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Generation of recombinant metapneumovirus nucleocapsid protein as nucleocapsid-like particles and development of virus-specific monoclonal antibodies

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ABSTRACT

Human metapneumovirus (hMPV) is a member of the *Pneumovirinea* subfamily within the *Paramyxoviridea* family. Since its discovery in 2001, hMPV has been isolated in several continents, which suggests its prevalence worldwide. hMPV resembles human respiratory syncytial virus with regard to disease symptoms and its ability to infect and cause disease in young infants as well as individuals of all ages. The aim of the current study was to construct an efficient high-level yeast expression system for the generation of hMPV nucleocapsid (N) protein and to develop monoclonal antibodies (MAbs) suitable for hMPV detection. The genome of hMPV was isolated from oral fluid of an infected patient by using specific primers and reverse transcriptase polymerase chain reaction (RT-PCR). DNA sequence corresponding to the N protein gene was inserted into yeast expression vector under inducible *GAL7* promoter. SDS-PAGE analysis of crude lysates of yeast *Saccharomyces cerevisiae* harbouring recombinant plasmid revealed the presence of a protein band of approximately 43 kDa corresponding to the molecular weight of hMPV N protein. Electron microscopy analysis of purified N protein revealed nucleocapsid-like structures with typical herring-bone morphology: rods of 20 nm diameter with repeated serration along the edges and central core of 5 nm. Recombinant hMPV N protein was reactive with human serum specimens collected from patients with confirmed hMPV infection. After immunization of mice with recombinant hMPV N protein, a panel of MAbs was generated. The specificity of newly generated MAbs was proven by immunofluorescence analysis of hMPV-infected cells. Epitope mapping using truncated variants of hMPV N revealed localization of linear MAb epitopes at the N-terminus of hMPV N protein, between amino acid residues 1 and 90. The MAbs directed against conformational epitopes did not recognize hMPV N protein variants containing either N- or C-terminal truncations. The reactivity of recombinant hMPV N protein with hMPV-positive serum specimens and the ability of MAbs to recognize virus-infected cells confirms the antigenic similarity between yeast-expressed hMPV N protein and native viral nucleocapsids. In conclusion, recombinant hMPV N protein and hMPV-specific MAbs provide new diagnostic reagents for hMPV infection.

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1. Introduction

Human metapneumovirus (hMPV), first isolated in the Netherlands in 2001, is a member of the *Paramyxoviridea* family. It has been tentatively assigned to the *Metapneumovirus* genus of the *Pneumovirus* subfamily (van den Hoogen et al., 2001). Since its discovery, hMPV has been found to infect humans worldwide. hMPV has been recognized as a common cause of respiratory infections, ranging from upper respiratory tract infections to severe lower respiratory tract infections in very young children, elderly individuals and immunocompromised patients (Khan, 2006; Hermos et al., 2010; Mullins et al., 2004). Similar to other members of *Paramyxoviridea* family, hMPV is an enveloped single-stranded negative-sense RNA virus. Its genome is approximately 13 kb in length and contains nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), second matrix (M2), small hydrophobic (SH), attachment (G) and RNA-dependent RNA polymerase (L) genes in the order 3′-N-P-M-F-M2-SH-G-L-5′. Extensive sequence analysis of virus isolates from around the world indicates that there are two major genetic lineages of hMPV. With the notable exception of the SH and G proteins, the polypeptides encoded by each of these genetic lineages are highly related at the amino acid (aa) level. For

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Table 1
Oligonucleotides used for cloning of DNA sequences encoding full-length hMPV N protein gene and its truncated variants.

Oligonucleotide name[a]	Sequence[b]	Position[c]
1-F	5′-GCA TCT AGA ACA ATG TCT CTT CAA GGG ATT C-3′	1–20 nt
90-F	5′-GCA TCT AGA ACA ATG CAA GCG GTT TTA ACC AGA AC-3′	271–290 nt
395-R	5′- TCT ACT AGT TCA TTA CTC ATA ATC ATT TTG ACT GTC-3′	1162–1186 nt
365-R	5′-CTT TCT AGA TTA GTT GAT TTT ATT ACT TTC TTT C-3′	1074–1095 nt
335-R	5′-CTT TCT AGA TTA TAT TAT GCC TAA GCC TGA G-3′	987–1006 nt
305-R	5′- CTT TCT AGA TTA CCT TAA ATG TAG GAG CCC AG -3′	896–916 nt
275-R	5′- CTT TCT AGA TTA ATG TCC TAA CAT TAT ATT G -3′	807–826 nt
245-R	5′- CTT TCT AGA TTA GAC GAA TAA ACT TTC TGC -3′	718–736 nt

[a] Primer name indicates aa position in full length hMPV N protein sequence, F–forward, R–reverse primers.
[b] Restriction site sequences are underlined.
[c] Position according to Genbank ID: JF509957.

example, the aa sequence identity of the predicted encoded proteins between these two groups of hMPV strains is 96% for the N protein (Khan, 2006). The diagnosis of hMPV infection usually requires virus isolation and the detection of viral RNA in clinical specimens. Isolation of viruses in cell cultures is considered to be the "gold standard" for hMPV detection (Khan, 2006; Kikuta et al., 2008). However, hMPV is difficult to detect by this assay, because virus poorly propagates in cell cultures (Khan, 2006). Reverse transcriptase polymerase chain reaction (RT-PCR) is concluded to be the most sensitive and specific procedure for hMPV detection. Several genes of hMPV have been selected as targets for the amplification (Kikuta et al., 2008). However, RT-PCR requires special laboratory conditions and therefore has limited use in routine diagnostic settings. Immunofluorescence assay (IFA) based on the use of virus-specific antibodies is a simple and rapid method commonly used in diagnostic laboratories for detecting respiratory viruses, although it is less sensitive than RT-PCR (Khan, 2006; Kikuta et al., 2008). Antibody-based chromatographic sandwich immunoassay has been recently developed and employed for hMPV detection in research settings (Kikuta et al., 2008). Serological assays using isolated viral antigens or direct antibody-based detection of the virus in respiratory secretions may provide new possibilities for a routine laboratory diagnosis of hMPV infection and wide epidemiological studies. Virus recombinant proteins are promising tools for development of vaccines and diagnostics. Advances in gene expression technology have made the production of recombinant viral proteins in both eukaryotic and prokaryotic host systems easier and more efficient. The ease with which genetic modification can be carried out; the yield of recombinant proteins and the host's capability for post-translation modification often governs the choice of host systems. Yeast are unicellular eukaryotic microorganisms that are capable of performing eukaryotic processing steps on the polypeptides expressed and are easy to manipulate genetically. Since yeast are eukaryotes, their intracellular environment is more suitable for the correct folding of eukaryotic virus proteins as compared to bacterial expression systems (Böer et al., 2007). A considerable body of data on formation of homologous and chimeric virus-like and nucleocapsid-like particles (NLPs) of various viruses in yeast has been reported to date (Hofmann et al., 1996; Sasnauskas et al., 1999; Gedvilaite et al., 2000; Hale et al., 2002; Samuel et al., 2002; Juozapaitis et al., 2007; Freivalds et al., 2006; Kucinskaite et al., 2007). The expression of recombinant N proteins with spontaneous assembly into NLPs has been demonstrated for a number of paramyxoviruses using both prokaryotic and eukaryotic systems (Spehner et al., 1991; Hummel et al., 1992; Fooks et al., 1993; Meric et al., 1994; Warnes et al., 1995; Bhella et al., 2002). It demonstrates that the N proteins produced in heterologous protein expression systems might provide researchers with valuable and low-cost material for complex studies of viral protein structure and functions. In addition, we have previously shown that N proteins of various paramyxoviruses self assemble very efficiently into NLPs in yeast (Slibinskas et al., 2004; Juozapaitis et al., 2005, 2007). The N protein of paramyxoviruses is one of the most abundant viral proteins and usually elicits a strong and long-lasting humoral immune response in patients. It was also demonstrated that yeast-derived recombinant NLPs of mumps and measles viruses share similar antigenic structure with native viral nucleocapsids and are excellent tools for serodiagnostics of these viruses in sera and oral fluid (Pumpens and Grens, 2002; Samuel et al., 2002, 2003; Zvirbliene et al., 2007; Warrener et al., 2010).

In the current study, we have generated in yeast expression system the hMPV N protein, demonstrated its structural and antigenic similarity with viral nucleocapsids, developed hMPV N protein-specific monoclonal antibodies (MAbs) and evaluated their diagnostic potential.

2. Materials and methods

2.1. Serum samples

Serum samples from children's with bronchial asthma exacerbations, induced by acute viral respiratory tract infections, were obtained from Vilnius University, Faculty of Medicine, Clinic of Childrens' Diseases (Lithuania) and used for ELISA. The clinical diagnosis of respiratory tract infections was confirmed by the presence of typical acute respiratory viral infection symptoms, including sneezing, nasal obstruction and discharge, sore throat, cough, moderate fever. The hMPV infection was confirmed by RT-PCR. Collection of specimens and clinical data was approved by the Lithuanian Bioethics Committee (No. 53, 2008-09-03). Serum samples were collected on day 2 after the onset of the disease.

For the control sera, samples from our previous study on hantavirus infection were analysed (Dargevicius et al., 2007). Two control serum pools, each pooled from 5 serum samples from dialysis patients collected in dialysis centers of Kaunas district (Lithuania) were used.

2.2. Viral RNA isolation and cDNA synthesis

Viral RNA was extracted from nasopharyngeal aspirates (NAs) from the asthmatic child with respiratory tract infection (specimen was obtained from Vilnius University, Faculty of Medicine, Clinic of Childrens' Diseases, Vilnius, Lithuania). NAs were collected with a saliva collection device Oracol (Malvern Medical Developments Ltd., Worcester, UK), suspended in RNA stabilization solution RNA later (Applied Biosystems/Ambion, Austin, USA) and stored at −70 °C until processed. Extraction of RNA from NAs was performed by using a commercial Qiamp Ultrasens Virus Kit (Qiagen GmbH, Hilden, Germany). The RNA extract was immediately frozen and stored at −70 °C until use. Extracted RNA and RevertAid™ H

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