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Article

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Prevalence of Anelloviruses in Nasopharyngeal Samples from SARS-CoV-2 Screened Individuals: A Cross-sectional Study

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Running title: Small DNA Viruses co-detection

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Abstract

The human virome encompasses both pathogenic and commensal viruses, with small circular DNA viruses such as Anelloviruses and Redondoviruses (ReDoVs). The objective of this study was to determine the prevalence of Anelloviruses and ReDoVs in nasopharyngeal samples, and to evaluate their potential clinical and oral health associations. In this cross-sectional study, 412 samples were collected. Demographic, clinical, and oral health data were obtained through interviews and clinical records. Viral DNA was extracted and ReDoVs were amplified by PCR targeting a conserved Capsid/Rep intergenic region. Anelloviruses were detected through a two-stage nested-PCR. Statistical analyses included chi-square, Fisher's exact test, logistic regression, and ROC curve modeling to assess associations between viral positivity, clinical symptoms, and oral health indicators. SARS-CoV-2 was detected in 26.5% and ReDoV in 22.1%. Anelloviruses were highly prevalent (TTV: 81.6%, TTMV: 47.8%, TTMDV: 43.9%). SARS-CoV-2 infection was associated with symptoms including fever, headache, and cough ($p < 0.0001$). ReDoV and TTMV were independently associated with self-reported gum problems ($p < 0.0001$ and $p = 0.015$, respectively). ReDoV showed intra-viral co-detection with TTMV and TTMDV. Logistic regression confirmed fever and gum problems as the only robust predictors of SARS-CoV-2 and ReDoV infection, respectively. Predictive models for these two viruses demonstrated good discrimination (AUC = 0.81-0.83). These findings highlight the ubiquity of Anelloviruses and the distinct clinical association of ReDoVs with oral health indicators. The results underscore the complexity of the respiratory virome and its potential as a biomarker of host–microbe interactions.

Keywords: Anelloviruses; Redondoviruses; SARS-CoV-2; Respiratory virome; Biomarkers

Introduction

The human virome encompasses not only classical pathogens but a vast array of commensal and persistent small DNA viruses whose roles in health and disease are increasingly recognized [1,2]. Metagenomic approaches have uncovered novel families, reshaped our understanding of orphan agents such as Anelloviruses, and identified emerging groups like the Redondoviridae, revealing that the respiratory tract harbours diverse viral communities with potential implications for host immunity, chronic inflammation, and acute infection outcomes [3–6]. Two viral families of particular interest are the Redondoviridae and Anelloviridae, both are highly prevalent, genetically diverse, and persistently colonize the oro-respiratory mucosa, yet their pathogenic significance remains unresolved.

The *Redondoviridae*, first described in 2019, are small circular single-stranded DNA viruses within the CRESS-DNA group that possess a distinctive tripartite genome encoding Rep, Cap, and a unique ORF3 [7]. Two genera, *Brisavirus* and *Vientovirus*, are consistently detected in human oral and respiratory samples, with prevalence ranging from 2-15% in Western cohorts to over 70% in some African populations [6,8–10]. Redondoviruses have been strongly linked to periodontitis, showing higher infection rates and independent odds of disease, with viral persistence in inflamed tissues and decline following clinical treatment [6,7,11,12]. Their diversity is largely driven by Rep-mediated recombination, but whether they actively contribute to disease or merely replicate opportunistically in inflamed niches remains uncertain.

The *Anelloviridae*, discovered in 1997 with Torque teno virus (TTV), are the most ubiquitous human viruses, infecting over 90% of the population [13,14]. Encompassing the genera *Alphatorquevirus* (TTV), *Betatorquevirus* (TTMV), and *Gammatorquevirus* (TTMDV), they encode multiple ORFs including immune-modulatory proteins and microRNAs, and are characterised by extraordinary genetic diversity, frequent intra-host co-infections, and lifelong persistence acquired in infancy via horizontal transmission [13–15]. Rather than mere presence, quantitative Anellovirus loads have emerged as critical immune-competence biomarkers: elevated titers are consistently observed in immunosuppressed individuals, transplant recipients, HIV patients, and those on chemotherapy, reflecting an inverse relationship between immune function and viral replication [13,16–21]. This has led to their exploration for predicting transplant rejection, guiding immunosuppression, and stratifying vaccine responses. In COVID-19, elevated respiratory TTV loads at admission have predicted intensive care needs, while low plasma loads, indicative of hyperactive immunity, have paradoxically also correlated with severe disease, and baseline TTV loads have been

inversely associated with SARS-CoV-2 vaccine antibody responses, especially in immunocompromised hosts [16,17,19,20].

Despite these advances, major gaps remain in understanding the ecology and interactions of Redondoviruses and Anelloviruses. Both families are enriched in states of immune dysfunction or chronic mucosal inflammation, including periodontitis, transplantation, and COVID-19, yet the extent to which they function as passive biomarkers, opportunistic replicators, or active contributors to pathogenesis is unknown. Moreover, their marked geographic variability underscores the need for region-specific studies [9,10,22]. We previously investigated the prevalence of Redondoviruses and their association with poor oral hygiene in a cohort of individuals screened for SARS-CoV-2 [11]. The present study extends that work by examining the co-occurrence of Anelloviruses and Redondoviruses in the same respiratory samples. It aims to elucidate their epidemiological interplay and clinical relevance within a contemporary screening setting, thereby contributing to the broader discourse on host-virome-disease relationships.

Materials

Study populations and data collection

This cross-sectional study included 412 nasopharyngeal swab samples (that we have used for detecting ReDoVs in our previous study [11] collected in viral transport medium (VTM) from individuals referred to health centers and hospitals affiliated with Iran University of Medical Sciences (Tehran, Iran) for SARS-CoV-2 diagnostic testing between 01/03/2023 and 30/08/2023 [23]. Demographic information such as age and sex, as well as SARS-CoV-2 RT-PCR results, were retrieved from institutional databases. Individuals who tested negative for SARS-CoV-2 and reported no acute respiratory symptoms were also included to provide a comparative group. Additional information was obtained through structured questionnaires and interviews, covering clinical symptoms, and oral health or dental problems. Written informed consent was obtained from all participants prior to inclusion, and ethical approval was granted by the institutional review board

Viral DNA extraction of the specimens

The viral DNA was isolated from the oropharyngeal and nasopharyngeal specimens using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's protocols, and then the quantity and quality of the extracted viral nucleic acid assessed by a Nano-Drop spectrophotometer (Thermo Scientific, Wilmington, MA) instrument, and then preserved at -80 °C until testing.

ReDoV amplification by PCR

Nasopharyngeal samples stored at -80°C were thawed, and viral DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. The DNA quality and concentration were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). PCR amplification targeted a conserved genomic region of Redondovirus using the primer pair Redondo-Forward (5′-ATAGTACCATCAGAAACAGGTG-3′; nucleotides 1709-1730) and Redondo-Reverse (5′-GTTTCACAAGTGACAACGAAA-3′; nucleotides 1895–1875), yielding a 187 bp product. Reactions were performed in a 25 µL mixture containing 2.5 µL of 10× Ex Taq buffer (Mg^{2+} free), 2.0 µL of 25 mM dNTP mix, 2.0 µL of 25 mM $MgCl_2$, 10 pmol of each primer, 1.0 U of Ex Taq DNA polymerase (TaKaRa Biotechnology, Japan), and 5 µL of extracted DNA as template. The cycling conditions consisted of an initial denaturation at 95°C for 10

min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 35 s, with a final extension at 72 °C for 5 min. PCR efficiency was verified by serial 10-fold dilutions of strongly positive samples, and relative quantification was further confirmed using a Rotor-Gene Q system (Qiagen, Germany), demonstrating high amplification efficiency. Amplicons were visualized by agarose gel electrophoresis, confirming specific detection of ReDoV DNA [6,11].

PCR detection of anelloviruses

The DNA of Anelloviruses was amplified using a two-stage PCR assay with specific primers (Table 1) as reported before [15]. In the first stage, a conserved genomic sequence of Anelloviruses was amplified with universal primers NG779/NG780 (sense) and NG781/NG782 (antisense). Reactions were carried out in a 25 µL mixture containing 2.5 µL of 10× Ex Taq buffer (Mg^{2+} free), 2.5 µL of a 25 mM dNTP mix, 2.5 µL of 25 mM $MgCl_2$, 1.0 U Ex Taq DNA polymerase (TaKaRa Biotechnology, Japan), 10 pmol of each primer, and 5 µL of extracted DNA template. Cycling conditions consisted of an initial denaturation at 94 °C for 10 min, followed by 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 8 min. The second stage consisted of semi-nested or nested PCRs using 5 µL of the first-round product as template and primers specific to each anellovirus species. For TTV, primers NG779/NG780 (forward) and NG785 (reverse) were used; for TTMDV, NG795 (forward) and NG796 (reverse); and for TTMV, NG792/NG793/NG794 (forward) with NG791 (reverse). Reaction cycling included an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension of 72 °C for 5 min. Amplified products, along with positive and negative controls and a 50 bp DNA ladder, were electrophoresed on 3.0% agarose gels stained with safe stain and visualized under UV transillumination. Expected amplicon sizes were 112-117 bp for TTV, 88 bp for TTMDV, and 70-72 bp for TTMV, enabling accurate differential detection of the three anellovirus species.

Table 1. Primers used in the current study to determine the presence of three species of anellovirus gemome in participants samples

Primer	PCR Round	Polarity	Nucleotide sequence (5' to 3')	Nucleotide position	Amplicon size/nt
			Universal		
NG779	First	Sense	ACWKMCGAATGGCTGAGTTT	99-118	
NG780	First	Sense	RGTGRCGAATGGYWGAGTTT	99-118	128 bp
NG781	First	Antisense	CCCKWGCCCGARTTGCCCCT	208-227	
NG782	First	Antisense	AYCTWGCCCGAATTGCCCCT	208-227	
			TTV-Specific		
NG779	First and Second	Sense	ACWKMCGAATGGCTGAGTTT	99-118	
NG780	First and Second	Sense	RGTGRCGAATGGYWGAGTTT	99-118	112-117 bp
NG785	Second	Antisense	CCCCTTGACTBCGGTGTGTAA	192-212	
			TTMDV-Specific		
NG795	Second	Sense	SGABCGAGCGCAGCGAGGAG	78-97	88 bp
NG796	Second	Antisense	GCCCGARTTGCCCCTAGACC	146-165	
			TTMV-Specific		
NG792	Second	Sense	TTTATGCYGCYAGACGRAGA	195-214	
NG793	Second	Sense	TTTAYCMYGCCAGACGGAGA	195-214	70-72 bp
NG794	Second	Sense	TTTATGCCGCCAGACGRAGG	195-214	
NG791	Second	Antisense	CTCACCTYSGGCWCCCGCCC	247-266	

Statistical analysis

Continuous variables were summarized as mean ± standard deviation (SD) or median with interquartile range (IQR), as appropriate, whereas categorical variables were reported as counts and percentages. To assess associations between categorical variables, contingency tables were constructed and analyzed using the Pearson chi-square test. For 2×2

contingency tables with small expected frequencies (<5), Fisher's exact test was applied. Effect sizes were quantified using Cramér's V. Associations between continuous and categorical variables were tested using Welch's *t*-test (with Cohen's *d* as effect size) when normality assumptions were satisfied (Shapiro–Wilk test, $p > 0.05$). In cases where normality was violated, the non-parametric Mann-Whitney *U* test was used, and rank-biserial correlation was reported. Correlations between two continuous variables were tested using Pearson's correlation when normality was met, or Spearman's rank correlation otherwise. All statistical analyzes were done using MS-Ofiice Excel v2019's XLSTAT v2019 add-in as reported before [24–26].

Multivariate analyses were conducted using logistic regression models to evaluate the independent association of different variables with viral positivity. Binary outcomes were modeled using maximum likelihood estimation. Results were reported as odds ratios (OR) with 95% confidence intervals (CI). Model performance was assessed using predicted probability distributions, receiver operating characteristic (ROC) curves, and area under the curve (AUC). All statistical tests were two-tailed, and a significance level of $\alpha = 0.05$ was applied. No correction for multiple comparisons was performed unless otherwise stated.

Ethical statement

This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and national guidelines for medical research in Iran. The research protocol was reviewed and approved by the Research Ethics Committees of School of Medicine, Iran University of Medical Sciences (Approval ID: IR.IUMS.FMD.REC.1404.061). Written informed consent was obtained from all participants prior to sample collection.

Results

Study population and characteristics

A total of 412 respiratory samples from individuals screened for SARS-CoV-2 were analyzed for the presence of Anelloviruses (Table 2 and Table 3). The mean age of participants was 41.1 ± 14.7 years, and the cohort was 36.9% male and 63.1% female. SARS-CoV-2 RNA was detected in 109 of 412 samples (26.5%), while ReDoV DNA was identified in 91 samples (22.1%). Anelloviruses were highly prevalent in this population. TTV was present in 336 samples (81.6%), TTMV in 197 (47.8%), and TTMDV in 181 (43.9%). Clinical symptoms were frequently reported by participants.

Table 2. *Descriptive statistics of data*

Variable	Mode	Categories	Rel. frequency per category (%)
Gender	Female	Female	63.11
		Male	36.89
Age categories	30-39	10-19	2.67
		20-29	17.48
		30-39	33.50
		40-49	24.27
		50-65	12.62
		>65	9.47
SARS-CoV-2	Negative	Negative	73.54
		Positive	26.46
ReDoV	Negative	Negative	77.91
		Positive	22.09
TTV	Positive	Negative	18.45
		Positive	81.55
TTMV	Negative	Negative	52.18
		Positive	47.82
TTMDV	Negative	Negative	56.07
		Positive	43.93
Gum Problem	Negative	Negative	74.51
		Positive	25.49
Fever	Negative	Negative	62.86
		Positive	37.14
Headache	Negative	Negative	65.53

		Positive	34.47
Confusion	Negative	Negative	88.83
		Positive	11.17
Chills	Negative	Negative	76.70
		Positive	23.30
Bone ache	Negative	Negative	73.54
		Positive	26.46
Dry cough	Negative	Negative	72.82
		Positive	27.18
Expectoration	Negative	Negative	87.62
		Positive	12.38
Chest pain	Negative	Negative	91.50
		Positive	8.50
Dyspnea	Negative	Negative	90.53
		Positive	9.47
Runny nose	Negative	Negative	82.52
		Positive	17.48
Rhinorrhea	Negative	Negative	86.65
		Positive	13.35
Bleeding stomach	Negative	Negative	99.51
		Positive	0.49
Anosmia	Negative	Negative	86.89
		Positive	13.11
Ageusia	Negative	Negative	90.53
		Positive	9.47

Table 3. *Baseline characteristics of the study population (N = 412). Values are given as number of individuals (% of total).*

Characteristic	Value
Total samples analyzed	412
Age (years)	41.1 ± 14.7 (mean ± SD)
Gender	152 male (36.9%), 260 female (63.1%)
SARS-CoV-2 positive	109 (26.5%)
ReDoV positive	91 (22.1%)
TTV positive	336 (81.6%)
TTMV positive	197 (47.8%)
TTMDV positive	181 (43.9%)
Fever	153 (37.1%)
Headache	142 (34.5%)
Dry cough	112 (27.2%)
Bone ache	109 (26.5%)
Gum problems	105 (25.5%)

Viral prevalence and co-detection patterns

Anelloviruses were generally distributed in the sample set. Chi-square tests revealed no significant associations between SARS-CoV-2 infection status and the presence of ReDoV or any of the three Anelloviruses (all $p > 0.05$). By contrast, several significant co-detection relationships were observed among the DNA viruses themselves. In particular, ReDoV positivity was correlated with the presence of TTMV ($p = 0.003$, Cramér's $V = 0.146$) and TTMDV ($p = 0.004$, $V = 0.142$) in samples (*see* and Figure 1). Strong intra-Anellovirus associations were also noted. Accordingly, TTV co-occurred with TTMDV more often than expected by chance ($p = 0.0078$, $V = 0.131$), and there was a distinct co-detection between TTMV and TTMDV ($p < 0.0001$, $V = 0.435$).

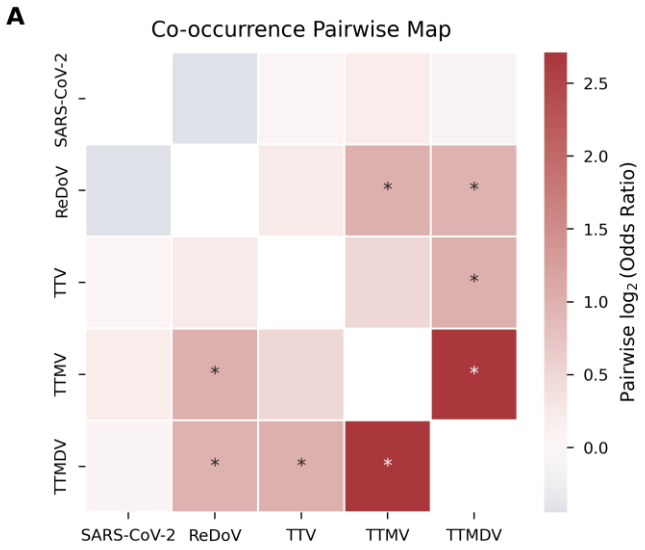


Fig. 1 *Co-detection/co-occurrence patterns among viruses. This network diagram/correlation matrix illustrates significant pairwise associations between the presence of different viruses in the same sample. Lines connecting virus pairs indicate a positive co-detection relationship. Particularly, ReDoV co-occurred frequently with TTMV and with TTMDV, and there were also significant links between TTV and TTMDV as well as a very strong association between TTMV and TTMDV*

Viral associations with clinical features

Consistent with clinical expectations, SARS-CoV-2 infection was significantly associated with multiple acute respiratory symptoms (Table 3). Patients who tested positive for SARS-CoV-2 were much more likely to report fever (approximately 68.8% of SARS-CoV-2-positive individuals had fever, vs. 25.7% of SARS-CoV-2-negative individuals; $p < 0.0001$, Cramér's $V = 0.393$). Similarly, headache (62.4% vs 24.4%; $p < 0.0001$, $V = 0.352$), chills (45.9% vs 15.2%; $p < 0.0001$, $V = 0.320$), bone ache (50.5% vs 17.8%; $p < 0.0001$, $V = 0.326$), and dry cough (48.6% vs 19.5%; $p < 0.0001$, $V = 0.289$) were all significantly more prevalent among SARS-CoV-2–positive cases than among those who tested negative for SARS-CoV-2. In addition to these very strong associations, several other symptoms showed moderate associations with SARS-CoV-2 status. Accordingly, expectoration was more common in the infected group (20.2% vs 9.6% in negatives; $p = 0.004$), as were chest pain (15.6% vs 5.9%; $p = 0.002$) and dyspnea (15.6% vs 7.3%; $p = 0.011$). Although these latter symptoms had smaller effect sizes (Cramér's V 0.13-0.15), they were still significantly associated with SARS-CoV-2 infection (Table 4).

Table 4. *Significant associations between viral detection and clinical symptoms (chi-square tests, N = 412). Effect size is measured by Cramér's V.*

Virus	Symptom	Symptom frequency if virus+ vs virus–	p-value	Cramér's V
SARS-CoV-2	Fever	68.8% vs 25.7%	< 0.0001	0.393

Virus	Symptom	Symptom frequency if virus+ vs virus–	p-value	Cramér's V
SARS-CoV-2	Headache	62.4% vs 24.4%	< 0.0001	0.352
SARS-CoV-2	Chills	45.9% vs 15.2%	< 0.0001	0.320
SARS-CoV-2	Bone ache	50.5% vs 17.8%	< 0.0001	0.326
SARS-CoV-2	Dry cough	48.6% vs 19.5%	< 0.0001	0.289
SARS-CoV-2	Expectoration	20.2% vs 9.6%	0.004	0.142
SARS-CoV-2	Chest pain	15.6% vs 5.9%	0.002	0.153
SARS-CoV-2	Dyspnea	15.6% vs 7.3%	0.011	0.126
ReDoV	Gum problems	63.7% vs 14.6%	< 0.0001	0.467
TTMV	Gum problems	31.0% vs 20.5%	0.015	0.120

In contrast, among the small circular DNA viruses, only two significant symptom relationships were observed. ReDoV positivity was associated with gum problems. In this regard, 63.7% of individuals positive for ReDoV reported gum issues, compared to only 14.6% of those without ReDoV ($p < 0.0001$, $V = 0.467$). Similarly, TTMV detection was associated with a higher frequency of gum problems (31.0% in TTMV-positive vs 20.5% in TTMV-negative individuals; $p = 0.015$, $V = 0.120$), although this association was modest in magnitude. No other acute respiratory symptom was consistently linked to the presence of TTV or TTMDV.

Predictors of infection and forest plots

Multivariable logistic regression was used to identify independent predictors of virus detection when considering all demographic and symptom variables together. Figure 2 presents a forest plot of the adjusted odds ratios (ORs) and 95% CI for all predictors across the five viral infection models. In the SARS-CoV-2 model, fever emerged as a strong independent predictor of infection, with an adjusted OR = 3.37 (95% CI 1.69-6.74, $p = 0.0006$). Age also showed a significant association with SARS-CoV-2 status (OR = 1.03 per year increase in age, 95% CI 1.01-1.05, $p = 0.0037$). Both fever and age remained significant predictors of SARS-CoV-2 positivity after controlling for multiple comparisons (false discovery rate adjusted q-values < 0.05). No other clinical or demographic predictors reached statistical significance for the SARS-CoV-2 model after FDR correction. In the ReDoV model, the presence of gum problems was a strong predictor of ReDoV detection (adjusted OR = 12.19, 95% CI 6.85-21.70, $p < 1\times10^{-16}$), and this association remained highly significant after FDR adjustment ($q < 0.001$). By contrast, for the TTV, TTMV, and

TTMDV models, none of the many candidate symptoms or demographic factors achieved significance level after correcting for multiple testing (all q > 0.05).

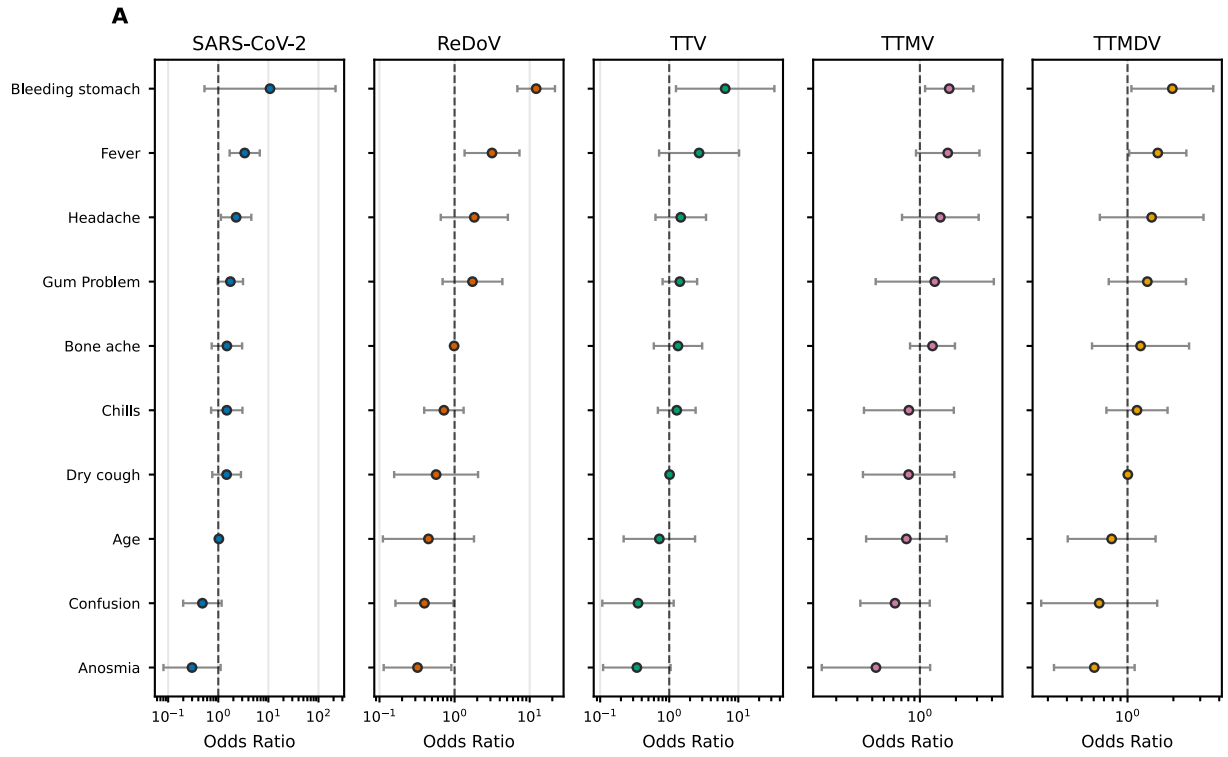


Fig. 2 *Multivariable logistic regression/forest plots results. The adjusted odds ratios (with 95% confidence intervals) for all predictor variables are summarized in each virus-specific logistic regression model. Each panel (or colored segment) corresponds to one virus, and each predictor's effect is plotted on a logarithmic OR scale. Predictors that met the significance threshold after multiple-comparison correction are highlighted. The lack of significant predictors in the TTV, TTMV, and TTMDV models is reflected by their forest plots, where no predictor's confidence interval is entirely above or below the null line*

In addition to identifying predictors, we evaluated the overall performance of each logistic model. Figure 3 shows the ROC curves for the five virus prediction models. The SARS-CoV-2 model achieved the highest discrimination with an AUC of 0.812, closely followed by the ReDoV model (AUC = 0.817). Both of these models show good classification ability, substantially better than chance. In contrast, the models for TTV, TTMV, and TTMDV demonstrated much poorer discriminatory power, with AUC values in the range of 0.62-0.66. Figure 4 also includes precision-recall (PR) curves for each model, which similarly show that the SARS-CoV-2 and ReDoV models outperform the others in identifying true positives with higher precision across a range of recall levels.

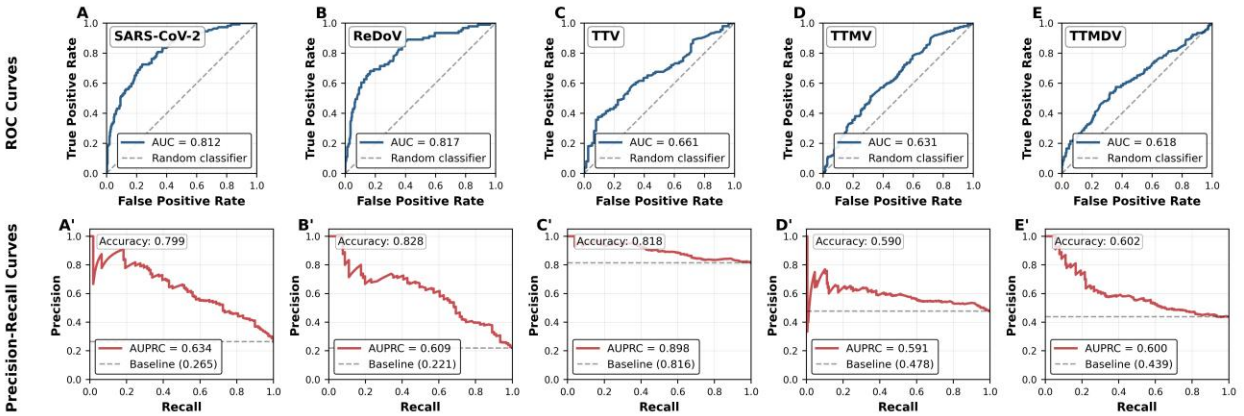


Fig. 3 *Model discrimination via ROC curves. This panel presents the receiver operating characteristic curves for the logistic regression models predicting infection status for each virus. The curves for SARS-CoV-2 (blue) and ReDoV (orange) rise furthest toward the top-left, corresponding to the highest AUC values. The TTV, TTMV, and TTMDV models (green, red, and purple curves, respectively) hover closer to the 45° diagonal, with AUCs around 0.62–0.66, denoting weak discrimination. Dotted lines illustrate the 95% confidence bands for each ROC curve. Inset plots or additional curves show the precision-recall performance, where again the SARS-CoV-2 and ReDoV models lead with higher precision across recall levels compared to the essentially flat, chance-level performance of the other virus models*

Predictor significance heatmap

To visualize the pattern of predictor importance across all viruses, a heatmap of the association significance was constructed (Figure 4). This heatmap with cells colored by $-\log_{10}$ p-value or FDR q-value for each predictor-virus combination highlights the distinctiveness of the predictors for each virus. Fever was a significant predictor only in the SARS-CoV-2 column, and gum problems stands out only in the ReDoV column. No other symptom or factor shows a notable signal for any of the viruses once the multiple-comparison adjustments are considered. In particular, there are no predictors that are consistently significant across more than one virus, each virus has a unique profile. The heatmap thus confirms that SARS-CoV-2 and ReDoV have one strong, specific predictor each, whereas TTV, TTMV, and TTMDV lack any significant predictors, aligning with the results of the individual logistic regressions.

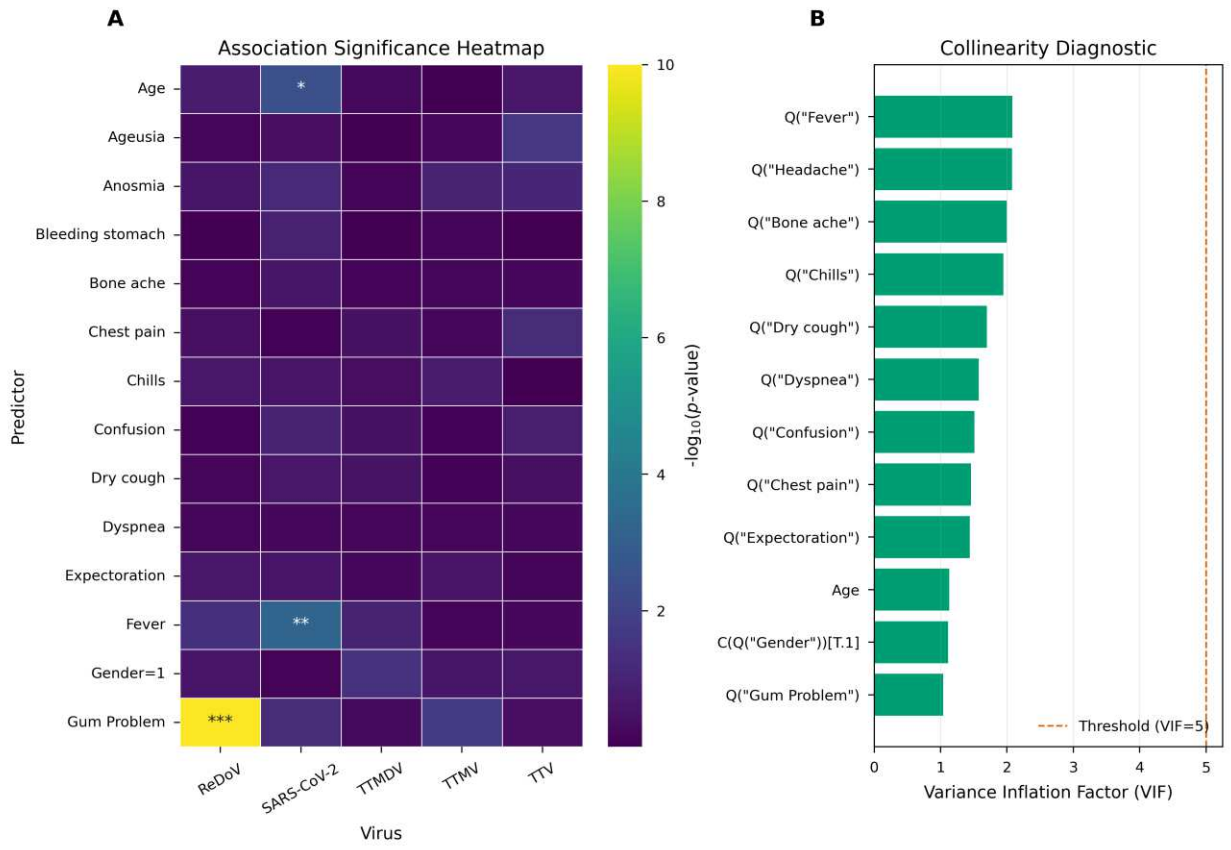


Fig. 4 *Heatmap of predictor significance across viruses. Each row in the heatmap corresponds to a clinical or demographic predictor, and each column corresponds to one of the five target viruses. The color intensity represents the statistical significance of the association between a given predictor and virus. This underscores that each virus has a distinct pattern of predictor significance and that no predictor/symptom is universally associated with multiple viruses in this study*

Predicted probability distributions

We further examined the distributions of predicted infection probabilities output by each logistic model, comparing the distributions for actual positive cases versus actual negative cases. Figure 5 displays these probability distributions for each virus model. For the SARS-CoV-2 model, the distribution for true positive cases was clearly shifted toward higher predicted probabilities compared to the distribution for negatives. SARS-CoV-2-positive individuals had a median predicted infection probability of about 0.50 and many had values well above 0.8, whereas SARS-CoV-2-negative individuals had a median predicted probability around 0.12. Similarly, the ReDoV model produced higher probability estimates for ReDoV-positive cases than for ReDoV-negatives. These shifts indicate that the models for SARS-CoV-2 and ReDoV assign substantially higher likelihoods of infection to those who truly are infected, reflecting decent model calibration and discrimination. In contrast, for TTV, TTMV, and TTMDV, the predicted probability distributions for positives and negatives overlap extensively, with both groups' probabilities largely

clustering around 0.5. In those models, a typical positive case is just as likely to receive a mid-range, around 50%, predicted probability as a negative case is. This supports the earlier ROC findings.

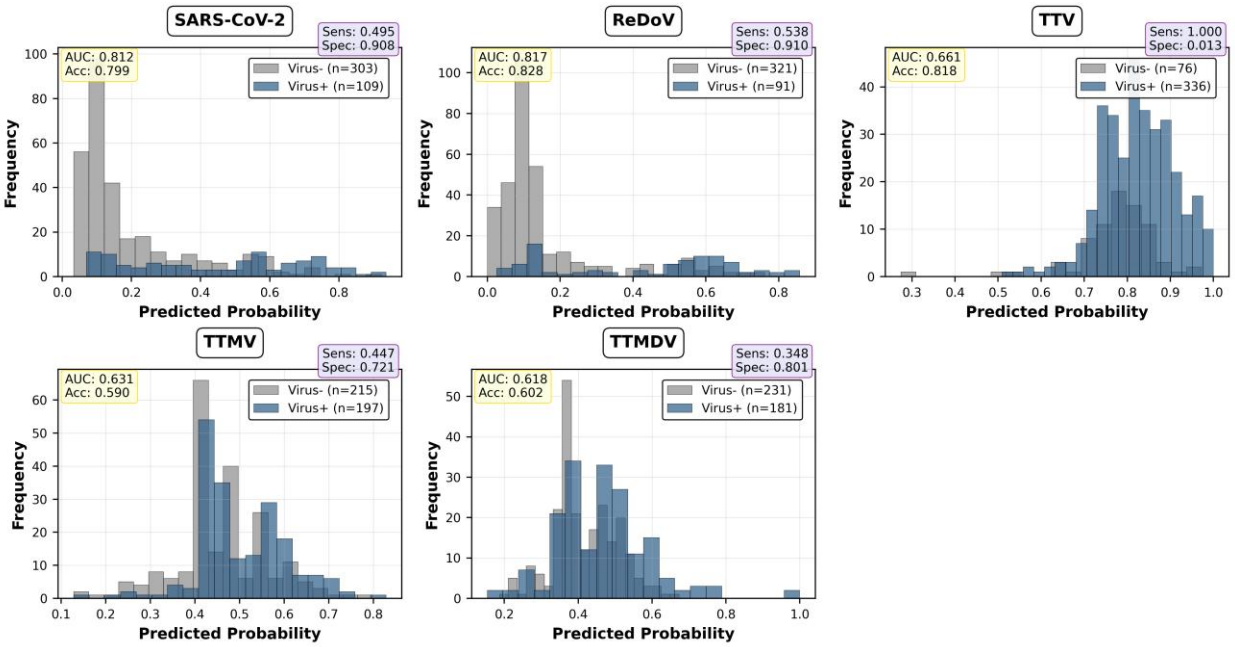


Fig. 5 *Predicted probability distributions by actual infection status. For each virus, the model's output probabilities have been plotted for the group of true-positive cases and for true-negative cases. SARS-CoV-2 and ReDoV models show a clear separation. The probability density for positives (solid bars) is skewed toward higher values compared to that for negatives (shaded bars), indicating that infected individuals tended to receive higher risk scores from the model. For instance, many SARS-CoV-2 cases have high predicted probabilities, whereas most uninfected individuals have probabilities below 0.3. In contrast, the TTV, TTMV, and TTMDV panels show nearly overlapping distributions for positives and negatives (both centered around 0.5), demonstrating the models' lack of discriminatory power*

Discussion

In this study, the prevalence and clinical correlations of Anelloviruses was investigated in respiratory samples collected during SARS-CoV-2 screening that was already used for detecting ReDoVs in the previous study [11]. Anelloviruses were prevalent among individuals with or without co-occurance of ReDoVs or SARS-CoV-2. Since it has been previously reported that oral hyegien could be a predictor of ReDoVs [6], a multivariate analysis was performed. Accordingly, SARS-CoV-2 infection was significantly associated with classic respiratory symptoms, age, and systemic features, but not with ReDoV or Anellovirus infections. Instead, ReDoV, and, to a lesser extent, TTMV, were correlated significantly with self-reported gum problems. Especially, intra-family associations emerged among small circular DNA viruses, particularly ReDoV with TTMV and TTMDV. Multivariate analysis confirmed no significant predictor for Anellovirus presence in the clinical respiratory samples.

The ReDoV prevalence (22%), as part of our previously reported finding [11], aligns with earlier PCR-based studies that placed detection in respiratory tracts between 4%-11% [7,8]. In contrast, global metagenomic surveys report ReDoV presence in up to 30-70% of saliva samples depending on population [9,10]. A significant association between ReDoV presence and gum problems was observed ($p < 0.0001$, $V = 0.467$), reinforcing a growing consensus that ReDoV is enriched in periodontitis and inflammatory oral environments [12]. Quantitative and longitudinal studies have even demonstrated that ReDoV levels decrease following periodontal treatment [9,27]. The detection of TTV (81.6%) and high rates for TTMV and TTMDV mirror established epidemiological patterns. Accordingly, these viruses are acquired early in childhood and persist lifelong [15,28]. The findings of the present study align with reports showing 80–90% adult prevalence and frequent concurrent infections across Anellovirus genera [13,14]. furthermore, ReDoV co-occurred significantly with TTMV and TTMDV, and these Anelloviruses likewise associated with gum problems. These patterns may reflect shared tissue tropism in the oral mucosa, a known niche for Anellovirus shedding [14]. The gum problems were identified as a strong predictive factor for presence of ReDoVs, especially in oral samples. This is accompanied by the fact that the *Entamoeba gingivalis* (*E. gingivalis*), a well know dental complication-associated amoeba is the known host for the virus [29]. Accordingly, investigating the *E. gingivalis* will most probably show a strong correlation with the prevalence of ReDoVs in patients with self-reporting gum/dental isses, and most likely increase the predictive power of the reported model.

Anelloviruses are increasingly aknowledged as surrogate markers of immune competence, with viral loads inversely related to immune status in transplant and immunodeficient populations [16,17]. Acute viral infections, including SARS-CoV-2, may transiently suppress TTV replication, consistent with some studies showing decreased Anellovirus loads early in infection, followed by rebound [30]. While the present study did not quantify viral loads, the high detection rates indicate preserved replication and reinforce TTV's role as a steady-state immune biomarker. Furthermore, the results emphasize the complexity and independence of respiratory virome components. Global studies have shown ReDoV diversity and persistence within individuals, with recombination shaping viral genomes [10]. On the other hand, Anellovirus diversity is maintained through mixed-genotype persistence and rapid evolution [14]. Thus, these two viral families appear to form a stable core of the upper airway virome, modulated by host immunity, microbial ecology, and possibly oral health. A major strength of this study lies in its comprehensive screening of a large, clinically relevant, respiratory sample cohort. By adding this study to previous reports, and correlating them with symptoms, and oral health, a relative interconnection between two small DNA virus with the causative agent of COVID-19, SARS-CoV-2 is addressed. However, some limitations should be acknowledged. Accordingly, viral presence was inferred by PCR detection rather than quantitative loads [31]; thus, we cannot distinguish between high- and low-abundance states, which may carry differing implications. Additionally, cross-sectional design limits inference of temporality; longitudinal sampling would better assess dynamics of viral replication in relation to symptom onset and immune state. Moreover, self-reported gum problems may under-report oral disease, which we have already addressed in the previous study [6].

Anelloviruses, particularly TTV, have been marked as biomarkers of immune status in transplantation and autoimmunity settings [32–34]. Monitoring their respiratory shedding may offer insights into local mucosal immunity, integrating quantitative load measures, immunophenotyping, and functional assays. Additionally, the coexistence of ReDoV and Anelloviruses underscores that the human respiratory tract harbors a stable, non-pathogenic DNA virome. Disentangling its contributions to health, immune regulation, and susceptibility to other infections remains a frontier. Broader integration of respiratory virome profiling with clinical metadata could enrich disease models, inform biomarker development, and ultimately deepen our understanding of host-virome interplay.

Conclusion

This study demonstrated the high prevalence of Anelloviruses in respiratory samples and confirms their widespread persistence regardless of SARS-CoV-2 or ReDoV status. ReDoVs were identified in approximately one-fifth of individuals and showed a robust association with gum problems, reinforcing their putative link with oral health and periodontal disease. In contrast, Anelloviruses exhibited strong intra-family co-detection but no significant clinical predictors, suggesting their role as commensal virome constituents rather than active contributors to acute respiratory illness. Together, these findings emphasize the distinct ecological niches of small circular DNA viruses in the human respiratory tract and highlight their value as potential biomarkers of mucosal and immune status. Future studies should incorporate quantitative viral load analyses and longitudinal sampling to disentangle the dynamic interactions between these viruses, host immunity, and oral–respiratory health.

Statements and Declarations

Declaration of competing interests: We have nothing to declare.

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Data Availability: The authors confirm that the data supporting the findings of this study are available within the article and its supplemented materials.

Ethical statement: This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and national guidelines for medical research in Iran. The research protocol was reviewed and approved by the Research Ethics Committees of School of Medicine, Iran University of Medical Sciences (Approval ID: IR.IUMS.FMD.REC.1404.061, Approval Date: 27 April 2025). Written informed consent was obtained from all participants prior to sample collection.

Consent for publication: Not applicable.

Clinical trial: Not applicable.

Supplementary material

S1 CSV. The raw data of the study. Full data used for the presented study.

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