

There is evidence for recombination in anelloviruses. Using the popular recombination detection tool RDP4 [88], which identifies recombination using an ensemble of different detection methods, Fahsbender et al. [89] found that 89% of torque teno Leptonychotes weddellii virus (TTLwV)-1 and 26% of TTLwV-2 had at least one and one putative recombination event, respectively. They also found that the recombination breakpoints detected in TTLwV-1 tend to fall within the UTR region. This agrees with a previous study [82], which found a significantly higher density of recombination breakpoints outside coding regions and at the edges of genes for almost all of 14 ssDNA viruses spanning 6 families (including TTV and TTMV). Other studies have similarly detected recombination within the different human TTV species [90,91] and within TTSuVs isolated from wild boars and domestic pigs [65]. While no studies to date have formally estimated the rate of recombination in anelloviruses, multiple studies have found a high frequency of co-infections of different anellovirus species [92–96], one even reporting a 48% frequency of dual or triple infection of TTV, TTMV and TTMDV in infants. This high frequency of co-infection, together with the strong recombination signals detected by the abovementioned studies, suggest that anelloviruses recombine frequently and that recombination may be a major contributor to the accumulation of genomic diversity.

# Transmission routes: anelloviruses can go anywhere (761)

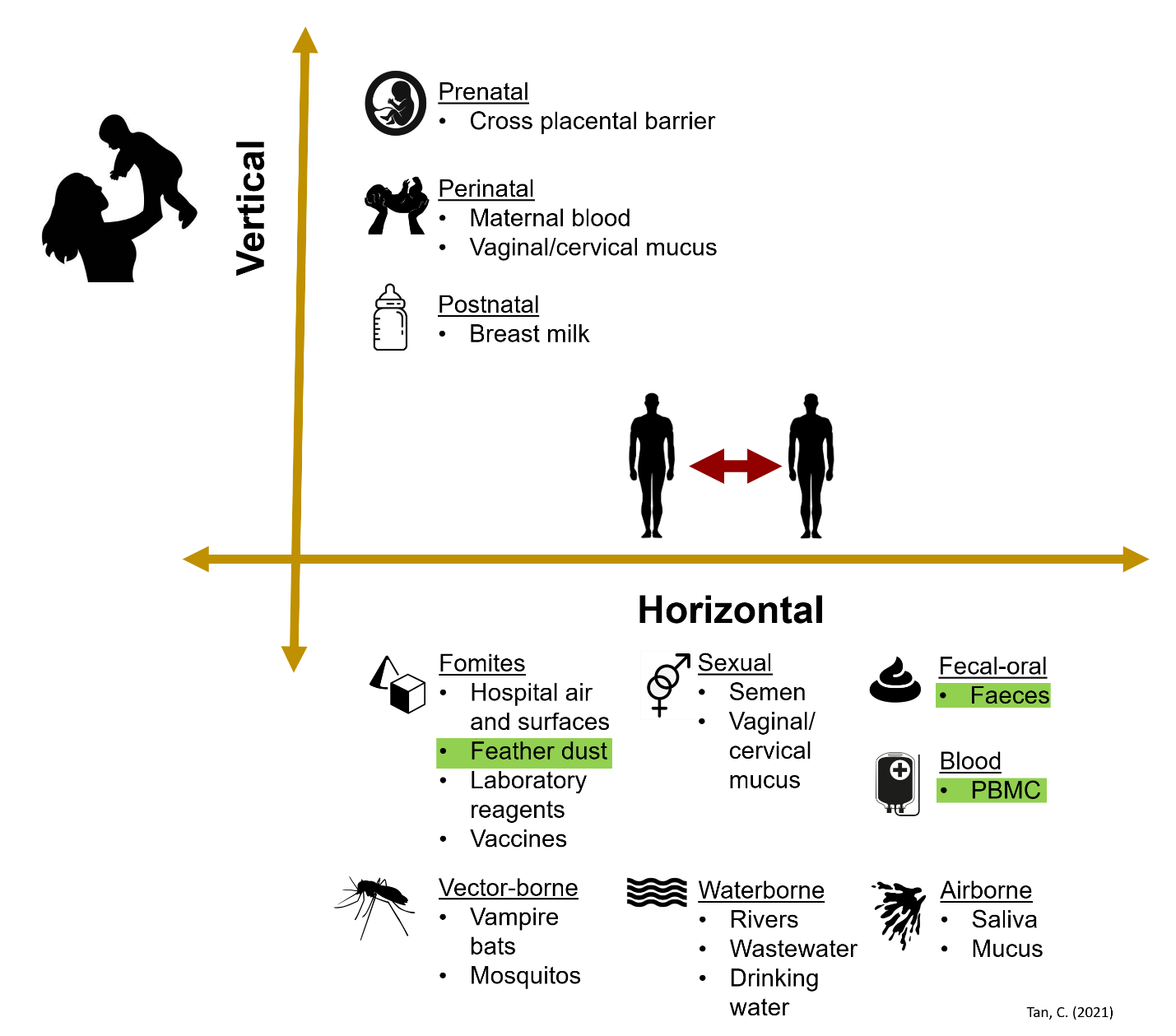
## Horizontal transmission

In line with its high prevalence, anelloviruses may potentially be transmitted via multiple routes, both vertically (*i.e.* from mother to offspring) and horizontally (*i.e.* between organisms not in a mother-offspring relationship) (**Figure 7**). In general, most studies of transmission thus far have employed PCR-based methods of detecting anelloviral DNA. In such methods, PCR with anellovirus-specific primers is used amplify viral genomic fragments, which are then visualised via agarose gel electrophoresis. Suitably sized gel bands corresponding to the length of the PCR target are extracted and subsequently sequenced to confirm that the correct genomic sequences were amplified. Successful amplification of the PCR target is generally regarded as a positive result. However, even if anelloviral DNA is detected, this does not entail that infectious virions are present. Since most studies did not demonstrate the viability of detected anelloviral DNA and its ability to infect naïve hosts, only a few transmission routes have been confirmed (highlighted in green; **Figure 7**). The strength of evidence provided for the different transmission routes will be discussed in this section.

Anelloviral DNA has been detected in bodily fluids like blood, bile, saliva, mucus, semen and faeces [97–103]. This suggests that anelloviruses may potentially be transmitted horizontally via sexual, faecal-oral and airborne routes, or via direct contact with bodily fluids. However, since most studies did not confirm the viability of virions in the samples, the presence of viral DNA in samples could have been due to transient shedding of inactivated virus from a distal source that cannot infect a new host. Exceptionally, Itoh et al. showed that mature virions could be extracted from faeces and visualised using electron microscopy, providing stronger evidence for faecal-oral transmission [99]. Additionally, anellovirus-positive blood was shown to be able infect peripheral blood mononuclear cells (PBMCs) and proliferate [98,103]. Moreover, persistent viremia of TTV and TTMDV from blood donors was found in blood transfusion recipients [104], providing confirmatory evidence for blood as a vehicle of transmission.

Anelloviruses may potentially be transmitted via water or fomites, which are inanimate objects or surfaces that can become contaminated with pathogens [105]. Anelloviral DNA has been detected in multiple environmental sources. Studies have demonstrated their persistence in rivers [106–108], wastewaters [109], drinking water [110,111], shed feathers [112], air and surfaces in hospitals [113] or even laboratory reagents [114] and vaccines [115]. Of these studies, only one demonstrated the viability of detected anelloviral DNA. Davidson et al. [112] showed that dripping feather shaft extracts obtained from the feathers of CAV-infected chicks into the eyes and mouths of unexposed chicks resulted in infection, confirming that feathers can act as fomites to transmit CAV. However, while confirmation of infectivity is lacking, given the ubiquity and persistence of anelloviral DNA in the environment, the detected viral DNAs are likely to be stably packaged in their capsid protein shells and potentially infectious. Otherwise, ‘naked’ viral DNAs quickly degrade when exposed to the environment. For example, Zhu demonstrated that plasmid DNA in river and ground water is degraded beyond detectable levels after 48-96 h [116]. In contrast, the viral load of infectious, encapisdated human adenovirus remains stable even after 40 days in surface waters as quantitated by a plaque assay [117]. The relatively higher stability of DNA in infectious virions is likely due to the presence of the viral capsid protein which shields the DNA from degradation by chemical agents, UV irradiation or other means.

Alternatively, anelloviruses may potentially be transmitted via vectors. Shi et al. [118] and Hameed et al. [119] both found that anelloviral sequences extracted from mosquitos in pig farms were not restricted to a mosquito-associated lineage, but shared common ancestry with multiple lineages of pig-associated TTSuVs. These phylogenetic relationships may reflect the random acquisition of TTSuVs from porcine hosts during mosquito blood-feeding (*i.e.* hematophagy). However, both studies did not confirm that the viral DNA detected in mosquitos were infectious, which may have provided stronger evidence for vector-borne transmission. Another study by de Souza et al. found that anellovirus sequences extracted from the liver of a vampire bat (*Desmodus rotundus*) and serum of an opossum (*Didelphis albiventris*) were of the same species at approximately 79% nucleotide sequence identity. Additionally, phylogenetic analysis suggests that these anelloviruses are likely to share a common ancestor that was transmitted between the two host types [46]. This is considerable evidence that vampire bats may be a vector of transmission for anelloviruses between the bats’ mammalian hosts.



**Figure 7. Summary of potential transmission routes for anelloviruses**. Transmission routes that are supported by substantial evidence in the literature are highlighted in green.

## Vertical transmission

There is strong evidence for vertical transmission of anelloviruses, although the exact route(s) of infection – whether infection occurs before (prenatal), during and immediately after (perinatal), or after birth (postnatal) – is not known. Gerner et al. [120] detected human TTV in human breast milk, and umbilical cord blood. Separately, Matsubara et al. [121] found TTV and TTMV DNA in breast milk, cord blood and amniotic fluid. Both studies employed polymerase chain reaction (PCR) followed by agarose gel electrophoresis to test the fluid samples. PCR amplicons were also sequenced via Sanger sequencing to rule out non-specific amplification. These studies suggest that breastfeeding might be a potential transmission route. However, cross-sample contamination during collection of cord blood or amniotic fluid is difficult to rule out and so these studies may not be sufficient to prove transplacental transmission. In fact, Tyschik et al. [122] did not find TTV in the cord blood from TTV-positive mothers using a highly sensitive quantitative PCR (qPCR) assay that could detect viral loads as low as 1000 viral DNA copies/ml of blood. The authors also detailed stringent measures to prevent cross-contamination, which the previous groups did not do. This suggests that the previous findings could have been due to contamination, which must be accounted for in future studies. Nevertheless, Gerner et al. [120] provided evidence for vertical transmission by showing that follow-up samples taken from infants with TTV-positive cord blood had persistent viremia.

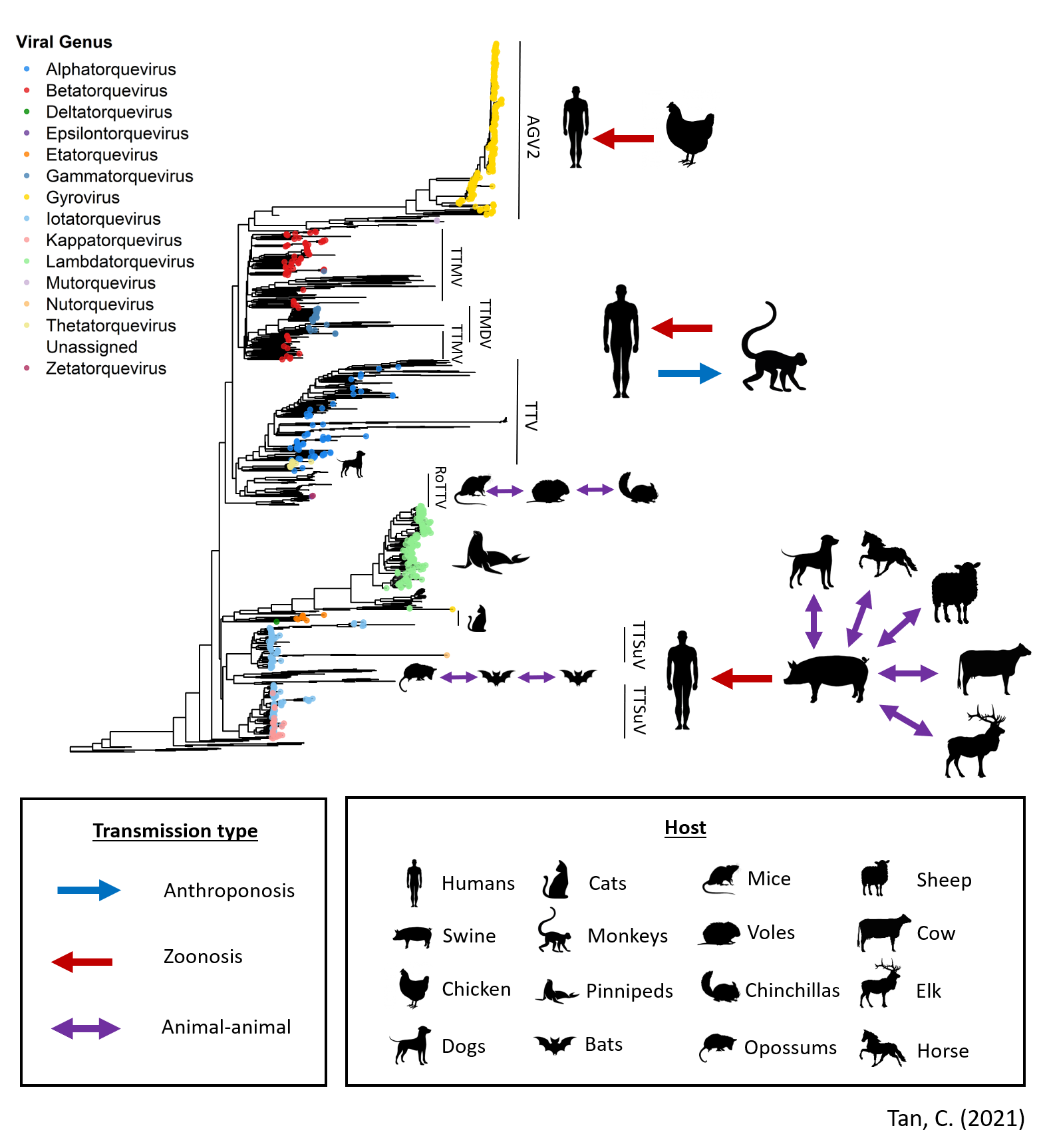
Vertical transmission has also been demonstrated in animals. In particular, Martinez-Guino et al. showed in separate studies that sows, their breast milk and their aborted foetuses [123] or stillborns [124] were PCR positive for TTSuV. They also showed that TTSuV sequences in mother-offspring pairs had nucleotide sequence identities of 91-98%, implying that sample cross-contamination is unlikely. Indeed, it is likely that samples that were cross-contaminated at the point of collection should share identical sequences. As such, these findings may suggest that infections of the foetuses were acquired prenatally and that TTSuV may be transmitted through breast feeding. Vertical transmission of TTSuV through breast milk can be further confirmed in future studies by feeding of TTSuV-positive milk to TTSuV-negative offspring. Separately, human TTMDV was detected in domestic hens and the yolks of their eggs [125], providing evidence for vertical transmission. Confirmation that chicks that developed from TTMDV-positive eggs were also persistently infected would have provided stronger evidence for vertical transmission. Nevertheless, the studies discussed above provide substantial evidence for vertical transmission of anelloviruses.

# Potential for the emergence of infectious diseases

## Anelloviruses are everywhere (485)

After the discovery of TTV, the first anellovirus, in 1997 [7], multiple studies have detected a diverse range of anelloviruses in humans and animals, including rodents [3], primates, birds, marine mammals [89,126,127], bats [4] and other wild or domesticated animals [46,128–131]. An alignment-free phylogeny of 1143 complete anellovirus genomes isolated from at least 43 annotated host species (NCBI Virus; accessed 20 Oct 2020) is presented here (**Figure 8**). There is significant clustering of the viral sequences by viral genus. The phylogeny demonstrates significant clustering by viral genus and a broad diversity of host types that are segregated by phylogenetically distinct clades (**Figure 8**). Given the extensive sampling of the broad diversity of anelloviruses from a variety of hosts, it may be feasible to investigate the selective pressures involved in viral speciation events (e.g. the emergence of TTMDV from a TTMV lineage; **Figure 8**) or changes to host-specificity. While unexplored in the literature, probing these ideas may yield valuable insights as to how new pathogens can emerge.

Studies that have explored the biodiversity of anelloviruses in humans and animals can be non-exhaustively categorised into virus-specific and metagenomic efforts. The former category refers to studies that performed PCR using anellovirus sequence-specific primers, followed by sequencing of the PCR amplicons. For example, Ye et al. amplified and sequenced a 346bp region of avian gyrovirus 2 (AGV2) in chickens from live poultry markets and healthy human blood [128]. Similarly, Singh et al. used TTSuV-specific primers to amplify and subsequently characterise anellovirus sequences in bovine, equine, ovine, canine and elk hosts [129]. The latter category refers to studies that perform unbiased sequencing of all DNA sequences in samples (*i.e.* ‘shotgun’ metagenomic sequencing), followed by *de novo* assembly[[2]](#footnote-2). For example, studies have employed this approach for samples obtained from mosquitos [118,119,126], rodents [3,132] and pinnipeds [127,133]. To explore the host range and biodiversity of anelloviruses in different animal reservoirs, metagenomic-based approaches appear to yield much more information. Indeed, *a priori* knowledge of viral sequences is required for virus-specific approaches. Given the large sequence divergence within the family, species-specific PCR primers are unlikely to be able to amplify novel anellovirus sequences. In contrast, metagenomic-based approaches do not require *a priori* sequence information and therefore can detect novel anelloviruses. Furthermore, because anellovirus genomes are small, lesser sequencing reads are required for sufficient genomic coverage to produce reliable genome assemblies. This entails that while the genomic fragments retrieved via virus-specific PCR is restricted by the amplicon length, longer or even full-length genomic sequences can be assembled via metagenomics-based approaches. In short, metagenomics may capture more genomic information from a larger proportion of the anelloviral diversity.



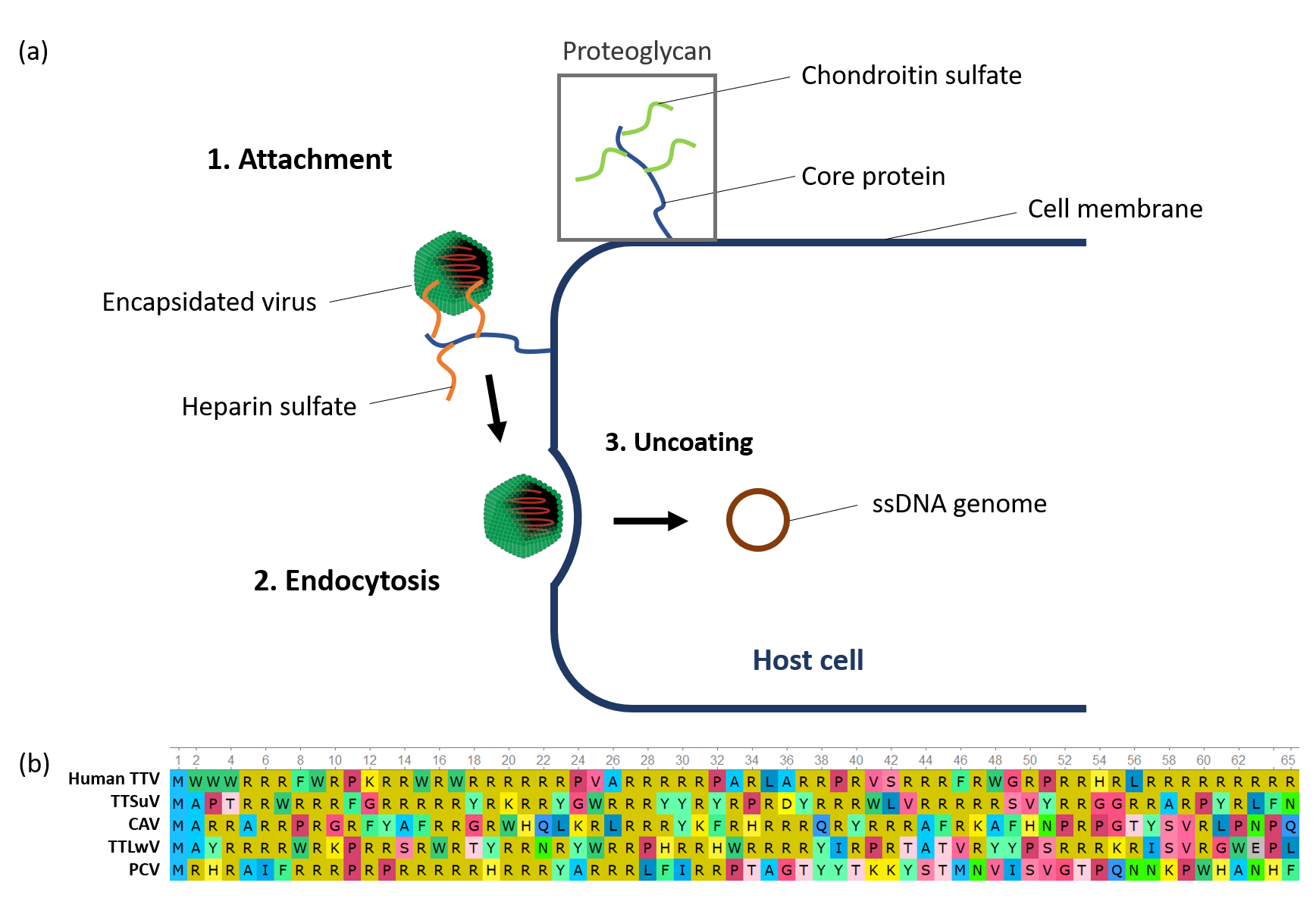
**Figure 8. Genomic diversity of the Anelloviridae and summary of evidence for cross-species transmission**. The genomic diversity of 1143 Anelloviridae genome sequences is represented here as a whole-genome neighbour-joining phylogeny, which was reconstructed using alignment-free Mash distances [134] and rooted with the genome accession MK012481 (see **Appendix**). This phylogeny is annotated with symbols illustrating the host range and cross-species transmission of various viral species.

## Evidence for cross-species transmission

There has been considerable evidence supporting the ability of anelloviruses to transmit between host species. Singh and Ramamoorthy [129] detected both TTSuV and human TTV sequences in canine, bovine, ovine, equine, and elk sera using species-specific PCR primers. They further detected TTSuV-specific antibodies in bovine, ovine and equine samples using enzyme-linked immunoabsorbant assays (ELISA). Since a virus must sustain a sufficiently high viral load for a sufficiently long duration to trigger this adaptive immune response, the results confirm the presence of productive TTSuV infection in these animals. Notably, the authors did not find TTSuV-specific antibodies in elk and canine samples despite the presence of TTSuV DNA, suggesting that either the magnitudes of antibody responses were below the limit of detection for the ELISA assay used, or that viral replication was insufficient to trigger the production of TTSuV-specific antibodies. The former possibility implies considerable variability in the strength of adaptive immune responses to anellovirus infection while the latter suggests that TTSuV may not be adapted for efficient replication in said hosts. Nonetheless, these findings provide strong evidence for the ability of TTSuV to infect multiple animal hosts and possible cross-species transmission of both TTSuV and human TTV. Separately, rodent TTVs have also been shown to infect a broad range of hosts. A large metagenomic surveillance study on rodent samples collected from 20 provinces in China found that rodent TTV lineages were found in multiple hosts from the same family, or even hosts from three different families (*Circitidae*, *Muridae* and *Chinchillidae*) [132]. Additionally, as mentioned previously, de Souza et al. [46] found two phylogenetically related anelloviruses in a vampire bat and an opossum. A third anellovirus sequence obtained from the frugivorous (i.e. fruit-eating) bat, *Carollia perspicillata*, was also estimated to share a common ancestor with the other two sequences [46]. Moreover, the node where the three sequences diverged from the other anelloviruses had a high bootstrap support[[3]](#footnote-3) value, suggesting that their evolutionary relationships can be confidently inferred. Given that *D. rotundus* and *C. perspicillata* are known to share roosts in caves [135,136], horizontal transmission of anelloviruses between the two hosts may have occurred during roosting. Collectively, these studies suggest that anelloviruses, or at least those associated with mammalian hosts, tend to be ‘generalists’ that have retained the ability to infect a broad range of hosts.

Understanding how anelloviruses are internalised by cells during infection may help to account for the broad host range of anelloviruses. However, the exact mechanism for viral entry is currently unknown. Generally, non-enveloped viruses, which include anelloviruses, enter host cells by the binding of capsid proteins to cell surface [137]. These viruses subsequently penetrate the cell membrane and release their genomes into the host cell where it proliferates. A potential mechanism for anellovirus viral entry may be modelled off that of porcine circovirus (PCV), a circular ssDNA virus from the family *Circoviridae.* A viral particle must first attach to extracellular host cell receptors and induce uptake (**Figure 9a**). PCV was shown to use proteoglycans, which consist of a core protein chain that is covalently attached to several glycosaminoglycan chains such as heparin sulfate (HS) and chondroitin sulfate-B (CSB), as their receptor for viral entry [138]. Additionally, an arginine-rich region at the N-terminus of the PCV capsid protein was shown to act as a cell-penetrating peptide (CPP) that triggers endocytosis and hence uptake of its cargo into the cell [139]. While a direct binding interaction between the N-terminal sequence of the PCV capsid and proteoglycan receptors has not been described, most CPPs are understood to allow the translocation of macromolecules into cells by proteoglycan-binding [140,141]. Visualisation of PCV and anellovirus capsid protein sequences found a similar arginine-rich region conserved in anellovirus capsid proteins (**Figure 9b**). This suggests that proteoglycans are possible candidates for receptors used by anelloviruses during viral entry. Future studies may consider investigating whether the anellovirus-derived N-terminal capsid sequences can function as CPPs to probe the mechanism(s) of viral entry.

Interestingly, PCVs were found to be able to infect cells lacking both HS and CS proteoglycans [138]. Additionally, inhibition of major endocytotic pathways such as clathrin-mediated endocytosis or macropinocytosis was found to reduce but not abolish uptake of PCV-derived N-terminal sequences [139]. These findings suggest that multiple cellular pathways are involved in PCV viral entry. This, in addition to the fact that HS proteoglycans are expressed on the surface of most animal cells, [142] may explain why PCV can infect multiple cell types and host species [143]. Similarly, the promiscuous cell- [144] and host-specificity of anelloviruses points to the involvement of common host cell receptors during viral entry. Arguably, with a better understanding of viral entry mechanisms, it may be possible to estimate the full host range of anelloviruses and their potential for cross-species transmission.



**Figure 9. Potential mechanism of viral entry** (a) Schematic of a possible mechanism for anelloviral entry modelled off that of PCVs. (b) UGene [22] visualisation of the first 65 amino acid residues in the capsid protein of Human TTV (QHS01920.1), TTSuV (YP\_003587901.1), CAV (AYN79688.1), TTLwV (YP\_009388631.1) and PCV (YP\_009315911.1). A meaningful alignment of these sequences could not be produced.

## The risk of zoonoses

The ‘generalist’ nature of anelloviruses is a risk factor for the emergence of zoonotic infectious diseases. Statistical modelling of 1415 human-associated pathogens, which includes viruses, bacteria and other types of pathogens, found that pathogens capable of infecting multiple hosts were at least 1.29 times more likely to emerge as zoonotic infectious diseases [145]. This echoes another study of 162 zoonotic viruses, which found that 63% of zoonotic viruses can be found in hosts from at least two different taxonomic orders [146]. These findings may be explained, in part, by the importance of virus-receptor interactions during viral entry that was highlighted in the previous section. In alpharetroviruses, a single amino acid substitution was sufficient for the extension of host range from avian cell types to include human, canine, feline, and rodent cells [147]. If receptor binding is the first barrier to viral entry, pathogens that already possess a promiscuous host-specificity may require the accumulation of fewer mutations to overcome this barrier. Since anelloviruses have a broad host range, there is a higher risk that they can emerge as novel pathogens responsible for infectious diseases that affect humans and livestock.

In fact, several studies have already demonstrated the potential for zoonotic transmission of anelloviruses. For example, AGV2 was detected in the blood of 2/178 (1.1%) individuals and shared at least a 90% nucleotide sequence identity with AGV2 isolated from chickens [128]. Additionally, simian-associated TTVs (SiTTV) were detected in 6.8% of 487 human serum samples [148]. Another study detected CAV and AGV2 in 8.6% and 8.7%, respectively, out of 748 human faecal samples from three separate cohorts [149]. However, in contrast to the extremely high prevalence of TTV, TTMV or TTMDV detected in humans, the proportions of samples that tested positive for these anelloviruses are low. This suggests that SiTTV, CAV or AGV2 may not yet be adapted for efficient replication and transmission in humans. There is stronger evidence for zoonosis of TTSuV. Ssemadaali et al [150] showed that out of 40 human serum samples, TTSuV DNA was detected in 67.5% and 80% of samples in two separate PCR assays targeting different regions of the TTSuV genome. Additionally, PBMCs transfected with TTSuV DNA could be used to sustain infection in unexposed PBMCs for three serial passages, suggesting that TTSuV is capable of proliferating in human cells. Most importantly, TTSuV-specific antibodies could be found in 40% of the human samples, confirming that TTSuV can productively infect human hosts. These results highlight the zoonotic potential of animal anelloviruses. Warning statement?

## Animal reservoirs

Animal reservoirs are groups of animals that harbour viral pathogens, some of which may be transmitted to humans (*i.e.* zoonoses), resulting in the emergence of novel human infectious diseases. The rich biodiversity of anelloviruses across host species suggest that there may be multiple animal reservoirs where zoonotic spillover may occur. Animal reservoirs that co-exist with humans such as livestock, rodents, pigeons, cats and dogs are of particular concern. As discussed above, anelloviruses seem to be ‘generalists’ that have the ability to infect multiple host species. However, even if an anellovirus manages to infect humans, it must be able to proliferate and transmit efficiently in humans for the emergence of a zoonotic disease in the human population. In fact, upon acquiring the ability to jump into a host species, there is a period of viral adaptation to its new host, where the virus accumulates mutations that potentially improves its transmissibility [151]. Animal reservoirs that are in close contact with humans may provide ample opportunities for such adaptation to occur. This is in line with the suggestions that a 20% geographical overlap is required for viral sharing between host species [152] and that the probability of sharing increases with increasing overlap up till a 50% overlap [153]. Furthermore, a network analysis of the number of viruses shared between different hosts showed that wild rodents and domestic animals such as cattle, pigs, cats and dogs were centrally located and highly connected [146]. That is, viruses in said animals are shared with the greatest number of host types. This reflects the potential of such animals to act as vectors that facilitate the movement of viruses between humans and wild-animal reservoirs that we may not be in direct contact with. Finally, the biodiversity of anelloviruses within host species makes it more likely to cause an emerging infectious disease. A study of 415 viruses found that the number of zoonotic viruses is proportional to the number of viral species maintained by each host type [154]. That is, the richer the diversity of viruses in an animal reservoir, the higher the likelihood of observing viruses that cause zoonotic infectious diseases. This is the case for anelloviruses, where metagenomic-based studies often detect different species of anelloviruses, sometimes from different viral genera. For instance, a survey of anelloviruses in civets (*Paguma larvata*) found viral species from at least four genera [155], while that in rodents spanned at least two viral genera [132].

Given the vast amount of metagenomic sequencing data from bats [156–158], rodents [159,160] and livestock [161–163], a meta-analysis of these datasets may shed light on the current diversity of anelloviruses, the novel anelloviruses that are emerging in these animal reservoirs and their potential for zoonotic emergence.

# Conclusions and Perspectives (500 words)

* If anelloviruses are arguably not pathogenic to humans, why should we care?

1. Kills livestock
2. Generalist nature potentiates cross-species transmission and possibly the emergence of pathogenic strains

* Stress importance of viral surveillance in animal reservoirs

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# Checklist

1. good use of figures and appropriate use of legends and references
2. “general-to-specific” structure
3. Understandable to the non-specialist
4. Introduction with relevance of the review topic, aims and roadmap present
5. Logical transitions between topics
6. Conclusions and perspectives present
7. Highlights limitations / challenges / questions in field.
8. Provides (few) directions for future experimental work based on the limits and challenges mentioned
9. Clear and relevant figures

1. *Bidnaviridae*, *Parvoviridae*, *Bacilladnaviridae*, *Circoviridae*, *Geminiviridae*, *Genomoviridae*, *Nanoviridae*, *Redondoviridae*, *Smacoviridae*. [↑](#footnote-ref-1)
2. Joining sequencing reads into longer sequences without a reference sequence with the aim of producing full genomes. [↑](#footnote-ref-2)
3. A statistic assigned to each node in a phylogeny to assess the robustness and accuracy of the observed tree topology. [↑](#footnote-ref-3)