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Recombinant expression and immunological characterisation of proteins derived from human metapneumovirus

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ABSTRACT

Background: Human metapneumovirus (HMPV) has been shown to cause respiratory infection, accounting for approximately 7% of all such disease, and contributes to the development of asthma in humans. HMPV has a worldwide distribution with infectivity rates approaching 100%, and immunocompromised patients are particularly at risk from viral exposure. No anti-HMPV vaccine is available and diagnosis is primarily based on in-house molecular or serological tests, in part due to limited availability of recombinant HMPV antigens.
Objective: To generate a panel of HMPV-derived recombinant antigens, develop standardised ELISA systems for HMPV IgG detection and explore the nature of B cell memory against HMPV to underpin future vaccine studies.
Study design: HMPV viral RNA was isolated from a clinical specimen and RT-PCR was conducted. The HMPV M and P genes were cloned and expressed in *Escherichia coli*. The HMPV N gene was cloned and expressed in insect cells using the baculovirus expression system. Each purified recombinant antigens was subsequently employed in HMPV-specific ELISA.
Results: High-level expression, and purification, of both HMPV matrix (M) (10 mg/g cells) and phosphoprotein (P) (3.82 mg/g cells) were achieved in an *E. coli* expression system. Recombinant HMPV (N) was successfully expressed in, and purified from the baculovirus expression system. Overall, a 99% HMPV IgG seroprevalence was observed ($n = 96$) using HMPV M-, N- and P-ELISA, respectively. The M antigen proved to be the most diagnostically useful with 99% of specimens tested exhibiting anti-M protein reactivity. A high correlation was observed between anti-M and N IgG reactivity ($r = 0.96$), with significant correlation also evident for anti-N and P IgG reactivity ($r = 0.74$). Lowest correlation was evident for anti-M and P IgG reactivity ($r = 0.57$). Finally, the first demonstration of HMPV-specific B cell memory (ranging 1–15 spot forming cells (SFC)/million cells) was achieved against M and P antigens in 40% of individuals tested.
Conclusion: This work describes robust diagnostic systems for HMPV and new insight into antigen-specific B cell memory against HMPV.

1. Background

Viral respiratory diseases are a major health problem. They affect people of all ages and exert a great economic impact on the health care system. The viruses most often associated with respiratory tract illness include influenza virus, parainfluenza virus, respiratory syncytial virus (RSV), adenovirus, rhinovirus and coronavirus. In 2001, Van den Hoogen and colleagues first reported the discovery of a new respiratory virus (human metapneumovirus, HMPV) in the Netherlands.[1] Serological studies have revealed that virtually all children have been exposed to HMPV by the age of 5 years.[1–5] Similar studies seem to support this hypothesis.[6–10] However, in other case studies HMPV infections have been reported in the elderly[11–14] and immunocompromised individuals,[15–19] which suggests that the virus is not strictly limited to infecting infants or children. Since its discovery, the occurrence of HMPV has been reported in many countries, such as Australia, Canada, Finland, United States, United Kingdom, Spain, Ireland, Israel and Japan. It is now thought to be prevalent worldwide, indicating that it is a common and ubiquitous human pathogen.[20–22]

The clinical manifestations of HMPV include tachypnea; rhinorrhea; nasal congestion; cough; fever; hypoxia; pharyngitis; hyperinflation; peribronchial cuffing; wheezing; bronchiolitis; pneumonia and respiratory failure.[23–25] Earlier recognition of this virus was delayed because it had been difficult to detect in cell

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culture due to its slow growth and mild cytopathic effect, and therefore awaited the development of reverse transcriptase-PCR (RT-PCR).

Considerable effort has been directed towards the elucidation of the nature of the T cell response to HMPV,[26–30] yet the nature of B cell memory directed against HMPV remains unclear. Memory B cells make a significant contribution to protective immunity and are characterised in terms of (i) a rapid proliferative response, accompanied by cellular differentiation upon antigen re-exposure, to produce affinity-matured, antibody-secreting plasma cells, (ii) a lower activation threshold relative to naïve B cells, in response to cytokine and antigen presence and (iii) an absence of spontaneous Ig secretion.[31] Evaluating B cell memory may have considerable importance with respect to the investigation of immunological memory to HMPV and prove useful in the elucidation of virus-specific B cell mediated immunity to HMPV.

2. Objectives

To generate a panel of HMPV-derived recombinant antigens, develop standardised ELISA systems for HMPV IgG detection and explore the nature of B cell memory against HMPV to underpin vaccine studies.

3. Study design

3.1. Isolation of RNA from a clinical specimen and RT-PCR

Briefly, RNA was isolated from a bronchoalveolar-lavage (BAL) from a 48 year old female patient (ROI135). This HMPV isolate was genotype A2. RT-PCR was conducted using a Qiagen one step RT-PCR kit. Three genes the HMPV Matrix (M) 0.8 kb; HMPV phosphoprotein (P) 0.9 kb and the HMPV nucleoprotein (N) 1.2 kb were amplified.

3.2. Cloning of HMPV M and P genes in Escherichia coli

The HMPV M and P gene sequences were amplified using oligonucleotide primers for the selected regions (M-For: GAGAAGGCCTATG GAGTCCTACCTAGTAGAC and M-Rev: GAGACTCGAGTCTGGACTTCAGCAC; P-For: GAGAAGGCCTATGTCGTTCCCTGAAGGA and P-Rev: GAGACTCGAG CATAATTAACTGGTAGATGTC, restriction sites *Stu*I and *Xho*I are underlined), ensuring that optimal directional cloning into the pProEX^{TM} HTb expression vector (Fig. 1). The HMPV M PCR cycling conditions were 95 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min, and a final extension step of 72 °C for 10 min in a Perkin-Elmer (Warrington, Cheshire, U.K.) 2400 model thermal cycler. The HMPV P PCR cycling conditions were identical to that of the M gene with the exception of an annealing temp of 63.5 °C.

3.3. Expression and purification of HMPV M

Expression of HMPV M protein was induced by the addition of isopropyl β-D-thiogalactoside (IPTG; 0.6 mM final) under the control of the *lac* promoter (Fig. 1). The M protein was highly insoluble and was present in the cell pellet as determined by SDS-PAGE and Western blot analysis using monoclonal antibody reactivity against a His_6 tag present on recombinant M protein and was therefore purified from inclusion bodies using a differential protein extraction method. Briefly, 3 h post-induction, cells were lysed by incubation with lysozyme (90 μg/ml) and sodium deoxycholate (0.04% (w/v)), in the presence of protease inhibitor cocktail. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min. Inclusion bodies were washed twice in 25 mM Tris, 1 mM EDTA (containing Triton X-100) pH 8.0, followed by a third 25 mM Tris, 2 M urea pH 8.0 wash. Centrifugation was performed as described above. The final protein pellet was solubilised by the addition Tris (25 mM; pH 8.0) containing 8 M urea, 1 mM EDTA and 2 mM dithiothreitol (DTT) with agitation for 30 min at room temperature. Aliquots of purified HMPV M protein were stored at −20 °C. Recombinant HMPV M (250 μg/ml) was serially dialysed from the 8 M urea buffer to 50 mM sodium carbonate, pH 9.4.

3.4. Expression and purification of HMPV P

Expression of HMPV P protein was induced similar to that of the M protein. The HMPV P protein was expressed with an N-terminal His_6-tag to aid protein purification (Fig. 1). The recombinant HMPV P protein was purified by Ni-NTA chromatography (Qiagen, West Sussex, U.K.) under denaturing conditions.

3.5. Cloning and expression of HMPV N in Spodoptera frugiperda 9 (Sf9) insect cells

It was necessary to design primers to amplify these regions for molecular cloning into the pBlueBac 4.5 V5-His vector. Oligonucleotide primers were designed for the selected region (N-For: ACAGGATCCGATGTCTCTTCAAGGGATTCAC, N-Rev: TATGAATTCGCCTCATAATCATTTTGACTG, and the PCR product was digested and ligated into the *Bam*HI/*Eco*RI sites (restriction sites are underlined) of the pBlueBac 4.5/V5-His vector (Fig. 1). The HMPV N PCR cycling conditions were 95 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 54.2 °C for 1 min, 72 °C for 2 min 30 s, and a final extension step of 72 °C for 10 min.

Recombinant HMPV N protein was purified from whole cell suspensions (2×10^8 cells), lysed in 16 ml lysis buffer (20 mM Tris–HCl, 8 M urea, 300 mM NaCl, pH 8.0), by His_6-affinity chromatography under denaturing conditions.

3.6. MALDI–ToF mass spectrometry (MS)

MALDI–ToF MS was carried out using an Ettan MALDI–ToF mass spectrometer (Amersham Biosciences (Europe) GmbH, Freiburg, Germany). Protein samples were excised from the gel and processed as described.[32]

3.7. Immunoblot and immunosorbent assay (ELISA) analysis

Immunoblots were conducted to assess IgG reactivity to the denatured form of each HMPV antigen. Briefly, each recombinant antigen was solubilised in SDS sample buffer (0.15 M Tris–Cl, pH 6.8, 4.6% SDS, 23% glycerol, and 0.2 M DTT in 0.1% (w/v) bromophenol blue) and heated at 100 °C for 5 min, prior to layering onto a SDS-PAGE gel (12.5%). After SDS-PAGE, proteins were electrophoretically transferred to nitrocellulose (NCP, Schleicher & Schuell, 0.45 μm pore size). Proteins were transferred at 120 mA in a transblotting chamber (Bio-Rad Instruments), for 1 h at 4 °C, using 25 mM Tris–HCl, 150 mM glycine, 20% (v/v) methanol. After transfer, the blots were blocked by incubation with 5% (w/v) non-fat milk powder in PBS for 1 h at room temperature. The membranes were then cut into strips and incubated for 1 h with human serum (1/100). Anti-His_6 monoclonal antibody was used as positive control for protein presence. The immuno-strips were washed 3 times in PBS, containing 0.05% v/v Tween-20 (PBST) and then incubated with horseradish peroxidase (HRP)-conjugated anti-human IgG (Dako A/S, Glostrup, Denmark) for 1 h. Following a wash step (4 times with PBST), immuno-reactive strips were visualized using 3, 3′-diaminobenzidine (DAB).