

*DEVELOPMENT OF A NOVEL
IMMUNOASSAY FOR THE DETECTION
OF HUMAN METAPNEUMOVIRUS IN
CLINICAL SPECIMENS.*

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Abstract

Human metapneumovirus (hMPV) has been described as a causative agent for acute respiratory tract infections and is considered one of the main causes of hospitalisation for such infections in young children worldwide. Here we describe an antigen detection system utilising a combination of monoclonal antibodies directed to major hMPV proteins. The performance characteristics of the immunoassay system were evaluated using panels of confirmed hMPV positive or negative respiratory specimens. The prototype assay results exhibited close correlation to PCR data. A high level of specificity was demonstrated with a panel of potentially cross-reactive, non-hMPV, specimens. The newly developed immunoassay has been found to be reactive with the four main genotypes of hMPV. This efficient, specific assay may have useful implications in hMPV detection and/or in culture confirmation. Details of sensitivity and specificity are presented.

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Introduction

Acute respiratory tract infections (ARTI) are a leading cause of illness and mortality worldwide⁽¹⁾. In 2001, Van den Hoogen et al. reported the discovery of a novel human virus associated with respiratory tract disease⁽²⁾. The new virus was classified as the first member of the human Metapneumovirus genus (hMPV). hMPV has been associated with both upper and lower ARTIs and primary infection is believed to cause the most severe symptoms^(3, 4). The most severe cases are to be found in pediatric patients, although cases have been identified in all age groups, with heightened concern for elderly patients and immunocompromised hosts⁽⁵⁾.

Viral agents responsible for ARTIs often remain unidentified. The aetiology of approximately 40% to 60% of community-acquired ARTIs remains undetermined⁽⁶⁾. Biotrin has developed an antigen capture assay for the detection of hMPV viral proteins from respiratory specimens. The test is specific for two major structural hMPV viral proteins, the matrix (M) and fusion (F) proteins.

In this study a panel of 21 hMPV positive specimens were tested to determine assay sensitivity as compared to PCR data. A panel of confirmed hMPV PCR negative respiratory specimens including potential cross-reactive specimens (RSV, Adenovirus, Influenza and Parainfluenza) was also tested to assess assay specificity.

References

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Methods

Monoclonal antibodies directed against hMPV matrix and fusion proteins were coated onto microtitre plates at varying concentrations to determine an optimum antigen capture coating ratio. In initial studies hMPV viral cultured material was detected on the coated plates using a pool of monoclonal antibodies conjugated to HRP; again at optimal ratios.

hMPV positive Vero cell cultures of genotypes A and B (including subtypes 1 and 2) were tested at varying dilutions on the assay to determine the range of specificity of the monoclonal antibodies utilised. A hMPV positive culture specimen from a patient NPA (TCID50 of 3.1×10^5 / ml, ct value = 23) was also tested on the assay undiluted and at further dilutions (1:2, 1:10 and 1:20 in PBS).

A panel of 42 hMPV positive and negative respiratory specimens (primarily nasal wash (n =35), with some bronchoalveolar lavage / aspirate (n = 3), nasopharyngeal aspirate (NPA) (n = 1) and tracheal aspirate (n = 3), including potential cross-reactive specimens, was tested by PCR and on the hMPV EIA for comparison. Potential cross-reactive specimens included in the negative sample group were positive for RSV, ADV, IFA, PIV respiratory viral culture.

The 42 specimens were screened with a 1-step RT-PCR procedure, with the F2 primer set⁽⁷⁾. PCR products were analyzed by electrophoresis (BioRad, Hercules, CA, USA) in a 1.2% (wt/vol) agarose gel stained with ethidium bromide. Screened specimens that gave bands within 200 bp of the expected 347-bp product were further tested with a 2-step RT-PCR with F1-, F2- and N-gene primer sets⁽⁷⁾. PCR products were analysed by gel electrophoresis. Specimens were designated RT-PCR-positive if the confirmatory N-gene primer set and at least 1 of the confirmatory F-gene primer sets yielded a band within 50 bp of the expected size (for primer sets refer to Table 1).

A simple immunoassay procedure was adhered to in which 75ul sample was mixed with 25ul extraction buffer immediately before addition to the well. After a wash step the anti hMPV

monoclonal-HRP conjugate was incubated on the plate, after which a TMB substrate was added. When the reaction was stopped the signal was measured at an absorbance of 450nm (FIGURE 1). The Cut Off for the assay was determined by obtaining the mean OD of the negative specimens tested, this figure was then added to the standard deviation (X2) of the same data set. The resultant **Cut Off was 0.19 OD units**. Assay sensitivity and specificity were calculated based on the Cut Off value and on a correlation to PCR data.

To ascertain the limit of detection for recombinant matrix (M) protein, and to determine if there was reactivity with nucleoprotein (N), two separate recombinant proteins for each group were assessed. Proteins at a starting concentration of 1ug/ml each were diluted serially and tested on the hMPV antigen assay.

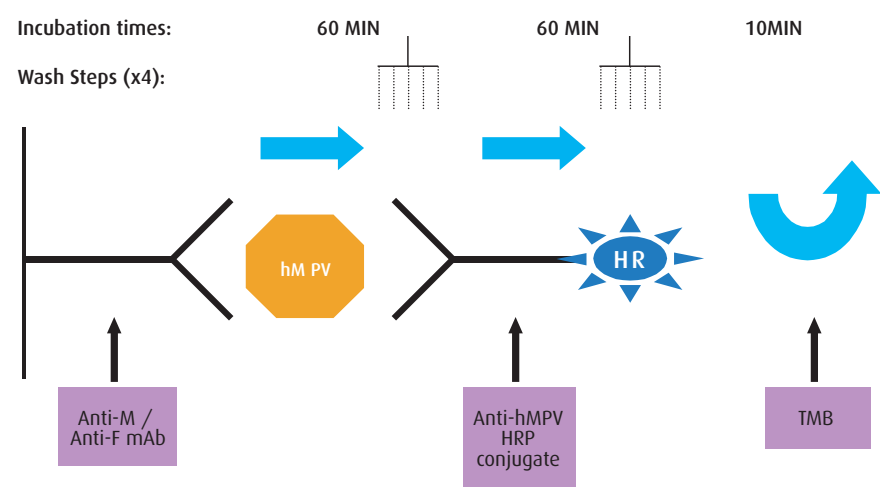


Figure 1. Biotrin hMPV antigen assay format.

Results

- hMPV infected Vero cells were harvested and tested on the hMPV antigen assay. Results show reactivity of the four types of viral strains at varying dilutions (FIGURE 2).
- A hMPV patient specimen was cultured and tested on the hMPV assay. Results indicate that the cultured specimen is reactive at TCID50 3.1×10^5 / ml equivalence (FIGURE 3).
- Of the 21 RT-PCR characterised positive specimens tested on the Biotrin hMPV antigen assay 100% reacted with an OD greater than the Cut Off (0.19). 20 of the 21 RT-PCR characterised specimens were below the Cut Off OD value (0.19) (FIGURE 4). The data was analysed by boxplot for comparison of sample groups, i.e., hMPV RT-PCR positive vs. negative specimens (FIGURE 5).
- Limit of detection was determined for recombinant matrix (M) proteins 1 and 2. The Limit calculated was ~ 0.32ng. Nucleoprotein (N2) at 1ug/ml gave an OD value just above the Cut Off at 0.249 OD units. (FIGURE 6).

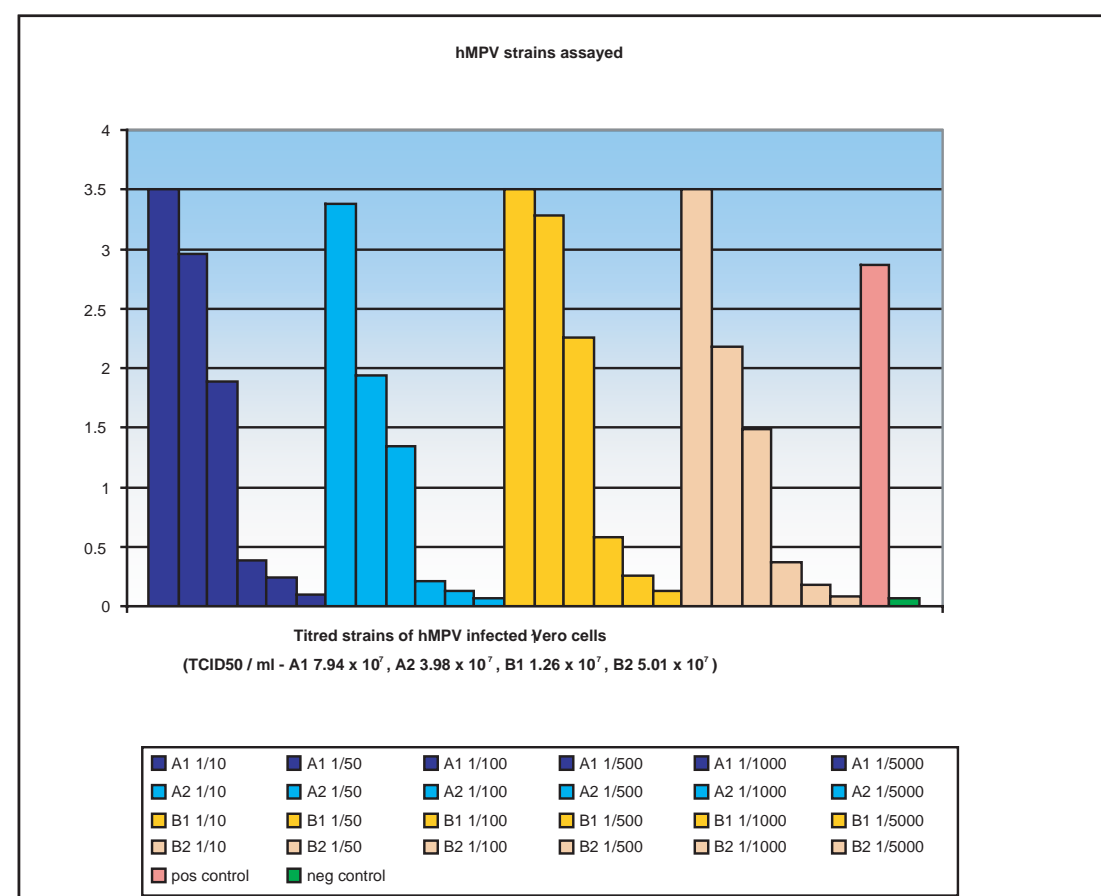


Figure 2. Strain detection of hMPV viral culture material on the Biotrin hMPV antigen assay.

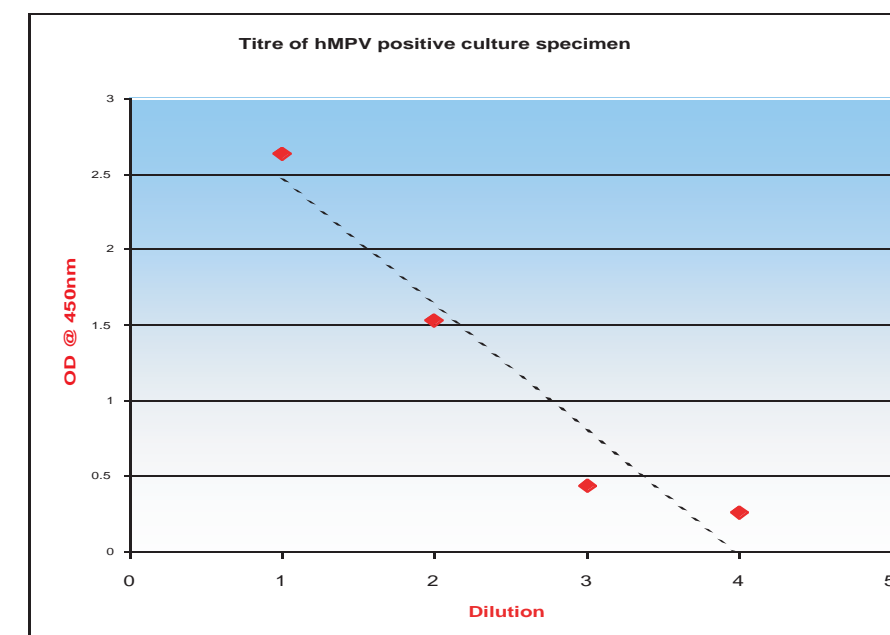


Figure 3. hMPV positive culture specimen (TCID50 of 3.1×10^5 / ml) serially diluted on the Biotrin hMPV antigen assay.

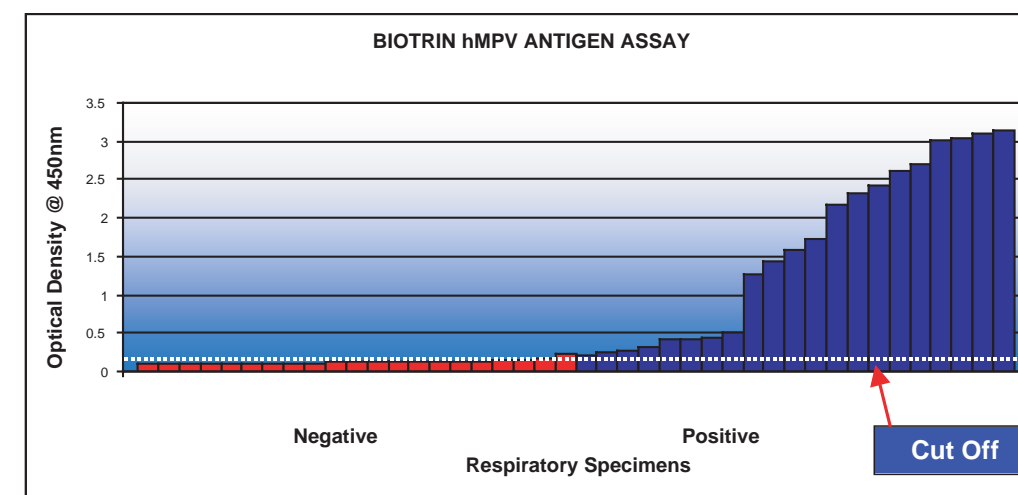


Figure 4. 21 RT-PCR hMPV positive and 21 RT-PCR hMPV negative specimens tested.

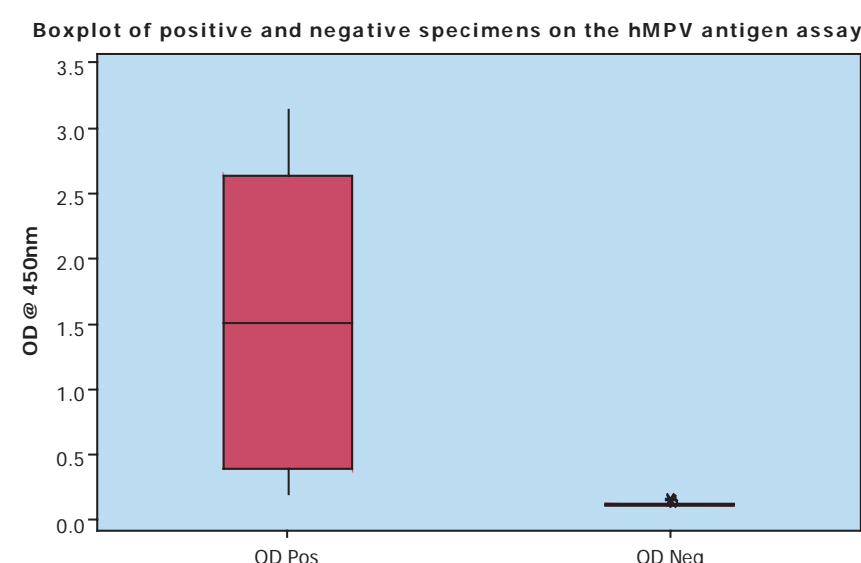


Figure 5. Boxplot of hMPV RT-PCR characterised specimens.

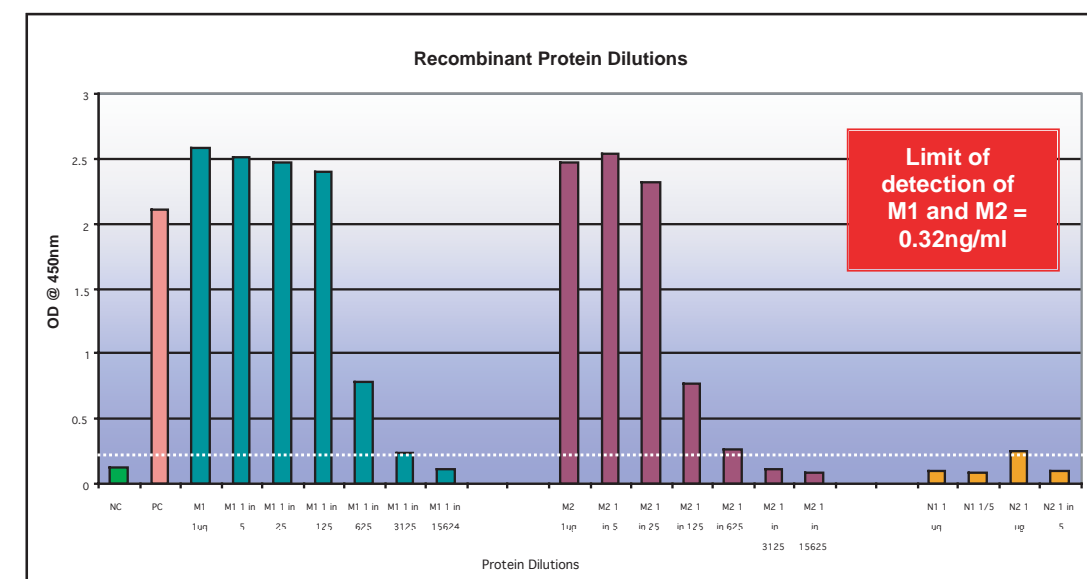


Figure 6. Limit of detection of Biotrin hMPV antigen assay for Matrix recombinant protein.

Conclusion

The assay has been shown to detect hMPV infected Vero cells for all four strains (A1, A2, B1 and B2) at a TCID50/ml of $\sim 1 \times 10^4$ equivalence (as calculated from the dilution series of cultured virus tested). The assay has also detected a positive patient specimen cultured for diagnosis. This isolate tested positive, assuming the Cut Off to be 0.19 OD units, at a TCID50 of $\sim 3.1 \times 10^4$ / ml showing a similar level of sensitivity as for the infected Vero cell cultures. Results indicate that the assay could potentially be useful in the interpretation of cultured virus methods to determine hMPV infection.

Of samples tested 100% of positive specimens gave an optical density (OD) reading of greater than the mean negative OD value (n=21) plus two standard deviations (0.19 OD units). Of the 21 negative specimens including the cross-reactives tested, one sample gave an OD reading of 0.242, which was greater than the limit of the assigned assay cut off (0.19). This translated to an assay sensitivity of 100% and an assay specificity of 95.24%.

The sample that gave a potential false positive result was found to be hMPV negative by RT-PCR and negative for viral culture for other respiratory viruses, therefore this sample was not identified as a potential cross-reactive specimen. The inclusion of the panel of potential cross-reactive specimens has indicated a high level of specificity of the monoclonal antibodies utilised. Results have been found to be reproducible with the same sample population, R² 0.957.

The limit of detection for recombinant matrix material is calculated at 0.32ng/ml or 0.032ng in the well indicating the assay's degree of sensitivity. The matrix protein is ubiquitous for hMPV and is one of the major structural proteins.

Overall the Biotrin hMPV antigen assay has shown a high level of sensitivity and specificity with excellent correlation to PCR for clinical respiratory specimens. The assay would be potentially invaluable to the clinician in determining the hMPV profile of fresh or banked respiratory specimens and/or as a culture confirmation assay. Its fast and easy to use format should make this assay the hMPV antigen assay of choice.

Table 1. Oligonucleotide primers used for amplification and sequencing of hMPV.

Primer set name	Target	Amplicon size (bp)	Sequence
F1 [13]	F gene	450	Forward 5' - CTTTGACTTAATGACAGATG-3'
			Reverse 5' - GTCTTCCTGTGCTAACITTTG-3
F2 [25]	F gene	347	Forward 5' - GAGCAAATTGAAAATCCAGACA-3'
			Reverse 5' - GAAAACCTGCCACAACATTAG-3
N1 [2]	N gene	210	Forward 5' - AACCGTGACTAAGTAGTGCACTC-3'
			Reverse 5' - CATTGTTTGACCCGCCATAA-3'

Abbreviations

- RSV = Respiratory Syncytial Virus
- RT-PCR = reverse transcription-Polymerase Chain Reaction
- M = Matrix protein
- N = Nucleoprotein
- OD = Optical Density
- HRP = Horse Radish Peroxidase
- TMB = Tetramethylbenzidine

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