VETERINARIA ITALIANA
RIVISTA DI SANITÀ PUBBLICA VETERINARIA

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Low pathogenic human coronaviruses during the first waves of COVID-19 in Italy

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Veterinaria Italiana, Vol. 60 No. 2 (2024) DOI: 10.12834/VetIt.3521.3521.1
Available on line: 21.04.2024

Abstract

Low-pathogenic human coronaviruses (HCoVs) infect the upper respiratory tract and cause mild, cold-like respiratory illness. Although several studies have shown evidence of the global distribution of HCoVs, information about their distribution in Italy are often focused only on hospitalized children and elderly with respiratory symptoms. In this study, a total of 916 swab samples collected during the first two SARS-CoV-2 pandemic waves in Abruzzo region (central Italy) was selected for molecular screening of low pathogenic HCoVs by real-time RT-PCR. We identified low-pathogenic HCoV in nine samples. Positive samples underwent whole genome sequencing for genome characterization; indeed, we also report the whole genome sequence of a HCoV-229E strain.

Keywords

Genomic characterization, Human respiratory low-pathogenic coronaviruses

Introduction

Members of the family *Coronaviridae*, a monophyletic group of viruses in the order *Nidovirales*, are enveloped, positive-sense RNA viruses that are known to infect four of the seven classes of vertebrates: mammals and birds (orthocoronaviruses), amphibians (letoviruses) and bony fish (pironaviruses) (Woo et al., 2023). Within the subfamily *Orthocoronavirinae*, four well-separated monophyletic clusters can be distinguished using a rooted maximum-likelihood tree generated from amino acid sequence alignments of the RNA-dependent RNA polymerase and helicase domains. These clusters correspond to the genera *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus* and *Gammacoronavirus*. Orthocoronaviruses have been detected in diverse mammalian and avian animals. Three members belonging to two viral species of the genus *Betacoronavirus* namely *Severe acute respiratory syndrome-related coronavirus* and *Middle East respiratory syndrome-related coronavirus* are highly pathogenic to humans and were responsible for SARS and COVID-19 (SARS-CoV-1 and SARS-CoV-2), and MERS (MERS-CoV), respectively (Decaro & Lorusso, 2020).

In terms of genome size and genetic complexity, members of the family *Coronaviridae*, are among the largest RNA viruses identified so far. The genome of CoVs is between 26 to 32 kilobases in length (Brian et al., 2005). It has a structure strictly conserved characterized by a 5'-end leader sequence involved in discontinuous subgenomic replication; the replicase gene consisting of two overlapping open reading frames (ORF 1a and 1b); four genes coding for structural proteins [spike (S), envelope (E), membrane (M) and nucleocapsid (N) proteins], and additional ORFs encoding for accessory genes involved in pathogenesis (Chen et al., 2020). Due to this genomic structure and replication features, CoVs show high mutation and recombination rates (Wells et al., 2023).

Currently, eight CoVs are recognized as human pathogens. In addition to the high-pathogenic zoonotic strains SARS-

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CoV-1, SARS-CoV-2 and MERS-CoV, there is a group of human coronaviruses (HCoVs) composed by four viruses usually responsible for common cold namely HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU1. Moreover, a novel canine-feline recombinant alphacoronavirus (CCoV-HuPn-2018) was isolated from pneumonia outbreaks in humans (Vlasova et al., 2022); next to these, a porcine deltacoronavirus (Hu-PDCoV) was identified in plasma samples of three Haitian children with acute febrile illness (Lednicky et al., 2021).

Several studies suggested that the common cold HCoVs also known as low-pathogenic HCoVs originated from an ancestor virus circulating in animals (such as bats and rodents). HCoV-229E derived from an alphacoronavirus from alpacas, which in turn derives from 229E-related CoVs identified in hipposiderid bats (Hamre et al., 1966; Decaro & Lorusso, 2020), while bovine coronavirus (BCoV) has been suggested to be the possible ancestor of HCoV-OC43, pointing cattle as intermediate host (Corman et al., 2018). As far as HCoV-NL63 and HCoV-HKU1 are concerned, the origin of their ancestor virus are bats and rodents, respectively; however, the role of a mammalian intermediate host is still unclear (Woo et al., 2005; Decaro & Lorusso, 2020).

HCoVs may induce severe manifestations in frail subjects (children, elderly, and immunocompromised individuals) stimulating severe acute respiratory illness only occasionally (Paules et al., 2020; Su et al., 2016). Their identification is challenging since targeted molecular screening methods are not frequently employed, and usually focused only on sub-population groups (Gagneur et al., 2008; Minosse et al., 2008; Hand et al., 2018; De Conto et al., 2019; Raoult et al., 2020; Gaunt et al., 2010; Ciotti et al., 2020).

Here, we describe the results of a screening for low-pathogenic HCoVs (lp- HCoVs) out of respiratory swab samples collected from individuals hospitalized or screened for SARS-CoV-2 RNA. These samples have been stored during the first waves SARS-CoV-2 pandemic in Abruzzo region (central Italy). In addition, we report the whole genome sequence of a HCoV-229E strain.

Materials and Methods

Ethics

The study was conducted according to the guidelines of the Declaration of Helsinki. Ethical approval was not provided for this study on human participants. After diagnostic routine for SARS-CoV-2, nucleic acids resulting from the biological material were stored to be processed for further analyses. For this study, no relevant personal data were collected from each specimen, inhibiting any correlations of these fully anonymized samples with the respective patients. Thus, according to national regulations and the institutional rules for Good Scientific Practice, the requirement for submission to an ethical committee and for obtaining patients' informed consent was waived.

Samples

A total number of 916 swab samples collected during the SARS-CoV-2 pandemic in Abruzzo region (central Italy) was selected for HCoVs molecular screening. Among 916 swabs, a total of 427 specimens was enrolled at the beginning of the first SARS-CoV-2 pandemic wave (from mid-March to mid-April 2020), while the remaining 489 at the second wave of SARS-CoV-2 (from November 2020 to April 2021). Swab samples were collected from the respiratory tract of individuals, which were either hospitalized or screened for a contact with SARS-CoV-2 infected subjects. The only exclusion criterion was a SARS-CoV-2 real-time RT-PCR positive result. Age and gender, such as any other patient characteristics, were not assessed to ensure anonymity.

Nucleic acid purification, molecular analysis and whole genome sequencing.

Nucleic acid purification was performed following established protocols previously described (Lorusso et al., 2020). Briefly, swabs were inactivated (PrimeStore® MTM, Bethesda, MD, USA) in BSL-3 conditions. RNA samples were purified by using the MagMax™ CORE kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's instructions. Eluted samples underwent SARS-CoV-2 screening by the TaqPathTM COVID-19 RT-PCR Kit (Thermo Fisher Scientific) for the detection of three portions of SARS-CoV-2 genome (ORF1ab, S and N protein-encoding genes). Samples that tested negative for SARS-CoV-2 were stored at -80°C and further processed for molecular detection of the four lp-HCoVs. Two different real-time RT-PCR procedures were used on QuantStudio 6 and 7 Flex Real-Time PCR Systems (Thermo Fisher Scientific). Samples collected during the first pandemic period were screened by the VIASURE Real Time PCR Coronavirus Detection Kit (Certest Biotec S.L., San Mateo de Gállego Zaragoza, Spain) following manufacturer's instructions. Swabs from November 2020 to April 2021 were analyzed with procedures previously described (Theamboonlers et al., 2007; Niu et al., 2016). In this case, two duplex Real-time RT-PCR were optimized, one for the detection of HCoV-NL63 and 229E and one for OC43 and HKU1 (Table I). Sequence

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targets were amplified by using the QuantiTect Probe RT-PCRs Kit (QIAGEN, Hilden, Germany) in a 25µl reaction volume containing 12.5µL of RT-PCR Master Mix, 0.25µL of RT Mix, 5µL of template RNA, as well as primers and probes following concentrations showed in Table I. The thermal profile consisted of 30min at 50°C for reverse transcription, 15min at 95°C for denaturation, and 45 cycles at 95°C for 15s with 60°C for 60s. After amplification, RNA from swabs which tested positive for low-pathogenic HCoVs were subjected to whole genome sequencing by NGS. Firstly, RNA samples were treated with TURBO DNase (Thermo Fisher Scientific, Waltham, MA, USA) at 37°C for 20min and purified by RNA Clean and Concentrator-5 Kit (Zymo Research, Irvine, CA, USA) according to manufacturer's instructions. RNA underwent the assessment of sequencing independent single primer amplification protocol (SISPA). cDNA was obtained by reverse-transcription (RT) using SuperScript® IV Reverse Transcriptase (Thermo Fisher Scientific) and a combination of two primers including the random-tagged primer FR26RV-N 5'-GCCGGAGCTCTGCAGATATCNNNNNN-3′ and a poly-A tagged primer FR40RV-T 5'-GCCGGAGCTCTGCAGATATCTTTTTTTTTTTTTTTTTTT-3′. The reaction was incubated at 23°C for 10min and at 50° C for 50min. After an inactivation step at 80°C for 10min, 2.5units of Klenow Fragment (3→5′ exo-) (New England Biolabs, Ipswich, MA, USA) was directly added to the reaction to perform the second strand cDNA synthesis. The incubation was performed as follow: 37°C for 1h and 75°C for 10min. Then 5µl of ds-cDNA was added to PCR master mix containing 1 × Q5 Reaction Buffer, Q5 High-Fidelity DNA Polymerase, dNTPs mix and the primer-tag FR20RV 5'-GCCGGAGCTCTGCAGATATC-3'. This is the thermal profile used: 98°C for 1min, 40 cycles of 98°C for 10s, 65°C for 30s and 72°C for 3min followed by a final extension step of 72°C of 2min. After amplification, PCR products were purified by ExpinTM PCR SV (GeneAll Biotechnology CO., LTD Seoul, Korea) and quantified using the QuantiFluor One dsDNA System kit (Promega, Madison, Wisconsin, USA). Libraries were prepared by using the Nextera DNA Flex Library Prep (Illumina Inc., San Diego, CA, USA) following the manufacturer's protocol. Finally, sequencing was performed on the MiniSeq (Illumina Inc., San Diego, CA, USA) using the MiniSeq Mid Output Kit (300-cycles) with standard 150bp paired-end reads. After quality check and trimming of the raw reads performed by FastQC and Trimmomatic, host depletion using Bowtie2 (human reference genome GCF_000001405) and de novo assembly by SPAdes v. 3.12.0 were accomplished. The sequence evolutionary history was inferred by using the maximum-likelihood analysis (Tamura-Nei model). In this way a phylogenetic tree depicting with the highest log likelihood (-46829.79) was retrieved. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances that was estimated through the Tamura-Nei model (by selecting the topology with the superior log likelihood value). Tree was drawn to scale with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted by using the MEGA software.

Duplex n.1	Primer/probe	Sequence (5'-3')	Final concentration (nM)
NL63-HCoV	Fwd	AGGACCTTAAATTCAGACAACGTTCT	100
	Rev	GATTACGTTTGCGATTACCAAGACT	50
	P	FAM-TAACAGTTTTAGCACCTTCCTTAGCAACCCAAACA-TAMRA	25
229E-HCoV	Fwd	CGCAAGAATTCAGAACCAGAG	50
	Rev	GGCAGTCAGGTTCTTCAACAA	75
	P	VIC-CCACACTTCAATCAAAAGCTCCCAAATG-TAMRA	25
Duplex n.2	Primer/probe	Sequence (5'-3')	Final concentration (nM)
OC43-HCoV	Fwd	GCTCAGGAAGGTCTGCTCC	50
	Rev	TCCTGCACTAGAGGCTCTGC	25
	P	FAM-TTCCAGATCTACTTCGCGCACATCC-TAMRA	25
HKU1-HCoV	Fwd	CCTTGCGAATGAATGTGCT	50
	Rev	TTGCATCACCACTGCTAGTACCAC	375
	P	VIC-TGTGTGGCGGTTGCTATTATGTTAAGCCTG-TAMRA	25

Table I. Primers and probes sequences (5'-3) for two duplex real-time RT-PCRs employed for HCoVs screening. Fwd, forward primer; Rev, reverse primer; P, probe; nM, nanomolar.

Results

Low pathogenic HCoVs were detected in 9 samples (0.98%): 8/9 were identified in swabs collected during the first wave, while only one during the second wave, from November 2020 to April 2021. In details, one HCoV-229E, one HCoV-OC43, two HCoV-NL63 and five HCoV-HKU1 were detected by the real-time RT-PCRs (Table II).

All nine positive samples underwent NGS. Nevertheless, a whole genome sequence was obtained only from the sample which was characterized as HCoV-229E. For the remaining samples, only a limited number of reads were obtained so their sequences were not processed for downstream analyses. The sequence HCoV 229E/human/ITA/TE5146/2020 has been deposited in the GenBank database with the acc. no MW039392. The

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sample produced a total of 508.388 raw reads with a mean quality score of 36.29. The consensus sequence of 26811 bp was then used for the phylogenetic analysis (Figure I). The sequence reported in this work shares a nt sequence identity ranging from 98.03 and 99.46% with all HCoV-229E included in the tree, many of which derived from samples collected in the USA. HCoV 229E/human/ITA/TE5146/2020 sequences showed the highest nucleotide identity with the strain 229E/Haiti-1/2016 (nt id 99.46%).

Sample ID	Real-Time RT-PCR results				
	SARS-CoV2	229E-HCoV	OC43-HCoV	HKU1-HCoV	NL63-HCoV
2020TE5146	–	+ (CT 22/45)	–	–	–
2020TE5834	–	–	+ (CT 34/45)	–	–
2020TE5003	–	–	–	+ (CT 24/45)	–
2020TE5467	–	–	–	+ (CT 21/45)	–
2020TE5824	–	–	–	0	–
2020TE5803	–	–	–	+ (CT 33/45)	–
2020TE5766	–	–	–	+ (CT 30/45)	–
2020TE6359	–	–	–	–	+ (CT 37/45)
2021TE127844	–	–	–	–	+ (CT 30/45)

Table II. Details of samples tested positive to low pathogenic HCoVs screening. Real-time RT-PCR results (CT, cycle threshold) are reported. ID, identification number of each sample.

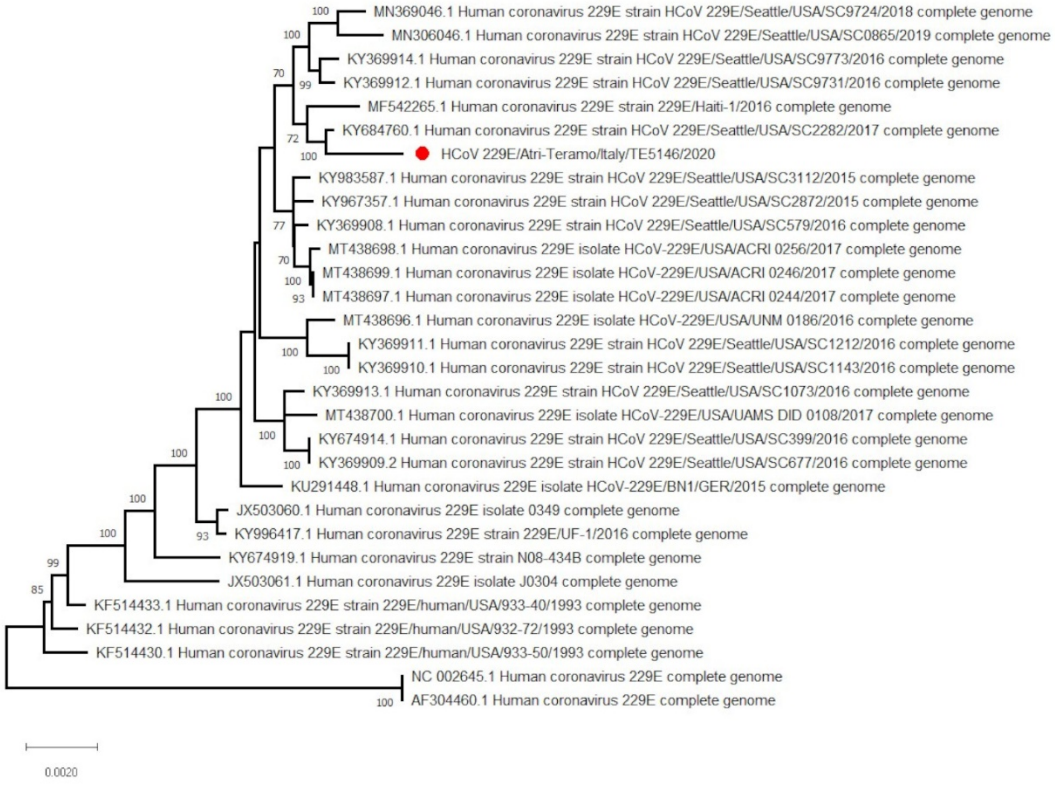

Table III. Phylogenetic analysis of the strain HCoV 229E/human/ITA/TE5146/2020 (GenBank acc. no MW039392) reporting 30 whole-genome sequences of 229E-HCoV in GenBank. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Tree was drawn to scale with branch lengths measured in the number of substitutions per site.

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Discussion and Conclusions

Low-pathogenic HCoVs are globally endemic with a prevalence that varies greatly at the global level. However, over 70% of the global population has seroconverted towards all four low-pathogenic HCoVs with primary infection in childhood (Zhou et al., 2013) and reinfections that occur during life (Gaunt et al., 2010). These viruses occasionally can stimulate severe respiratory diseases among children, elderly and immunocompromised individuals (Gagneur et al., 2008; Hand et al., 2018; Raoult et al., 2020) but, generally, induce asymptomatic or mild flu-like syndromes. For this reason, the differential diagnosis among HCoVs and other respiratory pathogens is important. This aspect acquires significant relevance for unexpected zoonotic CoVs which may cross the host-species barrier.

Our data show an overall lower incidence of lp- HCoVs than that reported by previous studies focused on hospitalized children and elderly with respiratory symptoms in Italy (Minosse et al., 2008; De Conto et al., 2019; Ciotti et al., 2020). However, we need to consider that samples were collected from an anonymous cohort in terms of age, gender and symptoms, in a period characterized by the forced adoption of facial masks, by social distancing and by lockdown restrictions due to COVID-19 pandemic. Our results are more similar those obtained from a large study performed in Edinburgh (UK), where almost twelve thousand respiratory samples were analyzed over 3 years; lp-HCoVs were detected from 0.30% to 0.85% of specimens in all age groups (Gaunt et al., 2010). In any case, our work has clearly potential bias due to the non-representative nature of the samples. Indeed, participants were gathered from individuals who were either hospitalized or had contact with subjects positive to SARS-CoV-2, rather than being randomly selected from population. Reasonably, this aspect influences the extent to which results can be generalized to the entire community.

Nevertheless, the main aim of our work was to genetically characterize HCoVs, so we employed all positive HCoVs samples to next generation sequencing. Out of nine samples, we obtained only a single whole HCoV-229E genome sequence (HCoV 229E/human/ITA/TE5146/2020) which showed a sequence nt-identity ranging from 98.03 and 99.46% with extant HCoV-229E sequences available in GenBank. This is the second HCoV-229E strain whole genome sequence published from Italy, after the first released by Farsani and colleagues (Farsani et al., 2012). Out of the remaining eight lp-HCoVs-positive RNA samples, only limited reads were obtained by the combination of SISPA and NGS. This underscores the importance of employing target-specific sequencing protocols similarly to what was developed for SARS-CoV-2 during the pandemic (Curini et al., 2023). This is essential because CoVs require rigorous monitoring through enhanced molecular screening and extensive sequencing efforts. This is crucial to follow their evolution and prevent other health emergencies.

Funding data

We declare no conflicts of interest. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the IZSAM. Funding was provided by the Italian Ministry of Health (Ricerca Corrente 2022 "OneCoV: coronavirus animali emergenti e impatto nella Salute Pubblica", recipient Alessio Lorusso). This research was also partially supported by EU funding within the NextGenerationEU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT).

Author contributions

IP, PR, and VC carried out the experiment. IP wrote the manuscript with support from VC, EC, SP, RI, FB, MC. PR, AL, SB supervised the project. AL, PR, SB, and FB conceived the original idea.

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How to cite: Puglia et al. Low pathogenic human coronaviruses during the first waves of COVID-19 in Italy. Veterinaria Italiana, Vol. 60 No. 2 (2024) DOI: 10.12834/VetIt.3521.3521.1