

Understanding Human Coronavirus HCoV-NL63

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Abstract: Even though coronavirus infection of humans is not normally associated with severe diseases, the identification of the coronavirus responsible for the outbreak of severe acute respiratory syndrome showed that highly pathogenic coronaviruses can enter the human population. Shortly thereafter, in Holland in 2004, another novel human coronavirus (HCoV-NL63) was isolated from a seven-month old infant suffering from respiratory symptoms. This virus has subsequently been identified in various countries, indicating a worldwide distribution. HCoV-NL63 has been shown to infect mainly children and the immunocompromised, who presented with either mild upper respiratory symptoms (cough, fever and rhinorrhoea) or more serious lower respiratory tract involvement such as bronchiolitis and croup, which was observed mainly in younger children. In fact, HCoV-NL63 is the aetiological agent for up to 10% of all respiratory diseases. This review summarizes recent findings of human coronavirus HCoV-NL63 infections, including isolation and identification, phylogeny and taxonomy, genome structure and transcriptional regulation, transmission and pathogenesis, and detection and diagnosis.

Keywords: Human coronavirus HCoV-NL63, clinical features, pathogenesis, diagnosis.

1. INTRODUCTION

Regardless of geographic location, respiratory tract infections rank among the top three killers of children under five years of age [1]. A significant proportion of these respiratory tract infections have no known cause. Recently, however, a number of novel coronaviruses have been identified as the causative agents for some of these infections [2, 3].

Coronaviruses (CoVs) belong to the family *Coronaviridae* in the order *nidovirales*. Members of the Coronavirus family are positive-strand RNA viruses with large genomes ranging in size from 27–33 kb. The coronavirus genome encodes for a 5' replicase polyprotein (ORF1a and ORF1b) that, in turn, encodes for all the enzymes required for viral RNA replication. The genome also encodes for the 3' structural proteins, including spike (S), envelope (E), membrane (M) and nucleocapsid (N), which are common to all coronaviruses. The structural proteins are involved in various viral processes, including virus particle formation [4]. Additional subgroup-specific accessory genes are found interspersed among the structural genes, which vary in number and location. Recent studies have shown that the proteins encoded by these genes could be modulators of pathogenicity in the natural host [5-7].

Five human coronaviruses have been identified to date, four of which are known to continuously circulate in the human population, especially in young children [8, 9]. HCoV-OC43 and HCoV-229E, first identified in the mid-1960s [10, 11], were shown to cause the common cold [12], but rarely infections of the lower respiratory tract [3]. A third human coronavirus, which causes severe acute respiratory syndrome, SARS-CoV, was identified in 2003 [13, 14]. This virus had a worldwide spread, causing acute respiratory illness with a mortality rate of ~10% [15]. The last reported SARS-CoV infections were laboratory acquired in 2004, and the virus has not been detected in the human population since [16, 17]. More recently, two additional human coronaviruses were identified; HCoV-HKU1 was isolated from a 71-year-old man who presented with fever and cough [3], and HCoV-NL63 isolated from a seven-month-old baby [2]. The latter is the topic of this review.

Several groups have studied different aspects of HCoV-NL63 infections, including its worldwide distribution, its association with human disease, and the replication characteristics of the causative virus. In this review we summarize recent findings of human coronavirus HCoV-NL63 infections, including virus isolation and identification, phylogeny and taxonomy, genome structure and transcriptional regulation, transmission and pathogenesis, and detection and diagnosis.

2. ISOLATION AND IDENTIFICATION OF HCoV-NL63

HCoV-NL63 was first isolated in Amsterdam in 2004 from the nasopharyngeal aspirate of a seven-month old child; the patient presented with symptoms suggesting respiratory

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tract infection (coryza, conjunctivitis, and fever), while his chest X-ray showed typical features of bronchiolitis. The aspirate tested negative for all known respiratory viruses. A group of Dutch scientists found that the virus initiated a cytopathic effect when inoculated onto tertiary monkey kidney cells. The group used a new technique, VIDISCA, to clone and amplify the viral genome. VIDISCA is a novel approach that provides a fast and effective tool for amplification of unknown genomes based on cDNA-amplified fragment length polymorphism [2, 18]. The virus was identified as a member of the *Coronaviridae* family. It was shown to be a novel member of Group I coronaviruses because of the similarity of its genome sequence to HCoV-229E. Another group in the Netherlands [19] reported the independent isolation and identification of, essentially, the same virus at about the same time.

3. PHYLOGENY AND TAXONOMY OF HCoV-NL63

Based on antigenicity, genome organization and sequence homology, coronaviruses are divided into three distinct groups [20]. Group 1 contains transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus (PEDV), feline infectious peritonitis virus (FIPV), canine coronavirus and HCoV-229E, among others. Group 2 contains mouse hepatitis virus (MHV), bovine coronavirus, haemagglutinating encephalomyelitis virus, HCoV-HKU1 and HCoV-OC43, to name a few, with Bat SARS-CoV and SARS-CoV considered distantly related Group 2b coronaviruses. Group 3 contains the avian coronaviruses [21-23]. Based on phylogenetic analysis, HCoV-NL63 belongs to the Group I coronaviruses [2, 24]. Interestingly, evidence

of recombination during the evolution of HCoV-NL63 has been reported, and viral isolates have, in fact, a mosaic genome structure. The authors speculate that HCoV-NL63 diverged from a HCoV-229E ancestor in the past, followed by a separation into two distinct HCoV-NL63 lineages. These two lineages recombined during co-infection, giving rise to the two currently observed genotypic subgroups [24-27]. In fact, recombination between different HCoV-NL63 isolates has been suggested, resulting in a mixture of clinical virus variants circulating in the human population [8, 27-30].

3.1. Genome Structure and Transcriptional Regulation

HCoV-NL63 has a single-stranded RNA genome that is capped and polyadenylated [27]. The genome is 27553 bases in size, with the genome order 5'-ORF1a-ORF1b-S-ORF3-E-M-N-polyT-3' (Fig. 1). Seven distinct ORFs are produced from six distinct mRNAs, which include the full-length genomic RNA and a nested set of five subgenomic (sg) mRNAs [4]. Coronavirus mRNAs are generated in the membrane-associated replication centers [31]. The five sg mRNAs encode for the viral structural and accessory proteins S, ORF3, E, M and N. With the exception of ORF E, a common transcription regulatory sequence (TRS), with core sequence AACUAAA, is located upstream of all the ORFs; this TRS is crucial for sg mRNA formation [27, 32]. HCoV-NL63 uses a discontinuous replication strategy to generate sg mRNAs during the minus strand synthesis [4, 27, 32], which are then copied into plus strand mRNAs. All plus strand mRNAs share a common ~70 nucleotide leader sequence at their 5' ends that is identical to the sequence at the 5' end of the genomic RNA [27].

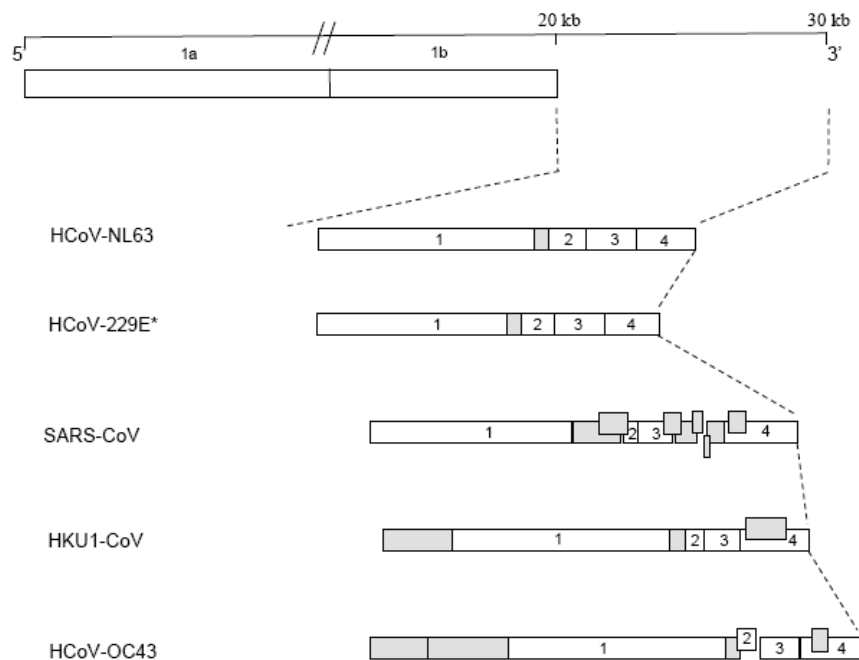


Fig. (1). Schematic comparison of the genome organization of coronaviruses infecting humans. Genomic maps shown are based on the complete genome sequences (NCBI accession numbers are shown in brackets): HCoV-NL63: Human coronavirus HCoV-NL63 (NC_005831); HCoV-229E: Human coronavirus 229E (NC_002645); SARS-CoV: Severe acute respiratory syndrome coronavirus (NC_004718); HKU1-CoV: Human coronavirus HKU1 (NC_006577); HCoV-OC43: Human coronavirus OC43 (NC_005147). ORFs S (1), E (2), M (3) and N (4) are shown and open reading frames encoding for accessory genes are shaded in grey. *ORF4 of HCoV-229E is shown as a single open reading frame [34].

3.1.1. Protein 1a/1b

HCoV-NL63 ORF1a/1b contains a putative elaborated pseudoknot structure that triggers a -1 ribosomal frameshift to translate the complete 1ab polyprotein; for a mini-review of the putative ORF1a/1b products and functions see Pryc *et al.* (2007) [27] and Van der Hoek *et al.* (2006) [8]. Chen and colleagues identified processed products nsp3 and nsp4 of the HCoV-NL63 replicase polyprotein, which could be detected at 24 hours post-infection. These products localize in the peri-nuclear sites of virus infected cells. Also, the group identified and characterized two viral papain-like proteases, PLP1 and PLP2, which process the viral replicase polyprotein. Interestingly, the PLP2 protease has deubiquitinating activity [9], although the function of this is not clear in viral replication.

3.1.2. Spike Protein

The species tropism and virulence of a particular coronavirus are largely determined by the spike (S) glycoprotein. Coronavirus S proteins mediate attachment to the cellular receptors and subsequent fusion of the virus and cell membrane [20]. The N-terminal portion of HCoV-NL63 S contains a unique 179 amino acid domain not present in other coronaviruses. This region represents the most variable region of the HCoV-NL63 genome and a role in immune evasion for this region has been proposed [8, 27]. HCoV-NL63 S is a single-chain glycoprotein and consists of an N-terminal receptor-binding domain (S1) and a C-terminal transmembrane fusion domain (S2). S2 consists of two highly conserved heptad-repeat (HR) sequences that are larger than the corresponding regions for the group II and group III coronaviruses [20, 27, 33]. Initial proteolytic studies of the S2 fusion core identified an α -helical domain consisting of a trimer of the HR segments N57 and C42. The resolved crystal structure of this trimeric complex shows distinctive high-affinity conformations of interacting cross-sectional layers of six helices. It has been suggested that the larger HR regions of the group I coronaviruses may be required to prime the S proteins for their fusion-activating conformational changes during entry of the virus [20].

3.1.3. Accessory Protein ORF3

Coronavirus genomes contain accessory genes found interspersed among the structural genes, which vary in number and location between the different coronavirus groups (Fig. 1). Both HCoV-NL63 and HCoV-229E are group I coronaviruses and encode for only one accessory gene product between the S and E genes [34]. Accessory genes are poorly characterized and the functions of the gene products are not well understood. Initial research into the functions of these genes has shown that they are non-essential and dispensable for virus growth in cell culture [5, 6, 35]. More recent studies have shown that the accessory genes are required for *in vivo* infection and pathogenicity in the natural host [6, 7, 36-38].

Unlike the SARS-CoV genome, the HCoV-NL63 genome encodes for only one accessory protein, ORF3 (Fig. 1). ORF3 is expressed from distinct subgenomic (sg) mRNA 3, which is one of at least six distinct mRNAs [4]. The ORF3 gene encodes for a putative 225 amino acid protein, about 25.6 kDa in size. Pryc *et al.* (2004) reports that the HCoV-

NL63 ORF3 gene has a unique nucleotide composition and appears as a U-rich and A-poor region within the genome, indicating a recent gene transfer event from another viral or cellular origin [4]. The ORF3 protein of HCoV-NL63 is homologous to proteins of the other Group I coronaviruses, with an amino acid sequence most similar (43% identity and 62% similarity) to HCoV-229E ORF4. Based on comparative *in silico* analysis of HCoV-NL63 ORF3 and other human coronavirus ORF3-like homologues, Fielding and Suliman speculate that the protein could contribute to pathogenesis in the natural host [39]. More recently, studies have shown that HCoV-NL63 localizes along the secretory pathway (ERGIC, Golgi, plasma membrane) and co-localizes with the structural proteins M and E in the ERGIC. Also, studies have shown that this N-glycosylated protein is incorporated into virions during assembly. This further suggests an important function for HCoV-NL63 ORF3, particularly in virus assembly and/or budding from the infected cell [40].

4. TRANSMISSION AND PATHOGENESIS

4.1. Mode of Entry into the Cell

It is well documented that SARS-CoV and HCoV-NL63 use the same receptor, angiotensin converting enzyme (ACE)-2, for entry into the host cell [41, 42]. However, the consequences following the entry are very different; SARS-CoV causes severe respiratory distress, while HCoV-NL63 might lead to a mild respiratory infection. This was attributed to the ability of each virus receptor-binding protein, spike or S protein, to bind to ACE-2 on the cell surface. It was found that the HCoV-NL63 S protein has a weaker interaction with ACE-2 than the SARS-CoV S protein. This lower-affinity interaction with ACE-2 might partly explain the different pathological consequences of infection by SARS-CoV and HCoV-NL63 [43].

Nevertheless, high pathogenicity is expected to evolve with coronaviruses. As for HCoV-NL63, the possibility of the development of a recombinant virus variant is high because of its high prevalence and the possibility of a recombination event through co-infection [24]. Moreover, the virus is able to survive for up to seven days in an aqueous solution and respiratory secretions and remains infective at room temperature. In heavily populated regions, direct person-to-person transmission is considered the major route of HCoV-NL63 spread [42, 44].

4.2. Seasonal Incidence

The virus has been shown to have a worldwide distribution and was observed primarily in the winter season in temperate climates. On the other hand, countries with extreme weather, like Canada, have also shown virus activity around January to March, although milder symptoms were reported [45]. Interestingly, seasonal variations have been reported in China where infection with HCoV-NL63 appeared mainly in spring and summer [26]. Also, a recent study of coronaviruses in Thailand did not show any seasonal predilection [46], while Wu *et al.* (2007) reported that the virus is detected during the autumn season in Taiwan [47]. It is evident that the virus has no predilection to a particular season and is not affected by temperature variations as infections can occur throughout the year (Table 1).

4.3. Prevalence

The human coronaviruses account for a significant number of hospitalization for children under 18 years of age, the elderly and immunocompromised individuals. In fact, a one-year study of children hospitalized in Hong Kong (China) has shown that respiratory tract infection with coronaviruses accounts for 4.4% of all admissions for acute respiratory infections. Of these, HCoV-NL63 was the most common coronavirus identified with an incidence of 2.6% [48]. Moreover, a study in Japan has shown that out of 419 specimens that tested negative for common respiratory viruses, five (1.2%) were positive for human coronavirus HCoV-NL63 [49]. Another Japanese report has indicated that out of 118 nasopharyngeal swab samples obtained from hospitalized children younger than two years of age, three (2.5%) were positive for HCoV-NL63 [50].

In Europe, high prevalence was also noted. In Italy, a study conducted on 322 infants suffering from acute respiratory disease has shown that 8.7% of the cases examined were caused by coronaviruses, with HCoV-NL63 accounting for 21.4% of the latter [51]. In France, 300 respiratory specimens were checked for HCoV-NL63 presence; of the 300 samples, 28 (9.3%) were positive [29]. Likewise, seven cases were reported in a one-year study in Belgium [28]. Interestingly, the Dutch group that first described the virus has obtained 949 samples from a German group who conducted a population-based study on lower respiratory tract infection in children under three years of age [52]; the group re-analyzed the samples and detected a 5.2% incidence of HCoV-NL63 [53]. In Australia, the virus was detected in Melbourne, where a study conducted on 543 patients with respiratory symptoms has shown 18 cases (3.3%) of human coronavirus HCoV-NL63 infection [54]. Moreover, in the USA and Canada, the virus was reported in 79 (8.8%) out of 895 and 19 (3.6%) out of 525 patients, respectively.

Currently, the accuracy of the percentage of detection is hampered by two main problems: firstly, the suitability of the samples examined: a recent study has shown that there are differences between respiratory samples collected by nose/throat swabs and by nasopharyngeal aspirates, specifically regarding their potential to detect and identify respiratory pathogens [55]. The second problem is that diagnostic tests for HCoVs are not frequently used in the routine testing for viruses, which probably results in the percentage of HCoVs infections being greatly underestimated [56]. Moreover, throughout the years several methods of variable sensitivity were used to determine the incidence of the virus [57-59].

4.4. Co-Infection

Many groups have reported that the occurrence of co-infections with HCoV-NL63 and other respiratory viruses, including other human coronaviruses, influenza A virus, respiratory syncytial virus (RSV), parainfluenza virus and human metapneumovirus (hMPV), are common [26, 30, 46, 47, 51, 53, 54, 60, 61]. Also, co-infected patients are more likely to be hospitalized, indicating the severity of this kind

of superinfection. In a study from Germany, RSV-A and HCoV-NL63 was the most common co-infection identified in children less than three years of age. This is probably due to the high incidence of RSV-A in winter and the overlap in seasonality of the viruses [53]. Also, in Italy, HCoV-NL63 circulates as a mixture of variant strains and is often associated with other viral infections [30]. In South Africa, co-infection of patients with HCoV-NL63 and bocavirus in hospitalized children is reported. Nasopharyngeal and bronchoalveolar lavage samples from 341 patients were screened for common respiratory viruses, and the co-presence of HCoV-NL63 and bocavirus in at least one sample was reported [62].

Interestingly, the viral load of HCoV-NL63 is lower in co-infected patients than in patients infected with HCoV-NL63 only. There are various possible explanations for this phenomenon [53]:

1. HCoV-NL63 might be the initial infection that weakens the immune system enough for a second infection to gain a foothold. By the time this second infection shows symptoms, the HCoV-NL63 infection might have already have been brought under control by the host immune system,
2. the two viruses may be in competition for the same receptor or target cell in the respiratory organs,
3. the elevated activation of the innate immune response triggered by the second respiratory virus may cause inhibition of HCoV-NL63 or,
4. prolonged persistence of HCoV-NL63 at low levels.

The high prevalence of co-infections of HCoV-NL63 and other respiratory viruses increases the chances of genetic recombination with these human or zoonotically transmitted viruses. In fact, Pryc *et al.* (2006) states HCoV-NL63 resulted from a recombination event between PEDV and an ancestral HCoV-NL63 strain. Theoretically, these types of recombination events could enable highly pathogenic virus variants to arise [24].

4.5. Clinical Features

Recent scientific and clinical evidence has indicated that the virus is found during upper and lower respiratory tract infections, causing symptoms and signs that do not differ greatly from the symptoms described for the 'old' viruses HCoV-229E and HCoV-OC43. Other systems involvement is still controversial [63].

Table 1 shows that patients diagnosed with the virus have presented with mild symptoms, indicating upper respiratory tract infection such as fever, cough and rhinorrhoea. On the other hand, the disease is also known to cause significant more alarming lower respiratory tract infection. One of the most alarming symptoms is bronchiolitis, an inflammation of the membranes lining the bronchioles. This symptom was reported by several research groups [25, 50, 64], and although a population-based study in China did not report an association of HCoV-NL63 with bronchiolitis [26], it is still believed to be one of the presenting symptoms. Several research groups have linked HCoV-NL63 to croup [26, 53].

Table 1. Epidemiological, Demographic, Clinical and Virological Characteristics of HCoV-HCOV-NL63 Infections

Country	Specimen Type	Method of Detection	Seasonal Prevalence	Type of Study	Number of Patients Involved in the Study (Sample Size)	Age	Incidence of HCoV-NL63	Co-Infection	Symptoms	Refs.
Australia	Throat and nose swab	RT-PCR	Winter	Community based	234	> 5 years	3.3%	ND	Mild No croup, No bronchiolitis	[54]
Belgium	NR	RT-PCR	Winter (January-February)	Hospitalized patients	NR	Variable (6 cases under 2 years)	2.3%	Absent	Fever, cough, wheezing, respiratory distress, diarrhoea	[28, 88]
Canada	Throat swab, nasopharyngeal swab, lung autopsy	RT-PCR	Winter (January-March)	patients with ARI	525	1 month-100 years	3.6%(13 males and 6 females)	Absent	Fever, rhinitis cough, sore throat, bronchiolitis	[45]
France	NR	RT-PCR	Winter (February)	Hospitalized patients for ARI	300	Variable (18 cases under 2 years)	9.3%	Absent	Fever, rhinitis, bronchiolitis, digestive problems, otitis, pharyngitis and conjunctivitis	[29]
Germany	Nasopharyngeal secretions	Real-time RT-PCR	Winter	Population-based (Inpatients+outpatients)	949	< 3 years	5.2%	Present	Croup Bronchitis Bronchiolitis	[52, 53]
China, Hong Kong	Nasopharyngeal aspirate	RT-PCR	Spring and summer	Hospitalized patients for ARI	587	6-57 months	2.6%	Present	Croup Fever Rash Sore throat Cough rhinitis shortness of breath Hoarseness Asthma exacerbations Seizures	[26]
Italy	Nasopharyngeal aspirate	RT-PCR	Winter (December-February)	Hospitalized infants for ARI	150	<2 years	1.86%	Present	Bronchiolitis Pneumonia Bronchospasm Wheezing rhinitis Bronchiolitis laryngitis	[51]
Japan	NR	RT-PCR	January-March	NR	419	NR	5 cases	ND	ND	[49]
Korea	Nasopharyngeal aspirate	RT-PCR	April-May	Children with lower respiratory tract infection	515		1.6%	25%	Fever, rales wheezing Pneumonia Croup Asthma exacerbation	[71]
Taiwan	Nasopharyngeal aspirate	Quantitative RT-PCR	Peak in October	Hospitalized patients for ARI	539	< 3 years	1.3%	Present	Fever, cough, croup, and stridor	[47]
Thailand	Nasopharyngeal aspirate	RT-PCR	Autumn	Hospitalized patients with pneumonia+out patients with flu like illnesses	1890	1 month-65 years	7/734 (first year) 1/1156 (second year)	Present	Cough, fever, stridor, croup	[46]

(Table 1) contd.....

Country	Specimen Type	Method of Detection	Seasonal Prevalence	Type of Study	Number of Patients Involved in the Study (Sample Size)	Age	Incidence of HCoV-NL63	Co-Infection	Symptoms	Refs.
South Africa	Nasal swab	RT-PCR	NR	ND	238	Median age= 12.4 months	8.3%	Present	Wheezing	[89]
Switzerland	Nasopharyngeal swab	RT-PCR	Cold months	outpatients	82	Neonates (less than 1 year)	7%	ND	Cough, wheeze, fever, rhinitis, otitis, tonsillitis	[60]
Sweden	Nasopharyngeal aspirate	RT-PCR	NR	Oupatients (Children attending to pediatric department)	212	ND	6%	ND	ND	[90]
USA	Respiratory specimens	RT-PCR	Mostly January-February	Both ambulatory and hospitalized patients for ARI	895	< 5 years	8.8%	Present	Cough, rhinorrhoea, tachpnea, fever, abnormal breath sounds, hypoxia	[91]

NR: Not Reported.
 ND: Not Done.

Croup children present with pharngitis, sore throat and hoarseness of voice, and are considered for hospitalization. Of the rare findings, a group has reported the association between HCoV-NL63 and Kawasaki disease, a form of childhood vasculitis that is presented as fever, polymorphic exanthema, oropharyngeal erythema and bilateral conjunctivitis [65]. However, others fail to report on this association [66, 67].

It is noteworthy to say that the report of symptoms in young children, who represent the majority of patients, is based mainly on parental observations, where other possible subjective signs and symptoms fail to be recognized by the parents. Moreover, most of the studies were conducted on patients reporting to hospitals suffering from acute respiratory tract infection. To date, there are only few population-based studies and the question arises whether larger numbers of such studies might reveal the involvement of other body systems.

5. LABORATORY DIAGNOSIS AND DETECTION

It is difficult to distinguish clinical symptoms caused by HCoV-NL63 from those caused by many of the other human viruses, which could make diagnosis and detection of the virus complex. Treatment of patients without a clear diagnosis could result in implementation of expensive and disruptive public health measures, as well as to an increased spread of the disease [68]. Therefore, improved surveillance and diagnosis of respiratory illness could reduce health-care costs drastically [68-70].

5.1. Detection of Viral RNA

Due to its high specificity and sensitivity, reverse transcription polymerase chain reaction (RT-PCR) and other nucleic acid tests are the preferred methods for diagnosis of coronavirus infections, such as SARS-CoV [15]. The

majority of nucleic acid amplification tests is designed with the ORF1a/1b, which is genetically stable in coronaviruses, and the nucleocapsid (N) or spike (S) genes (Table 1) [25, 26, 28, 30, 45, 46, 64, 67, 71-73]. Based on *in vitro* coronavirus expression studies, the N gene has the theoretical advantage of being more abundant in infected cells and therefore of higher sensitivity for PCR assays, but this has not been clearly proven in clinical studies [74]. RT-PCR of nasopharyngeal samples, both frozen and fresh, is the most popular choice for detection of HCoV-NL63 (Table 1) and viral culture is frequently used for confirmation of infection [67, 73].

5.2. Antigen and Antibody Detection Assays

Serum samples have been screened for antibodies against HCoV-NL63 using enzyme-linked immunosorbent assays (ELISA) [75, 76]. An ELISA assay based on the N protein showed high seropositive results for serum samples from children younger than 20 years old [76]. Interestingly, the presence of maternally-acquired N-directed antibodies was detected in serum by ELISA [75, 76], usually decreasing within the first 4-5 months of life [76]. Potent neutralizing activity directed against HCoV-NL63 S protein was detected in virtually all serum samples from patients eight years of age or older, suggesting that HCoV-NL63 infection of humans is common and usually acquired during childhood [42]. Alarmingly, SARS-CoV infections appear to stimulate cross-reactive antibody responses to other human coronaviruses, including HCoV-NL63 [77]. Such cross reaction between human coronaviruses has been reported previously for immunoflouescence and complement fixation tests [78, 79]. In fact, false positive results were also reported for SARS-CoV detection using ELISA tests based on the recombinant N antigen [80]. This cross-reactivity is most likely due to the presence of cross-reactive antigenic epitopes of the coronaviruses [77]. An awareness of this

cross-reactivity is important when developing antigen- and/or antibody-diagnostic assays and selecting the most suitable antigen or antigen target is important.

6. CONCLUSION

The discovery of the HCoV-NL63 and other novel human coronaviruses does not necessarily represent a sudden increase in emerging infections by 'new' coronaviruses. In fact, in the early 1960's, viral agents causing upper respiratory tract infections were isolated from patients and were subsequently shown to be coronaviruses by electron microscopy. At least four of these were shown to be serologically distinct from the known coronaviruses at the time [10, 11, 81]. However, with the clinical samples of these no longer available for study, it will never be known whether these 'old' viruses and the 'new' viruses represent the same strains [82].

Molecular clock analysis is the average rate at which a species' genome accumulates mutations and is used to measure that species' evolutionary divergence. The reliability of molecular dating is dependent on the validity of the molecular clock hypothesis, which assumes that the substitution rate is roughly constant [83], with an average substitution rate for coronaviruses estimated to be 10^{-4} substitutions per year per site [84, 85]. With the lack of sufficient sequence data available for HCoV-NL63, the substitution rate for HCoV-229E, using partial sequences of the S gene from different known dates, was calculated. Then, assuming a constant evolutionary rate in time and a constant evolutionary rate between the branches for HCoV-NL63 and HCoV229E, the time to their most recent common ancestor was dated to the 11th century [24]. This shows that HCoV-NL63 has been present in the human population for centuries. In support of this finding, the virus described by Fouchier *et al.* was originally isolated from an eight-month old child in 1988 [19]. More recent studies in the Netherlands also supported the notion that only SARS-CoV has recently been introduced to the human population from an animal source, while HCoV-NL63 has been circulating in humans for a while [86]. With the exception of SARS-CoV, the coronaviruses infecting humans are not well studied. This is partly due to the prevailing view that they are only involved in mild respiratory tract infections. The introduction of sensitive molecular and cell biology techniques have aided in identifying three 'new' human coronaviruses, which has improved our understanding of the classification of the coronaviruses. These modern tools now need to be developed further to make the sensitive and accurate detection of coronaviruses in clinical samples possible. This would increase our understanding of the extent to which coronaviruses affect human health. Importantly, Donaldson and colleagues has recently reported the assembly of a full-length infectious HCoV-NL63 clone, which will make the study of this virus easier, improving our understanding of the role of each of the proteins encoded by the HCoV-NL63 genome [87]. In the long run, molecular and cell biology tools could help elucidate the link, if any, between human coronaviruses and human diseases of the respiratory tract, the vascular system, the central nervous system and the gastrointestinal tract [82].

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ABBREVIATIONS

HCoV	= Human coronavirus
HCoV-NL63	= Human coronavirus HCoV-NL63
CoVs	= Coronaviruses
Kbs	= Kilobases
S	= Spike protein
E	= Envelope protein
M	= Membrane protein
N	= Nucleocapsid protein
SARS-CoV	= Severe acute respiratory syndrome coronavirus
ORFs	= Open reading frames
VIDISCA	= Virus discovery cDNA-ALFP
TGEV	= Transmissible gastroenteritis virus
PDEV	= Porcine diarrhea virus
FIPV	= Feline infectious peritonitis virus
MHV	= Mouse hepatitis virus
sg	= Subgenomic
HR	= Heptad repeat
kDa	= KiloDaltons
ACE2	= Angiotensin converting enzyme 2
RSV	= Respiratory syncytial virus
hMPV	= Human metapneumovirus
RT-PCR	= Reverse transcription polymerase chain reaction
ELISAs	= Enzyme-linked immunosorbent assays

REFERENCES

- [1] Murray CJL, Lopez AD, Mathers CD, Stein C. The global burden of disease 2000 project: aims, methods and data sources. Global programme on evidence for health policy. Geneva: World Health Organization. 2001.
- [2] van der Hoek L, Pyrc K, Jebbink MF, *et al.* Identification of a new human coronavirus. *Nat Med* 2004; 10: 368-73.
- [3] Woo PC, Lau SK, Chu CM, *et al.* Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. *J Virol* 2005; 79: 884-95.
- [4] Pyrc K, Jebbink MF, Berkhout B, van der Hoek L. Genome structure and transcriptional regulation of human coronavirus NL63. *Virology* 2004; 1: 7.
- [5] Casais R, Davies M, Cavanagh D, Britton P. Gene 5 of the avian coronavirus infectious bronchitis virus is not essential for replication. *J Virol* 2005; 79: 8065-78.
- [6] de Haan CA, Masters PS, Shen X, Weiss S, Rottier PJ. The group-specific murine coronavirus genes are not essential, but their deletion, by reverse genetics, is attenuating in the natural host. *Virology* 2002; 296: 177-89.

- [7] Haijema BJ, Volders H, Rottier PJ. Live, attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. *J Virol* 2004; 78: 3863-71.
- [8] van der Hoek L, Pyrc K, Berkhout B. Human coronavirus NL63, a new respiratory virus. *FEMS Microbiol Rev* 2006; 30: 760-73.
- [9] Chen Z, Wang Y, Ratia K, Mesecar AD, Wilkinson KD, Bake SC. Proteolytic processing and deubiquitinating activity of papain-like proteases of human coronavirus NL63. *J Virol* 2007; 81: 6007-60.
- [10] Tyrrell DA, Bynoe ML. Cultivation of a novel type of common-cold virus in organ cultures. *Br Med J* 1965; 1: 1467-70.
- [11] Hamre D, Procknow JJ. A new virus isolated from the human respiratory tract. *Proc Soc Exp Biol Med* 1966; 121: 190-3.
- [12] Bradburne AF, Bynoe ML, Tyrrell DA. Effects of a "new" human respiratory virus in volunteers. *Br Med J* 1967; 3: 767-9.
- [13] Drosten C, Gunther S, Preiser W, *et al.* Identification of a novel coronavirus in patients with severe acute respiratory syndrome. *N Engl J Med* 2003; 348: 1967-76.
- [14] Rota PA, Oberste MS, Monroe SS, *et al.* Characterization of a novel coronavirus associated with severe acute respiratory syndrome. *Science* 2003; 300: 1394-9.
- [15] Fielding BC, Tan Y-J. The Singapore contribution in the battle against SARS. *Issues Infect Dis* 2007; 4: 1-22.
- [16] Kahn JS. The widening scope of coronaviruses. *Curr Opin Pediatr* 2006; 18: 42-7.
- [17] Yip CW, Hon CC, Shi M, *et al.* Phylogenetic perspectives on the epidemiology and origins of SARS and SARS-like coronaviruses. *Infect Genet Evol* 2009; 9: 1185-96.
- [18] Pyrc K, Jebbink MF, Berkhout B, van der Hoek L. Detection of new viruses by VIDISCA. Virus discovery based on cDNA-amplified fragment length polymorphism. *Methods Mol Biol* 2008; 454: 73-89.
- [19] Fouchier RA, Hartwig NG, Bestebroer TM, *et al.* A previously undescribed coronavirus associated with respiratory disease in humans. *Proc Natl Acad Sci USA* 2004; 101: 6212-6.
- [20] Zheng Q, Deng Y, Liu J, van der Hoek L, Berkhout B, Lu M. Core structure of S2 from the human coronavirus NL63 spike glycoprotein. *Biochemistry* 2006; 45: 15205-15.
- [21] Cheng VC, Lau SK, Woo PC, Yuen KY. Severe acute respiratory syndrome coronavirus as an agent of emerging and reemerging infection. *Clin Microbiol Rev* 2007; 20: 660-94.
- [22] Lau SK, Woo PC, Li KS, *et al.* Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci USA* 2005; 102: 14040-5.
- [23] Ren W, Li W, Yu M, *et al.* Full-length genome sequences of two SARS-like coronaviruses in horseshoe bats and genetic variation analysis. *J Gen Virol* 2006; 87: 3355-9.
- [24] Pyrc K, Dijkman R, Deng L, *et al.* Mosaic structure of human coronavirus NL63, one thousand years of evolution. *J Mol Biol* 2006; 364: 964-73.
- [25] 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: 455-62.
- [26] Chiu SS, Chan KH, Chu KW, *et al.* Human coronavirus NL63 infection and other coronavirus infections in children hospitalized with acute respiratory disease in Hong Kong, China. *Clin Infect Dis* 2005; 40: 1721-9.
- [27] Pyrc K, Berkhout B, van der Hoek L. The novel human coronaviruses NL63 and HKU1. *J Virol* 2007; 81: 3051-7.
- [28] Moes E, Vijgen L, Keyaerts E, *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.
- [29] Vabret A, Mourez T, Dina J, *et al.* Human coronavirus NL63, France. *Emerg Infect Dis* 2005; 11: 1225-9.
- [30] Minosse C, Selleri M, Zaniratti MS, *et al.* Phylogenetic analysis of human coronavirus NL63 circulating in Italy. *J Clin Virol* 2008; 43: 114-9.
- [31] Brockway SM, Clay CT, Lu XT, Denison MR. Characterization of the expression, intracellular localization, and replication complex association of the putative mouse hepatitis virus RNA-dependent RNA polymerase. *J Virol* 2003; 77: 10515-27.
- [32] Pyrc K, Berkhout B, van der Hoek L. Antiviral strategies against human coronaviruses. *Infect Disord Drug Targets* 2007; 7: 59-66.
- [33] Bosch BJ, Martina BE, Van Der Zee R, *et al.* Severe acute respiratory syndrome coronavirus (SARS-CoV) infection inhibition using spike protein heptad repeat-derived peptides. *Proc Natl Acad Sci USA* 2004; 101: 8455-60.
- [34] Dijkman R, Jebbink MF, Wilbrink B, *et al.* Human coronavirus 229E encodes a single ORF4 protein between the spike and the envelope genes. *Virol J* 2006; 3: 106.
- [35] Hodgson T, Britton P, Cavanagh D. Neither the RNA nor the proteins of open reading frames 3a and 3b of the coronavirus infectious bronchitis virus are essential for replication. *J Virol* 2006; 80: 296-305.
- [36] Cavanagh D, Casais R, Armesto M, *et al.* Manipulation of the infectious bronchitis coronavirus genome for vaccine development and analysis of the accessory proteins. *Vaccine* 2007; 25: 5558-62.
- [37] Netland J, Ferraro D, Pewe L, Olivares H, Gallagher T, Perlman S. Enhancement of murine coronavirus replication by severe acute respiratory syndrome coronavirus protein 6 requires the N-terminal hydrophobic region but not C-terminal sorting motifs. *J Virol* 2007; 81: 11520-5.
- [38] Pewe L, Zhou H, Netland J, *et al.* A SARS-CoV-specific protein enhances virulence of an attenuated strain of mouse hepatitis virus. *Adv Exp Med Biol* 2006; 581: 493-8.
- [39] Fielding BC, Suliman T. Comparative analysis of human coronavirus-NL63 ORF3 protein homologues. *Afr J Biotechnol* 2009; 8: 3175-8.
- [40] Muller MA, van der Hoek L, Voss D, *et al.* Human coronavirus NL63 open reading frame 3 encodes a virion-incorporated N-glycosylated membrane protein. *Virol J* 2010; 7: 6.
- [41] Kuba K, Imai Y, Rao S, *et al.* A crucial role of angiotensin converting enzyme 2 (ACE2) in SARS coronavirus-induced lung injury. *Nat Med* 2005; 11: 875-9.
- [42] Hofmann H, Pyrc K, van der Hoek L, Geier M, Berkhout B, Pohlmann S. Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. *Proc Natl Acad Sci USA* 2005; 102: 7988-93.
- [43] Mathewson AC, Bishop A, Yao Y. Interaction of severe acute respiratory syndrome-coronavirus and NL63 coronavirus spike proteins with angiotensin converting enzyme-2. *J Gen Virol* 2008; 89: 2741-5.
- [44] Muller A, Tillmann RL, Muller A, Simon A, Schildgen O. Stability of human metapneumovirus and human coronavirus NL63 on medical instruments and in the patient environment. *J Hosp Infect* 2008; 69: 406-8.
- [45] Bastien N, Anderson K, Hart L, *et al.* Human coronavirus NL63 infection in Canada. *J Infect Dis* 2005; 191: 503-6.
- [46] Dare RK, Fry AM, Chittaganpitch M, *et al.* Human coronavirus infections in rural Thailand: a comprehensive study using real-time reverse-transcription polymerase chain reaction assays. *J Infect Dis* 2007; 196: 1321-8.
- [47] Wu PS, Chang LY, Berkhout B, *et al.* Clinical manifestations of human coronavirus NL63 infection in children in Taiwan. *Eur J Pediatr* 2008; 167: 75-80.
- [48] Chan KC, Tang NL, Hui DS, *et al.* Absence of association between angiotensin converting enzyme polymorphism and development of adult respiratory distress syndrome in patients with severe acute respiratory syndrome: a case control study. *BMC Infect Dis* 2005; 5: 26.
- [49] Suzuki A, Okamoto M, Ohmi A, Watanabe O, Miyabayashi S, Nishimura H. Detection of human coronavirus-NL63 in children in Japan. *Pediatr Infect Dis J* 2005; 24: 645-6.
- [50] Ebihara T, Endo R, Ma X, Ishiguro N, Kikuta H. Detection of human coronavirus NL63 in young children with bronchiolitis. *J Med Virol* 2005; 75: 463-5.
- [51] Canducci F, Debiaggi M, Sampaolo M, *et al.* Two-year prospective study of single infections and co-infections by respiratory syncytial virus and viruses identified recently in infants with acute respiratory disease. *J Med Virol* 2008; 80: 716-23.
- [52] Forster J, Ihorst G, Rieger CH, *et al.* Prospective population-based study of viral lower respiratory tract infections in children under 3 years of age (the PRI.DE study). *Eur J Pediatr* 2004; 163: 709-16.
- [53] van der Hoek L, Sure K, Ihorst G, *et al.* Group is associated with the novel coronavirus NL63. *PLoS Med* 2005; 2: e240.
- [54] Lambert SB, Allen KM, Druce JD, *et al.* Community epidemiology of human metapneumovirus, human coronavirus NL63, and other respiratory viruses in healthy preschool-aged children using parent-collected specimens. *Pediatrics* 2007; 120: e929-37.
- [55] Kleines M, Scheithauer S, Rackowitz A, Ritter K, Hausler M. High prevalence of human bocavirus detected in young children with

- severe acute lower respiratory tract disease by use of a standard PCR protocol and a novel real-time PCR protocol. *J Clin Microbiol* 2007; 45: 1032-4.
- [56] van Elden LJ, van Loon AM, van Alphen F, *et al.* Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reverse-transcriptase polymerase chain reaction. *J Infect Dis* 2004; 189: 652-7.
- [57] van de Pol AC, van Loon AM, Wolfs TF, *et al.* Increased detection of respiratory syncytial virus, influenza viruses, parainfluenza viruses, and adenoviruses with real-time PCR in samples from patients with respiratory symptoms. *J Clin Microbiol* 2007; 45: 2260-2.
- [58] Pilger DA, Cantarelli VV. Human metapneumovirus and human coronavirus NL63. *Pediatrics* 2008; 121: 445-6; author reply 446-7.
- [59] Lu RJ, Zhang LL, Tan WJ. Development and comparison of real-time and conventional RT-PCR assay for detection of human coronavirus NL63 and HKU1. *Bing Du Xue Bao* 2008; 24: 305-11.
- [60] Kaiser L, Regamey N, Roiha H, Deffernez C, Frey U. Human coronavirus NL63 associated with lower respiratory tract symptoms in early life. *Pediatr Infect Dis J* 2005; 24: 1015-7.
- [61] Yoo SJ, Kuak EY, Shin BM. Detection of 12 respiratory viruses with two-set multiplex reverse transcriptase-PCR assay using a dual priming oligonucleotide system. *Korean J Lab Med* 2007; 27: 420-7.
- [62] Smuts H, Hardie D. Human bocavirus in hospitalized children, South Africa. *Emerg Infect Dis* 2006; 12: 1457-8.
- [63] Vabret A, Dina J, Brison E, Brouard J, Freymuth F. Human coronaviruses. *Pathol Biol (Paris)* 2008; 57: 147-60.
- [64] Bastien N, Robinson JL, Tse A, Lee BE, Hart L, Li Y. Human coronavirus NL-63 infections in children: a 1-year study. *J Clin Microbiol* 2005; 43: 4567-73.
- [65] Esper F, Shapiro ED, Weibel C, Ferguson D, Landry ML, Kahn JS. Association between a novel human coronavirus and Kawasaki disease. *J Infect Dis* 2005; 191: 499-502.
- [66] McIntosh K. Coronaviruses in the limelight. *J Infect Dis* 2005; 191: 489-91.
- [67] Shimizu C, Shike H, Baker SC, *et al.* Human coronavirus NL63 is not detected in the respiratory tracts of children with acute Kawasaki disease. *J Infect Dis* 2005; 192: 1767-71.
- [68] Fox JD. Respiratory virus surveillance and outbreak investigation. *J Clin Virol* 2007; 40(Suppl 1): S24-30.
- [69] Esposito S, Bosis S, Niesters HG, *et al.* Impact of human coronavirus infections in otherwise healthy children who attended an emergency department. *J Med Virol* 2006; 78: 1609-15.
- [70] Halasa NB, Williams JV, Wilson GJ, Walsh WF, Schaffner W, Wright PF. Medical and economic impact of a respiratory syncytial virus outbreak in a neonatal intensive care unit. *Pediatr Infect Dis J* 2005; 24: 1040-4.
- [71] Choi EH, Lee HJ, Kim SJ, *et al.* The association of newly identified respiratory viruses with lower respiratory tract infections in Korean children, 2000-2005. *Clin Infect Dis* 2006; 43: 585-92.
- [72] Han TH, Chung JY, Kim SW, Hwang ES. Human Coronavirus-NL63 infections in Korean children, 2004-2006. *J Clin Virol* 2007; 38: 27-31.
- [73] Kistler A, Avila PC, Rouskin S, *et al.* Pan-viral screening of respiratory tract infections in adults with and without asthma reveals unexpected human coronavirus and human rhinovirus diversity. *J Infect Dis* 2007; 196: 817-25.
- [74] Cheng VC, Lau SK, Woo PC, Yuen KY. Severe acute respiratory syndrome coronavirus as an agent of emerging and reemerging infection. *Clin Microbiol Rev* 2007; 20: 660-94.
- [75] Dijkman R, Jebbink MF, El Idrissi NB, *et al.* Human coronavirus NL63 and 229E seroconversion in children. *J Clin Microbiol* 2008; 46: 2368-73.
- [76] Shao X, Guo X, Esper F, Weibel C, Kahn JS. Seroepidemiology of group I human coronaviruses in children. *J Clin Virol* 2007; 40: 207-13.
- [77] Chan KH, Cheng VC, Woo PC, *et al.* Serological responses in patients with severe acute respiratory syndrome coronavirus infection and cross-reactivity with human coronaviruses 229E, OC43, and NL63. *Clin Diagn Lab Immunol* 2005; 12: 1317-21.
- [78] McIntosh K, Chao RK, Krause HE, Wasil R, Mocega HE, Mufson MA. Coronavirus infection in acute lower respiratory tract disease of infants. *J Infect Dis* 1974; 130: 502-7.
- [79] Monto AS, Rhodes LM. Detection of coronavirus infection of man by immunofluorescence. *Proc Soc Exp Biol Med* 1977; 155: 143-8.
- [80] Woo PC, Lau SK, Wong BH, *et al.* False-positive results in a recombinant severe acute respiratory syndrome-associated coronavirus (SARS-CoV) nucleocapsid enzyme-linked immunosorbent assay due to HCoV-OC43 and HCoV-229E rectified by Western blotting with recombinant SARS-CoV spike polypeptide. *J Clin Microbiol* 2004; 42: 5885-8.
- [81] Almeida JD, Tyrrell DA. The morphology of three previously uncharacterized human respiratory viruses that grow in organ culture. *J Gen Virol* 1967; 1: 175-8.
- [82] Kahn JS. The widening scope of coronaviruses. *Curr Opin Pediatr* 2006; 18: 42-7.
- [83] Bromham L, Penny D. The modern molecular clock. *Nat Rev Genet* 2003; 4: 216-24.
- [84] Sanchez CM, Gebauer F, Sune C, Mendez A, Dopazo J, Enjuanes L. Genetic evolution and tropism of transmissible gastroenteritis coronaviruses. *Virology* 1992; 190: 92-105.
- [85] Vijgen L, Lemey P, Keyaerts E, Van Ranst M. Genetic variability of human respiratory coronavirus OC43. *J Virol* 2005; 79: 3223-4; author reply 3224-5.
- [86] van der Hoek L. Human coronaviruses: what do they cause? *Antivir Ther* 2007; 12: 651-8.
- [87] Donaldson EF, Yount B, Sims AC, Burkett S, Pickles RJ, Baric RS. Systematic assembly of a full-length infectious clone of human coronavirus NL63. *J Virol* 2008; 82: 11948-57.
- [88] Vijgen L, Moes E, Keyaerts E, Li S, Van Ranst M. A pancoronavirus RT-PCR assay for detection of all known coronaviruses. *Methods Mol Biol* 2008; 454: 3-12.
- [89] Smuts H, Workman L, Zar HJ. Role of human metapneumovirus, human coronavirus NL63 and human bocavirus in infants and young children with acute wheezing. *J Med Virol* 2008; 80: 906-12.
- [90] Koetz A, Nilsson P, Linden 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; 12: 1089-96.
- [91] 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.

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