Eur J Med Res (2007) 12: 134-138

Two Cases of Severe Obstructive Pneumonia Associated with an HKU1-like Coronavirus

B. Kupfer[2]*, A. Simon[1]*, C. M. Jonassen[4], S. Viazov[3], V. Ditt[3], R.-L. Tillmann[2], A. Müller[1], B. Matz[2], O. Schildgen[2]

[1]Department of Pediatrics, [2]Institute for Medical Microbiology, Immunology, and Parasitology, University of Bonn, [3]Institute for Virology, Essen University Hospital, [4]National Veterinary Institute, Norway

Abstract

Background: During the last few years a number of previously undescribed viruses, including human metapneumovirus, coronaviruses SARS, NL63 and HKU1, and bocavirus, were identified in nasopharyngeal samples from patients with signs of respiratory infections. These viruses may cause mild to life-threatening infections.

Objectives: Nasopharyngeal samples from hospitalized pediatric patients with respiratory disease were analysed for the presence of coronaviruses and other well known and newly identified respiratory viruses.

Results: Two clinical cases of a severe obstructive pneumonia, which were associated with the presence of RNA of a novel variant (subtype) of HKU1 coronavirus in the nasopharyngeal aspirates, were identified.

Discussion: The detection of a HKU1-like coronavirus in pediatric patients in the current study complement the most recent independent finding of similar or closely related coronaviruses in patients with respiratory diseases in France (Vabret et al. 2006) and Norway (Jonassen et al., see accompanying manuscript). These observations indicate a wide dissemination of HKU1-like coronaviruses in Europe.

Introduction

Beside the commonly known viruses that cause respiratory tract infections in children like respiratory syncytial virus (RSV), rhinoviruses, adenoviruses, and the human coronaviruses OC43 and 229E, an increasing number of respiratory viruses were detected recently. These pathogens include human metapneumovirus (van den Hoogen et al. 2001), human bocavirus (Allander et al. 2005) and three different coronaviruses. In 2003 one single outbreak of the SARS coronavirus evolved to a worldwide problem with high mortality rates of the infected patients (Drosten et al. 2003). Two other coronaviruses, namely the human coronavirus NL-63 (van der Hoek et al, 2004) also known as coronavirus NL (Fouchier et al. 2004) and the human coronavirus HKU1 (Woo et al. 2005) have been identified recently.

The coronaviruses NL63 and HKU1 seem to be distributed worldwide and in contrast to SARS coronavirus, they occur more frequently. Infections with these pathogens could lead to mild to severe respiratory tract infections (RTI) within all age groups. Despite some reports from different parts of the world have described the occurrence of HKU1 coronavirus (Esper et al. 2006; Sloots et al. 2006; Vabret et al. 2006), there is still little information about the virus, its distribution, and its phylogeny. Here we describe the detection of RNA from a new variant of HKU1 coronavirus in two children hospitalized with respiratory tract infections in Germany.

Patients and Methods

Clinical Samples and PCR

Nasopharyngeal aspirates were collected from hospitalized pediatric patients between October 2003 and May 2006 in the Department of Pediatrics of the University Hospital in Bonn, Germany. A total number of 296 samples were analysed for the presence of human coronaviruses.

Therefore, RNA was extracted from nasopharyngeal aspirates with the RNeasy protect kit (QIAGEN, Hilden, Germany) and subjected to a single-step RT-PCR (QIAGEN, Hilden, Germany) using a set of primers designed on the basis of comparison of pol ORF1b sequences of known human coronaviruses. Basically, this approach was a modification of the technique described by Stephensen et al. (1999) and Moes et al. (2005). RT was carried out for 30 min at 42°C followed by a PCR with one initial step of Taq-Polymerase activation (95 °C for 15 min.), 35 cycles of amplification (95 °C for 30 sec., 50 °C for 30 sec., 72 °C for 30 sec) and a final elongation step (72 °C for 5 min). The primers used for the pan-coronavirus PCR were sv387as (5' – TCA CAY TTW GGA TAR TCC CA), sv388s (5' – ACT CAR ATR AAT CTT AAR TAY GC), and sv389s (5' – ACT CAA ATR AAT TTR AAR TAY GC) at final concentrations of 0.6µM each. PCR-amplicons with a length of 251 basepairs were electrophoresed on an agarose gel and visualized by ethidium bromide staining.

Samples were also screened for the presence of human metapneumovirus (HMPV) (Viazov et al. 2003; Schildgen et al. 2004), RSV (Kupfer et al. 2006; Müller et al., submitted; Wilkesmann et al. 2006), influenza viruses A and B (Müller et al., submitted for publication; Wilkesmann et al. 2006), and bocavirus (Allander et al. 2005).

* The first two authors contributed equally to this paper.

Sequencing of Coronaviral PCR-amplicons and Phylogenetic Analysis

Amplicons were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and subjected to sequencing in both directions (BigDye Terminator DNA sequencing kit, Applied Biosystems, Darmstadt, Germany) as previously described (Schildgen et al. 2004). BlastN (http://www.ncbi.nlm.nih.gov/BLAST) was used for comparison of the obtained sequences to nucleotide sequence databases. Viral sequences were aligned to the corresponding sequences of coronavirus HKU1 (accession number: AY597011), SARS coronavirus (AY515512), coronavirus OC43 (AY585229), coronavirus NL63 (AY567487), and a very recently described coronavirus strain (NO/0053/04; AM261821) using ClustalX version 1.8 (Thompson et al. 1997).

Phylogenetic trees of the newly derived sequences were constructed by the neighbour-joining method (Saitou & Nei, 1987). Reliability of the topology of the tree was evaluated by the bootstrap resampling method using 1000 replicates. The generated tree was visualized with the program NJplot (Perrière et al. 1996).

Results

In the present study 296 nasopharyngeal samples from hospitalized pediatric patients suffering from respiratory symptoms were analysed by RT-PCR for coronaviruses. As controls, specimens from 15 healthy adult volunteers were tested.

The most frequent pathogens detected were human paramyxoviruses RSV and HMPV (manuscript in preparation). Coronaviruses were detected in five cases (1.7%). In three out of the five episodes of coronavirus-associated respiratory disease, the well characterized human coronavirus OC43 was identified by sequence analysis. Surprisingly, sequence analysis of coronavirus PCR amplicons derived from the two remaining individuals revealed in both cases a novel coronavirus variant.

Clinical Cases

The first patient with a coronavirus infection was a 16 months old boy admitted to the hospital with wheezing, cough, fever (38.5°C), and a reduced general condition. He was hospitalized for 14 days, and due to his reduced general condition and elevated inflammatory laboratory markers (CRP level 13.8 mg/L; leukocyte count 14.2×10^9/L) he received inhalative treatment with beta-mimetics, ipratropium bromide, budesonide (7 days each) in addition with inhalative corticoids for 6 days and systemic steroids for 3 days. No antibiotics were given since the chest radiography revealed no signs of a bacterial pneumonia. The child required supplemental oxygen for 4 days; the clinical severity was 2 on the modified McIntosh scale (Wilkesmann et al. 2006), probably due to a coinfection with HMPV.

The second patient who suffered from a coronavirus infection (without evidence of other pathogens) was an 18 months old boy who also showed symptoms of an atypical pneumonia. His clinical picture was characterized by a severe obstructive bronchitis, cough, wheezing, and tachypnoea (52 per minute). Compared to patient 1 the clinical symptoms were less severe (no supplemental oxygen required), but severe enough to require a 7 days stay in hospital. At admission the body temperature (36.9°C) was normal and because of the severe respiratory symptoms he received beta-mimetics, ipratropium bromide for 3 days, and intravenous antibiotics for 4 days, despite the absence of signs of an bacterial infection (CRP 3.6 mg/l, leukocyte count 6300/µl).

In nasopharyngeal washes from both patients, RNA from a novel HKU1-like coronavirus could be identified by RT-PCR.

Phylogenetic Analysis

The nucleic acid sequences obtained from the direct sequencing of the coronavirus amplificons were initially submitted to a nucleotide-nucleotide blast (BlastN). This analysis revealed a similarity of 92% to the HKU1 group of coronaviruses. Most surprisingly, an exact match (100% identity) (Fig. 1A), and 99,5% identity, were found to coronaviruses NO/0053/04 and NO/0341/04, respectively, identified in Norway from infants presenting respiratory tract infections in winter 2004/5 (Jonassen et al., submitted). Phylogenetic analysis of the identified viruses (DE/0411 and DE/0415) from both patients together with NO/0053/04 sequence revealed a novel coronavirus variant within the HKU1 cluster (Fig. 2). However, no differences between HKU1, DE/0411 and DE/0415 were observed on the amino acid level (Fig. 1B). Of note, within the analysed amino acid sequence in this portion of the viral replicase gene, there is only one substitution between HKU1 and OC43 (Fig. 1B). Phylogenetic analysis showed higher divergence of the HKU1-like variants to the human pathogenic SARS coronavirus (branching off the root of the group II coronaviruses) and coronavirus NL63 (group I coronavirus) on the nucleotide, as well as on the amino acid level (Fig. 1 and Fig. 2).

Discussion

During the last five years an increasing number of viral respiratory pathogens have been newly identified. This includes three new coronaviruses, namely the SARS coronavirus (Drosten et al. 2003), the human coronavirus NL63 (van der Hoek et al. 2004; Fouchier et al. 2004), and the coronavirus HKU1 (Woo et al. 2005). Nevertheless, there is still a certain proportion of RTIs without an identified pathogen. Some RTIs in our cohort were associated with human coronaviruses but neither the SARS coronavirus nor the human coronavirus NL63 could be detected in the nasopharyngeal samples. The first reason for this observation is probably that the SARS outbreak was a rather single zoonotic event whereas other coronaviruses circulate every year in the human population. Second, the coronavirus NL63 may be rare in our cohort of hospitalized patients.

In the present study and also in the work of

A

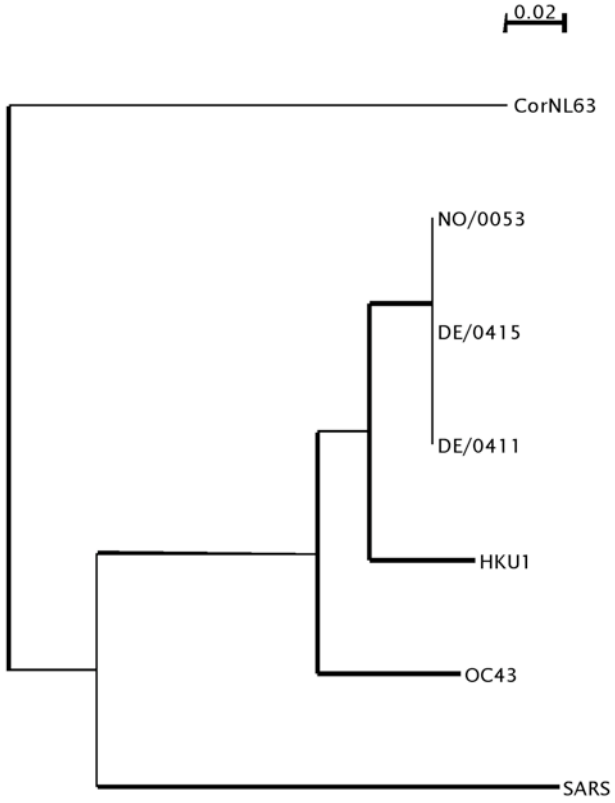
```
>DE/0411    ATTAGTGCTAAGAATAGAGCTCGTACTGTTGCAGGTGTTTCCATTCTTAGTACTATGACAGGTCGAATGTTTCATCAAAA
>DE/0415    ....................................................................................
>NO/0053    ....................................................................................
>CorHKU1    ..C.................C.....A..........T.................C........C........
>CorOC43    ................C..C........T..........A.............T..CA..............
>CorSARS    ........A.............C..C..A..T.....C..T..CTG.............AA.A..CA.........G..
>CorNL63    .......G...AG.AC.T...A.A.......GT........TC.GT.GTC...A.....CACAA..CAA.AC........

>DE/0411    ATGTTTGAAGAGTATAGCAGCTACTCGTGGTGTTCCTGTTGTTATAGGAACTACTAAATTTTATGGTGGCTGGGATGATA
>DE/0415    ....................................................................................
>NO/0053    ....................................................................................
>CorHKU1    ....................C..A.....................C.................T.....C....
>CorOC43    .........A.............A.............A........C..C.........................
>CorSARS    ..TA......TCA.....C..C...A.A..A.C.A....G..A..T.....A.GC..G.....C.........C..A...
>CorNL63    .CA.C.T..ATCC..T.TTAA...A..CAA..CCA.........T..T.....C.............T...A..A...

>DE/0411    TGTTACGCCATCTTATAAAGGATGTTGACAACCCTGTTCTTATGGGT
>DE/0415    ...........................................
>NO/0053    ...........................................
>CorHKU1    .......T...................................
>CorOC43    .........GC.....T..A...........T.....A.........
>CorSARS    .....AAAAC.G..TAC.GT.....A..A.CT..ACAC.........
>CorNL63    ....G..TAC.T.A..TG.T.G......A......A.G.........
```

B

```
DE/0411    ISAKNRARTVAGVSILSTMTGRMFHQKCLKSUAATRGVPVVIGTTKFYGGWDDMLRHLIKDVDNPVLMG
DE/0415    ............................................................................
NO/0053    ............................................................................
HKU1       ............................................................................
OC43       ...........................................................R................
SARS       ..............C....N.Q....L.........AT.....S......HN..KTVYS..ET.H...
NL63       ..G.E.....G...L.....T.QY...H....VN..NAT............NN...T..DG.E..M...
```



▲
Fig. 1. A. Nucleotide alignments corresponding to the phylogenetic tree. Sequences used for alignments were AY515512 (SARS), AY597011.2 (HKU1), AY585229 (OC43), AM261821 (NO/0053/04), and AY567487 (NL63) B. Alignments of the predicted amino acid sequences within the polymerase region from different coronaviruses. On the amino acid level no differences between the new variants NO/0053, DE/0411, DE/0415, and HKU1 were observed. Of note, the difference between HKU1 and OC43 within the analysed region is only one single amino acid exchange.

◀
Fig. 2. Phylogenetic tree of human respiratory coronaviruses. The novel variants NO/0053, DE/0411, and DE/0415 displayed a 92% homology to HKU1 on the nucleotide level and form a cluster of HKU1-like viruses, whilst reflecting a potential new genotype of HKU1. Sequences used for phylogenetic analyses were AY515512 (SARS), AY597011.2 (HKU1), AY585229 (OC43), AM261821 (NO/0053/04), and AY567487 (NL63)

Jonassen and colleagues (Jonassen et al., submitted) the identification of a novel variant of coronaviruses is described. These viruses were found in the nasopharyngeal secretions from children with acute respiratory tract infections and are closely related to the HKU1 coronavirus. However, the differences in the nucleotide sequences compared to HKU1 were rather high in this otherwise conserved part of the coronavirus genome, even though none of the observed substitutions was non-synonymous (i.e. 100% identity on the amino acid level between the novel viruses as compared to previously published HKU1). One may now speculate, however, that i) these viruses may represent a novel coronavirus variant with different pathogenic properties than the HKU-1 viruses identified in Asia or ii) the newly identified HKU1-like variant reflects the seasonability of the HKU1 variants found in different parts of the world at different times or III) the newly identified HKU1-like variant reflects a European clade as opposed to the Asian clade of the virus. In order to test these hypotheses, whole genome sequencing of newly identified coronaviruses is highly desirable.

Due to the identification of three coronaviruses in the last three years and the frequent observation of double infections we consequently started to test clinical samples from patients with RTI for the occurrence of these newly identified coronaviruses, even if another pathogen was detected. In fact, in one of our two patients infected by the novel HKU1-like coronavirus variant no further pathogens were detected, whereas the second patient was coinfected with HMPV. Furthermore, the two Norwegian infants where the HKU-1 variants were identified were both infected with RSV (Jonassen et al., submitted). Thus, the virus might have gone unidentified precedently due to apparently high frequencies of double infection, rather than being a novel pathogen to humans. The detection of the HKU1-like variant became possible by the use of pancoronavirus specific PCR primers. It now has to be clarified, how far the HKU1-like variants induce severe RTIs or whether they solely increase clinical severity of respiratory infections caused by other pathogens.

Finally, the present cases demonstrate that the HKU1-like viruses may have a long history of infecting humans.

Acknowledgement: This work was partially supported by grants from the Else Kröner-Fresenius-Stiftung (Grant Number A 01/05//F 00) and from the Herbert-Reeck-Stiftung (Bonn, Germany), and the BONFOR program of the Medical Faculty of the University of Bonn (Grant number O-151.0028).

REFERENCES

Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci U S A. 2005; 102:12891-6.

Drosten C, Gunther S, Preiser W, van der Werf S, Brodt HR, Becker S, Rabenau H, Panning M, Kolesnikova L, Fouchier RA, Berger A, Burguiere AM, Cinatl J, Eickmann M, Escriou N, Grywna K, Kramme S, Manuguerra JC, Muller S, Rickerts V, Sturmer M, Vieth S, Klenk HD, Osterhaus AD, Schmitz H, Doerr HW. Identification of a novel coronavirus in patients with severe acute respiratory syndrome. N Engl J Med. 2003; 348:1967-76.

Esper F, Weibel C, Ferguson D, Landry ML, Kahn JS. Coronavirus HKU1 infection in the United States. Emerg Infect Dis. 2006; 12:775-9.

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 U S A. 2004; 101:6212-6.

Jonassen CM, Kofstad T, Dudman SG, Jonassen TØ. Detection of different coronaviruses in Norwegian patients with respiratory symptoms, submitted to J. Clin. Virol.

Kupfer B, Vehreschild J, Cornely O, Kaiser R, Plum G, Viazov S, Franzen C, Tillmann RL, Simon A, Müller A, and Schildgen O: Severe pneumonia associated to new human bocavirus in a patient with a non-Hodgkin lymphoma (NHL); Emerg. Inf. Dis., in press

Moes E, Vijgen L, Keyaerts E, Zlateva K, Li S, Maes P, Pyrc K, Berkhout B, van der Hoek L, Van Ranst M. 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; 1;5(1):6.

Müller A, Kupfer B, Vehreschild J, Cornely O, Kaiser R, Seifert H, Viazov S, Tillmann RL, Franzen C, Simon A, Schildgen O. Fatal pneumonia associated with human metapneumovirus (HMPV) in a patient with myeloid leukemia and adenocarcinoma in the lung. Eur J Med Res 12(4) (accepted)

Perrière G, Gouy M. WWW-Query: An on-line retrieval system for biological sequence banks. Biochimie 1996; 78, 364-369.

Saitou N, Nei M. The neighbour-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 1987; 4:406-25

Schildgen O, Geikowski T, Glatzel T, Simon A, Wilkesmann A, Roggendorf M, Viazov S, Matz B. New variant of the human metapneumovirus (HMPV) associated with an acute and severe exacerbation of asthma bronchiale. J Clin Virol. 2004; 31:283-8.

Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, Mackay IM. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol 2006; 35:99-102.

Stephensen CB, Casebolt DB, Gangopadhyay NN. Phylogenetic analysis of a highly conserved region of the polymerase gene from 11 coronaviruses and development of a consensus polymerase chain reaction assay. Virus Res 1999; 60:181-9.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 94:6815-9.

Viazov S, Ratjen F, Scheidhauer R, Fiedler M, Roggendorf M. High prevalence of human metapneumovirus infection in young children and genetic heterogeneity of the viral isolates. J Clin Microbiol. 2003;41:3043-5.

Vabret A, Dina J, Gouarin S, Petitjean J, Corbet S, Freymuth F. Detection of the new human coronavirus HKU1: a report of 6 cases. Clin Infect Dis. 2006; 42:634-9.

Woo PC, Lau SK, Chu CM, Chan KH, Tsoi HW, Huang Y, Wong BH, Poon RW, Cai JJ, Luk WK, Poon LL, Wong SS, Guan Y, Peiris JS, Yuen KY. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J Virol. 2005; 79:884-95.

van den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD. A newly discov-

ered human pneumovirus isolated from young children with respiratory tract disease. Nat Med. 2001; 7:719-24.
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; 10:368-73.
Wilkesmann A, Schildgen O, Eis-Hubinger AM, Geikowski T, Glatzel T, Lentze MJ, Bode U, Simon A. Human metapneumovirus infections cause similar symptoms and clinical severity as respiratory syncytial virus infections. Eur J Pediatr. 2006 Mar 23; [Epub ahead of print]

Received: September 29, 2006 / Accepted: October 9, 2006

Address for correspondence:
Dr. rer. nat. Oliver Schildgen
Institute for Medical Microbiology, Immunology, and Parasitology
Department of Virology
University of Bonn
Sigmund-Freud-Strasse 25
53105 Bonn
Germany
Tel.: +49-(0)228-28711697
Fax: +49-(0)228-28714433