

novel human coronavirus and Kawasaki disease. *J Infect Dis* 2005; 191:499–502.

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Reprints and correspondence: Dr. Hideaki Kikuta, Dept. of Pediatrics, Hokkaido University Graduate School of Medicine, N-15, W-7, Kita-ku, Sapporo 060-8638, Japan (hide-ki@med.hokudai.ac.jp).

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Kawasaki Disease and Human Coronavirus

To the Editor—Esper et al. [1] recently reported the possible causal association between a novel human coronavirus (HCoV) and Kawasaki disease (KD). They reported that respiratory secretions from 72.7% of 11 patients with KD but from only 4.5% of 22 age-matched control subjects tested positive for an HCoV designated by Esper et al. as the “New Haven coronavirus” (HCoV-NH). This virus was reported to be closely related to HCoV-NL and HCoV-NL63, which were identified by 2 independent groups from The Netherlands [2, 3].

To determine if HCoV might be consistently associated with KD, we analyzed nasopharyngeal and oropharyngeal swab samples collected in 1999 as part of an etiologic investigation of KD in San Diego. The 1999 investigation focused on exploring the possible causal link between KD and *Chlamydia pneumoniae*, and the details of the investigation and case-control design are described by Schrag et al. [4]. After the 1999 investigation was completed, the pharyngeal swab samples were stored at -70°C . Pharyngeal swab samples were available for analysis from 10 patients with KD and from 6 control subjects. The patients, who had onset of KD between 9 February and 20 March 1999, had a median age of 3.6 years (range, 0.6–8.6 years). The patients met the epidemiologic case definition for KD: they had fever lasting for ≥ 5 days and had at least 4 of the 5 clinical features of KD [4]. The median age for the control subjects was 3.3 years

(range, 1.3–8.3 years). Pharyngeal swab samples were obtained within 10 days of the onset of illness in 6 of the patients with KD and on days 11, 15, 16, and 37 after the onset of illness in the remaining 4 patients.

All pharyngeal swab samples from the patients with KD and from the control subjects tested negative for HCoV by use of 2 different primer sets. Nucleic acid was extracted from 200 μL of the pharyngeal swab samples by use of the automated NucliSens Extractor (bioMérieux). Twenty-five-microliter reactions containing 5 μL of the extracted nucleic acid were prepared with the 1-step Access RT-PCR System (Promega). The first primer set used for amplification was an HCoV-NH/HCoV-NL63-specific primer described by Esper et al. [1] that had the following modification: a single nucleotide degeneracy was introduced into the sense-strand primer, 5'-GCGCTATGAGGGTGGTTG-YAAC-3', to accommodate a sequence variation among published sequences of HCoV-NH/HCoV-NL63 strains (the underlining indicates the modification). The amplification program consisted of a reverse-transcription (RT) step of 45 min at 45°C and 2 min at 94°C , to denature the reverse transcriptase; 40 cycles of 1 min at 94°C , 1 min at 55°C , and 1 min at 72°C ; and 10 min at 72°C , for final amplicon extension. The second RT-polymerase chain reaction (PCR) primer set had broadly reactive primers designed to target highly conserved regions of the HCoV RNA polymerase gene: sense-strand primer 5'-GGTTGGGATTATCC-TAARTGTGA-3' and antisense strand primer 5'-TATAACACACAACACCYTC-ATCA-3'. Amplification reactions were performed as described above, and the following program settings were used: an RT step of 45 min at 45°C and 2 min at 94°C , to denature the reverse transcriptase; 40 cycles of 1 min at 94°C , 1 min at 54°C , and 1 min at 72°C ; and 5 min at 72°C , for final amplicon extension.

The sense-strand primers for both sets were labeled with Cy5 fluorescent dye at

the 5' end to facilitate amplicon detection (the predicted sizes for the first and second primer sets were 215 and 454 bp, respectively) using the CEQ 8000 Genetic Analysis System (Beckman Coulter). Assays were performed using standard viral nucleic acid extracts (HCoV 229E and OC43) and nuclease-free water for positive and negative controls, respectively. All pharyngeal swab samples tested positive by RT-PCR for human glyceraldehyde-3-phosphate dehydrogenase enzyme, which indicated that there was adequate recovery of RNA from the samples and that RT-PCR inhibitors were absent.

Our findings do not support those of Esper et al. [1]. Methodologic differences in the type and timing of sample collection; in sample handling, storage, and processing; and in the selection of case patients and control subjects may explain our different findings. Alternatively, different etiologic agents could have been associated with KD in the 2 study populations. Further studies that include serologic testing and prospectively collected high-quality pharyngeal swab samples may be needed to determine the role, if any, that HCoVs play in the etiology of KD.

Ermias D. Belay,¹ Dean D. Erdman,¹ Larry J. Anderson,¹ Teresa C. T. Peret,¹ Stephanie J. Schrag,¹ Barry S. Fields,¹ Jane C. Burns,² and Lawrence B. Schonberger¹

¹National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia; ²Department of Pediatrics, School of Medicine, University of California at San Diego, La Jolla

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Reprints or correspondence: Dr. Ermias D. Belay, National Center for Infectious Diseases, Centers for Disease Control and Prevention, 1600 Clifton Rd., Mailstop A-39, Atlanta, GA 30333 (ebelay@cdc.gov).

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Reply to van der Hoek and Berkhout, Ebihara et al., and Belay et al.

To the Editor—The discovery of the human coronavirus (HCoV) we designated the “New Haven coronavirus” (HCoV-NH) was the result of a deliberate search for previously unknown HCoVs and was made without a priori knowledge of HCoV-NL63 [1] and HCoV-NL [2], the 2 HCoVs from The Netherlands that were recently described, and before the report of the discoveries of these viruses. Unlike HCoV-NL63 and HCoV-NL, which were initially identified in cell cultures, HCoV-NH was discovered by screening respiratory tract specimens with molecular probes. These probes targeted the sequences of the replicase 1a gene that are conserved among human, avian, and mammalian CoVs. Therefore, it is not surprising—perhaps it is predictable—that this region of the replicase 1a gene is highly conserved between HCoV-NH and HCoV-NL63. Until full genomic sequencing of HCoV-NH is complete, we believe that it is premature to state that HCoV-NH, HCoV-NL63, and HCoV-NL are the same virus. Nonetheless, as we clearly acknowledge in our study [3], it is likely that these viruses are closely related.

van der Hoek and Berkhout raise the issue of nomenclature [4]. Whether HCoV-NH and HCoV-NL63 are strains of the same species of virus remains to be determined. Sequence comparison between viruses is only one of many criteria used to differentiate between viral species [5]. In fact, the International Committee on Taxonomy of Viruses has established no

specific guidelines to address the issues of nomenclature and categorization of potentially closely related viruses that have been identified independently [5, 6]. Undoubtedly, this will continue to be an issue as new viral pathogens are identified.

We disagree with van der Hoek and Berkhout that the naming of HCoV-NH “needlessly complicates the HCoV literature” [4]. Rather, we believe that the identification of HCoV-NL63 and HCoV-NL by cell culture and genome amplification techniques and the identification of HCoV-NH by use of molecular probes exemplify the recent advances in methods to identify previously unrecognized viruses. As the full genomes of these viruses are described, the clarification of the nomenclature will follow.

We believe it is important to attempt to replicate in different populations our finding of the association between HCoV-NH infection and Kawasaki disease, and we appreciate the letters from Ebihara et al. [7] and Belay et al. [8]. We would be interested to know whether proper controls were included to demonstrate the integrity of the RNA that was used in the amplification assays by Ebihara et al. We agree with Belay et al. that the timing of sample collection and the type of sample screened (nasal vs. pharyngeal) may explain the discrepancy in results between their study and ours. Furthermore, more than one etiological agent may be linked to Kawasaki disease. Nonetheless, we are intrigued by the study by Graf that suggests the presence of a peptide, corresponding to the spike protein of HCoV-NL63, in tissue from individuals with Kawasaki disease [9]. These findings further support the association between HCoV-NH and Kawasaki disease.

Frank Esper,¹ Eugene D. Shapiro,^{1,2,3}
Marie L. Landry,⁴ and Jeffrey S. Kahn^{1,3}

¹Department of Pediatrics, Division of Infectious Diseases, ²Department of Pediatrics, Division of General Pediatrics, and ³Departments of Epidemiology and Public Health and ⁴Laboratory Medicine, Yale University School of Medicine, New Haven, Connecticut

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Reprints or correspondence: Dr. Jeffrey S. Kahn, Dept. of Pediatrics, Div. of Infectious Diseases, Yale University School of Medicine, 420 LSOG, PO Box 208064, New Haven, CT 06520-8064 (jeffrey.kahn@yale.edu).

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Reply to van der Hoek and Berkhout

To the Editor—In my Editorial Commentary [1], I certainly did not intend to detract from the novelty and importance of the findings by van der Hoek et al. [2] in early 2004. As I pointed out clearly, Esper et al. [3] were unaware of van der Hoek et al.’s discovery of the Netherlands strain of human coronavirus (HCoV-NL63) when they designed their polymerase chain reaction (PCR) and apparently also when they first found the HCoV they designated