Research article/Raziskovalni prispevek

HUMAN METAPNEUMOVIRUS FOUND IN CLINICAL MATERIALS OF CHILDREN WITH RESPIRATORY TRACT DISEASES

ČLOVEŠKI METAPNEVMOVIRUS, DOLOČEN V KUŽNINAH OTROK Z BOLEZNIMI DIHAL

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Key words *human metapneumovirus; paramyxoviruses; respiratory tract infections; RT-PCR*

Abstract

Background *Human metapneumovirus (hMPV) was first recognized in the Netherlands in 2001. Since then, it has been documented all over the world as a cause of human respiratory infections in all age groups. The objective of this study was to introduce and optimize an assay for detecting hMPV in clinical material of our patients. To date, there has not been a report that describes the detection of this virus in Slovenia.*

Methods *A total of 58 specimens, randomly collected during 2003/2004 from the patients ≤ 19 years old with respiratory disease and 20 specimens collected in 1997 were tested for hMPV. Extraction of RNA from frozen specimens and subsequent single-step reverse transcription-polymerase chain reaction (RT-PCR) were performed. Human metapneumovirus amplicons were determined by electrophoresis in a 2 % (w/v) agarose gel.*

Results *Human metapneumovirus was detected in 13/58 (22 %) specimens; 10/40 (25 %) specimens were from the upper and 3/18 (17 %) from the lower respiratory tract. The mean age of infected patients was 3.2 ± 2.2 years. Out of 13 of the hMPV-positive specimens, 9 were positive also for another respiratory virus. Two of 20 (10 %) archival specimens were hMPV-positive.*

Conclusions *This study is the first report about hMPV in Slovenia. Human metapneumovirus was detected as the second most frequent virus after RSV in children < 3 years of age. The virus was not found in the specimens from the children younger than 2 months. Based on the hMPV-positive results in archival clinical material, it is suggested that hMPV had circulated in Slovenia before the time it was discovered.*

Ključne besede *človeški metapnevmovirus; paramiksovirusi; bolezni dihal; RT-PCR*

Izvleček

Izhodišča *Človeški metapnevmovirus (hMPV) so prvič odkrili na Nizozemskem leta 2001. V človeški populaciji virus kroži zagotovo mnogo dlje, saj so v shranjenih serumih iz leta 1958 uspeli*

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dokazati protitelesa proti hMPV. Pozno so ga odkrili verjetno zato, ker se slabo in zelo počasi razmnožuje na celičnih kulturah, ki jih uporabljajo za diagnostiko drugih respiratornih virusov in ker je za njegovo optimalno razmnoževanje in vitro potreben tripsin, ki ga v zgodnjih raziskavah virusov, ki okužijo dihalne poti, niso uporabljali. Majhna skladnost v nukleotidnem zaporedju hMPV z drugimi sorodnimi virusi je tudi ovirala njegovo odkritje z enostavnim navzkrižnim pomnoževanjem z začetnimi oligonukleotidi, oblikovanimi za pomnoževanje zaporedij že znanih virusov. Od odkritja hMPV pa do danes je bil virus opisan po vsem svetu kot eden izmed pomembnejših povzročiteljev okužb dihal pri ljudeh vseh starosti. Klinična slika okužbe s hMPV zelo spominja na okužbo z respiratornim sincicijskim virusom (RSV), zaradi česar je bil hMPV tudi morda spregledan. Od človeških patogenih virusov je hMPV najbolj podoben RSV. Oba sta paramiksovirusa, vendar hMPV zaradi večje genske sorodnosti in podobne ureditve genoma s ptičjim pnevmovirusom uvrščamo v drug rod kot RSV, t. j. Metapneumovirus. Predpostavljali smo, da je virus razširjen tudi v naši populaciji. Cilj študije je bil, da uvedemo in optimiziramo metodo za določanje hMPV v kužninah iz dihal ter ugotovimo, če virus kroži tudi pri nas in kako pogosto ter v kateri starostni skupini bolnikov se pojavlja. Želeli smo tudi ugotoviti, če je bil hMPV v naši populaciji že pred odkritjem leta 2001.

Metode

V raziskavo smo vključili 58 naključno izbranih kužnin (brise nosu, brise žrela, brise nosnega žrela, brise tonzil, aspirate sapnika, aspirate bronhijev, bronhoalveolarne izpirke) bolnikov z boleznijo zgornjih ali spodnjih dihal, starih ≤ 19 let. Kužnine so bile odvzete v sezoni 2003/2004. V raziskavo smo vključili tudi 20 kliničnih materialov (bronhoalveolarnih izpirkov) bolnikov s kronično obstruktivno pljučno boleznijo dihal iz leta 1997, ki so bili še shranjeni v laboratoriju. Iz kužnin smo osamili celokupno RNK, nato pa virusno RNK pomnožili z verižno reakcijo s polimerazo s predhodno reverzno transkripcijo (RT-PCR). Za pomnoževanje 450 bp dolgega odseka virusnega gena za fuzijski (F) protein smo uporabili par začetnih oligonukleotidov, katerih nukleotidno zaporedje je bilo oblikovano na podlagi zaporedja genoma izolata NDL00-1 (referenčna št. v genski banki AF371337). Pridelke pomnoževanja smo določili z elektroforezo v 2-odstotnem (u/v) agaroznem gelu.

Rezultati

Človeški metapnevmovirus smo določili v 13/58 (22 %) kužninah; 10/40 (25 %) kužnin je bilo iz zgornjih dihal in 3/18 (17 %) iz spodnjih dihal. Povprečna starost bolnikov, okuženih s hMPV, je bila 3,2 ± 2,2 let. Od 13 kužnin, pozitivnih na hMPV, je bilo 9 pozitivnih še na en respiratorni virus: 2 na RSV, 6 na adenovirus in 1 na virus influence B. RNK hMPV smo določili tudi v 2/20 (10 %) arhivskih kužninah.

Zaključki

To je prva študija o okužbi s hMPV pri bolnikih z boleznijo dihal v Sloveniji. Rezultati raziskave kažejo, da je hMPV eden pomembnejših, do sedaj še neodkritih povzročiteljev okužb dihal, saj je bil glede pogostnosti okužb takoj za RSV pri otrocih, mlajših od 3 let. Pri otrocih, mlajših od 2 mesecev, ga nismo določili. Z naraščajočo starostjo bolnikov je število okužb s hMPV naraščalo. Glede na to, da smo virus dokazali tudi v kliničnem materialu, shranjenem več let pri –70 °C, sklepamo, da je virus v naši populaciji že dlje časa. V prihodnje bi lahko z genotipizacijo hMPV ugotovili, kateri sevi virusa in kako pogosto se pojavljajo v populaciji, ter tako dobili vpogled tudi v molekularno epidemiologijo tega virusa. Določanje hMPV pri bolnikih z boleznijo dihal je pomembno zaradi mnogih okužb dihal, ki jih po nepotrebnem zdravijo z antibiotiki, ter obetavnih dognanj, ki nakazujejo možnost zdravljenja okužb s hMPV v prihodnje. Študije in vitro so namreč pokazale, da ribavirin in imunoglobulini (za intravensko dajanje) ter nova sulfatirana lipidna molekula NMSO3 delujejo protivirusno. Njihovo zdravilno vrednost in vivo pa je potrebno še dokazati.

Introduction

Acute respiratory tract infections (ARTIs) are a leading cause of morbidity and mortality in infants and children worldwide (1). According to the World Health Organization, respiratory tract infections range as second among the leading causes of death in children less than 5 years old (2). The bacterial or viral agent responsible for an ARTI is often undetermined. In children with community-acquired pneumonia, the pathogenic agent can be detected in 43–85 % of cases (3, 4). The lack of an etiologic agent in the remaining cases may be due to insufficient sensitivity of current diagnostic tests or to unknown pathogens that are not detected by current methods. In 2001, a new respiratory tract viral pathogen, human metapneumovirus (hMPV), was described by the researchers from the Netherlands. Serological surveys showed that the virus had been present in that country since 1958 (5), suggesting that it is a newly recog-

nized rather than newly emerging respiratory virus. It has been associated with ARTI in all age groups (6), with more severe diseases, such as bronchiolitis and pneumonia in young children, elderly and immunocompromised patients (6–8). The clinical symptoms are similar to those of respiratory syncytial virus (RSV) infection (5, 8). Like the RSV, the hMPV belongs to the paramyxovirus family. Within that family of viruses, hMPV is genetically most similar to the avian pneumovirus, and so, it was assigned to the *Metapneumovirus* genus (5, 9). Two genetic lineages of hMPV have been identified, A and B, that can be further devided into subgroups A1, A2 and B1, B2 (9, 10). Both major lineages can circulate at the same time with no evidence of geographic clustering (10), however different subgroups may circulate at various rates during different seasons (11). Since its initial description, hMPV has been reported all over the world (5, 6, 9, 12–18).

A worldwide incidence of hMPV and the fact that there has not been a report about its presence in Slovenia inspired us to introduce and optimize a single-step reverse transcription-polymerase chain reaction (RT-PCR) for the detection of hMPV in the specimens from the patients with upper and lower respiratory tract disease. The hMPV results were considered as the addition to the results of routine clinical testing for other respiratory viruses and were compared with the hMPV results published round the world.

Materials and methods

Study design

Testing for hMPV on the respiratory specimens obtained from the patients ≤ 19 years old with respiratory disease, collected during the years 2003 and 2004, was performed. Most frequently defined clinical diagnoses of the patients were: chronic pulmonary disease (12 patients), acute bronchiolitis (11 patients, 3 of them with respiratory insufficiency, 4 with respiratory failure), adenovirosis (10 patients), upper respiratory infection (6 patients), acute obstructive bronchitis (4 patients, 2 of them with respiratory insufficiency), asthma (4 patients). Most of the patients were hospitalized. Forty clinical materials from the upper and 18 from the lower respiratory tract were included in the study. Clinical material comprised nose swabs, throat swabs, nasopharyngeal swabs, tonsil swabs, endotracheal aspirates, bronchial aspirates, and bronchoalveolar lavages (BALs). The specimens were pretested for other common respiratory viruses, like adenovirus, RSV, parainfluenza viruses types 1, 2, 3 and both influenza A and B virus by the use of direct immunofluorescence (DIF). A database, including patient identifying number, age, date of specimens collection, specimen type, clinical diagnosis and tests ordered, was generated from the computer data. Clinical diagnosis of the patients was defined only in some cases and was described as dyspnea, febrile state and bronchiolitis. Clinical picture of other patients was simply indicated as respiratory tract infection. Fifty-seven percent of samples were from male and 43 % from female patients. Seventy-seven percent of patients was less than 5 years old. Additionally, 20 archival specimens BALs, collected in the year 1997, of the patients of 5 to 17 years of age who all suffered from chronic obstructive pulmonary disease (COPD), were examined. Other data for these specimens were not available.

RNA extraction

RNA was extracted from 350 µl of each respiratory specimen using RNeasy® Protect Mini Kit (Qiagen, GmbH, Hilden, Germany) according to the instructions of the manufacturer.

Reverse transcription-polymerase chain reaction (RT-PCR) assay for hMPV

Viral RNA was amplified in a single-step RT-PCR using the Access RT-PCR Systems (Promega). The primers hMPV1f 5'-CTTTGGACTTAATGACAGATG-3' [bases 3704-3724] and hMPV1r 5'-GTCTTCCTGTGCTAACTTTG-3' [bases 4134-4153] (TIB Molbiol, Germany) were designed to amplify a 450 bp fragment of the hMPV fusion (F) gene (GenBank accession number AF371337). The process of RT-PCR optimization included the establishment of optimal reaction mixture and optimal amplification conditions. The optimized reaction mixture contained 1 µl of RNA, 10 µl of 5x AMV/Tfl puffer, 1 µl of 10 mM dNTP mix, 0.75 µl of 20 µM of each primer, 2 µl of 25 mM $MgSO_4$, 1 µl of 5U/µl of avian myeloblastosis virus reverse transcriptase (AMV RT), 1 µl of 5U/µl of *Thermus flavus* DNA polymerase; nuclease-free water was added to the volume of 50 µl. The following programme in thermocycler was established: 42 °C for 45 min for reverse transcription, 95 °C for 5 min for AMV RT inactivation and RNA/cDNA/primer denaturation; 45 cycles of 95 °C for 1 min, 54.1 °C for 1 min, 72 °C for 2 min, followed by an extension step of 10 min at 72 °C. Separation of amplified PCR products and molecular weight marker (MBI Fermentas, Maryland, USA) was performed by electrophoresis for 45–50 min at 100 V on a 2 % (w/v) agarose gel prestained with ethidium bromide. The gels were photographed using a Gel Doc 2000 programme (Biorad). Each run of RT-PCR included virus positive control and negative control (nuclease-free water) (19).

Propagation of hMPV in Vero cells

The original hMPV was obtained from Trondheim (Norway). The virus was propagated in cell culture in order to gain a virus stock for a positive control in RT-PCR. The original hMPV (200–400 µl) was in-oculated onto Vero (African green monkey kidney) cells (European collection of cell cultures, Salisbury, England), which were maintained in MEM (GIBCO, Denmark) supplemented with 2–5 % of fetal bovine serum (Dipro, Austria). After the inoculation, the cells were incubated at 37 °C. The medium overlying the cells was changed the next day. The cell monolayers were examined daily for cytopathic effect.

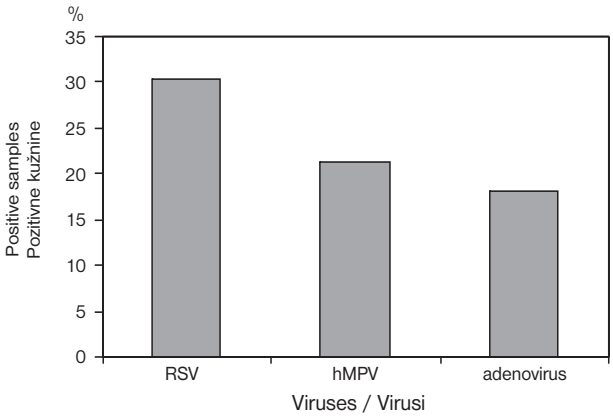

Figure 1. *Comparison between percentage of infections with human metapneumovirus (hMPV), respiratory syncytial virus (RSV) and adenovirus in children less than 3 years of age.*

Sl. 1. *Primerjava deleža okužb s človeškim metapnevmovirusom (hMPV), respiratornim sincicijskim virusom (RSV) in adenovirusom pri otrocih, mlajših od treh let.*

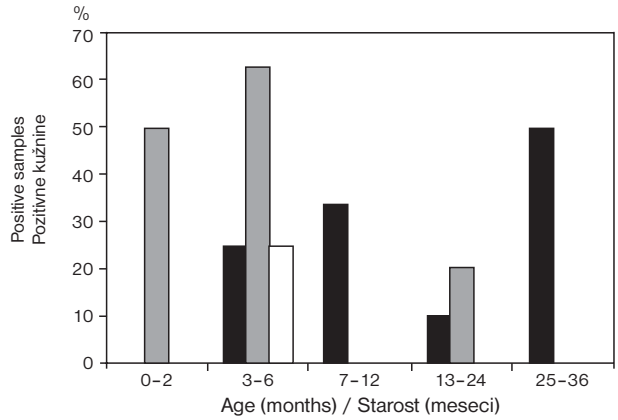

Figure 2. *Proportion of children with human metapneumovirus (hMPV), respiratory syncytial virus (RSV) infection and hMPV/RSV coinfection distributed by age. Bars: black for hMPV, grey for RSV, white for hMPV/RSV coinfection.*

Sl. 2. *Delež otrok okuženih s človeškim metapnevmovirusom (hMPV), respiratornim sincicijskim virusom (RSV) in sočasno okužbo hMPV/RSV glede na starost otrok. Stolpci: črni za hMPV, sivi za RSV, bel za hMPV/RSV sočasno infekcijo.*

Examining supernatants of Vero cells for the presence of hMPV by RT-PCR

To ascertain positive results of virus propagation, the supernatants of Vero cells were tested for the presence of hMPV by RT-PCR using the same protocol as for clinical material.

Results

Virus propagation in Vero cells

Human metapneumovirus was successfully propagated in Vero cells. The cytopathic effect, caused by the virus, appeared in scattered enlarged cells and was evident by day 5, and later progressed to a detachment of the affected cells from the monolayer. The hMPV RT-PCR-positive supernatants were used for positive control in testing clinical material for the presence of hMPV.

Evidence of hMPV infections

Out of 58 respiratory specimens, 13 (22 %) were positive for hMPV of which 10/40 (25 %) were from the upper respiratory tract and 3/18 (17 %) from the lower respiratory tract. Clinical diagnoses of the hMPV-positive patients were: 6 were suspected to have adenovirosis, 3 had acute bronchiolitis (2 of them with respiratory failure and 1 with respiratory insufficiency), 2 had asthma, 2 were chronic pulmonary patients, 1 had scarlet fever and 1 had sore throat. According to the type of clinical material, hMPV was detected in 6/20 (30 %) throat swabs, 2/3 (67 %) tonsil swabs, 1/3 (33 %) aspirate, 1/12 (8 %) nasopharyngeal swab from the upper respiratory tract and in 2/7 (29 %) aspirates and 1/8 (13 %) BAL from the lower respiratory tract. Out of 13 hMPV-positive specimens, 9 (69 %) were also positive for another respiratory virus: 2 for RSV, 6 for adenovirus and 1 for influenza B virus. Of the 24 specimens negative by DIF for other respiratory viruses, only 1 (4 %) was hMPV-positive. The median age of the hMPV-positive patients was 3.2 ± 2.2 years (age range 3 months to 7 years; 19-year-old patient was not included). A higher percentage (62 %) of patients with hMPV-positive specimens was determined among females. Human metapneumovirus was also detected in archival clinical material. Two of 20 (10 %) specimens were positive for hMPV; the data for the presence of other respiratory viruses in these specimens were not available.

hMPV and other respiratory viruses

In the specimens taken from the children aged less than 3 years, hMPV was found in 7/33 (21 %), RSV in 10/33 (30 %) and adenovirus in 6/33 (18 %) specimens, respectively (Figure 1). Human metapneumovirus was not detected in the children younger than 2 months. In children 3–6 months of age, it was detected in 2/8 (25 %) specimens, in the children of 7–12 months of age in 1/3 specimen (33 %), in the children of 13–24 months of age in 1/10 (10 %) specimen, and in the children of 25–36 months of age in 3/6 (50 %) specimens. RSV infection appeared to be most frequent in the children of 3–6 months of age; the virus was detected in 5/8 (63 %) specimens. On the contrary to hMPV, RSV was also found in 3/6 (50 %) specimens of children younger than 2 months. Co-infection of hMPV and RSV was found in children aged from 3 to 6 months (Figure 2).

Discussion

Viruses most frequently associated with respiratory infections include influenza viruses, RSV, parainfluenza viruses, rhinoviruses, coronaviruses, and adenoviruses (20–22). Investigations to date have shown that hMPV can now be added to the list of human pathogens causing respiratory infections in children and adults (6). In this study, we tried to find out whether the virus is present within our community, i.e. among the patients with upper or lower respiratory tract disease. We were also interested in the frequency of hMPV infections in different age groups of patients and in possible prevalence of the virus before the time it was discovered. For the detection of hMPV in clinical materials, a single-step reverse transcription-polymerase chain reaction (RT-PCR) was developed and optimized. The RT-PCR was designed to target a 450 bp fragment of the fusion (F) gene of hMPV. Conventional (7, 13, 15, 18) or real-time reverse transcription-polymerase chain reaction (RT-PCR) (23, 24) are currently the methods of choice for hMPV diagnosis due to the difficulty of culturing hMPV from clinical material (5, 6) and the lack of commercially available antigen detection test.

Using the RT-PCR assay, we were able to detect the virus in 13/58 (22 %) respiratory specimens, collected during the years 2003 and 2004. Out of all 58 tested specimens, 24 (41 %) were negative for all other respiratory viruses by direct immunofluorescence assay and out of these, 1 (4 %) was positive for hMPV. This is comparable to the results of previous studies in which hMPV was found in 2 % to 20 % of the specimens negative for other common respiratory viruses (12, 18, 25). Out of 13 hMPV-positive cases, 9 (69 %) coinfections with other respiratory viruses were observed: 2 with RSV, 1 with influenza B virus and 6 with adenovirus. What is the role of hMPV as a copathogen is still to be determined. Some studies suggest that hMPV/RSV coinfections may be an important risk factor for severe disease (26), whereas others do not (27). Two hMPV-positive archival samples from the year 1997 suggest that hMPV was circulating in our population already back then and probably even before.

Human metapneumovirus was found in 21 % of children aged less than 3 years, making it the second most prevalent respiratory virus in this age group following only RSV (30 %). Furthermore, the incidence of hMPV and RSV infections within this group was different according to patients' age. The proportion of hMPV-positive children tended to increase in correlation with increasing age (excluding the age group 13–24 months). In contrast, the proportion of children positive for RSV infection tended to decrease in older age groups. Similarly, Noyola et al. showed that the proportion of children infected with hMPV compared to RSV increased over the first 36 months of life (17). The mean age of our hMPV-positive patients was 3.2 ± 2.2 years (age range, 3 months to 7 years) and 6.2 ± 6.7 months (age range, 1 to 20 months) of RSV-positive patients, indicating that hMPV-positive patients were older than RSV-positive patients. The same was observed also in other studies (13, 28). RSV was detected only in the specimens from the children younger than 3 years, whereas hMPV was common also in older patients. We determined a somewhat high percent (30 %) of hMPV-positive patients in the age group of 5–15 years, with the mean age of patients of 6.5 years.

The male-female ratio of patients with hMPV infection was in favour of the latter (1:1.6). Many studies have found a higher proportion of patients with hMPV infection among boys than girls (8, 13), while some have not (30).

Whether hMPV detection varies by the type of specimen collected, as does the detection of RSV (31), is still unknown and awaits prospective studies.

Our relatively small number of hMPV-positive samples put limits to making any conclusions about preferred specimen type for diagnosing hMPV infections and also about the occurrence of hMPV infections according to the sex. Neither can we make any correlation between the hMPV infection and clinical diagnosis of the patients.

The majority of specimens tested for the presence of hMPV was collected in May and the virus was detected mostly in these specimens (10 cases of 13 hMPV-positive specimens), that is outside of the peak of hMPV infections in January through April (28, 32). The other 2 hMPV-positive specimens were obtained in September and one in January. Testing of respiratory specimens from every month of the year over many years should be done in the future to understand the seasonal distribution of hMPV infections in Slovenia. Moreover, sequencing of the hMPV-positive specimens should be introduced in diagnosing hMPV, so that we could determine, if there are certain strains more prevalent than others within our community.

In conclusion, the research is of major public health significance due to many respiratory infections that are going undiagnosed or patients being treated with unnecessary antibiotics. It is important for our physicians to know that hMPV has been circulating in Slovenia and can be detected. Furthermore, identifying hMPV in patients is relevant, because *in vitro* reports suggest that ribavirin, intravenous immunoglobulin and a new sulfated sialyl lipid molecule, NMSO3, have antiviral activity against hMPV (33, 34). Whether these agents have therapeutic value *in vivo* still needs to be demonstrated in future studies.

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