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Detection of subgenomic mRNA from endemic human coronavirus OC43 and NL63 compared to viral genomic loads, single virus detection and clinical manifestations in children with respiratory tract infections

Inger Heimdal\textsuperscript{a,\,*}, Hilde Lysvand\textsuperscript{a}, Sidsel Krokstad\textsuperscript{b}, Andreas Christensen\textsuperscript{a,\,b}, Henrik Døllner\textsuperscript{a,\,c}, Svein Arne Nordbo\textsuperscript{a,\,b}

\textsuperscript{a} Department of Clinical and Molecular Medicine, Norwegian University of Science and Technology (NTNU), Trondheim, Norway
\textsuperscript{b} Department of Medical Microbiology, St. Olavs hospital, Trondheim University Hospital, Trondheim, Norway
\textsuperscript{c} Children’s Clinic, St. Olavs hospital, Trondheim University Hospital, Trondheim, Norway

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\textbf{ABSTRACT}

\textbf{Background:} The importance of endemic human coronavirus (HCoV) in children has been insufficiently elucidated upon. Our aims were to develop subgenomic (sg) mRNA tests for HCoV species OC43 and NL63, and to evaluate the relationships to HCoV genomic loads, single HCoV detections and clinical manifestations.

\textbf{Methods:} We have used an 11-yearlong cohort study of children admitted with respiratory tract infection (RTI) and hospital controls. Nasopharyngeal aspirates were analyzed for HCoV subtypes OC43 and NL63 with in-house diagnostic PCR. Positive samples were tested with newly developed real-time PCRs targeting sg mRNA coding for the nucleocapsid protein.

\textbf{Results:} OC43 sg mRNA was detected in 86% (105/122) of available OC43-positive samples in the RTI group, and in 63% (12/19) of control samples. NL63 sg mRNA was detected in 72% (71/98) and 71% (12/17) of available NL63-positive patient and control samples, respectively. In RTI samples, sg mRNA detection was strongly associated with a \textit{Ct} value <32 in both diagnostic PCR tests (OC43: OR = 54, 95% CI [6.8–428]; NL63: OR = 42, 95% CI [9.0–198]) and single NL63 detections (OR = 6.9, 95% CI [1.5–32]). Comparing RTI and controls, only OC43 was associated with RTI when adjusted for age (aOR = 3.2, 95% CI [1.1–9.4]).

\textbf{Conclusion:} We found strong associations between OC43 and NL63 sg mRNA and high viral genomic loads. sg mRNA for OC43 was associated with RTI. The association between sg mRNA and clinical manifestations needs further evaluation.

1. Introduction

Six human coronavirus (HCoV) species are known to infect humans; the alphacoronaviruses 229E and NL63, and the betacoronaviruses HKU1, OC43, MERS-CoV, SARS-CoV and SARS-CoV-2. The non-SARS/MERS endemic HCoV species are commonly detected with sensitive PCR methods in children with lower respiratory tract infections (LRTI) [1–3]. However, their role in disease have been difficult to determine since they are also often co-detected with other respiratory viruses, in addition to being frequently detected in healthy children [4–9].

HCoV diagnosis has primarily been based on RNA detection with PCR methods that cannot differentiate RNA from active replicating viruses from RNA remains due to previous infections. Proof of active replicating HCoV in samples from children with LRTI will strengthen the suspicion of a causal link between endemic HCoV and severe LRTI. Usually, a growth in conventional cell lines is the gold standard for active viral replication, but it is inherently difficult to obtain for endemic HCoV [10]. Thus, we speculate that the detection of endemic HCoV subgenomic (sg) mRNA may be a better indicator of active virus replication than traditional RNA-based PCR methods, as the short sg mRNA is only produced during virus replication and should have a shorter half-life than the genomic RNA.

This study had three objectives: 1) to develop sg mRNA PCR tests for the two most frequently detected endemic HCoV species OC43 and NL63; 2) to investigate whether sg mRNA correlates with RNA genomic loads and the single detection of HCoV, and 3) to evaluate the associations between sg mRNA and clinical manifestations of respiratory tract infections.
infections (RTI).

2. Materials and methods

2.1. Study design and setting

This project is a part of a larger single-center prospective observational study of children hospitalized with RTIs, conducted at the Children’s Clinic at St. Olavs hospital, Trondheim University Hospital in Norway, from November 2006 to September 2017. In short, immunocompetent children <16 years of age with community acquired RTI admitted to the hospital were invited to participate. Healthy children undergoing elective surgery at the hospital were recruited as a control group during the whole study period. Nasopharyngeal aspirates (NPA) were collected from all participants and analyzed for 17 respiratory viruses. In total, 4312 virus positive children with RTI were included, and 668 controls. For the purpose of this study, we used available OC43 and NL63 positive NPAs from both the RTI group and the control group (Fig. 1).

2.2. Children with RTI and control group

Children in the RTI group were routinely examined and treated by physicians at the pediatric department. Clinical information regarding the current RTI episode and previous medical history were registered in standardized forms. RTI was classified as either an upper respiratory tract infection (URTI), or LRTI with or without URTI [11]. The disease severity was assessed based on a score of hospitalization length, the need for oxygen or respiratory support and treatment with supplemental fluids. A “severe disease” was defined as a score ≥75-percentile among all virus-positive children enrolled in the entire study [11]. Caregivers in the control group provided information about the child’s previous medical history, including symptoms of RTIs over the last 14 days, in standardized forms. For both groups, a “chronic disease” was defined as asthma, other lung disease, cerebral palsy, epilepsy, heart condition or gestational age <36 weeks.

2.3. Diagnostic analysis and definitions

The collected NPAs were placed into a standard virus transport medium without antibiotics, and analyzed for 17 viruses with in-house real-time PCRs and cultivation in conventional cell lines [12]. Diagnostic PCR for OC43 and NL63 was reported as cycle threshold (Ct) values, in which a Ct value >40 was regarded as negative. HCoV co-detections with “severe viruses” included respiratory syncytial virus, human metapneumovirus, influenza virus A/B or parainfluenza virus types 1–3. Co-detections with “mild viruses” included human rhinovirus, human adenovirus, human bocavirus, HCoV species 229E and HKU1, human enterovirus, human parechovirus or parainfluenza virus type 4.

2.4. Development of subgenomic mRNA tests for NL63 and OC43

Two real-time PCR tests for the detection of sg mRNA from NL63 and OC43 were developed. The primers were designed based on previous studies concerning HCoV replication targeting the sg mRNA coding for the nucleocapside (N) protein for each species [13–15]. Sequences and locations for primers and probes (TIB Molbiol, Berlin, Germany) tests are presented in Table 1.

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**Abbreviations**

| Abbreviation | Description |
|--------------|-------------|
| HCoV | Human coronavirus |
| LRTI | lower respiratory tract infection |
| NPA | Nasopharyngeal aspirate |
| RTI | Respiratory tract infection |
| sg | Subgenomic |

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Fig. 1. Flowchart of HCoV OC43 and NL63-positive samples analyzed for sg mRNA
In total, 58 samples were not available for testing for sg mRNA.
Definitions: RTI group, Children <16 years of age hospitalized with respiratory tract infections; Control group, children undergoing elective day surgery.
Abbreviations: HCoV, Human coronavirus; N, nucleocapsid gene; sg, subgenomic; TRS, transcription regulatory sequence.

Primers and probes for PCR assays targeting HCoV N sg mRNA OC43 and NL63.

Table 1

| Primer/Probe       | Sequence                          | Position | Location                                      |
|--------------------|-----------------------------------|----------|-----------------------------------------------|
| OC43 Forward primer| 5’-ATCCTCTGTATATCGTTCTTTGTGTA-3’ | 40-62    | Leader                                        |
| Reverse primer     | 5’-TTAGCCCTCTTACCCCTGG-3’         | 204-186  | N-gene                                       |
| Probe              | 5’-CAGTGTAGAGGGTCTCTCCTCGGAAAATGTC-3’ | 99-128   | N-gene                                       |
| NL63 Forward primer| 5’-GACTTTTGTCATCTCTTCTAACCT-3’   | 36-60    | Leader-body sg mRNA junction site             |
| Reverse primer     | 5’-TGGCTTATGACAAAAACCTACAC-3’     | 163-144  | N-gene                                       |
| Probe              | 5’-CTAACAAAAATGGCTAGTGAATTGGGC-3’ | 64-93    | End of TRS and start of N-gene                |

Available NL63- or OC43-positive samples stored at -80°C were re-extracted using NucliSens easyMag extractor (bioMérieux, Marcy l’Etoile, France). The total DNA and RNA was stored at -20°C until further processing.

Complementary DNA (cDNA) synthesis was performed with random hexamer primers (Riboclonex random primers, Promega Corporation, Fitchburg, WI, USA) and M-MLV Reverse Transcriptase (Life Technologies Corp. Carlsbad, CA, USA) according to the manufacturer’s recommendations using T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) at 37°C for 60 min and 94°C for 10 min before the templates were stored at -20°C.

The PCR reaction mixture for both NL63 and OC43 sg mRNA PCR consisted of 10 µL of Custom Multiplex PCR Supermix UNG (Quanta BioSciences Inc, Gaithersburg, MD, USA), 1 µl of reverse primer, 1 µl of forward primer, 2 µl of molecular-grade water and 5 µl of cDNA as a template. PCR was performed with a CFX96™ Real-Time System (Biorad). NL63 sg mRNA-PCR was performed with single steps of 45°C for 5 min and 95°C at 3 min, followed by 40 cycles at 95°C 10 s., 54°C 10 s. and 72°C 20 s. OC43 sg mRNA-PCR was performed with single steps of 45°C for 5 min and 95°C at 3 min, followed by 45 cycles at 95°C for 10 s., 56°C for 10 s and 72°C for 10 s. For both tests, a Ct value >40 was regarded as a negative result.

The amplicons generated with the sg mRNA PCR tests were evaluated with agarose gel electrophoresis and Sanger sequencing. We also determined the specificity against other endemic HCoV species (229E and HKU1).

2.5. Statistical analysis

Descriptive statistics were reported with counts, percentages, median (Mdn) and interquartile range (IQR) as appropriate. Comparisons of dichotomous variables were performed with a Pearson Chi-Squared test, and reported with odds ratios (OR) and 95% confidence intervals (CI). Associations of sg mRNA results to hospitalization lengths (<24 h) and participant status (RTI group vs. control) were also explored with age-adjusted multivariate logistic regression models (aOR). All analyses were performed using IBM SPSS Statistics version 27.

2.6. Ethics

The study was approved by the Regional Committees for Medical and Health Research Ethics (REC) Central in 2006 (No: 4.2006.2289) and 2012 (No: 2012.1042).

3. Results

3.1. Subgenomic mRNA PCR

Gel electrophoresis analysis revealed only one band for each PCR tests (Fig. 2). As expected, the product size was 170 bp for NL63 sg mRNA and 128 bp for OC43 sg mRNA. The amplicons were verified with Sanger sequencing (data not shown). Other HCoV species tested negative in the two sg mRNA PCRs, suggesting that both tests had a sufficient specificity. None of the PCR-positive specimens could be cultured in our conventional cell lines.

3.2. Association between diagnostic PCR Ct values and detection of subgenomic mRNA

Eighty-six percent (105 of 122) of OC43-positive samples in the RTI group were sg mRNA-positive, compared to 63% (12 of 19) in the control group (Fig. 1). The corresponding numbers for NL63 were 72% (70 of 97) in the RTI group and 71% (12 of 17) in the control group (Fig. 1). The distribution of sg mRNA-positive and -negative samples for both OC43 and NL63, compared to diagnostic PCR Ct values, is illustrated in Fig. 3. In the RTI group, diagnostic PCR for both OC43 and NL63 showed a bimodal pattern according to Ct values, with one cluster of samples with Ct values <32 and another with Ct values ≥32. There was a strong association between positive sg mRNA test results and low diagnostic Ct values <32, compared to Ct values ≥32 (Table 2). In the control group, the violin plots had a convex shape with somewhat higher Ct values. Higher percentages of samples with Ct values <32 tested positive for sg mRNA, compared to samples with Ct values ≥32 (OC43: 7 of 8 [88%] vs. 5 of 11 [45%]; NL63: 7 of 7 [100%] vs. 5 of 10 [50%]), but the groups were too small for statistical comparisons.

Fig. 2. Gel electrophoresis of sg mRNA N OC43 and NL63. Lane A-D, OC43 sg mRNA positive samples; lane E, negative control (water); lane F, 1 kb Plus DNA ladder; lane G, NL63 sg mRNA positive sample; lane H, negative control (water).
3.3. Comparing subgenomic mRNA-positive and -negative samples in the RTI group

Background characteristics, clinical data and virological data in sg mRNA-positive samples were compared to sg mRNA-negative samples in the RTI group (Table 2). For OC43-positive samples, there were no differences in background characteristics and clinical data between sg mRNA-positive and -negative samples. The proportion with sg mRNA OC43 detection and single OC43 detection was twice as high, but this difference was not statistically significant (OR = 3.0, 95% CI [0.8–11.0]).

For NL63, sg mRNA-positive samples were more likely to be obtained from children <2 years of age (OR = 12, 95% CI [4.0–33]), and they were more likely to be hospitalized >24 h (OR = 2.5, 95% CI [1.0–6.3]), compared to children with sg mRNA-negative samples, also when adjusting for age (aOR = 3.0, 95% CI [1.1–8.6]). NL63 sg mRNA-positive samples were more likely to have NL63 as the only virus detected (OR = 6.9, 95% CI [1.5–32]). They were also less likely to have a co-detection of a mild virus (OR = 0.4, 95% CI [0.1–0.96]).

Background characteristics and microbiological data for the control group are listed in Supplementary Table 1. Due to the small number of

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**Table 2**
Comparing sg mRNA OC43 and NL63-positive and -negative samples from children hospitalized with respiratory tract infections (RTI group).

|                      | RTI group: HCoV OC43 (n = 122) |   | RTI group: HCoV NL63 (n = 97) |   |
|----------------------|-------------------------------|---|-------------------------------|---|
|                      | sg mRNA + (n = 105)          | sg mRNA – (n = 17) | OR (95% CI)                  | sg mRNA + (n = 70) | sg mRNA – (n = 27) | OR (95% CI) |
| Background characteristics |                                |                            |                               |                          |
| Female gender        | 45 (43)                       | 6 (35)                     | 1.4 (0.5–4.0)                 | 26 (37)                  | 12 (44)             | 0.7 (0.3–1.8)       |
| Age in months, mdn, IQR | 13.9 (4.4–24.8)               | 17.0 (11.1–21.5)           |                               | 4.5 (1.6–14.9)          | 26.2 (11.3–39.6)    | *                  |
| Age <2 years old     | 77 (73)                       | 14 (82)                    | 0.6 (0.2–2.2)                 | 61 (87)                  | 10 (37)             | 11.5 (4.0–32.9)     |
| Chronic disease      | 94 (52)                       | 3 (18)                     | 2.3 (0.6–8.3)                 | 23 (33)                  | 13 (48)             | 0.5 (0.2–1.3)       |
| Clinical data        |                                |                            |                               |                          |
| Hospitalized >24 h   | 66 (63)                       | 13 (76)                    | 0.5 (0.2–1.7)                 | 49 (70)                  | 13 (48)             | 2.5 (1.0–6.3)       |
| Sole URTI            | 22 (21)                       | 6 (35)                     | 0.5 (0.2–1.5)                 | 25 (36)                  | 6 (22)              | 1.9 (0.7–5.5)       |
| LRTI                 | 83 (79)                       | 11 (65)                    | 2.1 (0.7–6.2)                 | 45 (64)                  | 21 (78)             | 0.5 (0.2–1.4)       |
| Severe disease       | 30 (29)                       | 5 (29)                     | 1.0 (0.3–3.2)                 | 19 (27)                  | 4 (15)              | 2.1 (0.7–7.0)       |
| Virological data     |                                |                            |                               |                          |
| HCoV Ct < 32         | 81 (77)                       | 1 (6)                      | 54 (6.8–428)                  | 54 (77)                  | 2 (7)               | 42 (9.0–198)        |
| Single HCoV detection | 41 (39)                       | 3 (18)                     | 3.0 (0.8–11.0)                | 25 (36)                  | 2 (7)               | 6.9 (1.5–31.8)      |
| Co-detected severe virus | 37 (35)                       | 8 (47)                     | 0.6 (0.2–1.7)                 | 31 (44)                  | 14 (52)             | 0.7 (0.3–1.8)       |
| Co-detected mild virus | 27 (26)                       | 6 (35)                     | 0.6 (0.2–1.9)                 | 14 (20)                  | 11 (41)             | 0.4 (0.1–0.96)      |

Abbreviations: IQR, Interquartile range; Mdn, Median; LRTI, Lower respiratory tract infection; URTI, Upper respiratory tract infection.

1 Either asthma, other lung disease, cerebral palsy, epilepsy, heart condition or gestational age <36 weeks.
2 A score ≥75% percentile, based on relevant clinical outcomes.
3 OR and 95% CI represent comparisons of single detections vs. all other samples with co-detected viruses.
4 “Severe virus” included either Respiratory syncytial virus, Human metapneumovirus, Parainfluenzavirus types 1–3 or Influenza virus. OR and 95% CI represents comparisons of samples with detected severe viruses to all other samples.
5 “Mild virus” included human rhinovirus, human adenovirus, human bocavirus, HCoV species 229E and HKU1, human enterovirus, human parechovirus or parainfluenza virus type 4. OR, and a 95% CI represents comparisons of samples with detected mild viruses to all other samples.
6 Comparisons were not conducted due to a lack of normally distributed data and non-equal variances.
samples, sg mRNA-positive and -negative controls are not compared with statistical tests.

3.4. Comparisons of the RTI group to the control group

Lastly, we compared sg mRNA results from children with RTI to controls. sg mRNA was more often detected in OC43-positive samples in the RTI group (86%, 105/122) compared to the control group (63%, 12/19), which was a statistically significantly difference (OR = 3.6, 95% CI [1.2–10.4]). The association remained significant when adjusting for age (aOR = 3.2, 95% CI [1.1–9.4]). There was no difference in NL63 sg mRNA detection rates between RTI and controls (72% (70/97) vs. 71% (12/17), OR = 1.1, 95% CI [0.4–3.4]).

4. Discussion

4.1. Main findings

We successfully developed PCR tests for the specific detection of sg mRNA from HCoV-NL63 and OC43. To the best of our knowledge, this is the first study to investigate the role of sg mRNA in endemic HCoV in samples from hospitalized children. The presence of OC43 and NL63 sg mRNA were strongly associated with high HCoV genomic loads, as evidenced by low Ct values and the single detection of NL63 RNA. OC43 sg mRNA was associated with RTI in age adjusted analyzes, but we were not able to confirm associations between sg mRNA and clinical manifestations and disease severity for NL63. However, due to a limited number of included controls, these observations should be re-assessed in larger studies.

4.2. Design of primers and probes

The endemic HCoV species OC43 and NL63 are single-stranded, positive-sense large RNA viruses of approximately 27–30 kb [16]. During replication, sg mRNAs coding for the viral structural proteins are synthesized with discontinuous transcription mechanism [17], resulting in several nested sg mRNAs with sequences corresponding to both the 5′ end and the 3′ end of the virus genome. We successfully designed primers and probes for detection of sg mRNA N. Previous studies have revealed that sg mRNA N has the shortest length, is highly expressed and is a key component to produce intact virions [14, 17, 18]. The short sg mRNA N is only produced during virus replication and is not packed into virions [19], and should theoretically have a short half-life. Thus, it is reasonable to assume that sg mRNA N is a good marker for active virus replication.

4.3. Clinical evaluation of HCoV OC43 and NL63 subgenomic mRNA detection

We found strong associations between OC43 and NL63 sg mRNA and high viral genomic loads. Most likely, these observations reflect that the detection of sg mRNA and high viral genomic load is a result of ongoing or recent viral replication, although it was not possible for us to confirm this by cultivation of the samples. For NL63, sg mRNA detection was also associated with single virus detection. We found similar tendencies for OC43, although this difference was not statistically significant. However, OC43 sg mRNA detection was associated with RTI. In our previous publications based on the same study population, we found that samples from hospitalized children with RTIs were associated with higher endemic HCoV genomic loads, but viral genomic loads were not related to upper or lower RTIs, nor to disease severity [11, 20].

In the present study, we observed a tendency that more of the symptomatic controls tested positive for sg mRNA compared to the asymptomatic controls, although we were not able to confirm that due to a limited number of HCoV-positive children in the control group. Nevertheless, we suggest that the presence of sg mRNA may reflect viral replication in both hospitalized children with RTI and in symptomatic controls with mild upper airway symptoms, as well as in asymptomatic controls in which the presence of sg mRNA may reflect an asymptomatic HCoV infection. Since sg mRNA and HCoV genomic loads may not be associated with disease severity, we speculate that the disease manifestations of pediatric endemic HCoV detections may be determined by the host immune response to a greater extent than the HCoV virulence.

Previously, we have made similar observations for human metapneumovirus [21], but not for RSV where high genomic load is associated with increased disease severity [22].

Several recent studies have investigated the clinical utility of SARS-CoV-2 sg mRNA detection. Sg mRNA for the E gene can be detected up to 17–22 days after the first SARS-CoV-2 detection, also after the virus culture is negative [23, 24]. Alexandersen et al. suggested that sg mRNA is protected from rapid degeneration in double membrane vesicles [23]. Still, it is implied that negative sg mRNA tests correlate with non-infectious individuals [25]. Contrarily, the detection of sg mRNA is positively correlated with virus culture growth [26, 27]. Wong et al. have recently published a paper stating that the ratio of sg mRNA to genomic RNA is associated with the presence of clinical symptoms [28]. However, the results concerning SARS-CoV-2 sg mRNA should be carefully interpreted with regard to endemic coronaviruses.

4.4. Limitations

This study has limitations. HCoV OC43 and NL63 could not be cultured in our conventional cell lines. Therefore, it was not possible to assess whether the presence of sg mRNA correlated with viral growth. The lack of longitudinal data should be emphasized in the interpretation of both the clinical and virological relevance of measuring sg mRNA. Since the sg mRNA kinetic during an infection is unknown, the absence of information about which stage during an infection the NPAs were collected may have an undetermined impact on the obtained results. The NPAs used to evaluate the clinical relevance of detecting sg mRNA had been stored at –80 °C for several years, hence we cannot guarantee for the quality of all specimens. In addition, some of the specimens had been freeze-thawed one or more times. Finally, the sample size is limited and the clinical relevance should be re-assessed in a larger population.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2022.105247.

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