Dear Dr. Astrid Vabret,

We would like to thank you for the swift reviewing process of our manuscript "A novel pancoronavirus RT-PCR assay: frequent detection of human coronavirus NL63 in children hospitalized with respiratory tract infections in Belgium", which we submitted to BioMed Central Infectious Diseases.

We have carefully considered the comments and recommendations of the reviewers and we have revised our manuscript accordingly. We include in the resubmission form a point-by-point response to the comments of the reviewers.

Minor essential Revisions

1: Reviewer's comment: "In the chapter 'Methods,' "isolates" have to be defined. Are they supernatants of cell culture, and what cell culture? It is important to know if there is a first amplification by culture before molecular diagnosis."

We agree with the reviewer and we included in the "Isolates and patients" section of the chapter "Methods" a statement that no prior amplification by cell culture was performed on the samples (page 12, lines 5-6).

2: Reviewer's comment: "It would be useful to know from which samples the positive results are obtained: pharyngeal swabs, nasopharyngeal aspirates, ..."

The nature of the specimens from which the positive results were obtained is described in Table 1, in the "specimen" section.

Discretionary Revisions

1: Reviewer's comment: "Why do you include patients with old age if you want to explore the incidence of HCoV-NL63 infection in children?"

Thank you for your thorough and constructive comments. We appreciate your efforts to improve our manuscript.
We agree with the reviewer, and in our analyses, we left out all patients older than 16. Changes in numbers and percentages were made accordingly throughout the manuscript.

2: Reviewer's comment: "It would be useful to know which other respiratory viruses circulated in this period, not only RSV, but also influenza viruses, rhinoviruses, ...do you have this data?"

In every winter season, there is correlation of RSV, influenza, parainfluenza, adenovirus and rhinoviruses in the winter months in Belgium. We have data about the yearly RSV and influenza epidemic collected by the National Institute of Public Health. However, we do not think that is is vital to include such a graph in this article.

Reviewer: Dr. Eric C.J. Claas

Major Compulsory Revisions

1: Reviewer's comment: "The manuscript lacks information on the analytical and clinical sensitivity of the pncoronavirus PCR. These issues are of particular importance when using highly degenerate primers."

The following information was included in the Results section, page 5, lines 13-17: "The sensitivity of the pncoronavirus RT-PCR assay was assessed by testing tenfold dilutions of HCoV-NL63 and HCoV-OC43 RNA. While 50 copies of HCoV-OC43 RNA copies per ul nasopharyngeal aspirate could be detected, the sensitivity for HCoV-NL63 was a bit lower i.e. 5 x 103 RNA copies per ul nasopharyngeal aspirate."

2: Reviewer's comment: "The manuscript would be improved by adding clinical data of the patients infected with NL63. The description URTI and LRTI in table 1 is too general. For the patient group that has been tested, terms as "serious respiratory symptoms" (page 5) and "relatively severe respiratory diseases" (page 9) is not informative either."

As suggested by the referee, a more detailed description of symptoms of the patients, infected with HCoV-NL63, is included in Table 1. On page 5, we omitted the term "serious" as well as the terms "relatively severe" on page 9.

3: Reviewer's comment: "Page 8, last line: 'These results...' needs to be put in perspective as these results are biased by the fact that sampling is only performed from January to May. This is also the case for the coinciding epidemic seasons for OC43 and NL63. In addition, the numbers are too small for a conclusion like that."

We agree with the reviewer, and we moderated the conclusion by replacing this sentence by the following sentences: "These results seem to support the tendency of human coronaviruses to circulate mainly during the winter season. However, in this study, sampling was only performed from January to May during the yearly RSV epidemic period, while no samples from the summer and autumn months were screened." A reference to a study describing the epidemic season of HCoV-OC43 and 229E, in addition to the already referred study by Larson and colleagues, has also been added (Hendley et al., 1972) (page 9, lines 2-5).

4: Reviewer's comment: "Some discussion should be added on the discordant phylogeny when comparing different genes. Based on the ORF1a sequences there appear to be four clades of viruses. However, within these clades different subtypes based on the spike region can be observed (specifically NL-p223 and HCoV-NL). Basically this means that the true phylogeny can only be established by analyzing full-length sequences."

We agree with the referee that some discordance is observed in the clustering pattern of the HCoV-NL63 isolates in both dendrograms. Therefore, we added the following discussion (page 10, lines 4-9): "When analysing the dendrograms based on ORF1a and S gene sequences, a discordance in the clustering pattern of some HCoV-NL63 isolates (e.g. HCoV-NL and NL-p223) can be observed, suggesting a possible recombination event. Further research of complete genome sequences of these isolates is required."
Drawing conclusions based on phylogenetic analysis of one single gene therefore requires caution as the true phylogeny can only be demonstrated by analysing complete genome sequences.

Minor Essential Revisions

1: Reviewer's comment: "The experimental procedure for detection of HCoV-NL63 is not clear. Using the pancoronavirus primers, every coronavirus results in a 251 bp fragment (figure 2). How are the viruses differentiated? The text reads that HCoV-NL63 positives are confirmed by amplification and sequence analysis of four other regions, but how the primary identification of NL63 (and also for the 229E and OC43) is made, remains elusive."

As suggested by the referee, we clarified the method of primary identification of the coronavirus type, and we introduced the following sentence in the Results section on page 5, lines 3-4 of second paragraph: "Samples, from which a 251 bp fragment could be amplified, were further identified by sequencing using the pancoronavirus primers."

2: Reviewer's comment: "Page 5: Discard the use of the “detection frequency” as the numbers are low: 7.7% when 1 out of 13 samples is positive is misleading. Figure 3A and B can be easily combined to one figure. This should also be adjusted in the discussion on page 8."

We agree with the reviewer that the term "detection frequency" can be misleading when this is calculated for such small numbers of samples. We left out this term when describing the number of positives out of the total number of samples tested in that month (page 6, lines 1-3). For clarity reasons, we prefer that Figure 3 remains in two parts (A and B).

3: Reviewer's comment: "Page 5 and figure 2: It is not clear what has been tested in figure 2: cell lysates, purified virus, something else? Do these degenerate primers generate specific PCR products on clinical specimens?"

We added the following information in the text on page 5, which refers to Figure 2: "To determine whether the newly designed pancoronavirus RT-PCR assay efficiently amplifies a broad range of coronaviruses the RT-PCR assay was tested on cell culture supernatant of the four known human coronaviruses and three animal coronaviruses: HCoV-NL63, HCoV-OC43, HCoV-229E, SARS-CoV, feline infectious peritonitis virus (FIPV), porcine hemagglutinating eencephalomyelitis virus (PHEV), and murine hepatitis virus (MHV)." For clinical specimens, all PCR products of the correct size were sequenced, and were shown to be coronaviruses, attesting to the specificity of this PCR protocol.

4: Reviewer's comment: "Page 11: If the samples are exclusively tested for RSV, indicate that no other pathogens were tested."

The samples were not exclusively tested for RSV, but also for influenza virus, parainfluenza virus and adenovirus. We introduced the following statement in the chapter Methods, in the Isolates and patients section: "Routine diagnostic testing was performed for respiratory syncytial virus (RSV), influenza virus, parainfluenza virus and adenovirus."

Discretionary Revisions

1: Reviewer's comment: "Page 2, line 8 and page 10, line 4: samples do not get infected."

We agree with the reviewer, and we replaced "infected" in these sentences by "positive".

2: Reviewer's comment: "Figure 1: it seems more logical to combine the sequences by phylogeny of the viruses. Now the primer sequence looks more heterologous by the scattered appearance of NL63, PDEV, TGEV and 229E in the alignment."
As suggested by the reviewer, we rearranged Figure 1 in a way that coronaviruses of the same group are put together.

3: Reviewer's comment: "Page 8, line 15: Provide some information on VATER as well (analogous to SLI)."

According to the reviewer's suggestion, we added the following information on VATER: "A second patient was diagnosed with VATER, a syndrome characterized by the sporadic association of specific birth defects or abnormalities such as vertebral and vascular anomalies, anal atresia, trachea and esophagus problems and renal anomalies." (page 8, lines 15-17)

4: Reviewer's comment: "Table 1: Remove RTI abbreviation in footnote and do not abbreviate it in the title."

The changes requested by the reviewer in this comment were performed.

Reviewer: Dr. Christian Drosten

1: Reviewer's comment: "Page 5, first paragraph: Was it observed experimentally that the primers of Stephensen et al. do not amplify NL63, or is this only assumed from the presence of 7 mismatches?"

It was indeed observed experimentally that the primers of Stephensen et al. do not amplify HCoV-NL63 RNA, extracted from cell culture supernatant as well as from HCoV-NL63 positive samples.

2: Reviewer's comment: "Next paragraph/Figure 2: Can the authors tell how much virus was amplified in each lane? Even a relative measure of some of the virus' quantities, including NL63, would be helpful since diagnostic use of the assay is proposed in the discussion section"

A similar comment was made by Dr. Eric Claas, and we addressed this issue in our reply to comment 1 of Dr. Eric Claas.

3: Reviewer's comment: "Page 10, 2nd last paragraph: Authors speculate that the current circulation of NL63 might suppress 229E. Is there any evidence for cross-neutralisation between the two viruses? Even if such data should not exist, it would be good to briefly address the phenomenon in the discussion."

We added the following discussion concerning the phenomenon of potential cross-neutralisation (page 10, lines 14-18): "At the moment, there are no data concerning cross-neutralization between HCoV-229E and HCoV-NL63. In theory, such cross-neutralization might be possible, since both viruses are relatively closely related species belonging to coronavirus group 1. Antigenic cross-reactivity has already been demonstrated between SARS-CoV and group 1 coronaviruses TGEV, FIPV and CCoV (Sun and Meng, 2004)."

We would like to thank the reviewers for their extensive review of our paper and their constructive comments. We appreciated this very much, and hope that our revised manuscript will now be acceptable for publication.

Best regards,

Prof. Dr. Marc Van Ranst