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Redondovirus DNA in human respiratory samples

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ABSTRACT

Background: Redondovirus (ReDoV) is a recently discovered circular, Rep-encoding single-stranded DNA (CRESS-DNA) virus in humans. Its pathogenesis and clinical associations are still completely unknown.
Methods: The presence of ReDoV DNA was investigated in biological specimens of 543 Italian subjects by in-house developed PCR assays.
Results: The overall ReDoV prevalence was about 4% (23 of 543 samples). The virus was detected in 22 of 209 (11 %) respiratory samples. One stool sample was also ReDoV positive. Viral DNA was not found in blood samples from immunocompetent and immunosuppressed subjects and cerebrospinal fluids from patients with neurological diseases. Genomic nucleotide differences were detected among the ReDoV isolates by sequencing a 582-nucleotide fragment of the capsid gene of the viral genome.
Conclusions: The results demonstrate that ReDoV is mainly present in the respiratory tract of infected people. Further investigations are needed to reveal possible clinical implications of this new CRESS-DNA virus in humans.

1. Introduction

While attempting to study human virome in bronchoalveolar lavage (BAL) samples of lung transplant patients [1,2], Abbas and colleagues identified sequence reads that aligned with low-coverage to a poorly characterized circovirus, named porcine stool-associated circular virus-5 (PoSCV-5) [3]. Further genomic characterization of these reads revealed that they belonged to a novel virus having a single, closed molecule of circular DNA approximately 3000 nucleotides (nt) in length called Redondovirus (ReDoV) [4]. It was proposed as the second most prevalent eukaryotic virus, after anelloviruses, in human respiratory samples from viral metagenomics studies. The circumstance that the genomic organization and homology of ReDoV differ from that of other known circular, single-stranded DNA viruses [including those belonging to the group of circular Rep-encoding single-stranded DNA (CRESS) viruses] [5,6] has suggested that ReDoV is the first member of the new viral family, named Redondoviridae [4,7]. To date, 22 ReDoV genomes have been completely sequenced [4,8] and, based on 50 % Rep protein identity [9–11], grouped in two species highly prevalent in the respiratory tract: *Vientovirus* and *Brisavirus* [4].

The ReDoV genome contains two ambisense major open reading frames (ORF) encoding capsid and Rep proteins [449–531 and 334–363 amino acids, respectively] [4]. The capsid protein, like that of other single-stranded DNA viruses [5,6,12], contains a basic amino terminus, while the Rep protein has two domains, similar to many small DNA and RNA viruses [13,14]. Surprisingly, the capsid protein is more conserved than Rep protein among ReDoV sequences, being the range of amino acid identities (67.5–99.6 % and 36.6–99.7 %, respectively). All ReDoV genomes also contain a third ORF (ORF3) overlapping the capsid gene. Epidemiology, biological properties, and pathogenic potential of ReDoV are still completely understood. Abbas and colleagues [4] have investigated ReDoV prevalence by re-analyzing for homology to the virus

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metagenomics sequence data of 7581 samples from 173 datasets covering 51 organisms or environments and 18 human body sites or fluids. ReDoV sequences were detected only in human samples, from oral cavity (3.8 %), lung (3.3 %), nasopharynx (0.95 %), and gut (0.59 %). The presence of ReDoV was also tested in oropharyngeal swabs from 129 individuals by real-time PCR. ReDoV DNA was detected in 12 %, at levels that in critically diseased patients were 10^4-fold higher than in healthy individuals. Additionally, consecutive samples from some subjects obtained at later times for 2–3 weeks remained ReDoV positive, suggesting the persistence of virus infection. These observations suggest that ReDoV is not part of the normal oral and/or respiratory microflora of humans, differently to other circular single-stranded DNA viruses [5, 15] and that its infection might be involved in clinically relevant disorders.

Thus far, the prevalence of ReDoV DNA in different human biological samples, distribution of infection to age and/or gender of infected hosts, and the clinical consequences of infection, also about the presence of other common respiratory viruses and/or microbial agents coinfecting, have been investigated only to a very limited extent. We have approached these issues by detecting ReDoV DNA in 543 Italian individuals by specific PCR assays. The results have indicated that ReDoV is present in our population, the overall mean prevalence is about 4%, with the highest positivity rate in the respiratory tract (11 %). Although we found no clear evidence that ReDoV was the direct cause of disease in the subjects studied, the most virus-positive patients had more severe respiratory diseases and no other common respiratory viruses and/or microbial agents, thus raising interesting questions about the pathophysiological significance of this virus.

2. Methods

2.1. Specimens and routine tests

A total of 543 human biological specimens were studied. Specimens had been submitted to our laboratory by local hospitals where they were processed for routine virological analysis. The study was run after ethical approval from Comitato Etico di Area Vasta Nord-Ovest (protocol numbers 39238, and 63,409). The most specimens (n. 443) were obtained from diseased patients: 209 respiratory specimens (151 nasopharyngeal swabs, 36 sputum samples, and 22 pharyngeal swabs obtained between April and July 2019) from individuals with acute and chronic respiratory diseases, 79 whole blood samples from transplant recipients, 105 stools from individuals with gastroenteric illness, and 50 cerebrospinal fluids from neurological patients. The remaining 100 plasma samples were obtained from healthy blood donors. All the respiratory specimens were submitted to systematic testing for common respiratory virus detection by commercial real-time PCR assays, according to the manufacturer's instructions.

2.2. ReDoV DNA detection

Viral DNA was extracted from 200 µL of samples by using the QIAamp DNA Mini kit (QIAGEN, Chatsworth, CA) manually or associated with a QIAsymphony SP/AS instrument. Extracted DNA was amplified with two different PCR protocols, both developed in our laboratories and targeting the capsid gene of the viral genome.

The first amplification was performed by a semiquantitative one-step real-time PCR based on SYBR-Green PCR (SY-PCR) coupled with a melting temperature analysis of 90 nucleotide-length fragment. The reaction was performed by SsoAdvanced Universal SYBR Supermix (Bio-Rad Laboratories, Hercules, CA, USA) using a total reaction volume of 25 µL (20 µL of reaction mix +5 µL of template DNA) and 0.4 µM of each primer (primer ReDoV_qF: 5′-TGATGTAACATTCTATACCAAATGGA-3′, nucleotide positions 1473–1498; primer ReDoV_R: 5′-ACACCTGTTTCTGATGGTACT-3′, nucleotide positions 1542–1562, according to isolate BrisaVirus-RC, MK059757). The reaction was run in a CFX Connect Real-Time PCR instrument (Bio-Rad Laboratories, Hercules, CA, USA) using a previously standardized program (95.0 °C for 3 min, 40 cycles of 10 s at 95.0 °C, and 57.5 °C for 30 s). Melting curves were produced by plotting the fluorescence intensity against the temperature as the temperature was increased from 60.0–95.0 °C at 0.5 °C/s. All samples with a threshold cycle value greater than 35 and those with a melting curve not compatible with the expected pattern were considered negative. To estimate the reproducibility of the assay, a ReDoV-negative sample spiked with a known copy number of a recombinant plasmid into which the target fragment of SY-PCR was inserted, was repeated in five independent experiments, and the coefficient of variation was calculated. The differences between input (830,000 copies) and calculated copy numbers were small, the latter ranging between 733,000 and 862,000 with the maximum inter-assay variation lower than 0.1 Log, thus indicating good reproducibility of the SY-PCR assay. The lower detection limit of the procedure was measured by testing serial dilutions of a known concentration of a recombinant plasmid and was found to be of 10 copies.

A 1547 base pairs fragment of the capsid gene of ReDoV genome was amplified by the second protocol of qualitative single-step PCR (ssPCR) by using sense primer (ReDoV_F: 5′-GCATCAAGAAAGAGAGTTTATCGTCG-3′, nucleotide positions 16–41) and antisense primer (ReDoV_R) under the following conditions: 95.0 °C for 3 min, followed by 40 cycles at 95.0 °C for 30 s, 52.0 °C for 30 s, 72.0 °C for 2.30 min and finally an extension at 72.0 °C for 5 min. The reaction was carried out in a 25-µl PCR mixture containing DreamTaq DNA Polymerase (Thermo Fisher Scientific, Carlsbad, California), each dNTPs at a concentration of 0.2 mM, primers (0.6 µM each), and optimized buffer components. The amplified product was analyzed by electrophoresis on a 2% agarose gel. The sensitivity of ssPCR was measured by testing serial dilutions of a positive sample and was about 1000 DNA copies. The specificity of both PCR assays was confirmed by sequencing a large number of the amplification products as well as by testing nucleic acids from ReDoV negative samples. To validate the amplification process, positive and negative controls (i.e. no template control such as sterile water and/or no amplification control omitting the DNA polymerase from the PCR reaction) were run in each PCR. To exclude the presence of carryover contamination, serum handling, DNA extraction, PCR amplification, and electrophoresis analysis was carried out in independent rooms. Appropriate negative controls were added during DNA extraction and PCR amplification. To assure the reproducibility of the results obtained excluding the presence of environmental and reagents contamination, tubes of water/buffer remained open and closed throughout the extraction phase. Additionally, some samples found to be positive were tested again starting from the second round of DNA extraction.

2.3. Rolling circle amplification (RCA)

RCA is a rapid, very sensitive, and isothermal single-stranded DNA amplification technique used for efficient amplification of circular DNA viral genomes without the need of specific primers [16]. RCA reaction was standardized carrying out in a 20 µL format by using an optimized mix with few ng of sample DNA, 25 µM of exonuclease-resistant random primer, 4 mM of dNTPs, and 10 U of φ29 DNA polymerase. Amplification was performed at 30 °C for 17 h, followed by inactivation of φ29 DNA polymerase at 65 °C for 10 min.

2.4. Sequence analysis

Several ReDoV PCR positive isolates were characterized by sequencing a 582-bp fragment (from nt 931–1512 of the capsid gene of the representative isolate BrisaVirus-RC, MK059757) encompassing the target region of ssPCR. After purifying the PCR amplicons from the gel (QIAquick Gel Extraction Kit, Qiagen, Chatsworth, CA), they were sequenced by the Big Dye Terminator Cycle Sequencing kit (Applied

Biosystems, Foster City, CA) and an automatic DNA sequencer (ABI model 3130, Applied Biosystems). Nucleotide sequences were aligned with those available at GenBank using the ClustalW algorithm. The evolutionary amino acid and nt analyses were performed by using the Maximum Likelihood method based on Kimura's two-parameter model and Poisson correction model, respectively. Phylogenetic trees were generated with MEGA X using Neighbor-Joining and BioNJ algorithms. A bootstrap resampling (1000 replications) was used to assess the reliability of individual nodes in each phylogenetic tree. Pairwise genetic distances between isolates were calculated using the Maximum Composite Likelihood model with pairwise deletions and uniform distributed site rate variation.

3. Results

3.1. Evidence of ReDoV infections

A sample was considered positive only when ReDoV DNA was detected by both PCR protocols. For avoiding the loss of virus-positive samples with a low load of ReDoV DNA, when a sample tested positive by SY-PCR but negative by ssPCR, extracted DNA was subjected to RCA and then re-amplified by ssPCR. If a PCR signal was seen, the sample was considered ReDoV DNA positive.

No blood and liquor samples yielded positive results. Of 23 samples in which ReDoV was detected, 22 (96 %) were from respiratory samples and one was from a stool sample (Table 1).

3.2. ReDoV DNA in patients with respiratory diseases

Most of the respiratory samples (n. 177) had undergone a systematic search for common respiratory viruses. Viral infections were found in 54 samples (31 %), with a total number of 57 viruses detected. Rhinovirus was the most common finding (33 %), followed by adenovirus (19 %), metapneumovirus (14 %), parainfluenza 3 virus (10 %), coronavirus (9%), respiratory syncytial virus (9%), bocavirus (3%), and influenza A virus (2%). As shown in Table 1, testing for ReDoV yielded 22 positive results (11 %). The virus was found in 18 nasopharyngeal samples (12 %), 3 sputum samples (8%), and 1 pharyngeal swab (4%) (Table 2). When the specimens were stratified according to the month of collection, the number of detections was uniformly distributed with the highest in May (17 %) and the lowest in June (11 %). Of the 22 subjects whose specimens tested ReDoV positive, 3 were not-hospitalized individuals affected by primary ciliary dyskinesia, while the remaining 19 had been hospitalized having bronchopneumonia or milder acute respiratory diseases. One of these latter was a patient with chronic lymphocytic leukemia. When the subjects were stratified according to the presence of other pathogens, 5 patients had tested positive for common viruses (two patients for metapneumovirus, one each for respiratory syncytial virus, parainfluenza virus type 3, and rhinovirus), and 4 for bacteria (two patients for *Haemophilus influenzae*, one each for *Streptococcus pneumoniae* and *Mycoplasma pneumoniae*). The other 13 subjects (59 %) had yielded no other infectious agent beyond ReDoV (Table 2).

3.3. ReDoV infection in stools

Only one stool specimen resulted positive for ReDoV. This was from a 60-year old woman receiving hematopoietic stem cell transplantation (HSCT) for a history of lymphoma. This patient's stool sample was also positive for rotavirus antigen.

3.4. Genetic analysis of ReDoV isolates

Sequencing was limited to 10 amplicons obtained from independent PCR runs, confirming the detection of ReDoV DNA, and phylogenetic analysis showed that all the isolates were related to the previously published strains (Fig. 1). Most of them were very closely related (overall mean distance: 0.104; range: 0.000−0.295). Again, our isolates differed little from the ones already in GenBank (Fig. 1).

Table 1
Prevalence of ReDoV DNA in 543 biological specimens.

Specimen	No. examined	No. (%) ReDoV DNA positive
Blood *	179	0 (0)
Respiratory secretions	209	22 (11)
Stools	105	1 (0.9)
Liquor	50	0 (0)
Total	*543*	*23 (4)*

* Blood samples included 100 plasma and 79 whole blood samples.

4. Discussion

The recent discovery of ReDoV inspired this retrospective study on biological specimens collected from diseased patients and healthy blood donors. The results obtained to extend the previous finding of the only study published so far [4], confirming that ReDoV may infect humans, and firstly raise other interesting points. The presence of ReDoV DNA had been investigated by Abbas et al. [4] in 129 oropharyngeal swabs and the virus prevalence was 12 %, thus opening to the hypothesis that ReDoV could have a specific tropism for the respiratory tract. In our study, respiratory specimens were taken from 209 subjects and ReDoV was demonstrated in 22 individuals, all with pathologies of their respiratory tract. This rate of ReDoV detection (11 %) is similar to the ones reported previously, thus indicating that ReDoV could be evenly distributed at least in developed countries. However, establishing whether a seasonality and/or an age gradient exist in the occurrence of ReDoV infection will have to wait for additional studies, examining a large number of subjects with respiratory diseases and possibly exploiting serological tests in addition to the molecular assays used to date. Evidence exists that the PCR protocol used may strongly influence the results of prevalence studies. Thus, it also probable that more sensitive PCR protocols can be developed with more capability of efficiently detecting the full spectrum of ReDoV isolates.

However, these data, associated with the absence of ReDoV detection in blood samples from immunocompetent and immunosuppressed subjects, raise the idea that the viral infection may be mainly restricted at the respiratory level and not be systemic or that, alternatively, the presence of circulating virus can be only intermittent. The idea is not unexpected since other CRESS DNA viruses, genetically similar to ReDoV, produce infections restricted to selected tissues and/or organs with no persistent viremia in infected animals [11,17].

In this study, the majority of virus-positive patients had more severe

Table 2
ReDoV DNA detection in 209 respiratory secretions.

Parameter	No. examined	No. (%) ReDoV DNA positive
Gender		
Female	103	7 (7)
Male	106	15 (14)
Mean age (yrs)[a]		
38 ± 17	90	9 (10)
66 ± 10	90	13 (14)
Respiratory sample		
Nasopharyngeal swabs	151	18 (12)
Pharyngeal swabs	22	1 (4)
Sputum	36	3 (8)
Common respiratory viruses[b]		
Negative	123	17 (14)[c]
Positive	54	5 (9)

[a] The patient age was available for 180 of 209 cases (86 %).
[b] The samples tested for common respiratory viruses were 177 of 209 (85 %).
[c] Four of 17 samples (24 %) were positive for bacteria other than ReDoV DNA.

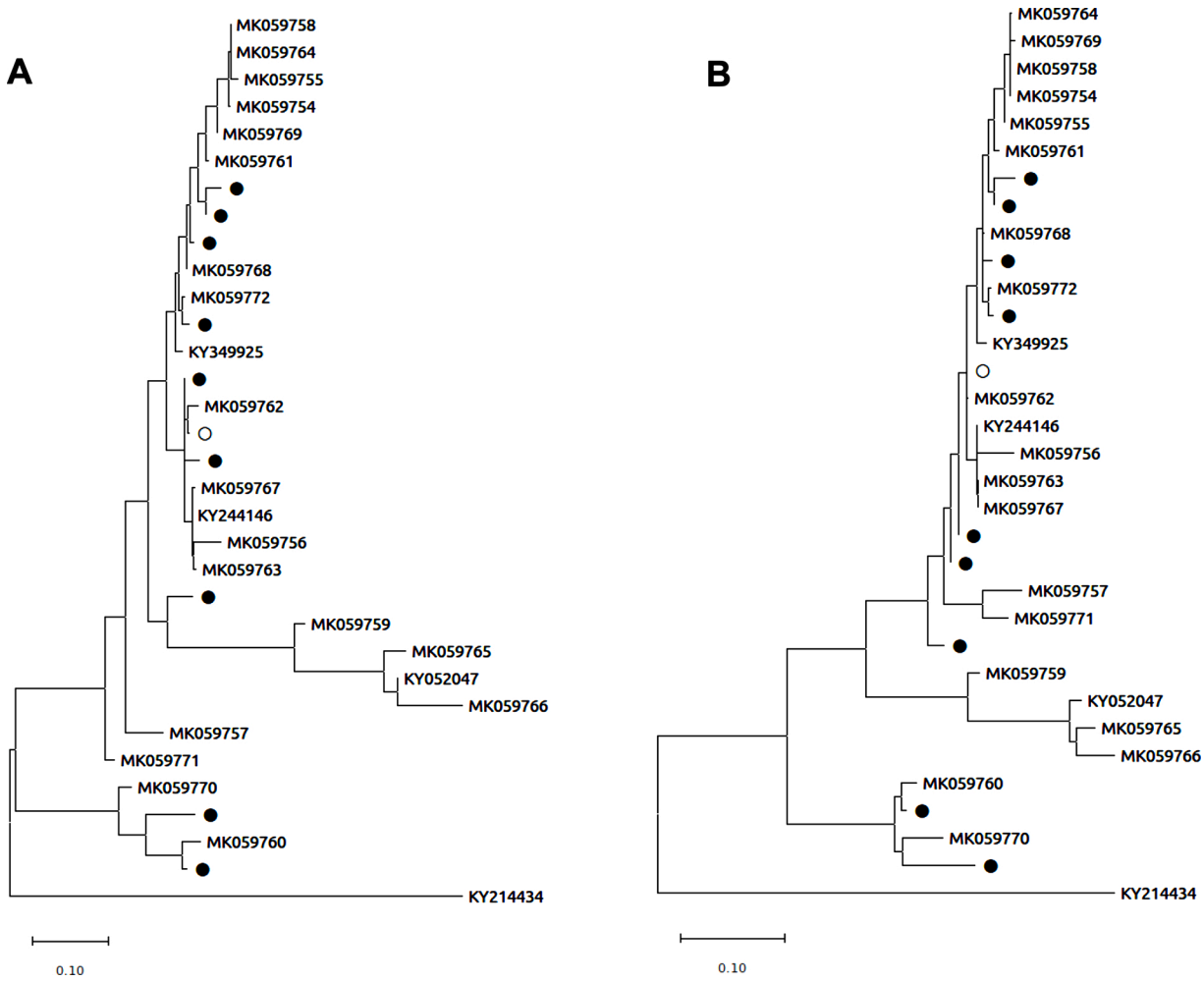

Fig. 1. Phylogenetic analysis of 10 ReDoV sequences from the present study. The aminoacidic (A) and nt (B) trees based on a 582-bp segment from the capsid gene of the viral genome was obtained by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using Jones–Thornton–Taylor (JTT) and Maximum Composite Likelihood (MCL) models, respectively. Bootstrap resampling (1000 replicates) was used to test the robustness of the trees. The tree was drawn by using MEGA X program (version 10.0.5). ReDoV sequences from the present study obtained by respiratory and stool samples are indicated by solid and open circles, respectively. The 22 sequences of ReDoV present in GenBank at the time of writing are indicated by accession number. The Porcine stool-associated circular virus/BEL/15V010 isolate 15V010 (accession number KY214434) was used as the outgroup. The bar represents the number of substitutions per site.

respiratory diseases, and most of them yielded no other common respiratory viruses and/or microbial agents that might have been responsible for the clinical forms from which the patients suffered. While the number of patients studied here is too small to be definitive regarding the association of ReDoV with severe respiratory tract disease, the observation of particularly higher ReDoV levels in critically ill patients than in healthy subjects [4] encourages to pursue the observation furtherly focusing on expanding the sample set and on specific pulmonary disease cohorts (e.g. asthma, cystic fibrosis, etc). After all, the finding suggests that ReDoV could be not a frequent commensal virus inhabiting the respiratory tract in the absence of symptomatic disease. Again, the follow-up of ReDoV positive patients will be important for understanding if the virus remains consistently persistent or is able only to give short-lasting acute infections in the infected host.

Of interest is the demonstration of ReDoV DNA in the feces of one subject. The finding might indicate that, similar to other respiratory viruses [18,19], ReDoV may not remain restricted to the respiratory tract. It is noteworthy that the subject in whom the observation was done receiving HSCT, suggesting that an immunosuppressed status of an infected host could favor this event. However, further studies will be needed to establish whether ReDoV can represent a further agent of viral enteritis or can just be excreted with the stools similar to other viruses primarily infecting the respiratory tract. Again, the short PCR fragment sequenced does not allow us to classify the ReDoV DNAs in one of the two species in which the virus has been genetically characterized. Sequencing of near full-length or full-length genomes will be necessary for better exploring the genetic variability of ReDoV and for improving its phylogenetic classification.

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Declaration of Competing Interest

I declare that the authors haven’t any financial or personal relationship with other people or organizations that could create a potential conflict of interest or the appearance of a conflict of interest with regard to the work.

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