

EVIDENCE OF HUMAN CORONAVIRUS-NL63 INFECTION IN JORDANIAN CHILDREN

*Nasser M. Kaplan MD**, *Winifred Dove BSc***, *Sawsan A. Abd-Eldayem BSc**,
Ahmad F. Abu-Zeid MD^, *Hiyam E. Shamoan MD^*, *C. Anthony Hart, PhD, FRCPath***

ABSTRACT

Objective: To detect and evaluate the role of the newly recognized human coronavirus (HCoV)-NL63 and HCoV-HKU1 as aetiologic agents of acute respiratory tract infections in hospitalized Jordanian children younger than 5 years of age.

Methods: Between December 2003 and May 2004, a total of 326 nasopharyngeal aspirates were collected from Jordanian children hospitalized with acute respiratory tract infections. Total DNA and RNA were extracted using Qiagen commercial kits. HCoV-NL63 and HCoV-HKU1 were detected by random reverse transcription-polymerase chain reaction using random hexamer primer for the reverse transcription step, and specific primers that target the replicase and polymerase genes to produce 215-bp and 392-bp amplicons respectively. Other potential respiratory pathogens were detected according to previously published protocols.

Results: HCoV-NL63 was detected in 4 (1.2%) out of 325 examined nasopharyngeal aspirates. HCoV-NL63 was detected in two children with severe, and in two with mild to moderate acute respiratory tract infections. HCoV-NL63 was the only pathogen detected in three patients, and mixed with adenovirus in one patient. HCoV-HKU1 was not detected in the 325 nasopharyngeal aspirates examined.

Conclusion: HCoV-NL63 is a significant causative agent of acute respiratory tract infections in hospitalized Jordanian children. HCoV-NL-63 can cause the respiratory disease either alone or in combination with other potential respiratory pathogens. Further studies are required to further characterize the clinical and epidemiological features of these newly recognized HCoVs in Jordan.

Key words: Children, Human coronavirus, Respiratory tract infections.

JRMS December 2008; 15(3): 17-21

Introduction

Acute respiratory tract infection is the major cause of death in children younger than five years of age, and occurs predominantly in the developing countries.⁽¹⁾ A variety of viruses, bacteria and fungi

are associated with respiratory tract illness. The viruses most frequently associated with respiratory tract infections include rhinoviruses, coronaviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses, adenoviruses, and the recently discovered human metapneumovirus and bocavirus.

From the Departments of:

*Microbiology, King Hussein Medical Centre (KHMC), Amman- Jordan.

**Medical Microbiology, University of Liverpool, Liverpool, England, United Kingdom.

^ Pediatrics, (KHMC)

Correspondence should be addressed to Dr. N. Kaplan, P. O. Box 140746 Amman-Jordan. E-mail: nassermkaplan@hotmail.com

Manuscript received December 8, 2007. Accepted June 26, 2008

Human Coronaviruses (HCoVs) are positive-stranded RNA viruses with the largest viral genome among the RNA viruses (27-33 kb). The enveloped pleomorphic virus particles (50-150 nm in diameter) carry extended spike proteins on the membrane surface, providing the typical crown (Latin, *corona*)-like structure seen by electron microscopy.⁽²⁾ HCoVs are predominantly associated with respiratory tract illnesses of varying severity. HCoVs are represented by 2 prototype strains, HCoV-229E and HCoV-OC43, which belong to antigenic groups 1 and 2 respectively. The emergence of severe acute respiratory syndrome (SARS) caused by a novel coronavirus (SARS-CoV) in 2003⁽³⁾ was followed by the recent identification of two novel HCoVs, namely HCoV-NL63 in 2004 and HCoV-HKU1 in 2005 respectively. HCoV-NL63, which is most closely related to HCoV-229E and belongs to antigenic group 1, was detected in a child with bronchiolitis in the Netherlands.⁽⁴⁾ HCoV-HKU1, which is most closely related to HCoV-OC43 and belongs to antigenic group 2, was detected in an adult with chronic pulmonary disease in Hong Kong.⁽⁵⁾ These two new HCoVs circulate worldwide causing infections that have been reported from a number of different countries.⁽⁶⁾ These viruses can be detected in 1-10% of patients with acute respiratory tract infections, and dual infections with other respiratory viruses are common.⁽⁷⁾

The purpose of this study was to detect and evaluate the role of HCoVs-NL63 and HCoV-HKU1, as aetiologic agents of acute respiratory infection in a hospital-based paediatric population younger than five years of age in Jordan.

Methods

The study was conducted over six consecutive months from December 2003 to May 2004. Children younger than five years of age with acute respiratory infection admitted to the paediatric wards of King Hussein Medical Centre and Queen Alia Hospital, Amman, Jordan were enrolled in the study irrespective of the severity of their illness. King Hussein Medical Centre, a tertiary referral hospital, and Queen Alia Hospital, a district general hospital, provide hospital paediatric care for Amman, the capital city of Jordan, and its surroundings. The clinical diagnosis of acute respiratory infection and assessment of its severity was made by using the

World Health Organization standard protocol for acute respiratory infection based on the presence of cough, tachypnoea, chest indrawing, and wheezing for <7 days duration.⁽⁸⁾ Severe disease was defined as present in children with a respiratory rate >60/min and chest indrawing. Oxygen saturation (pO₂) was measured by using pulse oximetry (Nellcor Puritan Bennett NPB-195, UK) and a pO₂ ≤ 85% was used as the cut-off for giving supplementary oxygen. Nasopharyngeal aspirates (NPAs) were collected from patients within 48 hours of admission to the hospital by instilling 1mL sterile phosphate-buffered saline through a sterile nasopharyngeal mucous extractor. The NPAs were frozen at -80°C until analyzed in the Department of Medical Microbiology, University of Liverpool, UK. The study was approved by the medical research ethical committee of King Hussein Medical Centre, Amman, Jordan and informed consent was obtained from each child's parents or legal guardians for participation in the study.

Total RNA and DNA were extracted separately from NPAs by using the commercial RNeasy and QIAamp DNA Mini Kits (Qiagen, Crawley, West Sussex, UK) respectively according to manufacturer's instructions. The genomes of HCoV-NL63 and HCoV-HKU1 were detected separately in the NPAs by random reverse transcription-polymerase chain reaction (RT-PCR).

The RT step was performed using random hexamer primer with a random 3' hexanucleotide sequence that can anneal to nearly any RNA. The 5' 20 nucleotides of the primer (tail) contain a unique sequence that serves as a template for subsequent PCR primer annealing. The primer that is annealed to the RNA template is extended by reverse transcriptase with an RNase H activity that allows the reattachment of the enzyme and insertion of the tailed random primer on both 3' and 5' sides. The cDNA product is PCR amplified using the unique region of the initial primer.⁽⁹⁾ A 5µl aliquot of the extracted viral RNA, serving as a template for cDNA synthesis, was added to 20µl of RT mixture. The final 25µl mastermix reaction contained 1X buffer, 2mM MgCl₂, 0.4mM deoxyribonucleotide triphosphates (dNTPs), 0.4µl random hexamer primer, 10units of RNase inhibitor, 12.5units of murine leukemia virus reverse-transcriptase. The RT was performed in an Applied Biosystems 2720 thermal cycler (Warrington, Cheshire, UK)

Table I. Comparison of the human coronavirus NL63-infected children

Patient	Age (months)	Sex	Date (sample collection)	Disease Severity	Co-infection
1	12	F	December	Severe	None
2	6	M	January	Severe	None
3	4	M	February	Mild-Moderate	Adenovirus
4	2	M	February	Mild-Moderate	None

according to the following program: 10min at 25°C, then 1hr reverse transcription at 42°C.

The HCoV-NL63 specific primers Forward (5'-GCG CTA TGA GGG TGG TTG TTA C-3') and Reverse (5'-CGC GCA GTT AAA AGT CCA GAA TTA AC-3') that target and amplify the replicase gene to produce a 215-bp amplicon were used.⁽¹⁰⁾ The HCoV-HKU1 specific primers LPW1926 Forward (5'-AAA GGA TGT TGA CAA CCC TGT T-3') and LPW1927 Reverse (5'-ATC ATC ATA CTA AAA TGC TTA CA-3') that target and amplify the polymerase gene to produce a 392-bp amplicon were used.⁽¹¹⁾ A 5µl aliquot of the cDNA templates was added to 45µl of PCR mixture. The final 50µl mastermix reaction contained 1X PCR buffer, 1.5mM MgCl₂, 0.2mM dNTPs, 0.4µM of each primer, and 2.5U of Amplitaq gold DNA polymerase (Applied Biosystems). The PCR was performed according to the following program: 5min DNA polymerase activation cycle at 94°C, then forty cycles (1 min denaturation at 94°C, 1 min of primer annealing at 50°C, and 1 min of primer extension at 72°C), and a final extension cycle of 72°C for 10 min. Using positive and negative controls, and a 100-bp molecular sizing ladder, 10µl volumes of each amplified DNA PCR product were separated by electrophoresis on a 2% (wt/vol) agarose-Tris-Borate-ethylene diamine tetracetic acid gel, stained with ethidium bromide, and visualized under UV light (Syngene Gel Documentation and Analysis System, Ingenius, Cambridge, UK).

Detection of other potential respiratory pathogens including adenovirus, human bocavirus, *Chlamydia* spp., and *Mycoplasma pneumoniae*, human respiratory syncytial virus, human metapneumovirus, human rhinovirus, influenza A and B viruses, and parainfluenza 1-4 viruses was performed by PCR and RT-PCR according to previously published protocols.

Results

A total of 326 hospitalized children (188, 58% male) with a median age of 5 months were recruited

to the study. All the patients had lower respiratory tract infections mainly bronchiolitis and bronchopneumonia, and routine cultures of their blood and respiratory secretions had detected no potential bacterial or fungal pathogens. A total of 326 NPAs were collected and analyzed, however the volume of some NPAs was inadequate for both DNA and RNA extractions. Only one NPA was inadequate for the detection of HCoV by RT-PCR. There was no HCoV-HKU1 detected in this study, however HCoV-NL63 was detected in 4 (1.2%) of 325 examined NPAs (Table I).

The median age of HCoV-NL63-infected patients was 5 months (range 2-12 months); and three (75%) were male. Two patients had mild/moderate and two had severe acute respiratory diseases.

HCoV-NL63 was the sole pathogen detected in the two patients with severe and in one patient with mild/moderate acute respiratory disease, however it co-infected with adenovirus in one patient with mild/moderate disease. Each of the four HCoV-NL63 isolates was identified during the December-February period of the winter season.

Discussion

Although numerous groups have reported HCoV-NL63 infections worldwide,⁽⁶⁾ this study has confirmed that HCoV-NL63 is a significant cause of acute respiratory infection in young children in Jordan. In this study, HCoV-NL63 was detected in 4 (1.2%) of 325 patients as compared to detection in 1 to 10% of patients with acute respiratory tract infections in other studies. The median age of HCoV-NL63-infected patients in this study was 5 months (range 2-12 months) suggesting that children under the age of 12 months were most at risk of infection. Each of the four HCoV-NL63 isolates in this study were identified during the December-February period suggesting that this virus circulates in Jordan primarily in the winter as reported in other temperate climate countries.⁽⁷⁾ However a spring-summer peak of activity was reported in Hong Kong⁽¹²⁾ indicating that the seasonality of HCoV-

NL63 in tropical and subtropical regions may not be restricted to the winter season. A summer peak cannot be excluded in our study which was only conducted from December to May.

Although all the currently circulating HCoV's can probably be classified as common cold viruses, a more severe lower respiratory tract infection is frequently observed in young children, patients with underlying disease and the elderly.⁽⁹⁾ However the four HCoV-NL63-infected patients in this study had no underlying illnesses or pre-existing lung diseases.

Although the first described cases of HCoV-NL63 infections were in young children with severe lower respiratory tract infections in hospital settings,^(4,13,14) a recent Canadian study showed that HCoV-NL63-infected patients had relatively mild symptoms like fever, cough, sore throat, and rhinitis.⁽¹⁵⁾ This demonstrates the broad clinical spectrum of HCoV-NL63 infections. It has also been reported that HCoV-NL63 infection is associated with high frequency of croup.⁽¹⁶⁾ In our study, two patients had mild/moderate and two had severe acute respiratory diseases; however there were no deaths or admissions to the intensive care unit.

Although HCoV-NL63 infections are often found in combination with a second respiratory virus, and the frequency of double infections can exceed 50%,⁽⁷⁾ in this study HCoV-NL63 was more likely to occur in the absence of another potential respiratory pathogen as it was the sole pathogen detected in the two patients with severe and in one patient with mild/moderate acute respiratory disease, however it co-infected with adenovirus in one patient with mild/moderate disease.

HCoV-HKU1 was not detected in this study; nevertheless this does not mean that this virus does not exist in Jordan. This may be explained by the relatively short period of the study and the variable seasonality of HCoV-HKU1 infection.

Conclusion

This study presents the first evidence that HCoV-NL63 circulates in Jordan and contributes to the hospitalization of children younger than 5 years of age. Whether this pathogen is responsible for a substantial proportion of respiratory tract infections in Jordanian children remains to be determined. Further studies over longer periods of time are required to better determine the epidemiologic

features and clinical spectrum of the two newly recognized HCoV's in Jordan.

References

1. **Bryce J, Boschi-Pinto C, Shibuya K, Black RE, WHO Child Health Epidemiology Reference Group.** WHO estimates of the causes of death in children. *Lancet* 2005; 365 (9465): 1147-1152.
2. **Lai MMC, Holmes KV.** Coronaviridae: The viruses and their replication. In: Knipe DM, Howley PM *et al* (Eds). *Fields Virology*. 4th edition. Lippincott Williams & Wilkins Publishers, PA, USA 2001; 1163-1185.
3. **Peiris JS, Lai ST, Poon LL, Guan Y, Yam LY, Lim W, *et al*.** Coronavirus as a possible cause of severe acute respiratory syndrome. *Lancet* 2003; 361(9366): 1319-1325.
4. **van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, *et al*.** Identification of a new human coronavirus. *Nat Med* 2004; 10 (4): 368-373.
5. **Woo PC, Lau SK, Chu CM, Chan KH, Tsoi HW, Huang Y, *et al*.** Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J Virol* 2005; 79(2): 884-895.
6. **Pyrc K, Berkhout B, van der Hoek L.** The novel human coronaviruses NL63 and HKU1. *J Virol* 2007; 81 (7): 3051-3057.
7. **van der Hoek L, Pyrc K, Berkhout B.** Human coronavirus NL63, a new respiratory virus. *FEMS Microbiol Rev* 2006; 30 (5):760-773.
8. **Pio A.** Standard case management of pneumonia in children in developing countries: the cornerstone of the acute respiratory infection programme. *Bull World Health Organ* 2003; 81 (4): 298-300.
9. **Pyrc K, Berkhout B, van der Hoek L.** Identification of new human coronaviruses. *Expert Rev Anti Infect Ther* 2007; 5(2): 245-253.
10. **Choi EH, Lee HJ, Kim SJ, Eun BW, Kim NH, Lee JA, *et al*.** The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000-2005. *Clin Infect Dis* 2006; 43(5): 585-592.
11. **Lau SK, Woo PC, Yip CC, Tse H, Tsoi HW, Cheng VC, *et al*.** Coronavirus HKU1 and other coronavirus infections in Hong Kong. *J Clin Microbiol* 2006; 44 (6): 2063-2071.
12. **Chiu SS, Chan KH, Chu KW, Kwan SW, Guan Y, Poon LL, Peiris JS.** Human coronavirus NL63 infection and other coronavirus infections in children hospitalized with acute respiratory disease in Hong Kong, China. *Clin Infect Dis* 2005; 40 (12): 1721-1729.

13. **Fouchier RA, Hartwig NG, Bestebroer TM, Niemeyer B, de Jong JC, Simon JH, Osterhaus AD.** A previously undescribed coronavirus associated with respiratory disease in humans. *Proc Natl Acad Sci USA* 2004; 101 (16): 6212-6216.
14. **Arden KE, Nissen MD, Sloots TP, Mackay IM.** New human coronavirus HCoV-NL63 associated with severe lower respiratory tract disease in Australia. *J Med Virol* 2005; 75(3): 455-462.
15. **Bastien N, Anderson K, Hart L, Van Caesele P, Brandt K, Milley D, et al.** Human coronavirus NL63 infection in Canada. *J Infect Dis* 2005; 191(4): 503-506.
16. **van der Hoek L, Sure K, Ihorst G, Stang A, Pyrc K, Jebbink MF, et al.** Croup is associated with the novel coronavirus NL63. *PLoS Medicine* 2005; 2(8): e240.