

Human coronavirus NL63 in children with acute upper respiratory tract infection by reverse transcription polymerase chain reaction (RT-PCR)

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Abstract: Background and aim: Human coronavirus NL63 (HCoV-NL63) is a recently discovered human corona virus that causes respiratory illness in children. In this study, we looked for HCoV-NL63 in children with acute upper respiratory tract infection by reverse transcription polymerase chain reaction (RT-PCR) and determined coinfections between HCoV-NL63 and other respiratory viruses. **Subjects and methods:** Nasal secretions collected from 160 children with acute upper respiratory tract infection and 80 controls were subjected to reverse transcription-PCR (RT-PCR) for detection of HCoV-NL63 virus and direct immunofluorescence (DIF) for detection of seven respiratory viruses (RSV, influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus). **Results:** We detected HCoV-NL63 RT-PCR in 24 out of 160 (15%) nasal secretion specimens collected from children with acute upper respiratory tract infection. All children with HCoV-NL63 infection were aged from 12- 32 months. Direct immunofluorescence assay detected RSV, influenza A, influenza B, and adenovirus in 42(26.3%), 23 (14.4%), 15 (9.4%) and 4(2.5%) respectively in the nasal secretion specimens of the patients. RSV was detected positive in 10 (43.4%) children with HCoV-NL63 infection, influenza A was detected positive in 4 (17.4%) patients with HCoV-NL63 infection, 4 (17.4%) patients with RSV infection and 3 (13%) patients with influenza B infection. While RSV was detected positive in 2 (8.7%) adenovirus infection. **Conclusion:** HCoV-NL63 is frequently detected human pathogen, often associated with other respiratory viruses and acute respiratory tract infections in children. Rapid DIF screening reagent for detection of multiple respiratory viruses within 1 to 2 hours is of great benefit in terms of patient management and infection control.

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1.Introduction:

Viral respiratory diseases are a major health problem and represent the leading cause of death due to infectious disease in Canada. They affect people of all ages and exert a great economic impact on the health-care system [1]. The viruses most frequently associated with respiratory-tract infections include rhinoviruses, coronaviruses, influenza viruses, parainfluenza viruses, respiratory syncytial viruses (RSVs), adenoviruses, and the recently identified human metapneumovirus (hMPV). However, the etiological agents for a large number of respiratory infections remain unknown. A new human coronavirus, NL63 (HCoV-NL63), has been discovered in the Netherlands [2].

Coronavirus, a genus of the Coronaviridae family, is an enveloped virus with a large plus- strand RNA genome [3]. Human coronavirus (HCoV) 229E and OC43 are members of groups I and II, respectively. HCoV infection is thought to be responsible for up to 30% of common cold cases in winter and occasionally causes acute lower respiratory tract disease in susceptible infants, elderly individuals, and immunocompromized adults [4, 5].

In 2003, the outbreak of severe acute respiratory syndrome (SARS) led to the preliminary identification of SARS corona virus [6]. In 2004, a new HCoV-NL63 was identified in clinical specimens from both infants and adults with acute respiratory tract infection (ARTI) in the Netherlands [3]. Sequence analysis of the complete genome of HCoV-NL63 revealed that the virus was more closely related to HCoV- 229E than to the other human coronaviruses [3].

Preliminary data suggested that HCoV-NL63 might be an important respiratory tract pathogen in children, similarly to respiratory syncytial virus and human metapneumovirus [3, 7-9]. The epidemiology and clinical features of HCoV-NL63 infection are largely unknown. Early diagnosis of infected patients and appropriate infection control measures will limit the spread in hospitals, while social distancing strategies may be necessary to control the outbreak in communities [10].

Aim of the work

In this study, we looked for HCoV-NL63 in children with acute upper respiratory tract infection by reverse transcription polymerase chain reaction

(RT-PCR) and determined coinfections between HCoV-NL63 and other respiratory viruses.

2. Subjects and methods

This case control study was conducted on 160 children with acute upper respiratory tract infection (104 males, 56 females; age range, 1 to 5 years; mean \pm SD age, 2.8 \pm 1.5 years) attending the Pediatric Outpatient Clinic in the period from September 2012 to January 2014. In addition to 80 apparently healthy children (55 males and 25 females) matched in age with the patients as a control group. The main symptoms on presentation were cough, rhinitis and fever ($>$ 38.5°C).

All patients and controls were subjected to the following after their written consent:

- Full history taking and thorough clinical examination.

- Total and differential leukocytes count.

- Chest radiograph.

- Nasal secretions collected from the patients and controls were subjected to direct immunofluorescence (DIF) for detection of seven respiratory viruses (RSV, influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus) and reverse transcription-PCR (RT-PCR) for detection of HCoV-NL63.

Nasal secretions collection

The nasal secretions of the patients and controls were quantified by weighing the swabs before and after collection and were then diluted 1:10 (wt/vol) with phosphate-buffered saline and clarified by centrifugation at 900 \times g for 10 min to pellet cells for DIF. Finally, the cell-free supernatant nasal fluid was stored in aliquots at -20°C until RNA extraction [11].

I. Direct immunofluorescence assay (DIF)

It permits detection of seven viruses in one cell spot by using a new respiratory screen reagent, SimulFluor Respiratory Screen (RS; Chemicon International, Temecula, Calif.), the viruses are: RSV, influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus. This reagent utilizes a reddishgold (rhodamine) label for RSV and an apple green (fluorescein) label for influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus. So, if green-stained cells are visualized, a second slide must be stained to determine which of the last group of viruses is in the sample.

1. Slide preparation.

Cell pellets were resuspended in a small amount of PBS, and 200 μ l of the suspension was applied per cell spot by cytocentrifugation (Cytospin 3; Shandon Inc., Pittsburgh, Pa.) at 800 rpm for 4 min. Slides were air dried and then fixed by acetone for 10 min. For the study, two to three cytospin slides were prepared depending on the viruses suspected.

2. Respiratory screen DIF.

Cell spots were stained with 40 μ l of SimulFluor RS reagent (Chemicon International) for 15 min at 37°C. Slides were washed for 30 seconds in PBS, then were mounted in glycerol, examined for the presence of fluorescein-labeled cells by the use of a fluorescein filter (for detection of influenza A and B viruses, parainfluenza virus types 1 to 3, and adenovirus), and then reexamined for the presence of rhodamine-labeled cells by the use of a rhodamine filter (for detection of RSV). When fluorescein-positive cells were detected, a second and, occasionally, a third cell spot were stained to determine the infecting virus. The staining pattern varied with the infecting virus and the stage of growth. Fluorescent staining was nuclear and/or cytoplasmic and often punctate; it was bright apple green for fluorescein-labeled antibodies and reddish gold for rhodamine-labeled antibodies.

II. Reverse transcription-PCR (RT-PCR)

For detection of HCoV-NL63 in nasal secretion samples of the patients and control.

1. Extraction: Total RNA was extracted by using the QIAamp viral RNA mini kit (Qiagen, Germany)

2. Primer sequences:

The primers used for the diagnosis of HCoV-NL63 were:

730 (147-AGATGAGCAGATTGGTTATTGG-168) and 731 (491-GAAGA

GTCTCGTGAGTTGTTAC-470), based on the sequence of the nucleocapsid gene, were designed using BEACON software.

RT-PCR:

Viral RNA was amplified in a one-step RT-PCR (QIAGEN) following the manufacturer's recommendations. Briefly: 5 μ l of RNA was added to the RT-PCR mixture containing 2 μ l QIAGEN OneStep RT-PCR enzyme mix, 10 μ l of 5 X QIAGEN OneStep RT-PCR buffer, 400 μ mol/L deoxynucleoside triphosphate, 0.6 μ mol/L of each primer, and 10 μ l of Q solution in a final volume of 50 μ l. The thermocycler conditions used were as follows: 50°C for 30 min for reverse transcription, 95°C for 15 min for the activation of the HotStart DNA polymerase and then 50 cycles of 95°C for 30 sec, 50°C for 1 min, 72°C for 30 sec, followed by an extension of 10 min at 72°C. Positive and negative RT-PCR controls containing standardized viral RNA extract and nuclease-free water, respectively, were included in each run. The PCR products were separated on 2% agarose gels containing 0.5 g of ethidium bromide per milliliter in Tris-borate-EDTA (TBE) buffer and were visualized under ultraviolet light. The size of the amplified products of the HCoV-NL63 corresponds to (169 bp) [12]. To know

the efficiency of nucleic-acid extraction, each sample was also tested for the presence of the RNase P housekeeping gene by use of a 1-step RT-PCR kit under the following conditions: 30 min at 50°C for reverse transcription, 15 min at 95°C for the activation of the HotStart DNA polymerase followed by 50 cycles of 15 s at 94°C, 30 s at 50°C, and 30 s at 72°C and an extension for 7 min at 72°C. All the specimens tested were positive for the RNase P gene.

3. Results

I. Reverse transcription-PCR (RT-PCR) results

Nasal secretion samples of the 160 patients and 80 controls were tested for HCoV-NL63 by RT-PCR, and 24 out of 160 (15%) patient samples yielded positive results while none of the controls yielded positive results (Table 1). Patients with HCoV-NL63 aged from 12- 32 months.

Table (1): Incidence of respiratory virus in children with respiratory tract infection

	Monoinfection	Coinfection	Total
HCoV-NL63	10(41.7%)	14(58.3%)	24/ 160(15%)
RSV	26(61.9%)	16(38.1%)	42/160 (26.3%)
Influenza A	12(52.2%)	11(47.8%)	23 /160(14.4 %)
Influenza B	12(80%)	3(20%)	15/160(9.4%)
Parainfluenza 1,2,3	0	0	0/160
Adenovirus	2(50%)	2(50%)	4/160(2.5%)

Table (2): The number and percentage of coinfection of viral agents

Coinfection	No. of patients (%)
HCoV-NL63+RSV	10/23(43.4%)
HCoV-NL63+Influenza A	4/23(17.4%)
RSV+Influenza A	4/23(17.4%)
Influenza A+Influenza B	3/23(13%)
RSV+Adenovirus	2/23(8.7%)

4. Discussion

Four human coronaviruses are known to exist: human coronavirus 229E (HCoV-229E), HCoV-OC43, severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV) and Human coronavirus NL63 (HCoV-NL63). The complete genome sequence indicates that HCoV-NL63 virus is not a recombinant, but rather a new group 1 coronavirus. The virus replicates *in vitro* on tertiary monkey kidney cells and the Rhesus monkey kidney epithelial cells (LLC-MK2) [3]. HCoV-NL63 is a recently discovered human coronavirus found to cause respiratory illness in children and adults [13].

In this study, HCoV-NL63 was detected by RT-PCR in 24 out of the 160 (15%) nasal secretion samples of the patients with lower respiratory tract infections. The HCoV-NL63 positive patients aged from 12- 32 months. In 2005, Esper *et al.*, [8]

II. Direct immunofluorescence assay (DIF) results

Out of the 160 nasal secretion samples of the patients, RSV was detected in 42 (26.3%) samples, influenza A was detected in 23 (14.4%) samples, influenza B was detected in 15 (9.4%) samples and adenovirus was detected in 4 (2.5%) samples by DIF (Table 1). In the controls, RSV was detected in 2 (2.5%) samples and influenza A was detected in 3 (3.8%) samples.

III. Co-infection of HCoV-NL63 with other viruses.

RSV was detected positive in 10 (43.4%) children with HCoV-NL63 infection, influenza A was detected positive in 4 (17.4%) patients with HCoV-NL63 infection, 4 (17.4%) patients with RSV infection and 3 (13%).

patients with influenza B infection. While RSV was detected positive in 2 (8.7%) adenovirus infection (Table 2).

reported a positivity rate for HCoV-NL63 (8.8%) in their study to respiratory specimens obtained from 895 children <5 years by polymerase chain reaction (PCR). Chiu *et al.*, [1] reported that 15 out of the 587 (2.6%) children hospitalized with fever and acute respiratory symptoms were positive for HCoV-NL63, nine (1.5%) were positive for HCoV-OC43, and two (0.3%) were positive for HCoV-229E by multiplex RT-PCR. While Xin *et al.*, [14] detected HCoV-NL63 by reverse transcriptional PCR (RT-PCR) in eight out of 878(0.9%) respiratory specimens freshly collected from hospitalized children with acute lower respiratory tract infections between April 2006 and March 2008 in Children's Hospital of Chongqing Medical University.

Simul Fluor respiratory screen reagent was used as a new rapid, economic, and sensitive direct immunofluorescence assay that can be used for

diagnosis of multiple respiratory viruses. It should be noted that the SimulFluor reagents used did not differentiate among parainfluenza virus types 1, 2, and 3.

Our results revealed that out of the 160 nasal secretion samples of the patients, RSV was detected in 42 (26.3%) samples, influenza A was detected in 23 (14.4%) samples, influenza B was detected in 15 (9.4%) samples and adenovirus was detected in 4 (2.5%) samples by DIF. Landry and Ferguson [15] reported that out of the 373 respiratory samples RSV was detected in 62 (16.6%) samples, influenza A was detected in 232 (62%) samples, influenza B was detected in 38 (10.2%) samples parainfluenza virus types 1 to 3 in 22 (5.9%) and adenovirus was detected in 18(4.8%) samples by DIF. Khandaker *et al.*, [16] reported in their study to nasopharyngeal aspirates and/or nose/throat swabs collected from 294 children in Children's Hospital at Westmead, Sydney, Australia, 51% had laboratory-confirmed influenza and 49% had other respiratory viral infections such as parainfluenza viruses (34%) and adenoviruses (15%) using Simulfluor respiratory screen reagent.

In our study, RSV was detected positive in 10 (43.4%) children with HCoV-NL63 infection, influenza A was detected positive in 4 (17.4%) patients with HCoV-NL63 infection, 4 (17.4%) patients with RSV infection and 3 (13%) patients with influenza B infection. While RSV was detected positive in 2 (8.7%) adenovirus infection. Kuypers *et al.*, [17] reported that HCoV-NL63 was detected in 11 (16.7%) out of the 66 coronaviruses positive nasal wash specimens obtained from children by real-time reverse-transcription polymerase chain reaction. When coronaviruses positive specimens tested for other respiratory viruses by RT-PCR, 30 (45.5%) of the 66 coronavirus-positive specimens also had another respiratory virus detected. RSV was the most common additional respiratory virus detected, accounting for 20 (66.7%) of the 30 coinfections. Other copathogens included 2 (6.7%) parainfluenza viruses, 2 (6.7%) influenza type A viruses, 3 (10%) metapneumoviruses, and 3 (10%) adenoviruses. Jin *et al.*, [18] reported in a study aimed to evaluate the overall prevalence of 10 respiratory viruses in 813 children with acute lower respiratory tract infections in China from 2006 to 2009 by RT-PCR, 73.47% of the HCoV-HKU1 and HCoV-NL63-positive samples tested positive for at least one other virus, most commonly human rhinovirus and RSV. Gharabagh *et al.*, [19] reported in their study to 750 nasopharyngeal swabs obtained from children from (birth to 17 years) and tested for 17 viral agents, 502 (66.9%) were positive for at least one virus by DIF. A single virus was detected in 420 specimens (83.7%), two viruses in 77 (15.3%), three in four specimens (0.8%) and

four viruses in one (0.2%). Among dual infections ($n = 77$) entero/rhinoviruses were most commonly associated with other viruses. Triple virus-infected specimens included adenovirus + enterovirus / rhinovirus + bocavirus ($n = 1$), parainfluenza 4 + adenovirus + enterovirus / rhinovirus ($n = 1$), RSVB + NL63 + bocavirus ($n = 1$) and RSVB + parainfluenza 4 + enterovirus / rhinovirus ($n = 1$). The unique quadruple-agent infected specimen was parainfluenza 3 + NL63 + enterovirus / rhinovirus + bocavirus ($n = 1$).

Conclusion:

HCoV-NL63 is frequently detected human pathogen, often associated with other respiratory viruses and acute respiratory tract infections in children. Rapid DIF screening reagent for detection of multiple respiratory viruses within 1 to 2 hours is of great benefit in terms of patient management and infection control.

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References:

1. 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 Jun 15; 40(12):1721-9.
2. Bastien N, Anderson K, Hart L, Van Caesele P, Brandt K, Milley D, Hachette T, Weiss EC, Li Y. Human coronavirus NL63 infection in Canada. *J Infect Dis.* 2005 Feb 15; 191(4):503-6.
3. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B. Identification of a new human coronavirus. *Nat Med.* 2004 Apr; 10(4):368-73.
4. Falsey AR, Walsh EE, Hayden FG. Rhinovirus and coronavirus infection-associated hospitalizations among older adults. *J Infect Dis.* 2002; 185:1338-41.
5. Pene F, Merlat A, Vabret A, Rozenberg F, Buzyn A, Dreyfus F, *et al.* Coronavirus 229E-related pneumonia in immunocompromised patients. *Clin Infect Dis.* 2003; 37:929-32.
6. Kuiken T, Fouchier RA, Schutten M, Rimmelzwaan GF, van Amerongen G, van Riel

- D, *et al.* Newly discovered coronavirus as the primary cause of severe acute respiratory syndrome. *Lancet*. 2003; 362:263-70.
7. 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; 191:503-6.
 8. Esper F, Weibel C, Ferguson D, Landry ML, Kahn JS. Evidence of a novel human coronavirus that is associated with respiratory tract disease in infants and young children. *J Infect Dis*. 2005; 191:492-8.
 9. Moës E, Vijgen L, Keyaerts E, Zlateva K, Li S, Maes P, *et al.* A novel pancoronavirus RT-PCR assay: frequent detection of human coronavirus NL63 in children hospitalized with respiratory tract infections in Belgium. *BMC Infect Dis*. 2005; 5:6.
 10. To KK, Hung IF, Chan JF, Yuen KY. From SARS coronavirus to novel animal and human coronaviruses. *J Thorac Dis*. 2013 Aug; 5(Suppl 2):S103-8.
 11. Maggi F, Pifferi M, Vatteroni M, Fornai C, Tempestini E, Anzilotti S, Lanini L, Andreoli E, Ragazzo V, Pistello M, Specter S, Bendinelli M. Human metapneumovirus associated with respiratory tract infections in a 3-year study of nasal swabs from infants in Italy. *J Clin Microbiol*. 2003 Jul; 41(7):2987-91.
 12. Koetz A, Nilsson P, Lindén M, van der Hoek L, Ripa T. Detection of human coronavirus NL63, human metapneumovirus and respiratory syncytial virus in children with respiratory tract infections in south-west Sweden. *Clin Microbiol Infect*. 2006 Nov; 12(11):1089-96.
 13. 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 Jun 15; 40(12):1721-9.
 14. Xin C, Yong ZZ, Yan L, Dong ZX. Human coronavirus NL63 in hospitalized children with respiratory infection: a 2-year study from Chongqing, China. *Indian Pediatr*. 2012 Oct; 49(10):825-8.
 15. Landry ML and Ferguson D. SimulFluor Respiratory Screen for Rapid Detection of multiple respiratory viruses in clinical specimens by immunofluorescence staining. *J Clin Microbiol*. 2000; 38(2):708.
 16. Khandaker G, Heron L, Rashid H, Li-Kim-Moy J, Lester-Smith D, Kesson A, McCaskill M, Jones C, Zurynski Y, Elliott EJ, Dwyer DE, Booy R. Comparing the use of, and considering the need for, lumbar puncture in children with influenza or other respiratory virus infections. *Influenza Other Respir Viruses*. 2013 Nov; 7(6):932-7.
 17. Kuypers J, Martin ET, Heugel J, Wright N, Morrow R, Englund JA. Clinical disease in children associated with newly described coronavirus subtypes. *Pediatrics*. 2007 Jan; 119(1):e70-6.
 18. Jin Y, Zhang RF, Xie ZP, Yan KL, Gao HC, Song JR, Yuan XH, Cheng WX, Hou YD, Duan ZJ. Newly identified respiratory viruses associated with acute lower respiratory tract infections in children in Lanzou, China, from 2006 to 2009. *Clin Microbiol Infect*. 2012 Jan; 18(1):74-80.
 19. Gharabaghi F, Hawan A, Drews SJ, Richardson SE. Evaluation of multiple commercial molecular and conventional diagnostic assays for the detection of respiratory viruses in children. *Clin Microbiol Infect*. 2011 Dec; 17(12):1900-6.

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