Phylogenetic analysis of torquetenovirus in Romania: possible evidence of distinct geographical distribution

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Abstract

Torquetenovirus (TTV) is highly prevalent and little is known of its circulation in humans. We aimed to assess the geographical distribution and phylogeny of TTV from Romanians. A fragment of TTV UTR B was sequenced in samples from volunteers from all seven development regions of Romania. Additional sequences from Romanian dialyzed patients were added for phylogenetic analysis. More than 80% of Romanian sequences clustered with isolates assigned to TTV1 and TTV3 (former genogroup 1), while the rest clustered with TTV19-SANBAN and TTV22-svi-1 (former genogroup 3) and TTV-Hebei-1. Phylogenetic analysis showed segregation between isolates from North-East and West Romania providing possible evidence of distinct geographical distribution.

Full Text

Torquetenovirus (TTV) is a small non-enveloped virus with a single-stranded circular DNA genome that infects primarily humans. Despite undergoing major taxonomic identity changes since its discovery in 1997, TTV is currently considered the type species of the *Alphatorquevirus* genus of Anelloviridae family [1, 2]. Its genome contains an untranslated region (UTR, divided into UTR A and UTR B) [3] and a coding region (with three main open reading frames). Of the two regions, the UTR is relatively conserved, and it has been hypothesized that it may play important roles in viral replication [4].

The high genetic variability formerly led to classifying the TT viruses in five main genogroups with at least 50% nucleotide sequence divergence in turn divided into genotypes [5]. The latest classification imposes a cut-off value of 35% nucleotide sequence identity for species demarcation, as a result of which the *Alphatorquevirus* genus is currently composed of 29 species designated TTV1-29 [2]. Besides its high genomic diversity, TTV is also ubiquitous around the world and is considered by some to be part of the human virome [6].

The extremely high prevalence of the virus initially led to searching for disease association, without any significant results. Genotype-specific pathogenicity was also considered for TTV; for instance, genotype 1 was associated with hepatitis of unknown etiology and increased ALT levels [1] and with poor outcome in patients with laryngeal cancer [7], while TTV genotype 4 is less common in healthy subjects and more prevalent in patients with inflammation-prone conditions [8]. Subtype-specific or clinically specific pathogenicity remains debatable as there is no strong evidence to support or to reject this hypothesis.

TTV does not seem to be pathogenic per se, contrary to initial assumptions, nevertheless, its capacity to modulate and evade immune response [9, 10] may predispose to multiple autoimmune diseases [11], or augment pre-existent conditions and / or conditions. Even more, the most recent TTV research showed that TTV DNA-aemia is a potential marker for monitoring kinetics of functional immune competence prior and following solid organ transplants [8, 12, 13].

In the last few years the attention shifted from the pathogenic potential of TTV to its potential use as a biomarker. However, discriminating between TTV variants that may be associated with pathology and

innocuous variants should not be overlooked, thus genotyping and tracking the distribution of TTV genotypes remains an important task.

Despite several studies investigating the prevalence and association with pathology of TTVs in Romania [14-16], information on molecular characterization and phylogeny of these viruses is limited [17-20].

This study aimed to determine the prevalence and geographical distribution of TTV in Romania and to describe the phylogenetic relationships between isolates found in Romanian healthy subjects, as well as hemodyalized patients.

Two hundred and thirty-six clinically healthy volunteers undergoing routine medical check-up (110 men and 126 women) were selected between April and May 2019 from Romania's major healthcare centers (Table 1). Three of the individuals were immigrants from Europe, Middle East and Far East respectively. Blood samples were collected after informed consent was signed. The study was approved by the National Institute of Research and Development for Food Bioresources Ethics Committee with the registration number 342/16.05.2014. Consent forms for underage subjects were signed by legal guardians. Analysis was performed in blind; samples were given codes, only information on age, gender and residence were available.

Table 1

Infection rate in tested population sample

Category	Number	Mean Age ±SD, age intervals(years)	TTV DNA prevalence
Adult Men	105	55.84 ±13.84, 20-94	75/105 (71.4%)
Adult Women	121	51.54 ±15.26, 19-84	76/121 (62.8%)
Boys	5	7.6 ±7.3, 1-17	3/5 (60%)
Girls	5	9.6 ± 7, 0-17	2/5 (40%)
Total	236	51.6 ±17.1, 0-94	**156/236 (66.1%)**

Briefly, genomic DNA was purified with a commercial kit (PureLink® Genomic DNA Mini Kit, Invitrogen) from whole blood and was used to amplify a region of viral UTR B using primers descried previously [3]. Amplicons purified from gel (PureLink® PCR Purification Kit, Invitrogen) were quantified with Qubit fluorometer (dsDNA HS Assay Kit, Invitrogen) and subjected to direct sequencing with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed by capillary electrophoresis on a 3130 Genetic Analyzer system (Applied Biosystems).

Chi squared and Fisher exact test were used to examine possible differences in the prevalence of TTV DNA among men and women, while independent samples Mann-Whitney U test was used to compare means. Statistical tests analysis was conducted in SPSS Statistics software v 20.0.0 (IBM).

The subjects selected for the study were residents of all eight development regions of Romania (NUTS II-level divisions in European Union member states): Bucharest metropolitan area (Bucharest and Ilfov County), South, South-East, North-East, Center, North-West, West and South-West development regions. Place of residence of study participants is presented in Fig. 1.

Of the 236 subjects analyzed, 156 (66%) were positive for TTV DNA. There was no difference between the distribution of TTV DNA in men and women (Chi squared $p>0.05$). A statistically significant difference in the distribution of TTV DNA in different age groups (independent-samples Mann-Whitney U test $p=0.002$) was identified, where prevalence increased with age. The geographical distribution of TTV DNA prevalence did not differ significantly and ranged between 54.5% (12/22) in the South-West and 85.7% (6/7) in the West region.

The TTV prevalence recorded in our study group was lower than those reported for the general population of other European countries (e.g. 84-88% in Italy, Finland, Poland and Greece [21]) and Asian countries (e.g. 72% in India [22], 83.4% Qatar [23], 93.3% in China [24]), yet higher than the TTV prevalence reported for Iran (49.3%) in 2018 [25]. However, these variations can be explained by the genomic target used for TTV detection.

Of the TTV-positive samples, 80 random samples (20 from Bucharest, 5 from the central region, 7 from north-east, 2 from north-west, 18 from south, 15 from south-east, 8 from south-west, 4 from west and one non-Romanian sample) were subjected to direct amplicon sequencing. Out of these, more than half (43/80, 54%) had mixed infection with at least two types of TTV providing poor quality electropherograms. Anellovirus mix-infections, intra- and inter- phylogenetic groups – both among healthy individuals and among patients with different conditions [14, 23, 26-29] – are frequently reported and have become characteristic for human TTV infections.

The sequences obtained from subjects with monotypic infections (n=37) were submitted to the European Nucleotide Archive (ENA) of EMBL under the accession numbers LR742476-LR742512, OU989706 and were used to build the phylogenetic tree of Romanian TTV isolates (Fig. 2). In order to maintain the resolution of the phylogenetic analysis, three of these sequences (shorter than 200bp) were further excluded from analysis. In addition to these samples, 12 other sequences obtained in a previous study [30] from Romanian obese patients with diabetic nephropathy undergoing hemodialysis were included. Also, two positive control samples from a subject known to have persistent TTV DNA-aemia collected in 2015 (LB809941) and, respectively, 2019 (LR742486) were added to the analysis. Phylogenetic analysis was conducted in MEGA X software [31] and information on isolates used for constructing the phylogenetic tree is found in Supplementary material.

sequences of TTV led to classifying TTV isolates into five distinctive genogroups [32], with the later addition of two new groups [27]. Each genogroup contained more genotypes. Current *Anelloviridae* taxonomy is based on the analysis of entire ORF1 region of TTV genome and imposes the following cut-off values for sequence divergence: genera >56%, species >35%, subspecies >20% and isolates <20% [2]. Given the lack of unity in taxonomical classification of TTV in literature, data obtained in this study was compared with both types of TTV classification. In order to integrate and analyze the sequence data obtained in this study compared to similar data in international databases, all type of classifications were considered (namely former genogroup/genotype classification and new species classification).

The phylogenetic tree (Fig. 2) clearly shows relationships between sequences obtained from Romanian subjects, as well as the relationships between these sequences and other assigned and unassigned TTV isolates. It presents six main clusters, all supported by bootstrap values greater than 90%. The first four of these clusters contained several isolates that have been formerly classified as genogroup 1 [33] and are currently assigned to different subspecies of TTV1 and TTV3.

The first three clusters were strongly supported monophyletic groups containing sequences highly similar to isolates belonging to different subspecies of TTV1 and TTV3: US32 in 1 and T3PB in group 2 (formerly classified as genotype 2 [34], respectively genotype 3 of genogroup 1 [35], but currently subspecies of TTV1 [2]), and isolate TTV3-HEL32 in cluster 3. Cluster 4 comprised isolates similar to P/1C1 and tth16 currently assigned to a subspecies of TTV3 [2]. The majority of sequences of Romanian origin were grouped in these clusters – 39 out of 45 (87%).

The fifth cluster was composed of a Romanian originated isolate derived from a dialysis patient and two unassigned TTV isolates, namely isolate TTV-Hebei-1 – obtained from a Chinese patient with fatal fever [36] and isolate TTVMY HB34 from a Malaysian hepatitis B patient. The sequences from this cluster varied significantly from all known TTV groups. The analysis of the entire genomic sequence of the TTV-Hebei-1 isolate [36] included in the seventh genogroup [27], as well as the ORF1 sequence (data not shown), strongly suggests that this isolate meets the current taxonomic criteria of demarcation for novel species, and the Romanian isolate (RO-od21) may belong to this new taxon.

The sixth cluster grouped sequences related to TTV19-SANBAN and TTV22-svi-1, formerly classified as genogroup 3. The Romanian isolates from this cluster exhibited high resemblance to unassigned isolates from hepatitis and/or HIV-1 infected patients from Uruguay (TTVH7UY) [37] and from a healthy Malaysian subject (TTVMYC39) (unpublished results).

Previous methodologies adopted in TTV phylogenetic analyses were based on N22 amplification protocols, which limited the possibility to amplify other isolates besides those belonging to genogroup 1 [38], thus data regarding the phylogenetic distribution of TTV in different populations is scarce and limited to studies from Italy and South America (Brazil and Uruguay). A study describing the phylogenetic relationships between TTV isolates from Iranian hepatitis patients [25] highlights the importance of choosing genomic regions with good phylogenetic resolution, because the tree obtained in the above mentioned study lacks support (bootstrap values for major branches below 10) and leads to artificial

grouping/dividing of known highly similar/dissimilar isolates into groups. A study from Japan aiming to monitor the presence of TTV in wastewater - since TTV DNA has been shown to be present in fecal samples [39] - also paints a picture of the prevalence of TTV genogroups in the human population [40].

Studies performed in Italy [41], Brazil [42], Uruguay [37] and Japan [40] reported TTV genogroup 3 as the most prevalent, followed closely by genogroup 1, while the least prevalent genogroup was 2. Two other studies performed on Brazilian subjects found genogroup 5 isolates to be the most frequent [28, 43].

The phylogeny studies performed on the N22 region of TTV genome (amplifying mostly genogroup 1 TTV) in different countries (Hungary [29, 44], Czech Republic [45], Egypt [46], Saudi Arabia [47], India [48], Japan, Korea, Shanghai, Mongolia, Colombia, Cameroon, Germany, UK [49]) revealed genotypes 1 and 2 to be the most prevalent - in some studies being the only ones found. The two genotypes are spread in populations worldwide. Also, there was evidence that genotypes 4, 5 and 6 were found mainly among Asians, while genotype 3 was found mainly among Europeans [49]. The most common genotype of genogroup 1 isolates found in this study was genotype 3 (similar to T3PB isolate).

Differently from previous studies reporting the highest prevalence of TTV genogroup 3, the results of this study showed that genogroup 1 TTV had the highest prevalence (39/45, 87%) followed by genogroup 3 (5/45, 11%). None of the obtained sequences resembled isolates belonging to genogroups 2, 4 or 5.

Migration rates have been shown to alter the epidemiology of viral infections (e.g. HCV genotype circulation in Turkey [50], dispersal of HIV from Uganda [51]). In the present context of global migration – mainly due to the refugee crisis–, Romania was not a preferred destination for Asian immigrants. Moreover, the Migration Policy Institute (https://www.migrationpolicy.org/programs/data-hub/charts/immigrant-and-emigrant-populations-country-origin-and-destination) and Eutostat (https://ec.europa.eu/eurostat/databrowser/view/tps00177/default/map?lang=en) data show that most immigrants in Romania come from the neighboring countries and Romanian efflux of population is higher than the influx. Considering these aspects, the particular distribution of TTV in Romania could be explained by the low immigration rates.

Sequences obtained from samples collected in Bucharest were found in all clusters, except for the fifth. Bucharest is the capital city of Romania with a population over 3,000,000 inhabitants and is the largest university center and employer in the country. Many of its inhabitants come from all over the country, thus explaining the diversity observed in the current study for the Bucharest metropolitan area.

All sequences from the north-east region (marked with yellow squares in Fig. 2) were found in cluster 4, together with isolates from known to belong to a subspecies of TTV3, namely P/1C1 and tth16, as well as two unassigned isolates from Malaysia (i.e. TTVMY HC9 – from the serum of a control subject, TTVMY HB25 – from the serum of a HBV infected patient).

Sequences obtained from samples collected from the west and center of the country (marked with blue and pink squares in Fig. 2) were aggregated in cluster 2, together with an unassigned isolate from

Malaysia (TTVMYC124) and isolate T3PB – formerly genotype 3, now known to belong to a subspecies of TTV1. The separation of sequences originating in subjects living in the north-east and west of Romania in distinct clusters may be explained by a geographical barrier – Carpathian Mountains – limiting the population exchange between these areas.

In samples collected from immigrants, two out of three were TTV-positive. Sequences obtained from the positive samples were, however, unfit for further analysis.

The sequences obtained from the subject with persistent TTV DNA-aemia in 2015 (LN809941) and 2019 (LR742486) grouped separately. The sequence obtained in 2015 aggregated within a cluster with high similarity isolates, including TTV1 US32 isolate, former genotype 2. The sequence obtained four years later shared the highest degree of similarity with an unassigned isolate derived from an HBV infected Malaysian (TTVMY HB25) and grouped closer with isolates currently belonging to TTV3. This result may indicate the possibility of periodically clearing and acquiring a different TTV isolate. Another explanation considers the known fact of TTV mixed infections [14, 23, 26-29] and viral dynamics in time [52]; due to the mentioned phenomena, at a point in time a certain isolate may have higher viral load and become the only one detected by the selected amplification strategy or Sanger sequencing.

Sequences derived from dialyzed obese patients with diabetic nephropathy were found scattered in 5 out of 6 clusters (Fig. 2). Despite the seemingly uniform distribution, a larger number of these sequences grouped together with TTV3-HEL32 (genotype 6) and TTV19-SANBAN. TTV3-HEL32 (genotype 6) has an overall low prevalence [53], and was more likely found in hepatitis patients [29, 46]. Isolate SANBAN (TTV19) was shown to produce a protein suppressing the NF-kB pathway contributing to TTV pathogenicity and relating it to autoimmune and/or inflammatory-prone conditions [54].

Inflammation plays a key role in many disorders. CpG motifs are known to effectively activate immune cells via TLR9. The bioinformatics analysis performed on the full genome of multiple isolates of each TTV genogroup (1-5) showed a high variation of CpG indexes intra- and inter- genogroup. Members of genogroup 4 seem to have a greater stimulatory effect compared to others [9].

Genogroups 4 and 2 are reported to be the rarest in all populations. Also, it is noteworthy that genogroup 4 TTV was found with higher rates in patients with conditions whose clinical severity depends on the degree of inflammation [55, 56], while a novel variant of TTV7 (genogroup 2) was associated with Kawasaki disease – a pediatric vasculitis of unknown etiology [57].

The most common TTV isolates among healthy Romanian individuals belong to genogroup 1. There seems to be a geographical distribution of TTV sub-species from east to west of the country, while in the metropolitan area of the capital and neighboring counties the circulation of TTV isolates appears boundless.

The issue of finding suitable uninfected control for the study of disease association is well debated. However, studies hinting to the potential involvement of certain species/genotypes/isolates in human

diseases make it worthwhile to distinguish between TTV phylogenetic groups and to focus on what stands out in phylogenetic analysis. Given that most subjects selected for this study were healthy individuals - except for the few sequences (12) obtained from dialyzed obese patients (who were likely to received blood transfusions from healthy individuals infected with TTV) – our results may support the idea that TTV from different genogroups do not have the same pathogenic potential. It is most likely that TTVs from genogroup 1 are less involved in the onset or modulation of diseases, and that TTV from the rarer genogroups should be further studied in association to pathological conditions.

Declarations

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Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Oana Apircioaie*,* Larisa Gogianu*,* Lavinia Berca*,* Silvia Nica*, and* Mihai Toma*.* Sonia Spandole-Dinu*,* Dănuț Cimponeriu and Remus Nica designed the study. *The first draft of the manuscript was written by* Sonia Spandole-Dinu *and all authors commented on previous versions of the manuscript.* Ileana Stoica *supervised and critically* revised the work*. All authors read and approved the final manuscript.*

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the National Institute of Research and Development for Food Bioresources Ethics Committee (342/16.05.2014). Consent forms for underage subjects were signed by legal guardians.

Conflict of interest

The authors declare that they have no conflict of interest

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Figures

Figure 1

Geographical distribution of analyzed samples. a) Overall analyzed samples (n=236). Each region is composed of 4-7 counties and represented with a different color. The black circled digits represent the number of samples analyzed from each county. The total amount of samples analyzed per region is represented in red. Samples from non-Romanian subjects are figured in red outside of Romania's borders. b) Geographical distribution of samples sequenced and submitted to ENA.

Figure 2

Phylogenetic tree of human TTV based on a fragment of UTR B region. The evolutionary history was inferred using the UPGMA with p-distance method. Values of 90-100% for replicate trees for which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown as dots next to the branches. Evolutionary analyses were conducted in MEGA X [31] and annotations were added using EvolView [58]. black circles – sequences obtained from healthy Romanians for this study; white circles – sequences obtained from Romanian obese dialyzed patients with diabetic nephropathy in 2015; black

triangles – sequences from positive control; taxonomically assigned isolates are marked in burgundy and unassigned isolates in grey; *proposed as genogroup 7 [27].

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