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Short communication

Evidence of human coronavirus HKU1 and human bocavirus in Australian children

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Abstract

Undiagnosed cases of respiratory tract disease suspected of an infectious aetiology peak during the winter months. Since studies applying molecular diagnostic assays usually report reductions in the number of undiagnosed cases of infectious disease compared to traditional techniques, we applied PCR assays to investigate the role of two recently described viruses, namely human coronavirus (HCoV) HKU1 and human bocavirus (HBoV), in a hospital-based paediatric population. Both viruses were found among Australia children with upper or lower respiratory tract disease during the autumn and winter of 2004, contributing to 21.1% of all microbial diagnoses, with individual incidences of 3.1% (HCoV-HKU1) and 5.6% (HBoV) among 324 specimens. HBoV was found to coincide with another virus in more than half of all instances and displayed a single genetic lineage, whilst HCoV-HKU1 was more likely to occur in the absence of another microbe and strains could be divided into two genetic lineages which we propose be termed HCoV-HKU1 type A and type B. Children under the age of 2 years were most at risk of infection by these viruses which contribute significantly to the microbial burden among patients with respiratory tract disease during the colder months.

Keywords: Human coronavirus; HCOV-HKU1; Human bocavirus; Epidemiology; Acute respiratory tract disease

1. Introduction

Acute respiratory tract infection (ARTI) is a frequent cause of paediatric morbidity and mortality and a common reason for both outpatient visits and hospitalizations. Among humans, RNA viruses are the most frequent agent to cause the common cold, usually a self-limiting upper respiratory tract illness (Heikkinen and Järvinen, 2003). Viruses are also frequently associated with paediatric lower respiratory tract disease (Klig and Shah, 2005; Hayden, 2004). Human rhinoviruses (HRV), respiratory syncytial virus (HRSV) and parainfluenza viruses (HPIV) are among the most commonly detected while human metapneumovirus (HMPV) and members of the genus *Coronavirus* also contribute to respiratory illness including severe disease (Hayden, 2004; Fouchier et al., 2005; van der Hoek et al., 2005; Selwyn, 1990; Hall, 2001).

Recently, two newly described respiratory viruses have been reported which account for an as yet undefined proportion of suspected ARTI. Human coronavirus (HCoV) HKU1 was first described in January 2005 following detection in a patient with pneumonia. Further studies in an HCoV-SARS-negative population identified a single case. Apart from the two cases in Hong Kong reported in 2005, only one other instance of HCoV-HKU1 has been reported (Woo et al., 2005; Allander et al., 2005). This suggested that the putative Group 2 coronavirus had a very low incidence (Woo et al., 2005).

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More recently, human bocavirus (HBoV) was described and tentatively classified as the first member of the family *Parvoviridae*, genus *Bocavirus* to cause disease among humans (Allander et al., 2005). HBoV occurred in 3.1% of hospitalized patients and in 17.6% of these detections, a coinciding microbe was also present.

Previous studies at our hospital of patients with suspected ARTI have shown that the highest number of undiagnosed cases occurs during autumn to spring. We initiated an investigation of specimens collected over the late autumn and winter of 2004 for the presence of HCoV-HKU1 and HBoV using PCR.

2. Materials and methods

2.1. Study population

The study population comprised 324 specimens from ill patients who had presented to Queensland hospitals or general practitioners during May to August 2004 with ARTI suspected of having an infectious aetiology. Specimens were predominantly nasopharyngeal aspirates (NPA; 99.1%), but also included bronchoalveolar lavage (0.6%), and bronchial washings (0.3%) collected either at the time of visit or following hospital admission. The majority of patients visited the Royal Children's Hospital and Royal Brisbane and Women's Hospitals in Brisbane, Queensland. Consecutive specimens were retrospectively selected by season, without prior knowledge of patient details or microbiological status. The subjects comprised 59.3% males and ranged in age from 7 days to 86.0 years (mean = 6.8 years, median = 1.1 years, mode = 0.1 years), with children 5 years of age or younger constituting 79.9% of the study population.

Prior to this retrospective study, specimens had been tested for common microbial respiratory pathogens and stored at −70 °C. The laboratory assays included a culture-amplified direct fluorescent assay and subsequent RT-PCR (Syrmis et al., 2004) to detect HRSV, human adenoviruses (HAdV), HPIV-1, 2 and 3 and influenza viruses A and B (IFAV, IFBV; (Syrmis et al., 2004)). An additional RT-PCR assay was used to detect all four subtypes of HMPV (Maertzdorf et al., 2004). Selective culture media were used to isolate bacterial pathogens including *Pseudomonas aeruginosa*, *Streptococcus* species, *Haemophilus influenzae*, *Neisseria* species, *Staphylococcus* species, *Legionella pneumophila* and *Candida albicans*. No microbes had been detected in 259 specimens (79.9%) while a suspected pathogen had been detected in 88 instances among 65 specimens (20.1%).

2.2. Virus PCR

Single-tube RT-PCR and PCR amplifications (OneStep RT-PCR and HotStarTaq kits, QIAGEN) utilized 0.3 μM of each primer for the pan-HCoV (Chiu et al., 2005, targeting ORF 1b), HCoV-HKU1 (Patrick Woo, personal communication), HCoV-OC43 (HCoVOC43_01.2, CGATGAGGCTATTCCGACTAGGT and HCoVOC43_02.4 TTTGGCAGTATGCTTAGTTACTT targeting the nucleoprotein gene) and HBoV (HBoV01.2, TATGGCCAAGGCAATCGTCCAAG and HBoV02.2, GCCGCGTGAACATGAGAAACAGA targeting the *NS1* gene) assays to examine 1 μL of purified nucleic acid. Reverse transcription was performed at 50 °C for 20 min. PCR was preceded by 15 min incubation at 95 °C, then followed by 45 cycles of 94 °C for 20 s; 56 °C for 20 s and 72 °C for 30 s.

2.3. Nucleotide sequencing and phylogenetic analysis

Amplicon was purified directly from the reaction mixture (QIAquick, QIAGEN, Australia) and nucleotide sequencing reactions were performed on both amplicon strands by applying the ABI PRISM™ BigDye cycle sequencing kit (Perkin Elmer Applied Biosystems Division, USA). Sequences were determined using an Applied Biosystems 3130 × l capillary electrophoresis (CE) genetic analyser.

Nucleotide sequences were aligned using BioEdit V7.0.0 (Hall, 1999) and presented in a topology tree prepared in MEGA 3 (Kumar et al., 2001). The nucleotide distance matrix was generated using the Kimura two-parameter neighbour-joining method. Nodal confidence values indicate the results of boot strap resampling ($n = 1000$). Sequences for the HCoV-HKU1 ORF 1b and HBoV *NS1* gene comparative phylogenetic analyses were obtained from GenBank (HCoV-NL63, AY567487; HCoV-229E, NC_002645; HCoV-OC43, AF124989; HCoV-HKU1, NC_006577; SARS-CoV, AY274119 and HBoV, DQ000495-6; canine minute virus (MVC), NC_004442; erythrovirus B19, NC_000883). Nucleotide sequences deposited into GenBank as a result of this study include DQ190472 and DQ200648.

3. Results

We investigated a population of 324 specimens predominantly collected during winter 2004, our peak months of viral ARTI, using a previously reported pan-coronavirus RT-PCR assay (Chiu et al., 2005). Nucleotide sequencing of coronavirus-positive specimens determined two instances of HCoV-HKU1. Using HCoV-HKU1-specific primers provided by Dr. Patrick Woo, Hong Kong University (personal communication) we re-screened the population and detected an additional eight instances of HCoV-HKU1 infection from NPAs of children less than 4 years of age and one 40 year old adult, resulting in a prevalence of 7.5% of whom 66.7% were female. One co-detection, representing 11.1% of HCoV-HKU1 positive specimens, was detected in which HMPV and HCoV-OC43 were also present in the same 13-month-old male presenting with bronchiolitis.

We next designed a new pair of PCR primers for HBoV diagnosis in order to avoid the non-specific amplification we noted when screening using a previously described assay (data not shown; (Allander et al., 2005)). When investigating the same specimen population we detected 18 instances of HBoV from the NPAs of children less than 3 years of age (59.3% male) indicating a prevalence of 5.6% comprising 13.5% of all microbial detections.

HRSV was the most frequently detected microbe in our population with 14.5% of specimens containing the virus ($n = 47$). HBoV was the second most frequently detected microbe followed by HMPV at 5.2% ($n = 17$) and HCoV-OC43 at 3.4% ($n = 11$). Seven instances of HCoV-OC43 (63.6%) were missed by the pan-coronavirus assay while one pan-coronavirus-positive was sequenced and found to be HCoV-229E, a target not included in this study. HCoV-HKU1 was the fifth most frequently detected microbe comprising 7.5% of all microbial detections and 3.1% of the study's specimen population. In eight instances, HBoV was the sole microbe detected and in two of these either bronchiolitis or viral pneumonia was suspected. In the remaining 10 instances, (55.6% of all HBoV detections) another microbe was detected, including eight instances of HRSV and one each of HAdV and HMPV type A2.

Children aged between 0 and 6 months (44.4% of all detections) were most at risk of infection by HCoV-HKU1, whereas children aged between 6 months and 2 years were the group most at risk of infection by HBoV. None of the 65 subjects older than 5 years were infected with HBoV whilst 1 HCoV-HKU1 infection occurred among this group.

Both viruses were detected throughout winter with HBoV occurring in late autumn but predominantly in early winter (61.1% of all microbial detections during June), and HCoV-HKU1 occurring solely in winter, predominantly late winter (55.6% of all detections occurred in August).

Detailed clinical reviews of virus-positive patients were not performed in this study; however, clinical notes made at consultation described presenting signs including rhinorrhoea, fever cough and wheeze and disease manifestations including bronchiolitis and pneumonia that were consistent with ARTI.

To better understand the nature of the two viruses under investigation and to confirm our PCR results we sequenced eight HCoV-HKU1 and 10 HBoV strains. Phylogenetic analysis of Queensland HCoV-HKU1 strains indicated that a single cluster existed, but that it represented a second lineage when compared to the prototype Hong Kong strain. We propose the lineages be termed HCoV-HKU1 A and B (Fig. 1). Queensland HCoV-HKU1 strains exhibited 97.5%–98% nucleotide and 100% predicted amino acid sequence homology with the prototype strain. Queensland HBoV strains (Fig. 2) shared greater than 99% nucleotide and 100% predicted amino acid homology with the HBoV prototype strains, st1 and st2.

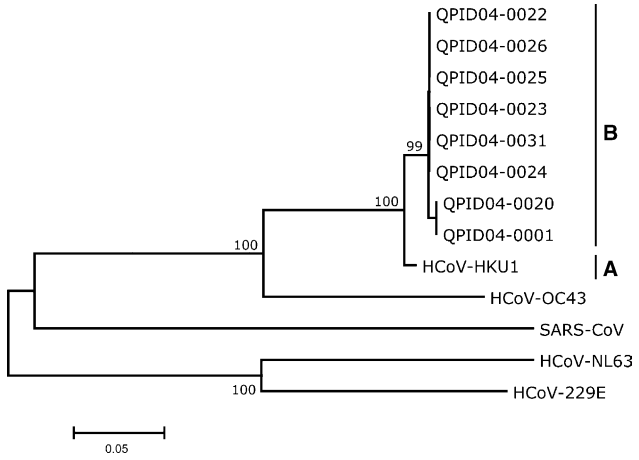

Fig. 1. Phylogenetic analysis of Queensland strains of HCoV-HKU1, (QPID04), detected during 2004 presented on a topology tree prepared in MEGA 3 and compared to the type strain obtained from GenBank (HKU1) and all other human respiratory coronaviruses. A 414 bp nucleotide alignment of a portion of the polymerase (1b) gene was prepared using BioEdit V7.0.0.0. The nucleotide distance matrix was generated using the Kimura two-parameter estimation. Nodal confidence values indicate the results of boot strap resampling ($n = 1000$). Proposed HCoV-HKU1 genetic lineages are indicated.

4. Discussion

This study presents the first evidence that HBoV circulates in ill children outside of Sweden, and that HCoV-HKU1 is present in patients in the Southern hemisphere, suggesting both are actively infecting humans on a global scale. Our data also describe the first detections of HCoV-HKU1 from children in a hospital environment and indicate that a recently published pan-coronavirus assay (Chiu et al., 2005) failed

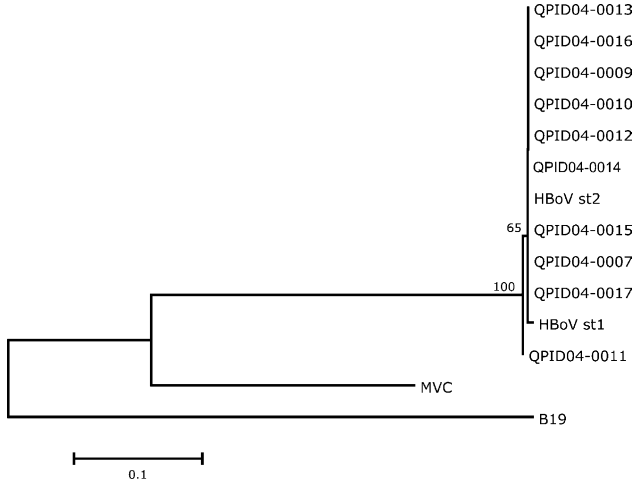

Fig. 2. Phylogenetic analysis of the Queensland strains of HBoV (QPID04), detected during 2004 presented on a topology tree prepared in MEGA 3. A nucleotide alignment of a 245 bp portion of the *NS1* gene was prepared using BioEdit V7.0.0.0. The nucleotide distance matrix was generated using the Kimura two-parameter estimation. Nodal confidence values indicate the results of boot strap resampling ($n = 1000$).

to detect 80% of HCoV-HKU1 and 63.6% of HCoV-OC43-positive specimens.

Together, HCoV-HKU1 and HBoV comprised 21.1% of all microbial detections in our population, found in 8.6% of all specimens tested. These values are significantly greater than those reported in the original publications describing HCoV-HKU1 in Hong Kong and HBoV in Sweden. Our detections of these two viruses provided a putative aetiologic cause for ARTI in 16 previously undiagnosed subjects. Based on these data we recommend the inclusion of HCoV-HKU1 and HBoV testing in routine laboratory screening of children and adults with suspected ARTI during the colder months. Co-detections were not common with HCoV-HKU1 infection and our data strongly suggests that apparent respiratory disease may often be the result of infection by this coronavirus. By comparison, 55.6% of all HBoV-infected patients were also infected with another virus, most frequently HRSV, which is a well-characterised cause of mild to severe ARTI. In the original description of HBoV, Allander et al. (2005) reported that 17.6% of positives were co-detected with another virus namely one incidence of HAdV and two of HRSV. However, it was unclear what other microbial targets had been screened for in that population; therefore, it is to be hoped that future studies can contribute to this issue (Allander et al., 2005). Currently neither HCoV-HKU1 nor HBoV can be associated with ARTI until more detailed, carefully controlled community and hospital-based studies are performed to elucidate the full spectrum of disease resulting from infection.

The region of the HBoV *NS1* gene amplified during this study displayed limited sequence variation among Queensland strains, and additional epidemiological studies on different genomic regions will be required to investigate the overall stability of the viral genome. Conversely, examination of HCoV-HKU1 strains detected in this study showed these could be subdivided into two genetic lineages, A and B, encompassing 8–10 nucleotide differences over the length of the 414 bp amplicon (1.9%–2.4%) generated from the Australian strains that were absent in the prototype strain. HCoV-HKU1 B predominated during the winter of 2004. It remains to be determined whether these two lineages differ in disease severity among infected patients and whether additional variation will be seen elsewhere in the world.

Further investigation will be required to define an association between infection by HCoV-HKU1 and HBoV and respiratory disease. Such conclusions will be reached more rapidly if comprehensive studies can define the annual incidence of these viruses and the prevalence of infection within the broader community. Additionally, targeted studies are needed to investigate the role of these putative pathogens among the elderly and the immunocompromised. Nonetheless, we have shown that HCoV-HKU1 and HBoV both occur in children with suspected acute respiratory tract disease and that the two viruses contribute significantly to the total microbial burden at our hospital during winter. We recommend that HCoV-HKU1 be considered in the laboratory diagnosis of ARTI during autumn and winter and that further studies are needed to define the role of HBoV in ARTI.

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