JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2005, p. 1950–1952 Vol. 43, No. 4
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.4.1950–1952.2005

Detection of Human Metapneumovirus in Clinical Samples by Immunofluorescence Staining of Shell Vial Centrifugation Cultures Prepared from Three Different Cell Lines

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Received 7 June 2004/Returned for modification 9 August 2004/Accepted 23 November 2004

Monoclonal antibody MAb-8 was evaluated for detection of human metapneumovirus (HMPV) in shell vial centrifugation cultures (SVCC). Detection of HMPV was similar in A549, HEp-2, and LLC-MK2 SVCC, and MAb-8 staining was optimal on day 2 postinoculation. Availability of SVCC for HMPV will be of significant benefit to clinical laboratories.

Since its recent discovery, human metapneumovirus (HMPV) has been recognized as a common cause of respiratory infections (4, 5, 8, 10, 11), especially in young children. To date, laboratory diagnosis of HMPV infection has been limited due to the difficulty in growing the virus and the lack of readily available diagnostic reagents. Although HMPV can be isolated in conventional cell cultures such as LLC-MK2, the appearance of recognizable cytopathic effects can take 2 weeks or more and immunoreagents have not been commercially available to identify HMPV isolates. Amplification of HMPV genomic RNA by reverse transcription-PCR (RT-PCR) is more sensitive and rapid than virus isolation, and many papers have been published on molecular methods (2, 7). However, many clinical laboratories do not presently have the capability of performing routine diagnostic RT-PCR for HMPV.

Immunofluorescence staining (IF) of clinical specimens and shell vial centrifugation cultures (SVCC) are methods commonly used in clinical virology laboratories for rapid diagnosis (6, 9). In order to perform these tests, sensitive and specific monoclonal antibodies are needed. In this study, we evaluated a monoclonal antibody (MAb-8) to HMPV matrix protein developed at the Centers for Disease Control and Prevention (CDC) for its utility in the rapid diagnosis of HMPV by both IF and SVCC methods.

Nasopharyngeal (NP) aspirates and swabs (MicroTest M4 medium; Remel, Lenexa, KS) submitted to the Clinical Virology Laboratory for respiratory virus testing on children less than 5 years old from January through March of 2003 were used for this study. All samples were tested for respiratory viruses as previously described (6). Extra slides and excess samples were stored at −70°C for 3 to 12 months until tested for HMPV.

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HMPV stock virus was obtained from Guy Boivin (10). A murine monoclonal antibody (MAb-8) to HMPV strain MPV75-1998/CAN98-75 (9) was developed at CDC by standard methods and was selected for this study, since it gave the best staining properties among antibodies tested. MAb-8 targets the HMPV matrix protein and was broadly reactive with HMPV isolates representing both major HMPV genogroups and was nonreactive with cultures of respiratory syncytial virus, parainfluenza virus types 1 to 4, influenza A and B, adenovirus, mumps and measles virus, rhinovirus, and herpes simplex virus (data not shown). MAb-8 is now available commercially as MAB8510 (Chemicon International, Temecula, CA).

For IF, NP samples were tested as described previously (6). For SVCC, LLC-MK2, A549, and HEp-2 cells in shell vials were each inoculated with 0.2 ml of patient specimen. Shell vials were centrifuged for 45 min at 2,000 rpm (700 × *g*) at room temperature, and then 1.0 ml of virus growth medium (albumin, porcine pancreatic trypsin, glucose, antibiotics) was added, followed by incubation at 37°C for 1 to 5 days. Medium was removed, monolayers were rinsed, and 1.0 ml cold acetone was added for 10 min. After air drying, 175 μl of MAb-8 working dilution (1:300) was added to each shell vial and incubated for 45 min at 37°C in a moist chamber. Coverslips were washed twice, and then 175 μl of conjugate working dilution (Alexa Fluor goat anti-mouse immunoglobulin G; Molecular Probes, Inc., Eugene, OR) (1:300) was added to each shell vial. After incubation for 45 min at 37°C in a moist chamber, coverslips were washed twice, mounted (cell side down) on glass slides containing one drop of mounting medium, and then examined using epifluorescence microscopy (×250).

A total of 159 clinical specimens were tested for HMPV RNA by RT-PCR (3, 5). All samples were also tested for GAPDH by an RT-PCR assay to insure adequate recovery of sample RNA and the absence of RT-PCR inhibitors (1). All RT-PCR-positive specimens were tested by a quantitative real-time TaqMan RT-PCR assay to determine the relative viral load. The primers and probe that were designed to target the

TABLE 1. Comparison of HMPV detection by shell vial centrifugation cultures in three cell lines stained with MAb-8 at days 1, 2, and 3 postinoculation

Day of shell vial culture	Highest dilution positive in cell line:		
	A549	LLC-MK2	HEp-2
1	10^{-4}	10^{-4}	10^{-4}
2	10^{-5}	10^{-5}	10^{-5}
3	10^{-5}	10^{-4}	10^{-5}

HMPV fusion protein gene were as follows: sense primer, 5′-CAA GTG TGA CAT TGC TGA CCT GAA-3′; antisense primer, 5′-ACT GCC GCA CAA CAT TTA GAA A-3′; and probe, 5′-TG GCY GTY AGC TTC AGT CAA TTC AAC AGA-3′. The assay was performed using TaqMan One-Step RT-PCR master mix (Applied Biosystems, Foster City, Calif.). Thermocycling conditions consisted of 30 min at 48°C for RT, 10 min at 95°C, and 45 cycles of 15 s at 95°C and 1 min at 60°C.

Cytospin slides prepared for the 159 NP samples were examined by indirect IF staining. Nonspecific background staining made interpretation difficult, and only four samples were definitively reported positive. After training on the recognition of HMPV-positive cells, blinded rereading of IF slides by two different technologists detected 10 (67%) and 12 (80%), respectively, of 15 RT-PCR-positive samples.

LLC-MK2 shell vials were inoculated with HMPV stock virus and stained with MAb-8 at 2 and 5 days postinoculation (p.i.). HMPV was detected with multiple fluorescent foci up to the 10^{-5} dilution at day 2 p.i., whereas staining at day 5 p.i. detected only questionable foci at the 10^{-3} dilution and reduced staining at 10^{-1} and 10^{-2}. Hence there was a 2 $\log_{10}$ reduction in sensitivity from day 2 to day 5. Two experiments were then performed with three cell lines, A549, LLC-MK2, and HEp-2, to determine the optimal time for staining with MAb-8. All three cell lines were similar in sensitivity, and incubation for 2 days was optimal (Table 1). Staining was brighter in HEp-2 and LLC-MK2 than in A549 shell vials. The number of positive cells was somewhat higher in LLC-MK2. However, since the HMPV stock virus had been serially passaged in LLC-MK2 this may represent positive selection. Specific staining was characterized in the early stages by cytoplasmic speckling and later by prominent cellular and extracellular brightly staining filamentous forms. Nonspecific staining was also seen but was more homogeneous, involved scattered discreet foci of cells, and appeared to be predominantly in the extracellular matrix and the cell membrane.

Limited sample remained from 10 of the 15 RT-PCR-positive clinical specimens for further study. These samples were serially diluted, inoculated into three cell lines, and incubated and stained 2 days p.i. In order to inoculate all three cell lines, reserve an aliquot for RT-PCR, and perform serial dilutions, the dilutions of the samples differed. For samples with lower virus titers and limited remaining sample amounts, serial dilutions of less than 10-fold were employed. As shown in Table 2, HMPV-infected cells were detected in six samples in two cell lines (LLC-MK2 and HEp-2) and seven samples in one cell line (A549). However, HEp-2 was positive at the highest positive dilution in six of eight samples, LLC-MK2 was positive at the highest positive dilution in four of eight samples, and A549 was positive at the highest positive dilution in three of eight samples. Intensity of staining was somewhat greater in LLC-MK2 and HEp-2 than in A549. Five randomly selected RT-PCR-negative clinical samples were also inoculated as known negative controls. Nonspecific staining occurred in the SVCC but could be readily distinguished from specific staining. RT-PCR testing of serial dilutions used to inoculate the SVCC determined that one sample (sample 9) was negative by RT-PCR at the 1:2 dilution and that one (sample 54) was positive by RT-PCR only at the 1:2 dilution. For the other eight SVCC-positive samples, virus could be detected at least one to two $\log_{10}$ higher by RT-PCR than by SVCC. Although trypsin was included in the SVCC medium, studies in our laboratory with laboratory-passaged virus indicated that trypsin did not enhance virus recovery (data not shown). However, insufficient clinical material was available for us to assess the value of trypsin for recovery of wild-type HMPV from clinical specimens.

TABLE 2. Sensitivity of shell vial centrifugation cultures stained at day 2 with MAb-8 compared with RT-PCR for detection of HMPV in clinical samples

Sample no. (C_T value)[b]	Highest sample dilution positive by method[a]:			
	A549 SVCC	LLC-MK2 SVCC	HEp-2 SVCC	RT-PCR of inoculum
46 (27)	1,000	1,000	≥10,000	≥10,000
101 (22.8)	1,000	1,000	1,000	≥10,000
127 (27.7)	6	6	36	≥1,296
116 (29.8)	2	10	10	≥10,000
15 (35.5)	2	10	10	100
138 (35.8)	2	2	Neg	≥1,000
135 (32.5)	Neg	Neg	2	≥432
118 (34.5)	2	Neg	Neg	≥72
54 (36)	Neg	Neg	Neg	2
9 (38.3)	Neg	Neg	Neg	Neg
No. positive	7	6	6	9

[a] Starting dilution 1:2; however, dilution series varied among specimens. Neg, negative results obtained at 1:2 dilution.

[b] C_T, cycle threshold for real-time HMPV RT-PCR result on original undiluted sample; the lower the C_T number, the higher the virus concentration in the sample.

Human metapneumovirus has recently been recognized as a common respiratory pathogen affecting all ages, but especially the very young and the elderly (4, 5, 11). To date, diagnostic testing remains largely confined to research settings where RT-PCR is the most widely used assay (2, 7). The development of anti-HMPV monoclonal antibodies (MAb-8) allowed evaluation of immunofluorescence-based methods that are widely used for the rapid diagnosis of other viral respiratory pathogens and can be readily implemented in clinical virology laboratories (9).

Use of MAb-8 in IF staining of clinical specimens was not successful. Nonspecific background staining made reading very tedious and interpretation difficult. In contrast, results from SVCC inoculated with both laboratory-passaged virus and clinical specimens were very encouraging. All three cell lines tested were acceptable, and the ability to detect positives by days 1 and 2 after inoculation is a great advantage over present conventional culture methods. Although nonspecific staining occurred in SVCC, with experience it could be distinguished

from specific staining. It is recommended that positive and negative controls stained with MAb-8 always be included for reference.

In the future, incorporating HMPV antibodies into antibody pools to screen for multiple respiratory viruses in SVCC, evaluating mixed cell cultures for recovery of HMPV, and obtaining a labeled primary anti-HMPV antibody to shorten assay time will facilitate diagnosis of HMPV in clinical laboratories. MAb-8 is now available commercially (MAB8510, Chemicon International, Temecula, CA). The availability of SVCC for rapid diagnosis of HMPV will be of significant benefit to clinical laboratories, especially those without molecular diagnostic capabilities.

HMPV stock virus was graciously provided by Guy Boivin. We thank George Gallucci and Shannon Emery (Respiratory and Enteric Viruses Branch, CDC) for performing the nucleic acid extractions and the PCR assays.

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