Acta Virologica

TYPE Brief Research Report
PUBLISHED 21 March 2025
DOI 10.3389/av.2025.13512

OPEN ACCESS

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RECEIVED 10 July 2024
ACCEPTED 05 March 2025
PUBLISHED 21 March 2025

CITATION
Nokhova AR, Dubovitskiy NA, Derko AA, Khozyainova AA, Kurskaya OG, Shestopalov AM and Sharshov KA (2025) Genetic characteristics of anelloviruses detected in individual viromes of children with acute respiratory symptoms using the metagenomic approach.
Acta Virol. 69:13512.
doi: 10.3389/av.2025.13512

Genetic characteristics of anelloviruses detected in individual viromes of children with acute respiratory symptoms using the metagenomic approach

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More than 20 years after the discovery of the first anelloviruses (AVs), they still remain a mysterious group of viruses. To date, there is no clear understanding of their impact on the host organism, although information is emerging about their participation in various pathologies. Unfortunately, the widespread distribution of AVs makes it difficult to determine their significance. However, it is well known that AVs are an important component of the human virome. We assessed the abundance of AVs in viromes from four individual nasal and pharyngeal samples from children with respiratory symptoms using a metagenomic approach. Three of samples were positive for bocavirus, influenza virus A and respiratory syncytial virus, while one sample was negative for any respiratory viruses in standard PCR diagnosis. AVs were detected in all samples, namely Torque teno mini virus (TTMV). The greatest abundance and diversity of AVs [Torque teno virus (TTV), TTMV and Torque teno midi virus (TTMDV)] were observed in a diseased patient who had an absence of respiratory viruses. It is suggested that an activated immune system to combat the main pathogens, against the background of which the number of AVs decreases, may explain the reduced diversity of anellome in other patients. We also compared the results of the metagenomic workflows and qPCR for major respiratory viruses, which were in agreement. In addition, metagenomic sequencing made it possible to obtain additional data on viral genomes in order to establish their taxonomic identification and characterize individual viral profiles in patients.

KEYWORDS

respiratory virome, torque teno virus, torque teno mini virus, torque teno midi virus, anellome, human anelloviruses, TTV

Introduction

Metagenomic studies, especially in the field of virome, have shown significant growth in recent years due to their advantages in investigating of viruses that are inaccessible to classical methods, characterizing viral diversity in various environments, identifying novel strains and studying viral evolution. This growth has led to detailed studies on the human respiratory virome (Freer et al., 2018; Mitchell and Allan, 2018; Van Rijn et al., 2019). It has been demonstrated that one of the ubiquitous members of the human respiratory virome is the enigmatic *Anelloviridae* family of viruses (Freer et al., 2018). These viruses are presented in up to 90% of individuals and acquired in childhood (De Souza et al., 2018; Spandole et al., 2015). TTV's DNA has been identified in a variety of biological samples, including blood, breast milk, saliva, nasal secretions, tears, sweat, bile, cerebrospinal fluid, urine, feces, semen, cervical secretions (Bagaglio et al., 2002; Itoh et al., 2000; Freer et al., 2018; Chan et al., 2001; Umemura et al., 2001; Neri et al., 2020). Various transmission routes have been described such as vertical, fecal-oral, parenteral, sexual (McElvania TeKippe et al., 2012; Taylor et al., 2022; Maggi et al., 2003a).

With age, the "anellome" [the composition of anelloviruses (AVs) in the body], representing the diversity of AVs within an individual, becomes stable; however, in childhood, the composition is variable with a predominance of TTMV (Torque teno mini virus) and TTMDV (Torque teno midi virus) (Kaczorowska and van der Hoek, 2020; Kaczorowska et al., 2022).

Anelloviridae family comprises 30 genera, four of which are detected in humans: *Alphatorquevirus* (Torque teno virus, TTV), *Betatorquevirus* (TTMV, previously known as TTV-like Mini virus, TLMV), *Gammatorquevirus* (TTMDV), and the recently discovered *Hetorquevirus* (Torque teno hominid virus) (Varsani et al., 2021). Currently, the most well-characterized genus is *Alphatorquevirus*. At present there is no effective and reproducible *in vitro* system available for culturing AVs and sustaining their replication. Successful propagation is typically limited to the first few passages, which presents significant challenges for further research on these viruses (De Villiers et al., 2011; Focosi et al., 2016). AVs include non-enveloped viruses with single-stranded negative-sense DNA circular genomes ranging in size from 1.6 to 3.9 kb (McElvania TeKippe et al., 2012; Varsani et al., 2021). This group exhibits a high degree of heterogeneity among DNA viruses (Kaczorowska and van der Hoek, 2020; Arze et al., 2021; Abbas et al., 2019). It is proposed to consider the species demarcation threshold as nucleotide sequence identity of ORF1 at 31% (Varsani et al., 2021). This means that if a new AV shows 69% or higher pairwise similarity with a classified member of the species, it is classified within that species.

While it is not definitively established whether AVs cause diseases, they are currently considered to be commensal viruses. However, their involvement in the development of a wide range of pathologies, including respiratory infections, hepatitis, multiple sclerosis, lymphoma, autoimmune diseases, and others, is also suspected (Van Rijn et al., 2019; De Souza et al., 2018; McElvania TeKippe et al., 2012; Jazaeri Farsani et al., 2013; Mancuso et al., 2013). It is reliably noted that an increase in viral load occurs in conditions of compromised immune function (such as HIV/AIDS) (Shibayama et al., 2001; Liu et al., 2021), and replication is controlled by the immune system (Timmerman et al., 2024a). Moreover, there is a growing number of studies investigating whether AVs are associated with respiratory diseases (Freer et al., 2018; McElvania TeKippe et al., 2012; Maggi et al., 2003a; Dodi et al., 2021; Moen et al., 2003; Pifferi et al., 2005; Bal et al., 2022; Del Rosal et al., 2023). In this study, we employed viral metagenomics to investigate the presence, diversity, phylogenetic relationship of AVs among pediatric patients exhibiting acute respiratory symptoms.

Materials and methods

Sample collection

The Ethics Committee of the Federal Research Center for Fundamental and Translational Medicine approved this study. Nasal and throat swabs were obtained from 1,310 pediatric patients hospitalized with respiratory symptoms in Novosibirsk, Russia, between October 2022 and May 2023. Clinical diagnoses and demographic data of patients were obtained from medical records. The nasal and throat swabs were combined. Samples were placed in tubes with transport medium [Dulbecco's modified Eagle's medium (Capricorn Scientific, Ebsdorfergrund, Germany)] with 0.5% bovine serum albumin, 100 μg/mL of gentamicin sulfate (BioloT, Saint-Petersburg, Russia), and 50 units/mL of amphotericin B (BioloT, Saint-Petersburg, Russia). Tubes were stored in liquid nitrogen immediately after collection. After transport to the laboratory, all samples were stored at −80°C for future studies.

Virus detection

All samples were tested for the presence of major respiratory viruses. RNA was extracted using the RIBO-sorb kit (Interlabservice, Moscow, Russia). Reverse transcription was conducted using the REVERTA-L kit (Interlabservice, Moscow, Russia). The resulting cDNA was used to detect

Abbreviations: AV, anellovirus; TTMDV, torque teno midi virus; TTMV, torque teno mini virus; TTV, torque teno virus.

TABLE 1 Characteristics of the samples. For each patient (E1–E4), a clinical diagnosis and respiratory pathogens were determined (according to qPCR, bacteria culture and genome binning).

Samples	Sex	Age, years	Clinical diagnosis	Respiratory pathogens, according to qPCR and bacteria culture	Respiratory viral contigs, according to blastn/virome (>500 nt)	Reads after processing, million	Viral contigs/ all contigs	Contigs of AVs (% of viral contigs)	Unique sequences of AVs[a]
E1	F	4	Pneumonia	Bocavirus; No bacteria	Bocavirus	2.213	551/967	3 (0.544%)	3
E2	M	2	Bronchitis	Influenza virus A(H1N1)pdm09; *Candida albicans*	Influenza virus A (H1N1) pdm09	2.152	236/497	1 (0.424%)	1
E3	M	4	Pneumonia	No viruses and bacteria	No respiratory viruses	2.231	407/944	25 (6.143%)	8
E4	M	2	Bronchitis	Respiratory syncytial virus	Respiratory syncytial virus B	3.955	730/962	4 (0.548%)	4

F, female; M, male.

[a]Unique sequences of AVs include consensus sequences of AVs obtained through reference-based assembly and 3 contigs (1 from E1 and 2 from E4) of 461–529 nucleotides that did not assemble based on the reference.

respiratory syncytial virus (HRSV); alphacoronaviruses and betacoronaviruses (HCoV); parainfluenza virus (HPIV); metapneumovirus (HMPV); rhinovirus (HRV); adenovirus (HAdV) and bocavirus (HBoV) using the AmpliSens AR-VI-screen-FL kit (Interlabservice, Moscow, Russia).

Detection of influenza virus A, influenza virus B and SARS-CoV-2 was carried out in a one-step real-time PCR reaction using the AmpliPrime Influenza SARS-CoV-2/Flu (A/B/H1pdm09) kit (Nextbio, Moscow, Russia).

Bacteria detection

Additionally, bacterial culture for opportunistic and pathogenic organisms (including *Acinetobacter baumannii, Candida albicans, Candida lusitaniae, Candida parapsilosis, Enterobacter cloacae, Escherichia coli, Klebsiella oxitoca, Klebsiella pneumoniae, Moraxella catarrhalis, Neisseria spp., Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus viridans, Streptococcus pneumoniae*) was performed in the clinical diagnostics laboratory of the Municipal Children's Infectious Diseases Hospital, Novosibirsk, Russia. During the microbiological study, the primary sowing of clinical material on nutrient media was carried out, followed by the re-sowing of grown colonies of microorganisms on selective media. The species identification of microorganisms was performed using the MALDI-TOF mass spectrometry method.

Four individual samples from children aged 2–4 years diagnosed with pneumonia or bronchitis were selected for further viral metagenomic analysis. We selected three samples in which respiratory viruses were present as a monoinfection and one sample without respiratory pathogens (for more details, refer to the Table 1).

Sample preparation for metagenomics

Enrichment of samples with virus-like particles

The sample preparation for viromic analysis was conducted in accordance with the NetoVIR protocol, with modifications (Conceição-Neto et al., 2018). Test tubes with transport medium and viscose swabs were mixed using a vortex for 3 min. An aliquot of each sample was then transferred to a clean tube and centrifuged for 3 min at 17,000 × g. The supernatant was filtered through a 0.8 μm filter (Sartorius Vivaclear, Göttingen, Germany) for 1 min at 17,000 × g. 20× enzyme buffer was added to the filtrate. Then benzonase and 1 μL of micrococcal nuclease were added and incubated for 2 h at 37°C. The reaction was stopped with 0.2 M EDTA and extraction began immediately.

RNA extraction, reverse transcription and amplification of double-stranded cDNA

Nucleic acids were extracted using a column-based extraction kit (Biolabmix, Novosibirsk, Russia). Complementary DNA was obtained using Sequence-Independent, Single-Primer-Amplification (SISPA) according to the protocol (Chrzastek et al., 2017). Briefly, first-strand cDNA was prepared using K-8N primer (5′-GACCATCTAGCGACCTCCACNNNNNNNN-3′). To do this, 50 pmol of the K-8N primer was added to the viral RNA and incubated for 2 min at 70°C. Then RT buffer, M-MuLV reverse transcriptase, and water from the M-MuLV-RH kit (Biolabmix, Novosibirsk, Russia) were added. The reaction

mixture was incubated at 25°C for 10 min, 42°C for 60 min and 70°C for 10 min.

To synthesize the second strand of cDNA, 10x Klenow buffer, 20 pmol K-8N primer and 250 mM of each dNTP were added to the entire volume of the previous reaction. The reaction mixture was incubated at 95°C for 3 min. Then it was cooled to 4°C, added Klenow fragment (SibEnzyme, Novosibirsk, Russia) and incubated at 37°C for 60 min. The reaction products were purified using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, United States).

Double-Stranded cDNA (ds-cDNA) amplification was carried out using the Encyclo Plus PCR kit (Evrogen, Moscow, Russia). To the 10 µL of ds-cDNA, 5 µL of 10x Encyclo buffer, 1 µL of dNTP mix (10 mM each), 5 µL of K-primer (5′-GACCATCTAGCGACCTCCAC-3′) 100 pmol/µL, 1 µL of 50x Encyclo polymerase mix and 28 µL of water were added. The reaction mixture was incubated at 95°C for 4 min, followed by 35 cycles at 95°C for 15 s, 55°C for 30 s, 72°C for 2 min, and a final extension at 72°C for 10 min. The reaction products were purified by Reaction mixtures DNA isolation kit (Biolabmix, Novosibirsk, Russia) and quantified through using a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, United States) with a Spectra Q HS Kit (Sesana, Moscow, Russia).

Library preparation and sequencing

Preparation of libraries was carried out using the KAPA HyperPrep Kit (Roche, Switzerland). Briefly, the size of cDNA fragments was assessed using a 4150 TapeStation automated gel electrophoresis platform (Agilent, United States). Then, the terminal sections of the cDNA fragments were repaired and the 3′ ends were adenylated. After this, the adapter was ligated and the target cDNA fragments were selected using KAPA Pure Beads (Roche, Switzerland). Next, the libraries were amplified with indexing primers and target fragments were selected using KAPA Pure Beads (Roche, Switzerland). Finally, the quality and quantity of the obtained cDNA libraries were assessed on a Qubit 4.0 (Thermo Scientific, Waltham, MA, United States) fluorometer using the dsDNA HS kit (Thermo Scientific, United States) and an automatic gel electrophoresis platform 4150 TapeStation (Agilent, Santa Clara, CA) using the HS D1000 ScreenTape kit.

Sequencing was performed on the GenoLab M platform (GeneMind, Shenzhen, China). The finished libraries were diluted to a concentration of 4 nM and pooled according to the operating manual of the GenoLab M sequencing system (GeneMind, Shenzhen, China). The library pool was denatured and diluted to a final concentration of 2.8 pM, and the volume fraction of PhiX in the pool was 1%. Datasets are deposited in the Sequence Read Archive (SRA) (accession number SRR28717243 – SRR28717246).

Bioinformatics processing

Based on the sequencing results approximately 13–17 million 150 bp paired-end reads per sample were achieved. Raw reads were trimmed to remove low-quality bases, ambiguous bases and adapter sequences using Trimmomatic v0.39 (Bolger et al., 2014). Trimmed reads were decontaminated from the human genome using Bowtie2 (Langmead and Salzberg, 2012). To reduce the deduplication level, we utilized fastp v0.23.4 (Chen, 2023), Clumpify [from the BBMap v39.06 package (Bushnell, 2024)], and FastUniq v1.1 (Xu et al., 2012). These reads were processed using the Virome Paired-End Reads (ViPER) pipeline[1]. For *de novo* assembly, we utilized metaSPAdes v3.15.5 (Nurk et al., 2017) and filtered the assembled contigs based on coverage (≥10x) and length (≥300 nt). The annotation and classification of the assembled contigs were performed using DIAMOND v2.0.15 (Buchfink et al., 2015) and KronaTools v2.8.1 (Ondov et al., 2011). Contigs from *Anelloviridae* were filtered out and handled individually. To assemble according to the reference, the contigs of AVs were mapped against the nr/nt database using BLAST to obtain the closest full genomic (or complete CDS) sequence, followed by consensus building from the libraries using BWA-MEM (Li and Durbin, 2009), SAMtools (Li et al., 2009). Sequences (>400 nt) obtained were analyzed by BLASTn and classified by *Anelloviridae* genus. Seven sequences of AVs from phylogenetic analysis are available in GenBank (accession numbers: PP666855 - PP666861).

Phylogenetic analysis

Phylogenetic analysis was performed based on the complete (or almost complete) amino acid ORF1 sequences of the seven AVs (five TTMVs, two TTMDVs) acquired in this study, along with their closest relatives based on BLASTx and other representative virus strains. MAFFT v7.520 (Katoh and Standley, 2013) was employed for multiple sequence alignment using the mafft-linsi option and a maximum of 100 iterations separately for TTMVs and TTMDVs. Subsequently, the alignments were merged ("--merge") and trimmed using TrimAl v1.2 with the "--gappyout" option (Capella-Gutiérrez et al., 2009). Maximum likelihood (ML) phylogenetic trees were constructed for the amino acid dataset using MEGA 11.0 under the best-fit substitution model (GTR + F + G + I) (Tamura et al., 2021). Bootstrap with 1,000 samples was performed to assess the robustness of tree topologies.

1 https://github.com/Matthijnssenslab/ViPER

Results

We examined four nasal and throat swabs from children aged 2–4 years with respiratory symptoms from Novosibirsk, Russia, collected between November and December 2022. Three swabs showed the presence of respiratory pathogens, while sample E3 exhibited no detection of either bacterial or viral respiratory pathogens (Table 1). Despite the absence of major respiratory pathogens, the patient exhibited symptoms of pneumonia, fever, malaise, nasal congestion, and cough.

To obtain comprehensive information on the prevalence of viruses in children with respiratory symptoms, a virome study was performed. This research utilized the virus-like particle enrichment protocol established by Conceição-Neto et al. (2018), which effectively reduces bacterial and host nucleic acids in samples. The study by Conceição-Neto et al., mock-virome and bacterial mock-community and real samples showed an effective reduction in non-target nucleic acids while maintaining viral diversity. Therefore, after enrichment of samples with virus-like particles, we obtained a mixed sample, which was then used for nucleic acid extraction and preparation for sequencing. The results of metagenomic analysis were consistent with the qPCR diagnostic data.

Characteristics of the obtained reads and contigs

Following sequencing, there were between 13–17 million reads obtained. After trimming, deduplication, and removal of host reads, there were approximately 2 million reads remaining in samples E1, E2, and E3, and 4 million reads in sample E4 (Table 1). Through *de novo* assembly, we obtained 967 contigs in E1, 497 in E2, 944 in E3, and 962 in E4. Among these, 3, 1, 25, and 4 contigs belonged to AVs in each respective sample. After reference-based assembly using these contigs, we obtained 13 sequences of AVs (length 1,165–2,832 nt). In addition, three contigs (one from E1, and two from E4) remained unchanged in the attempt to assemble them based on the reference. Thus, a total of 16 unique sequences of AVs were obtained, with 8 of these being were >2,000 nt (1 each in E1, E2 and 6 in E3), and 8 were <2,000 nt (2 in E1, 2 in E3, and 4 in E4).

Prevalence of viral families by samples

The majority of viral reads were assigned to bacteriophages (*Caudoviricetes, Crassvirales, Siphoviridae, Myoviridae, Inoviridae, Podoviridae, Herelleviridae*), comprising 97%–99% in PCR-positive samples (E1, E2, and E4) and 89% in PCR-negative sample (E3) (Figure 1). Among eukaryotic viruses (excluding bacteriophages), reads mapping to the family *Anelloviridae* predominated in samples E1 and E3 Meanwhile in the sample E2 the family *Orthomyxoviridae* predominated. Additionally, in the samples E1 and E4 we found reads mapping to the *Parvoviridae* (Human bocavirus) and *Pneumoviridae* respectively (Figure 1), which is corresponding to the respiratory viruses detected in the PCR analysis. The *Parvoviridae* family was also detected in samples E2 and E3; however, the identified representatives were not human pathogens. It is worth mentioning that in the sample E4, contigs belonging to the family *Astroviridae* were identified, specifically Astrovirus VA3. This finding is noteworthy as it is usually associated with gastrointestinal disorders (Finkbeiner et al., 2009; Kapoor et al., 2009), however there are also some studies reporting the presence of this virus in nasopharyngeal swabs from febrile children (Wylie et al., 2012; Cordey et al., 2016; Cordey et al., 2018).

Characterization of AVs sequences

Sequences of AVs were detected in all tested samples, with sample E3 exhibiting a significantly higher number of assembled contigs of *Anelloviridae* ($p < 0.05$) compared to the others (Figure 1; Table 1). While this abundance in E3 may be related to gender, as male typically have a richer anellome although this has primarily been demonstrated in adult males (Cebriá-Mendoza et al., 2023). Additionally the possibility that AVs may have played a role in the development of inflammation cannot be excluded, as the patient exhibited respiratory symptoms.

The overwhelming majority of AVs sequences identified across all patient samples belonged to the *Betatorquevirus* genus (TTMV) (11 out of 16, 68.8%). Meanwhile, three sequences (18.8%) were attributed to the *Gammatorquevirus* genus (TTMDV) and two sequences (12.5%) to the *Alphatorquevirus* genus (TTV). The higher content of TTMVs and TTMDVs in children is in line with studies reporting an increased prevalence of these AVs in younger age groups (Kaczorowska et al., 2022). In our study, all individuals had TTMVs; however, E2 and E4 exhibited monoinfections solely with TTMV, E1 showed double coinfections (TTM + TTMV), and E3 presented with triple coinfections (TTM + TTMV + TTMDV) (Figure 2).

Variability of AVs

To establish a genetic relationship between AVs strains, a phylogenetic analysis based on the amino acid sequence of ORF1 was conducted for seven assembled full-length or almost full-length genomes (the smallest being 2,520 nt for ORF1, which is 94% of the complete genome) (Figure 3). The remaining nine sequences had lengths less than 75% of ORF1; hence, they were not included in the phylogenetic analysis.

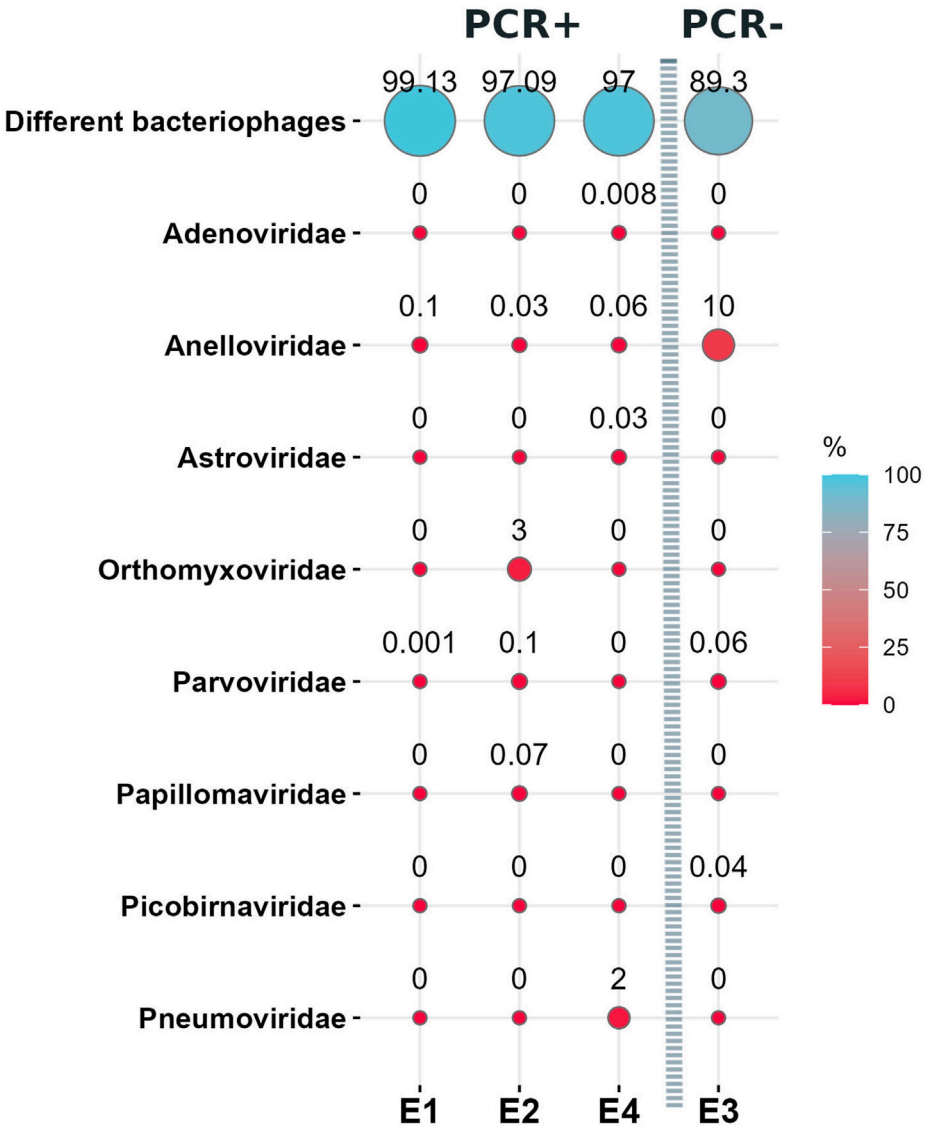

FIGURE 1
Relative viral composition of individual viromes in pediatric patients. The figure illustrates the relative number of reads in the viral group mapped to specific families for PCR-positive samples E1, E2, and E4 for major respiratory viruses, as well as the PCR-negative sample E3.

All of our sequences obtained from pediatric patients exhibit similarity to genomes identified in a large viromic study involving blood samples from febrile children in Tanzania (Cordey et al., 2021) (Figure 3, evolutionary distances). These genomes display significant differences from previously well-described species in NCBI and do not cluster with them. This feature is particularly evident in the genus *Gammatorquevirus* (Figure 3), where the branch with reference strains is separate from the group of our sequences and those from the virome study of febrile children. This may indicate a significant gap in the detection and well-characterization of TTMDV sequences, which could potentially fill the gap between these two branches.

Studied TTMV genomes differentiated into distinct clades from each other and demonstrated a high degree of divergence. The exceptions are NODEA508_E2 and NODE99_E3, which exhibit a high degree of identity (99% amino acid similarity of ORF1, Supplementary Table S1) despite being collected from different children. Due to the highly divergent TTMV sequences, it can be hypothesized that patient E3 was infected with multiple strains of *Betatorquevirus* that evaded host immune system's eradication. Two sequences of TTMDVs NODEA98_E3 and NODEA297_E3 are very similar (100% amino acid identity of ORF1, Supplementary Table S1) and cluster together on the phylogenetic tree.

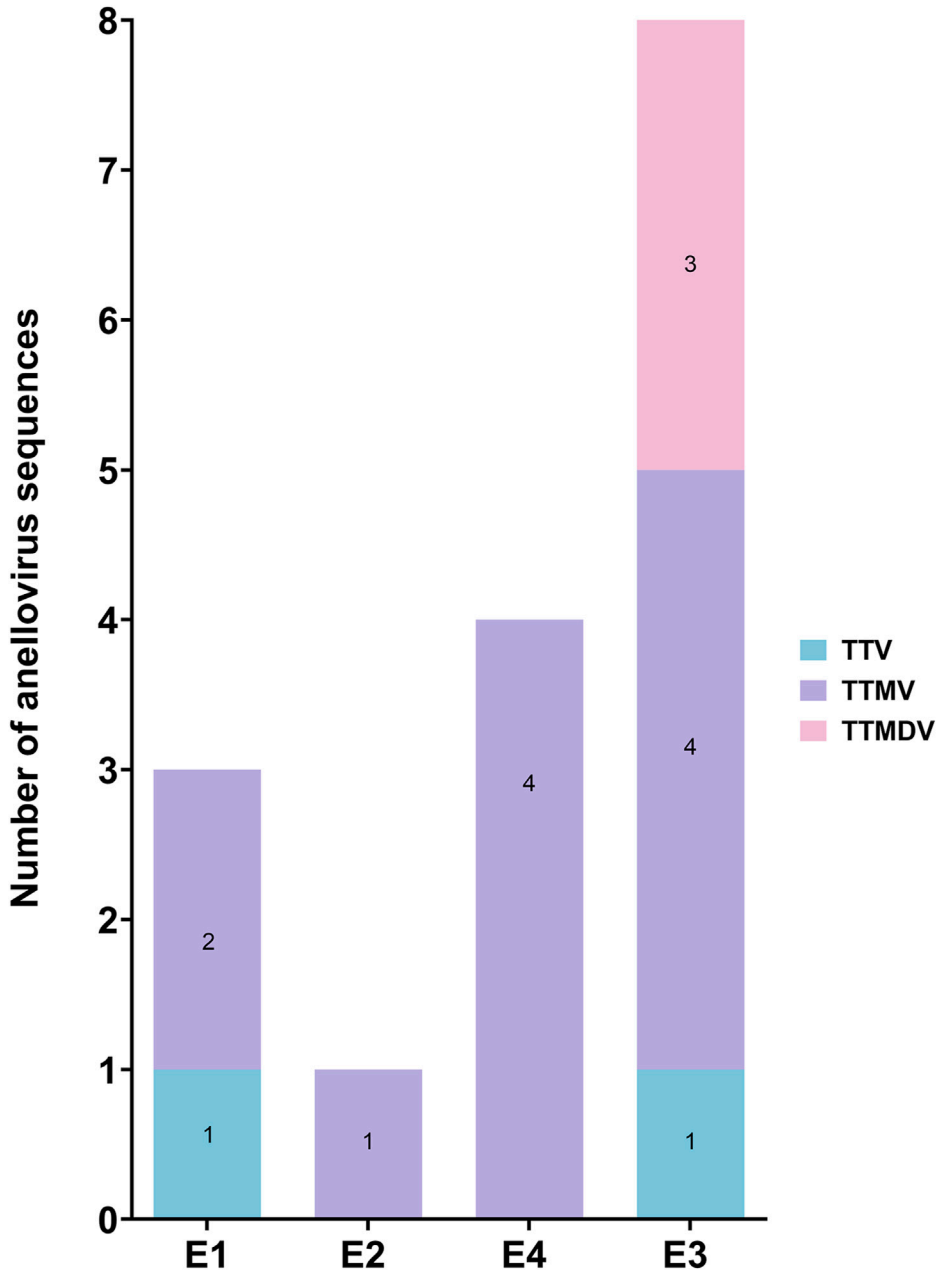

FIGURE 2
Distribution of AVs sequences generated for PCR-positive for major respiratory viruses (E1, E2, and E4) and PCR-negative (E3).

Additionally, a plot was constructed showing the similarity of ORF1 for TTMV sequences covering over 80% of the complete genome (Supplementary Figure S1). The alignment was performed against the prototype species for the TTMV genus - Torque teno mini virus 1-CBD279. The greatest distance from the prototype strain was demonstrated by NODE_28, NODE_58, NODE_98 from sample E3.

Discussion

Thus, the current metagenomic study provided individual viral profiles for children with respiratory symptoms. The analysis of the obtained data confirmed the results of PCR diagnostics for major respiratory pathogens. Additionally, it provided more detailed information on AVs in the examined pediatric patients. The research findings indicate that different

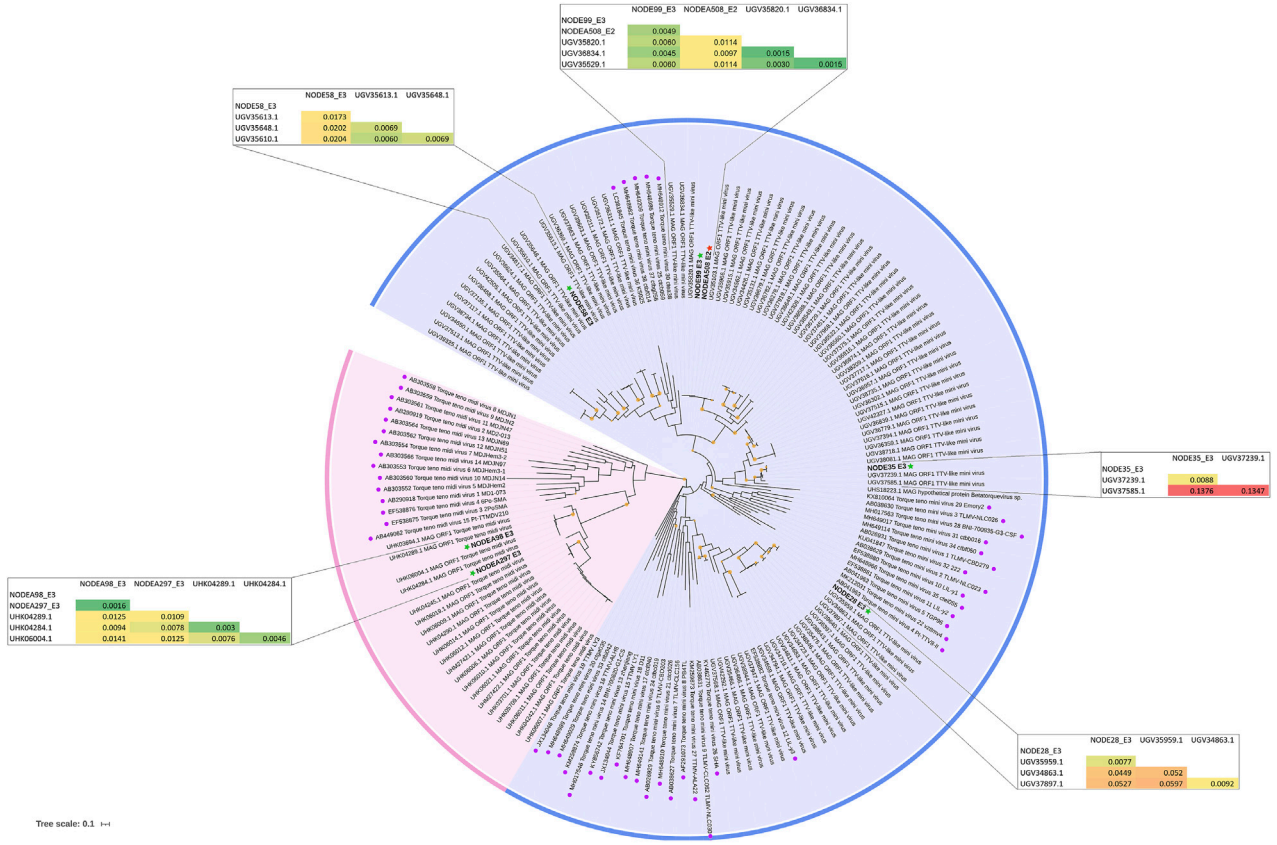

FIGURE 3
Phylogenetic tree for TTMV, TTMDV from aligned ORF1 sequences built by Maximum likelihood method based on the general time reversible + Freq. model implemented in MEGA with 1,000 Bootstrap replicates. The size of the orange circles on the branches indicates the bootstrap level from 70% to 100% The phylogenic tree includes obtained sequences in this study (marked with a red star from sample E2, a green star from E3), reference strains (labeled with a purple circle) of AVs, closest strains (from the non-redundant database BLASTp) to our samples. The pairwise comparison based on ORF1 protein among different strains within the same group is displayed adjacent in the tables. Visualized with iTOL (Letunic and Bork, 2021).

AVs are present in the virome of children with acute respiratory symptoms. The presence of viruses from the *Anelloviridae* family in all samples is in accordance with data on the prevalence of AV infections in children suffering from respiratory diseases (McElvania TeKippe et al., 2012; Maggi and Bendinelli, 2009).

Current research presents varying data concerning the composition of the respiratory tract anellome in both ill and healthy children, often demonstrating contradictory results due to the limited available data. Some studies have reported an increased viral load of TTV in children diagnosed with pneumonia (Maggi et al., 2003b) and those presenting with fever (McElvania TeKippe et al., 2012). Conversely, other investigations have failed to corroborate these findings, indicating no significant differences in TTV levels between children with respiratory symptoms and those without (Wang et al., 2016). In our study, TTVs were detected in the samples E1 and E3 which may be attributed to the more severe respiratory conditions observed in these children — pneumonia. Alternatively, this could reflect a tendency for anellome to shift toward the presence of *Alphatorquevirus* with age (Kaczorowska et al., 2022; Laubscher et al., 2022). The interaction of TTV PAMPs (Pathogen-associated molecular patterns) with receptors, has been documented, suggesting a modulation of inflammatory responses (Freer et al., 2018; Maggi et al., 2003a; Dodi et al., 2021; Maggi and Bendinelli, 2009; Zheng et al., 2007; Rocchi et al., 2009).

The literature regarding TTMDV remains limited. In the study by McElvania TeKippe et al., an increase in TTMDV (and TTV) viremia was demonstrated in febrile children compared to healthy controls, highlighting a potential association between TTMDV, TTV, and fever (McElvania TeKippe et al., 2012) In our investigation, TTMDVs were identified solely in the negative E3 sample. Given the aforementioned study, it cannot be dismissed that the presence of TTMDV and TTV in a sample lacking respiratory viruses but associated with pneumonia may contribute to the manifestation of respiratory symptoms.

Regarding TTMV, the opinions remain that there is insufficient evidence to assert that its levels increase during respiratory illnesses (McElvania TeKippe et al., 2012). However, some studies have reported a high prevalence of TTMV among samples from children with respiratory diseases of unknown etiology (Bal et al., 2022) and pneumonia (Romero-Espinoza et al., 2018).

In our study, all patients exhibited respiratory symptoms, and TTMVs were present in each case, aligning with existing literature documenting TTMV in children with respiratory tract diseases. Notably, the highest number of reads was observed in samples with pneumonia, both in those without respiratory viruses (E3) and in co-infection with RSV (E4). The ability of TTMVs to modulate the innate immune response via proinflammatory cytokines is also reported (Galmès et al., 2013). However, contrasting data exist, suggesting that human AV TTMV is not associated with fever and severe infection (McElvania TeKippe et al., 2012).

Interestingly, our research revealed that in the diseased patient without respiratory viruses, there was a significant representation of AVs in terms of both read numbers and genetic diversity. It is possible that this representation of AVs can activate immune and inflammatory responses, influencing the occurrence and maintenance of respiratory symptoms. Although a similar phenomenon has so far been well described only for TTV infection (Freer et al., 2018; Maggi et al., 2003a; Dodi et al., 2021; Maggi and Bendinelli, 2009; Zheng et al., 2007; Rocchi et al., 2009), there is also information about the possible influence of TTMV on the immune system (Galmès et al., 2013).

The low representation of AVs in patients with respiratory viruses (E1, E2, E4 as opposed to E3) prompts speculation about the conditions under which AVs have an opportunity for replication. It is likely that the immune system, activated to combat pathogens, suppresses the replication of AVs. After the elimination of the main pathogen, AVs may increase to their initial levels, and the anellome stabilizes. Similar observations in fluctuations of AV levels have been made in patients with COVID-19, where the anellovirus load significantly decreased in the first weeks of SARS-CoV-2 infection (Timmerman et al., 2024b).

Unfortunately, the widespread presence of AVs complicates the search for a definitive answer to whether this group of viruses truly worsens the clinical course of the disease.

Limitations

A technical negative control sample (i.e., a sample with transport medium and probe) that underwent metagenomic analysis was missing in our study. However, we compared the assembled contigs of respiratory viruses and found no identical contigs across different samples, indicating the absence of cross-contamination.

We acknowledge that our study was conducted using a small sample size (n = 4) and that it is challenging to draw comprehensive conclusions from this sample. This metagenomic investigation should be considered a pilot study, the results of which we definitely plan to expand in the future to obtain a complete picture, as we consider it interesting. Currently, it offers information on the feasibility and optimization of the metagenomic approach used for the respiratory virome. This is particularly important in the case of novel or complex methods where verification of the effectiveness and reliability of the approach is essential. Despite the sample size limitation, the results may still provide value as they represent the first attempt to shed light on the individual viral profiles of the respiratory tract in children from Novosibirsk. These findings can serve as an impetus for subsequent research and add information useful for the interpretation of related investigations in the future.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ Supplementary Material.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the Federal Research Center for Fundamental and Translational Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/ next of kin.

Author contributions

AN: Writing–original draft, Visualization, Software, Investigation, Formal analysis, Data curation; ND: Writing–review and editing, Software, Methodology, Investigation, Data curation; AD: Writing–original draft, Writing–review and editing, Methodology, Investigation; AK: Methodology, Investigation, Resources; OK: Writing–review and editing, Visualization, Supervision, Project administration, Resources, Funding acquisition, Conceptualization; AS: Supervision, Resources, Conceptualization; KS: Writing–review and editing, Formal analysis, Project administration, Resources,

Funding acquisition, Conceptualization. All authors contributed to the article and approved the submitted version.

Funding

The author(s) declare that financial support was received for the research, authorship, and/or publication of this article. This research was supported by project RSF 19-74-10055-P (sampling, laboratory diagnostics and analysis) and by the State-funded budget project 122012400086-2 (sample preparation for metagenomics).

Acknowledgments

We express our gratitude to the clinicians at Novosibirsk Children's Municipal Clinical Hospital No. 6 for their help with sample collection. This study received partial support from the Centers for Collective Use of Scientific Equipment for "Proteomic Analysis" (agreement No. 075-15-2021-691), as well as from the "Modern Optical Systems" of the Research Center for Fundamental and Translational Medicine in Novosibirsk, Russia. We also acknowledge the preprint titled "Genetic Characteristics of Anelloviruses Detected in Individual Viromes of Children with Acute Respiratory Infection Using the Metagenomic Approach" (https://www.preprints.org/manuscript/202404.1211/) for its contributions to this work.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontierspartnerships.org/articles/10.3389/av.2025.13512/full#supplementary-material

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