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MAJOR ARTICLE

Human Coronavirus NL63 Is Not Detected in the Respiratory Tracts of Children with Acute Kawasaki Disease

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Kawasaki disease (KD) is a self-limited, systemic vasculitis of children for which an infectious trigger is suspected. Recently, an association between KD and human coronavirus (HCoV)–New Haven (NH) was reported, on the basis of polymerase chain reaction (PCR) with primers that also amplified HCoV-NL63. We investigated the possible association between these HCoVs in the respiratory tract and KD by reverse-transcriptase (RT) PCR and viral culture in a geographically and ethnically diverse population. Only 1 (2%) of 48 patients with acute KD was positive by RT-PCR for HCoV-NL63/NH in a nasopharyngeal swab. These data do not support an association between these HCoVs and KD.

The mystery of the etiology of Kawasaki disease (KD), a self-limited, systemic vasculitis of children, remains unsolved after >30 years since its original English language description [1]. An infectious cause has long been suspected, because of discrete seasonal peaks [2], focal epidemics with a wavelike spread of illness [3], and the acute onset of such clinical features as fever, rash, conjunctival injection, and cervical adenitis resembling other infectious diseases. The peak incidence is in the toddler age group, with only rare cases in adults and in infants <3 months of age, which suggests a ubiquitous agent to which adults are immune and against which young infants are protected by passive maternal antibodies. A study by Rowley et al. suggests an antigen-driven immune response to an etiologic agent with a respiratory portal of entry [4]. However, conventional bacterial and viral cultures and serologic investigations have failed to establish an infectious cause [5–8]. These studies included 2 members of the human coronaviruses (HCoVs), HCoV-229E and -OC43 [5, 6], a diverse group of RNA viruses that cause respiratory tract disease [9].

Recently, an association between KD and HCoV–New Haven (NH) was reported [10]. Using reverse-transcriptase (RT) polymerase chain reaction (PCR) with primers that also amplified HCoV-NL63 (GenBank accession numbers NC_005831 and AY518894), the investigators detected viral sequences in respiratory secretions from 8 of 11 patients with acute KD but only 1 of 22 age-matched, contemporaneous control subjects. HCoV-NH and -NL63 have identical sequences in the primer binding sites for the spike glycoprotein gene [10], and phylogenetic analysis of a 126-bp region in the open-reading frame (ORF) 1a gene [11] shows minimal variation between isolates. Thus, we will refer to these viruses collectively as HCoV-NL63/NH, which was first detected by RT-PCR and culture in respiratory secretions from infants with lower respiratory tract dis-

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ease in The Netherlands [12, 13]. Our collaborative, multi-institutional investigation tested the possible association between HCoV-NL63 in the respiratory tract and acute KD by RT-PCR and viral culture of respiratory samples from a larger, geographically and ethnically diverse population.

PATIENTS, MATERIALS, AND METHODS

Two centers in the United States (Children's Hospital of San Diego, San Diego, California, and Children's Memorial Hospital, Chicago, Illinois) and 1 center in The Netherlands (the Academic Medical Center [AMC], Amsterdam) collected respiratory samples from patients with acute KD between December 2000 and March 2005. Respiratory samples included throat swabs, nasopharyngeal (NP) swabs, scraped NP epithelial cells, and nasal washes and were either archived or collected prospectively specifically for this study. Inclusion criteria for children with acute KD were >5 days of fever plus at least 4 of 5 standard clinical criteria (rash, conjunctival injection, cervical lymphadenopathy, changes in the extremities, and changes in lips or oral mucosa) [14] or 3 of 5 criteria with dilated coronary arteries by echocardiogram (z score > 2.5) [15]. The research protocol was reviewed and approved by the institutional review boards of each institution. Informed consent was obtained from the parents of all patients.

Throat and NP swabs were placed in viral transport medium (either TRIzol or TRIzol LS [Invitrogen]). Scraped NP epithelial cells were obtained with a Rhinoprobe curette (Arlington Scientific). Cells were eluted into viral transport medium and either were processed directly for RNA isolation or were washed twice in PBS, collected by centrifugation, and stored at −70°C until RNA extraction. Nasal washes were performed by forceful instillation of 2 mL of sterile saline (0.9% NaCl) into a nostril by use of a syringe and subsequent aspiration of the same nostril to obtain a deep NP sample. All but 2 samples were stored at −70°C in TRIzol reagent until RNA was extracted; the remaining 2 samples were stored for 1–2 weeks at 4°C before RNA extraction. Five centers (University of California, San Diego [UCSD], La Jolla, California; Vanderbilt University, Nashville, Tennessee; Loyola University, Maywood, Illinois; Northwestern University, Chicago, Illinois; and the AMC) extracted RNA from the samples and either (1) synthesized cDNA followed by PCR or nested PCR and analysis by ethidium-bromide–stained gel or (2) performed quantitative PCR using either a TaqMan probe or SYBR Green fluorescence (Applied Biosystems). Fifteen different primer (and probe) sets had binding sites located in the HCoV-NL63 nucleocapsid protein gene (primer sets A–C [13], N, O, and R), HCoV-NL63 ORF1b (primer sets D–F and M and primer set P with nested primer set Q [12]), HCoV-NH ORF1a (primer set J [10]), and the HCoV-NH spike glycoprotein gene (primer set K with nested primer set L [10]). Three additional degenerate primer sets (G–I) were designed on the basis of conserved regions of the RNA polymerase and helicase in ORF1b, which are shared by HCoV-NL63, severe acute respiratory syndrome (SARS)–CoV (GenBank accession number NC_004718), HCoV-E229 (GenBank accession number NC_002645), and HCoV-OC43 (GenBank accession number NC_005147). For details on assay conditions, reagents, and primer sequences, see Appendix A, in the electronic edition (letter code for primer pairs refers to the list in Appendix A). All samples were also tested using primers and probes for either human glyceraldehyde-3-phosphate dehydrogenase (Applied Biosystems), porphobilinogen deaminase (primer/probe set T), or β-2-microglobulin (primer set U [16]), to check the quality and quantity of extracted RNA. Each RT-PCR included appropriate positive and negative controls. Positive control templates for HCoV-NL63 PCR were either clinical isolates of HCoV-NL63 RNA [13] (gift from Ron Fouchier, Erasmus Medical Center, Rotterdam, The Netherlands) or Topo-NL63 nucleocapsid protein gene plasmid (gift from Ralph Baric, University of North Carolina, Chapel Hill). The positive control template for the degenerate CoV PCR assays was cDNA from SARS-CoV (gift from Michael Buchmeier and Benjamin Neuman, Scripps Research Institute, La Jolla, California). The sensitivity of the PCR assays was assessed by testing 10-fold serial dilutions of known quantities of either RNA derived from HCoV-NL63 culture supernatants, Topo-NL63 nucleocapsid protein gene plasmid, or PCR products from each primer set.

At UCSD, in an attempt to isolate HCoV-NL63 from the patients with acute KD, we followed previously published protocols for virus isolation [12, 17]. Throat or NP swabs were placed in viral transport medium that was subsequently inoculated onto 2 monkey kidney cell lines (LLC-MK2 and Vero E6) in Refeed medium (Diagnostic Hybrids) with 0.2% trypsin and without additional serum. Monolayers were observed for cytopathic effect daily for 2 weeks. Scraped NP epithelial cells underwent rapid screening for respiratory viral antigens (Rhinoprobe) at UCSD. Pooled monoclonal antibodies were used to detect adenovirus, respiratory syncytial virus (RSV), influenza viruses A and B, and parainfluenza viruses 1–3 by direct fluorescent antibody assay (DFA; Respiratory Screen, Light Diagnostics).

RESULTS

A total of 57 samples (33 throat swabs, 7 NP swabs, 10 NP epithelial cell scrapings, and 7 nasal washes) from 48 patients (42% non-Hispanic white, 15% Asian, 25% Hispanic white, 6% black, and 12% mixed) were tested at 5 centers (table 1). Of the 48 patients, only 1 had HCoV-NL63 detected in an NP swab. None of the remaining 47 patients had HCoV detected with any of the primer sets, including 19 patients tested with the primer set J and the primer set K with nested primer set L, which were used by Esper et al. [10]. The presence of PCR

Table 1. Results of human coronavirus (HCoV) reverse-transcriptase polymerase chain reaction (RT-PCR) on respiratory samples from patients with acute Kawasaki disease.

								Samples						
			HCoV RT-PCR		Patients			Source				Collection		
Location of sample collection	Location of sample analysis	No. of patients (no. of samples)	No. of positive patients	Primer sets[a]	Age range (median)	No. of males	CAA[b]	Throat swab	NP swab	Scraped NP cells	Nasal wash	Illness days[c] (median)	No. of post-IVIG samples	Date (%)[d]
San Diego, California	UCSD	17 (23)	1	A–J	2 m–9 y (30 m)	15	1/6	8	7	8	0	3–15 (6.5)	2	Dec. '04–Mar. '05 (100)
Chicago, Illinois	NU and LU	13[e] (15)	0	J–O	4 m–10 y (38 m)	11	1/5	13	0	2	0	4–9 (6)	0	Mar. '01–Jan. '05 (85)
	VU	12[e] (12)	0	A–C	4 m–6 y (12 m)	11	1/3	12	0	0	0	3–9 (6.5)	0	Dec. '00–Sep. '04 (25)
Amsterdam, The Netherlands	AMC	7 (7)	0	P–R, J–L	5 m–9 y (35 m)	5	1/2	0	0	0	7	4–9 (7)	2	Nov. '04–Mar. '05 (86)
Total	…	48 (57)	1	…	2 m–10 y (31 m)	42	4/16	33	7	10	7	3–15 (7)	4	Dec. '00–Mar. '05 (77)

NOTE. AMC, Academic Medical Center; CAA, coronary artery abnormality; IVIG, intravenous immunoglobulin; LU, Loyola University; m, months; NP, nasopharyngeal; NU, Northwestern University; UCSD, University of California, San Diego; VU, Vanderbilt University; y, years.

[a] Primer sets A–C, N, O, and R are for the HCoV-NL63 nucleocapsid protein gene; primer sets D–F and M and primer set P with nested primer set Q are for the HCoV-NL63 open-reading frame (ORF) 1b; primer set J is for the HCoV-NH ORF1a [10]; primer set K with nested primer set L are for the HCoV-NH spike glycoprotein gene [10]; and primer sets G–I are degenerate primer sets for conserved regions of ORF1b that are shared by HCoV-NL63, severe acute respiratory syndrome–CoV, HCoV-E229, and HCoV-OC43.

[b] No. of patients with aneurysms/no. of patients with dilatation (internal lumen *z* score >2.5) [15].

[c] Illness day 1 is defined as the first day of fever.

[d] Shown is the percentage of samples collected from December through March.

[e] One patient sample was tested at 2 centers.

inhibitors as a possible cause of negative results was excluded by successful amplification of cellular RNA from all samples.

The 1 positive sample yielded an amplification product with all of the primer sets used: HCoV ORF1b degenerate primer sets G–I, HCoV-NL63 nucleocapsid protein primer sets A–C, and HCoV-NL63 ORF1b primer sets D–F, as well as with the HCoV-NH ORF1a primer set J [10]. The sequences of the amplification products from the primer sets (for A, C, F, and J, 123 bp, 99 bp, 106 bp, and 215 bp, respectively) matched the sequence for HCoV-NL63. The patient, a 5-year-old Bangladeshi American boy, met 4 of 5 classic clinical criteria for KD. In addition, he had symptoms of an upper respiratory tract infection (cough and coryza). Throat culture for β-hemolytic group A streptococcus was negative. Viral cultures for common respiratory pathogens and monkey kidney cell cultures for HCoV were negative. An NP swab for PCR testing was obtained on the seventh day of illness, before administration of intravenous immunoglobulin and aspirin. The patient responded with complete defervescence, but his respiratory symptoms persisted. Echocardiography revealed transient dilation of the left main coronary artery, with a z score of 3.8 [15].

The sensitivity of the PCR assays was assessed, and all primer sets detected 1–100 copies by TaqMan and SYBR Green PCR (primer sets A–I and R), as did conventional PCR with gel analysis (primer sets M–O). The sensitivity of nested PCR using primer set P with nested primer set Q was 100–1000 copies. Quantitation of HCoV-NL63 RNA from the 1 positive patient was estimated to be 2.0×10^6 RNA molecules/swab with primer sets A and B (TaqMan analysis) and 0.1×10^6 RNA molecules/swab with primer set D (SYBR Green analysis), assuming that each genomic RNA or subgenomic mRNA molecule yielded 1 molecule of cDNA.

DFA screening of scraped NP epithelial cells for respiratory viruses was performed at UCSD for 9 patients, and none were positive during the acute phase of the illness. HCoV-NL63 viral cultures were negative for all 9 patients tested, including the patient who was positive for HCoV-NL63 by RT-PCR.

DISCUSSION

We detected HCoV-NL63 in only 1 (2%) of 48 patients with acute KD tested at 5 centers in the United States and The Netherlands using RT-PCR assays. Our study sample included an ethnically and geographically diverse population, with more than one-half of all respiratory samples (54%) collected prospectively during the winter and spring months, which is the peak season for HCoV-NL63 in Europe and North America [10, 12, 13, 18]. Thus, we would have expected to detect HCoV-NL63 in a higher number of patients using our sensitive RT-PCR assays if infection with this virus was indeed temporally related to KD.

The debate over whether HCoV-NL63 and HCoV-NH represent different isolates of the same virus has not yet been resolved, but all available data (albeit limited) indicate that they likely represent the same virus. To ensure detection of either virus, degenerate primers were designed (primer sets G–I), and 2 of the primer sets (primer set J and primer set K with nested primer set L) used in our study were identical to the primer sets used to detect HCoV-NH by Esper et al. [10]. The single positive HCoV-NL63 molecular isolate from a patient with acute KD yielded an amplification product with all primers tested, including the HCoV-NH ORF1a primers from Esper et al., making it unlikely that we missed HCoV-NH because of sequence divergence from HCoV-NL63. In all, 12 throat swabs, 2 scraped NP epithelial cell samples, and 1 NP swab were tested using the HCoV-NH ORF1a primers, and 12 throat swabs and 2 scraped NP epithelial cell samples were tested using the HCoV-NH spike glycoprotein primers [10].

Differences in geographic region, definition of cases, respiratory sites sampled, selection bias, and PCR methods may explain the differences between our findings and those of Esper et al. [10]. First, the Esper study included only frozen, archived nasal washes from New Haven, Connecticut, and 6 of 8 samples positive by RT-PCR were collected from January to April of 2004 (F. Esper, personal communication). This suggests the possibility of a CoV outbreak in New Haven that could have caused coincidental infection in children with KD. Arguing against this possibility is that control children sampled within 2 weeks of the patients with KD did not show a similarly high rate of HCoV detection.

Another difference was the method and site of sample collection in the upper respiratory tract. In the present study, 58% of samples were throat swabs, whereas all of the samples in the Esper study were nasal washes. Only 20 patients in the present study had an NP sample of some type tested, and the single positive sample was from 1 of only 7 NP swabs. It is possible that sample collection methods affect the rate of detection of HCoV-NL63. In Canada, HCoV-NL63 was detected in 6 (6.5%) of 92 NP swabs versus 8 (2.8%) of 284 throat or pharyngeal swabs collected from patients with acute respiratory tract infections [18]. However, in the case of SARS-CoV, rates of viral detection by RT-PCR did not differ significantly among NP swabs, throat swabs, and NP aspirates [17]. SARS-CoV was detected in 15 (26%) of 58 NP swabs, 13 (30%) of 43 throat swabs, and 60 (31%) of 192 NP aspirates collected during the first 4 weeks of illness.

In the Esper study, one primer set was located in ORF1a, and the other set was located in the spike glycoprotein gene, for which 80 cycles were reportedly required to detect PCR product resolved on an agarose gel. The sensitivity of the ORF1a PCR was not described in the original publication. We amplified HCoV-NL63 using 13 different sets of primers with TaqMan PCR, SYBR Green PCR, and conventional PCR, with a sensi-

tivity of 1–100 copies. Thus, the RT-PCR assays used in the present study should have been sufficiently sensitive to detect HCoV-NL63 in the respiratory samples.

Few studies describing the epidemiologic and clinical features of HCoV-NL63 are available. HCoV-NL63 has been detected by RT-PCR during the winter in 1.6%–3.6% of adults and children who were hospitalized or visited an outpatient clinic because of respiratory symptoms [12, 13, 18, 19]. Esper et al. detected HCoV-NH in 79 (8.8%) of 895 children with respiratory symptoms who were negative for RSV, influenza viruses A and B, human parainfluenza viruses 1–3, and adenovirus by DFA [11]. The detection rate for HCoV-NL63 was highest in children <5 years of age [18], which coincides with the peak age group for KD. Thus, coexisting HCoV-NL63 infection in children with KD may occur, especially during winter. A wide variety of respiratory viruses have been occasionally isolated from patients with KD [8, 20, 21]. The one patient with KD in the present study who was positive for HCoV-NL63 had prominent respiratory symptoms not characteristic of KD, including copious rhinorrhea and cough. Therefore, this patient had coexisting, symptomatic HCoV-NL63 upper respiratory tract infection.

In the Esper study [10], samples were archived only if patients had respiratory symptoms at the time of admission to the hospital, and this bias in patient selection may account for the high rate of HCoV-NH detection. Also, some patients may have been misdiagnosed as having KD in the Esper study, because a subjective measure of coronary artery wall brightness by echocardiography was used as a criterion to include 2 patients who met only 3 clinical criteria.

In conclusion, we found no association between detection of HCoV-NL63 in the respiratory tract and acute KD. Future studies should continue to address the possibility of this and other microbes with a respiratory portal of entry as the etiologic agent of KD.

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