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: GAGA<u>AGGCCT</u>ATG GAGTCCTACCTAGTA-GAC and M-Rev: GAGA<u>CTCGAG</u>TCTGGACTTCAGCAC; P-For: GAGA<u>AGGCCT</u>ATGTCGTTCCCTGAAGGA and P-Rev: GAGA<u>CTCGAG</u> CATAATTAACTGGTAGATGTC, restriction sites StuI and XhoI 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: ACA<u>GGATCC</u>GATGTCTCTTCAAGGGATTCAC, N-Rev: TAT<u>GAATTC</u>GCCTCATAATCATTTTGACTG, and the PCR product was digested and ligated into the BamHI/EcoRI 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).
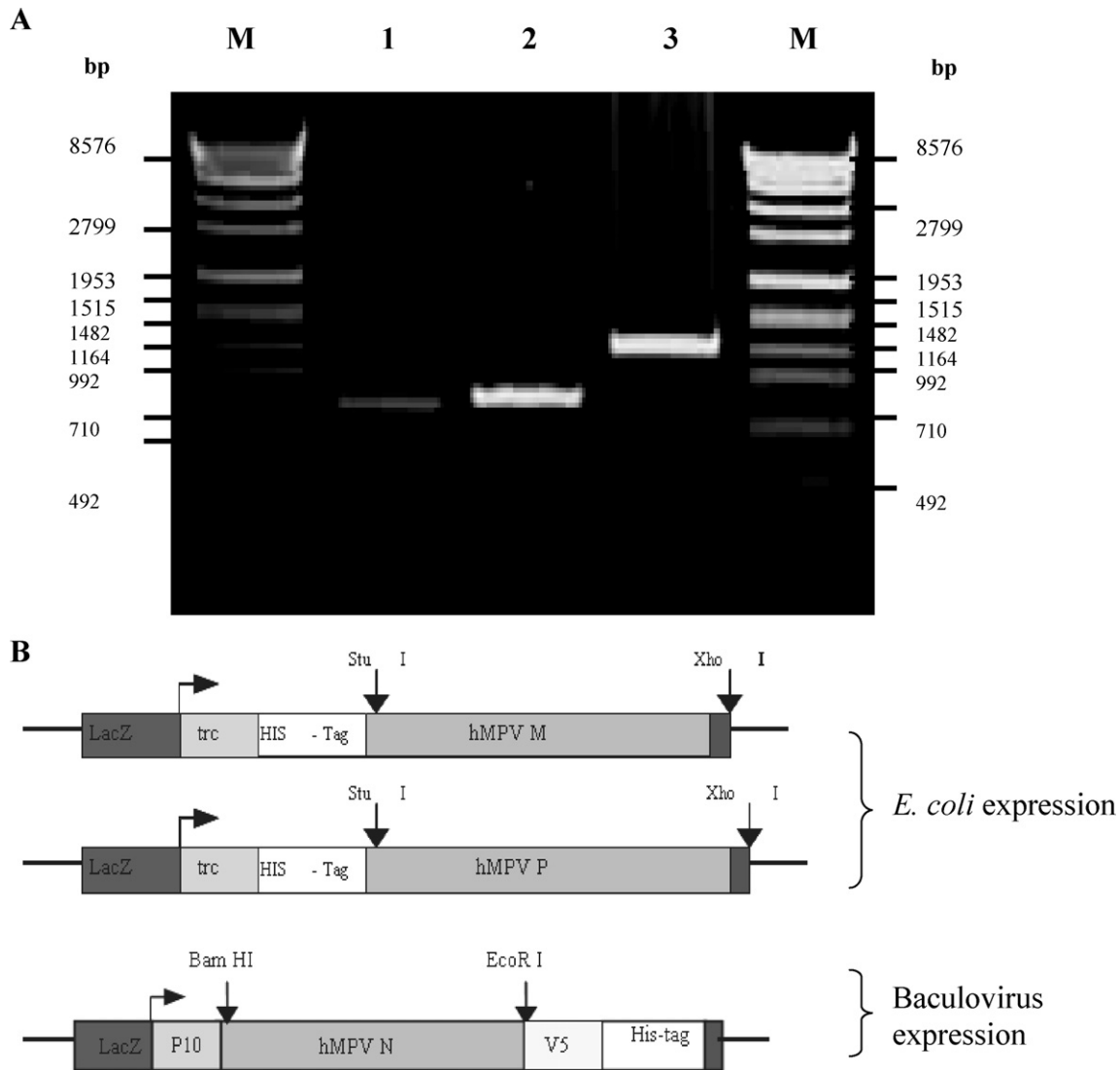
Fig. 1. (A) RT-PCR analysis of each of the HMPV genes. The PCR products for each gene were visualised on 1% (w/v) agarose gels. HMPV M cDNA product at 885 bp (lane 1); HMPV P cDNA product at 909 bp (lane 2); and HMPV N cDNA product at 1200 bp (Lane 3). DNA molecular weight Marker VII (Roche, lane M). (B) Schematic of the HMPV constructs used for recombinant protein expression in *E. coli* and insect cells. The HMPV/pProEX Htb constructs for recombinant protein expression in *E. coli*. A stop codon was inserted at the N-terminal end of the ORF of each gene to terminate translation (shaded box). The HMPV/pBlueBac 4.5/V5-His construct for recombinant protein expression in insect cells. A stop codon was inserted at the C-terminal end of the His$_6$ tag to terminate translation (shaded box).

Human sera were evaluated for HMPV IgG reactivity to the recombinant HMPV M, P, and N proteins by ELISA. Assay procedures were identical to that of Corcoran et al.[31] Immunoassay cut-offs were determined as the absorbance + 2 standard deviations greater than the mean absorbance obtained from a panel of HMPV IgG negative samples (negative by ELISA and Western immunoblots to all three antigens) and an index value (I.V.) less than 1.0 was considered seronegative. Seroreactivity were grouped into the following categories: seronegative (I.V. $\leq$ 1); weak seropositive (I.V. of 1–2); medium seropositive (I.V. of 2–4); high seropositive (I.V. $\geq$ 4).

3.8. B cell memory ELISpot assay

Briefly, peripheral blood mononuclear cells (PBMC) were isolated, quantified and cultured for five days in complete RPMI in the presence of heat-killed *Staphylococcus aureus* cells ((SAC); Cowan I strain) and interleukin-2 (IL-2). SAC and IL-2 jointly function to induce generalised antibody production in resting memory B cells. HMPV antigen-specific memory B cells were washed and quantified, as spot forming cells (SFC), by ELISpot technique as

previously described,[31] except that HMPV antigens were used for B cell capture.

4. Results

4.1. Expression of HMPV M

The expression of foreign proteins at high levels in *E. coli* often results in the formation of inclusion bodies of insoluble aggregates of the expressed protein. The HMPV M recombinant protein (32 kDa) was highly insoluble (present in the cell pellets), and was purified from inclusion bodies under denaturing conditions using differential extraction. The inclusion bodies were recovered from bacterial cell lysates by centrifugation and washing with Triton X-100 and EDTA to remove as much bacterial protein as possible from the aggregated protein. To obtain soluble HMPV M protein, the washed inclusion bodies were dissolved in denaturing agents and the released protein was refolded by gradual removal of the denaturing reagents by dilution and dialysis. A 32 kDa HMPV M band was observed following SDS-PAGE (Fig. 2), this was consistent with
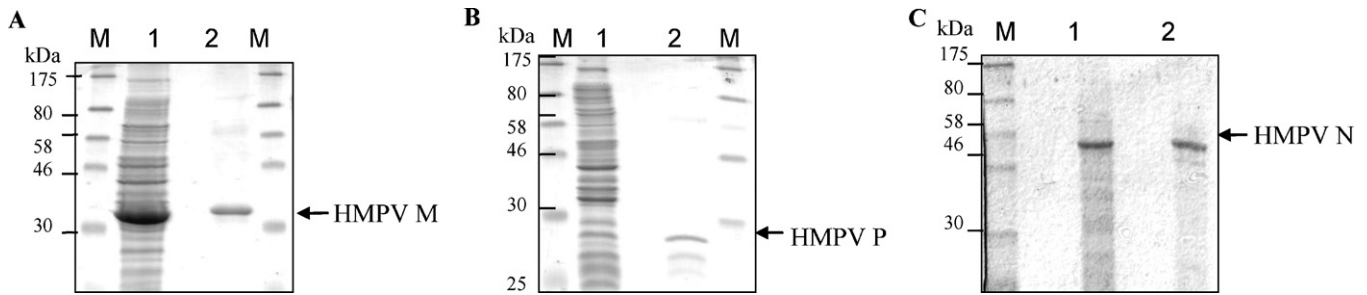
Fig. 2. SDS-PAGE analysis of each recombinant HMPV antigen. (A) HMPV M recombinant protein. Clarified cell lysate (lane 1), purified HMPV M at 32 kDa (lane 2). (B) HMPV P recombinant protein. Clarified cell lysate (lane 1), purified HMPV P at 29 kDa (lane 2). (C) HMPV N recombinant protein. Clarified supernatant from *Sf9* cells expressing HMPV N (lane 1), Ni-NTA affinity-purified HMPV N (47 kDa) (lane 2). Molecular Mass Marker (New England; P7703 (lane M)). SDS-PAGE was conducted according to Laemmli.[35] Briefly, samples were electrophoresed on 12% SDS-PAGE gels at 120 V under reducing condition for 1.5 h. Gels were stained with Coomassie Brilliant Blue R-250 (Sigma).

the predicted M_r of the M protein including the His$_6$ tag region at 31.6 kDa (Table 1). The purification process yielded 10 mg M protein/gram of cells. MALDI–ToF MS analysis of recombinant HMPV M following trypsin digestion confirmed the identity of recombinant HMPV M (GenBank accession no. gi|24429832) whereby 6/15 peptides (36% sequence coverage) was observed (Table 2).

Table 1
The predicted, versus actual M_r by SDS-PAGE, of each recombinant HMPV protein. The computation of the theoretical pI (isoelectric point) and M_r was determined using http://expasy.org/tools/pi_tool.html from the Swiss Institute of Bioinformatics.

Protein ID	Predicted pI	Predicted M_r (kDa)	Predicted M_r + His tag (kDa)	SDS-PAGE M_r (kDa)
M	8.2	27.6	31.6	32
P	4.81	32.71	36.7	29
N	6.73	43.51	47.5	47

Table 2
Mass spectrometry analysis of purified HMPV recombinant antigens. Sequence coverage of 35–56% confirmed the identity of each HMPV recombinant antigen.

Protein	Peptides identified	% Sequence coverage	Protein ID
HMPV M	K. TLTITTLYAASQSGPILK.V K.VNASAQGAAMSVLPK.K K.FEVNATVALDEYSK.L K.NTPVTIPAFIK.S K.ESESATVEAAISSEADQALTQAK.I K.TWSHQGTRYVLK.S	(6/15) 36%	gi|24429832
HMPV P	K. DILFMGNEAAK K.LAEAFQK K.VNTVSETLELPTISRPTKPTILSEPK K.KLAWTDK K.LKPSTNTKK K.LKPSTNTKKK K.KVSFTPNEPGK K.DALDLLSDNEEEDAESSILTFEER R.DTSSLSIEARLESIEEK R.LESIEEK K.LSMILGLLR	(12/28) 56%	gi|45388092
HMPV N	K.YAAEIGIQYISTALGSER R.VQQILR K.GEDLQMLDIHGVEK K.LASTIEVGLETTVR R.VLSDALKR R.SFYDLFEQK R.SLFIEYGK K.AESLFVNIFMQAYGAGQTMLR K.AESLFVNIFMQAYGAGQTMLR R.WGVIAR R.GRVPNTELFSAAESYAK R.VPNTELFSAAESYAK	(12/25) 35%	gi|38327234

4.2. Expression of HMPV P

Recombinant P protein was observed at 29 kDa following SDS-PAGE analysis (Fig. 2). The predicted M_r of the P protein plus the His$_6$ tag region is 36.7 kDa (Table 1). The discrepancy between the observed and predicted M_r may be attributed to the fact that the P protein has a low isoelectric point (pH 4.8) and therefore migrates further upon SDS-PAGE. The P Protein was purified by His$_6$ affinity chromatography under denaturing conditions. The relative yield of purified P protein was 3.82 mg/g of lysed *E. coli* (Fig. 2). MALDI–ToF MS analysis of recombinant HMPV P following trypsin digestion confirmed the identity of recombinant HMPV P (GenBank accession no. gi|4588092) with 56% sequence coverage (Table 2).

4.3. Expression of HMPV N in Sf9 insect cells

Insect cells were infected with recombinant baculovirus-encoding HMPV N at multiplicity of infection (MOI) = 10. The HMPV N recombinant protein was successfully purified by His$_6$ affinity chromatography under denaturing conditions using an AKTA chromatography system (Amersham). Purification resulted in a single major protein band a molecular mass of 47 kDa (Fig. 2). The purified protein yield was 1.5 mg/1×10^8 cells. MALDI–ToF MS analysis of the purified HMPV N protein confirmed identity with a 35% sequence coverage (Table 2).

4.4. Immunological analysis

To define the seroepidemiology of HMPV in an Irish population, ELISA systems were developed using three recombinant HMPV proteins as detection antigens. To investigate the seroprevalence of HMPV, a total of 96 human sera were screened by ELISA. Sera from this blood donor cohort ($n = 96$) were analysed in duplicate by ELISA against *E. coli*-expressed HMPV M and P antigens and the baculovirus-expressed HMPV N protein. To establish the immunoassay cut-offs for each of the HMPV ELISAs, a panel IgG negative sera from healthy individuals were identified by ELISA and Western immunoblots (Fig. 3). The cut-off was established as the absorbance + 2 standard deviations greater than the mean absorbance obtained from a panel of HMPV IgG negative samples (negative by ELISA and Western immunoblots to all three antigens) and an I.V. less than 1 was considered seronegative.

Of the total number of seropositive serum specimens (99%); 58% were weakly seropositive, 30% were showed medium seroreactivity and only 11% had high human IgG reactivity to the *E. coli*-expressed M antigen (Table 3). This extensive seroreactivity suggests that the M antigen binds IgG with high affinity or that the occurrence of specific anti-HMPV M IgG is common.

For the recombinant P ELISA, the overall total percentage seropositivity was 74% (70/96) and 26% were seronegative (Table 3).
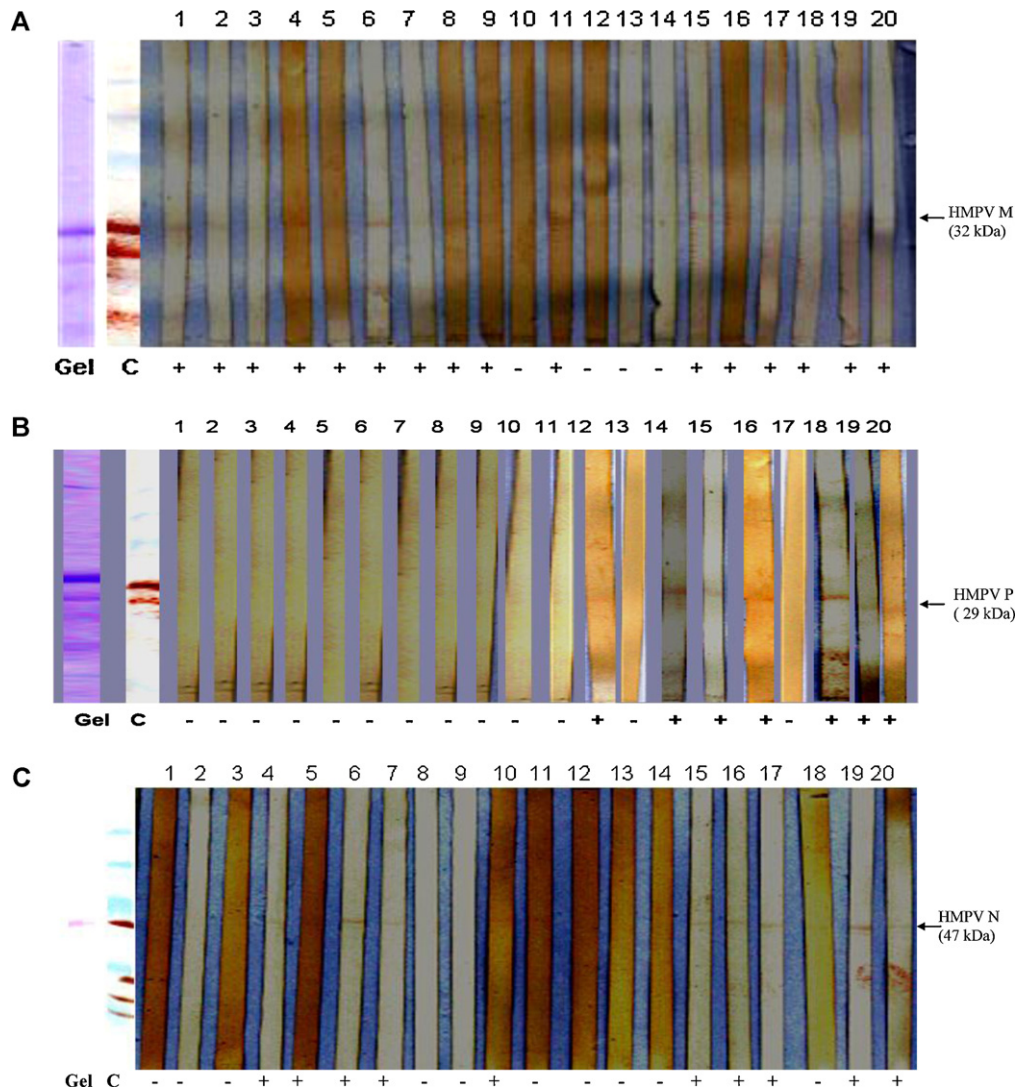
Fig. 3. Human sera ($n=20$) screened using IgG immunoblot containing denatured HMPV antigens. (A) HMPV M protein, (B) HMPV P protein and **C**. HMPV N protein. A positive control (C) was used (i.e., an immunostrip probed (1/1000)) with anti-His$_6$ monoclonal antibody which reacts with the His$_6$ region on each recombinant antigen. A representative set of 20 serum samples from the 96 sera tested by ELISA is shown. Prior to electrotransfer, a 1 mm strip was removed from the gel and stained with Coomassie Brilliant Blue R250, this was to ensure that 1 μg of each antigen was loaded per strip. The (+) and (−) signs indicate IgG positive and IgG negative specimens, respectively.

This suggests that the IgG in human sera is immunoreactive against the recombinant P protein. In fact, of all seropositive specimens (74%), 27% were weakly seropositive, 28% exhibited medium seroreactivity and only 19% had high IgG reactivity to the

Table 3
The percentage human IgG antibody reactivity to HMPV-M, P and N recombinant antigens. Seroreactivity is grouped into the following categories: seronegative (I.V. ≤ 1); weak seropositive (I.V. of 1–2); medium seropositive (I.V. of 2–4); high seropositive (I.V. ≥ 4). Each category was scored as a percentage of the total number of sera screened.

Protein ID	Seronegative	Weak Seropositive	Medium Seropositive	High Seropositive
		99% (95/96) Total Seropositive		
M	1% (1/96)	58% (55/96)	30% (29/96)	11% (11/96)
		74% (71/96) Total Seropositive		
P	26% (25/96)	27% (26/96)	28% (27/96)	19% (18/96)
		91% (88/96) Total Seropositive		
N	9% (8/96)	58% (56/96)	32% (31/96)	1% (1/96)

antigen. Therefore there was lower seroreactivity observed via ELISA within the sera cohort and may suggest that the P antigen is not as diagnostically useful as HMPV M.

For the recombinant N ELISA, the overall total percentage seropositivity was 91% (87/96), and 9% of specimens exhibited no reactivity against recombinant N antigen (Fig. 3). This data demonstrate that using the M protein results in a higher sensitivity than using the N protein, when subjected to ELISA analysis. Of the 96 specimens screened, 58% were weakly seropositive, 32% showed medium seroreactivity and only 1% had high IgG reactivity to the recombinant HMPV N antigen.

Table 4 shows the sequence identity between HMPV M, P and N proteins used in this study, compared to exemplar isolates representative of genotypes A1, B1 and B2.

4.5. Immunological memory analysis by B cell ELISpot

The use of an HMPV-specific B cell ELISpot offered the possibility to confirm past exposure to, and infection with, HMPV. It can be seen from Fig. 4 that all individuals ($n=10$) screened were HMPV IgG seropositive with the exception of one individual (E10) who
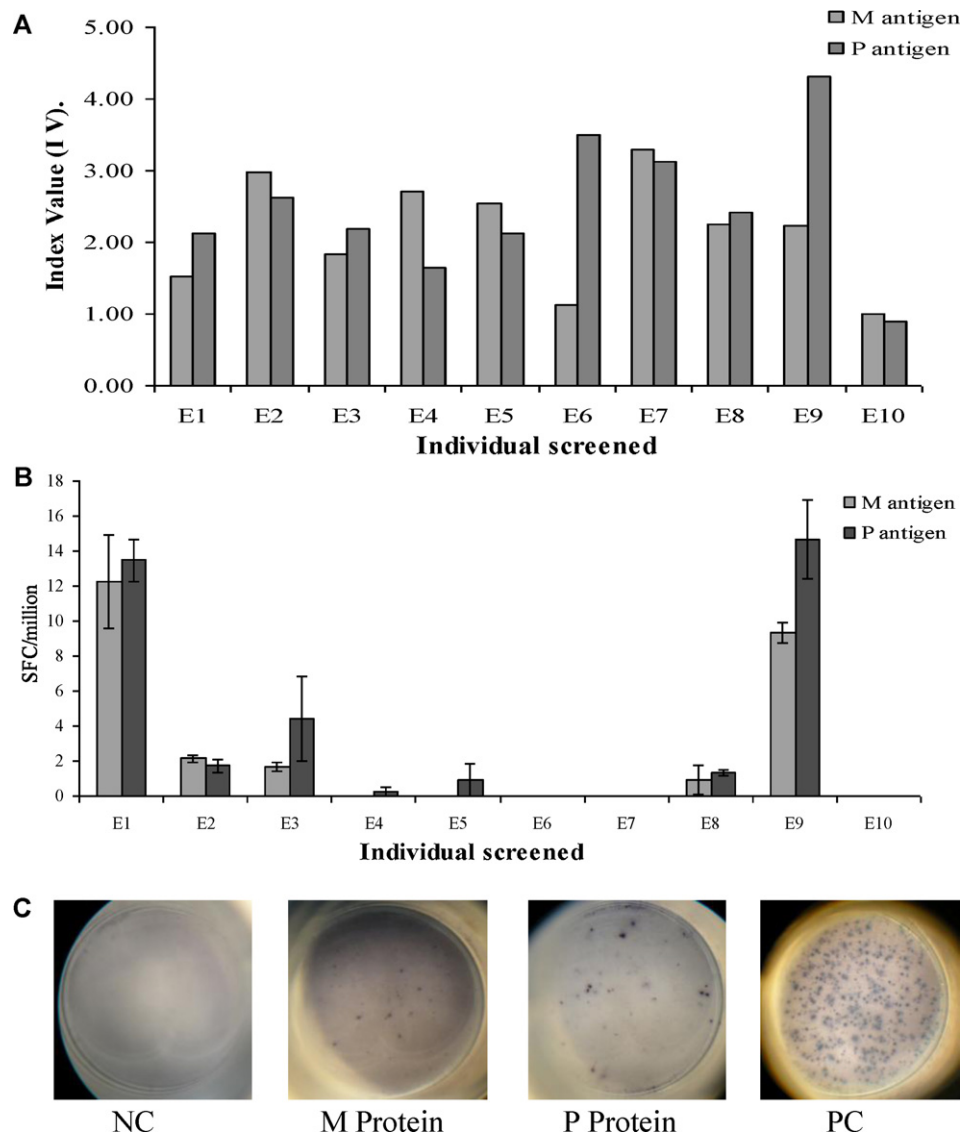
Fig. 4. ELISA and B cell ELISpot assay. (A) ELISA of human IgG response in plasma obtained from adult volunteers ($n = 10$) against the HMPV viral proteins M and P, expressed as index values (I.V.). An index value above 1 is considered seropositive. Serum E10 is from a seronegative individual (negative control). (B) B cell memory, directed against HMPV antigens, are expressed as SFCs/1 million starting cells and are the mean ± SD of the triplicate wells. ELISpot microplates were coated with either HMPV M protein (grey) or HMPV P protein (dark shaded) at $10\,\mu g/ml$, respectively. **C.** Reactive memory B cells to both HMPV M and HMPV P recombinant antigens. Buffer only (negative control (NC), no antigen was coated on the plate) and positive control (PC) (anti-human IgG on the plate to detect all activated memory B cell which secreted IgG). (Scale = $10\times$ magnification.)

was seronegative by ELISA. The seronegative individual (E10) and four seropositive individuals (E4, E5, E6 and E7) did not appear to harbour high levels of HMPV-specific memory B cells (Fig. 4). Five seropositive individuals (E1, E2, E3, E8 and E9) exhibited detectable

(range: 1–14.6 SFC/million B cells) HMPV-specific B cell memory against HMPV M and HMPV P antigens (Fig. 4).

5. Discussion

To define the seroepidemiology of HMPV in an Irish population, we developed enzyme-linked immunosorbent assays (ELISAs) based on the expression of HMPV M and P proteins in *E. coli* and the recombinant HMPV N produced in *Sf9* insect cells. A sero-epidemiological survey using these HMPV recombinant antigen-based ELISA systems was employed to determine the prevalence of antibodies to specific HMPV proteins in Ireland. The total antibody reactivity to HMPV M, P and N antigens correlated well, in particular there was significant correlation between the seroreactivity observed in both the M and N ELISA systems, ($r = 0.96$; Table 3). The correlation between the presence of HMPV antibodies determined by the M and P ELISAs was lower ($r = 0.57$) compared

Table 4
The percentage protein sequence identity for each HMPV antigen from isolate ROI135 genotype A2 compared to representative isolates from each of the other HMPV genotypes. The GenBank accession number of each isolate is as follows: JSP03 (AY530092); NL-1-99 (AY525843) and BJ1816 (DQ843658).

HMPV isolate	Genotype	% Sequence identity		
		M protein	P protein	N protein
JSP03	A1	98.8	96.3	99.5
NL-1-99	B1	97.2	85	95.2
BJ1816	B2	97.2	86.1	96.2

to the correlation between HMPV M and N ELISAs. Significant correlation was also observed between the N and P ELISAs ($r = 0.74$). The lack of correlation between the M and P protein-based ELISA systems may be due to variable levels of IgG to each recombinant antigen within the selected donor cohort. Ishiguro et al. tested for specific antibodies against nucleocapsid (N) and matrix (M) proteins in 97 sera by Western blot using recombinant N and M proteins of HMPV expressed in *E. coli*.[33] The results were compared with those of immunofluorescence assays (IFAs) based on HMPV-infected LLC-MK2 cells, which expressed the whole HMPV proteome. Their results indicated that the antibodies against N and M proteins are highly specific (100%) but less sensitive (42.1%, N protein; 40.8%, M protein) than those against whole proteins of HMPV detected by IFA. This would also suggest that a multi-HMPV antigen ELISA would increase the sensitivity of the detection of HMPV antibodies within human sera. However, the lower detection of P antigen-specific IgG may be due to the relatively low percentage sequence identity between HPMV P (from the A2 genotype) and genotypes A1 B1/2, respectively.[34] The HMPV genotype A2 proteins (M, P and N from ROI135) used in this study exhibited 97.2–98.8, 85–96.3, and 95.2–99.5% sequence identify to selected examples of genotypes A1 and B1/2, respectively (Table 4). Extensive validation of the HMPV ELISA systems described herein, whereby antigen coating on microtitre plates was optimised (2, 3 and 5 μg/ml for HMPV M, P and N, respectively) and assay reproducibility, in terms of % coefficient of variation, was determined to be 5.5, 8 and 8%, respectively, preclude sub-optimal assay performance as a reason for the observed differences in antibody detection. In summary, recombinant M, P and N proteins of HMPV were antigenic, and the responses to M, P, and N proteins differed across the study population. Assays based on multiple antigens provided higher sensitivities than assays based on single antigens.

The defining feature of the acquired immune system is its ability to generate immunological memory to a particular pathogen in defence of re-infection. Recently, considerable efforts have been directed towards the elucidation of the nature of the T cell response to HMPV and the definition of T cell epitopes,[26–30] yet the nature of B cells memory directed against HMPV remains unclear. All individuals ($n = 10$) screened were HMPV IgG seropositive with the exception of one individual E10, who was seronegative for HMPV specific IgG (Fig. 4). Five individuals (E1, E2, E3, E8 and E9) exhibited low, but detectable (range: 1–14.6 SFC/million B cells) B cell memory. There was a significant correlation between the number of memory B cells directed against HMPV M and HMPV P antigens ($r = 0.655$). However, the greatest numbers of SFCs were evident in two individuals (E1, mean ± SD: 13.5 ± 1.2 SFC/million cells and E9, 14.7 ± 2.2 SFC/million cells). B cell memory could not be detected in the absence of IL-2 and SAC stimulation prior to ELISpot analysis, which was an unstimulated B cell control (Fig. 4).

When the IgG reactivity of the ten specimens (E1–E10) against HMPV M was analysed by ELISA and compared against the B cell ELISpot data, no statistically significant positive correlation was observed between SFC/million cells and antibody reactivity ($r = 0.022$). Conversely, a correlation was observed between the ELISpot results and IgG reactivity to the HMPV P antigen ($r = 0.655$) for individuals exhibiting IgG specific antibody responses to the HMPV P antigen. This confirms that previous exposure to HMPV also results in B cell memory (mainly individuals E1 and E9).

The lack of correlation between the ELISpot data obtained for the M protein and that obtained for ELISA data is not unusual; the detection of specific serum antibodies is the most widely applied method to investigate immunity against diseases like HMPV, although a specific correlate of protection against HMPV has not been established yet. Besides antibodies, long-term memory B and T cell immunity might play an important role in protection against HMPV. In some individuals, high antibody levels with low or no numbers of memory B cells were found. This strengthens the idea that long-lived plasma cells maintain antibody levels and that memory B cells are a distinct population of cells. Memory B cells do not secrete antibodies prior to activation. Upon renewed antigen stimulation, memory B cells may rapidly respond, proliferate and differentiate in antibody secreting cells. The ongoing circulation of HMPV among the Irish population causing antigenic re-challenge might explain the low correlation between circulating HMPV-specific memory B cells and antibody levels measured in our study. Importantly, in some individuals no HMPV-specific memory B cells, as well as low HMPV-specific antibody levels, were detectable which might result in a higher susceptibility for infection with HMPV.

In conclusion, this work has provided multiple new detection systems for, and establishes the seroprevalence of, HMPV IgG in an Irish population. We also report the first demonstration of HMPV-specific B cell memory against HMPV M and P antigens which furthers our knowledge on the antigen-specific B cell memory against the HMPV viral proteins. Determination of antigen-specific B cell memory status may enhance the serological and molecular analyses of persistent HMPV infection. Further studies are warranted to elucidate the nature of differential seroreactivity to each viral antigen.

Competing interests

Funding

Ethical approval

Ethics permission to obtain and use material of human origin was obtained from the NUI Maynooth Ethics Committee.

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