

Short communication

Comparison of different cell lines and incubation times in the isolation by the shell vial culture of human metapneumovirus from pediatric respiratory samples

Jordi Reina^{a,*}, Francesc Ferres^b, Eva Alcoceba^a, Aina Mena^a,
Enrique Ruiz de Gopegui^a, Joan Figuerola^b

^a Virology Unit, Clinical Microbiology Service, University Hospital Son Dureta,
Andrea Doria 55, 07014 Palma de Mallorca, Spain

^b Pediatric Service, University Hospital Son Dureta, Palma de Mallorca, Spain

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Abstract

We report a prospective study concerning the efficacy of LLC-MK2 (continuous monkey kidney cell), Hep-2, MDCK (Madin–Darby Canine Kidney), Vero and MRC-5 cell lines, by shell vial assay, and incubation time in the isolation of hMPV from pediatric respiratory samples. The overall sensitivity of the cell lines studied were: 100% for the LLC-MK2, 68.7% for the Hep-2, 28.1% for the Vero, 3.1% for the MDCK and 0% for the MRC-5. Only one strain (3.1%) showed growth in the four cell lines studied and 10 (31.2%) strains only grew in the LLC-MK2 cell line. The analysis of incubation times showed that only 14 strains (43.7%) were able to grow after 3 days of incubation, while all strains (100%) showed growth after 5 days. The use of shell vials with commercial LLC-MK2 cells could be a method for isolating hMPV from respiratory samples in the pediatric population.

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1. Introduction

Human metapneumovirus (hMPV) is a new virus of the genus *Metapneumovirus*, of the subgroup *Pneumovirinae* belonging to the family *Paramyxoviridae*. The virus is a pleomorphic, enveloped virion with surface glycoproteins and an average diameter of approximately 200 nm, containing a 13,300 bases single-stranded RNA of negative polarity (Peret et al., 2002; Van den Hoogen et al., 2001, 2002, 2004).

hMPV was first isolated and identified in the year 2001 in Holland in the nasopharyngeal aspirates from children and adults with acute respiratory tract infection (Van den Hoogen et al., 2001). Subsequent studies showed that this virus is responsible for a significant part of community acquired viral

respiratory infections affecting the infant population throughout the world (Chan et al., 2003; Maggi et al., 2003; Peiris et al., 2003; Peret et al., 2002; Van den Hoogen et al., 2002, 2004). Co-infections between hMPV and respiratory syncytial virus are relatively frequent and appear to worsen the prognosis and morbidity in pediatric bronchiolitis (Boivin et al., 2002; Kahn, 2006).

The diagnosis of respiratory infections by hMPV is based on the amplification of hMPV genome RNA by reverse transcription-polymerase chain reaction (RT-PCR) (Van den Hoogen et al., 2001). This technique displays the greatest sensitivity and specificity and is actually considered as the gold standard method of diagnosis (Cote et al., 2003; Van den Hoogen et al., 2001, 2002). There are certain difficulties involved in the routine isolation (cell culture) of hMPV in clinical samples and its isolation is less sensitive than the RT-PCR method (Chan et al., 2003; Cote et al., 2003). One

* Corresponding author.

E-mail address: jreina@hsd.es (J. Reina).

alternative to viral isolation is direct antigen detection in the sample by means of an immunofluorescence or enzyme immunoassay assays, which shows a sensitivity of 70–75% against the RT-PCR (Barry-Murphy et al., 2006; Ebihara et al., 2005; Ingram et al., 2006). The routine use of antigen detection is complex and difficult when processing a large number of respiratory samples. For this reason isolation of hMPV in cell culture could be a useful alternative for those laboratories where the molecular methods are not available.

We have carried out a prospective study concerning the routine efficacy of different cell lines, by shell vial assay, and incubation time in the isolation of hMPV from pediatric respiratory samples.

2. Materials and methods

Over a 14-month period (5 December to 7 January) we studied all the nasopharyngeal aspirates sent to the virology laboratory for the diagnosis of viral respiratory infections in pediatric patients. The respiratory samples were taken by inserting a suction catheter into the nasopharyngeal area via the nostril and sent as soon as possible to the virology laboratory. The time interval between samples arrival at the laboratory to the time the samples were processed was no more than 3 h.

Each sample was homogenized with 3 ml of phosphate buffered saline (PBS, pH 7.4) without antibiotics to obtain a cell suspension. For the shell-vial cultures 200 µl of the sample was inoculated into a LLC-MK2 (continuous monkey kidney cell; for parainfluenza virus and hMPV), Hep-2 (RSV and adenoviruses), MDCK (Madin–Darby Canine Kidney; for influenza A and B), and MRC-5 (human lung embryonated cells; for enteroviruses and CMV) vials (Vircell, Ingelheim Diagnostica, Spain). The vials were then centrifuged at 700 × g for 45 min. They were allowed to rest at 36 °C for 60 min and the supernatant was discarded. To each sample 1 ml of maintenance medium (MEM with 1% fetal bovine serum and with 2 µg/ml of trypsin for LLC-MK2 and MDCK) was added. The vials were incubated for 3 and 5 (hMPV) days at 36 °C and subsequently stained with an indirect immunofluorescence assay by a monoclonal antibody against the different respiratory viruses (Monofluokit, Diagnostic Pasteur, France). The samples positive for the hMPV (stored at 4 °C) were inoculated again in the same cell lines (2 shell vials/line) and in the Vero cell line (Green Monkey Continuous Cell Line) and incubated for 3 and 5 days at 36 °C. After the incubation period the monolayers were stained with an indirect immunofluorescence assay by a monoclonal antibody specific for the hMPV (MAB8510, Chemicon International, Temecula, CA). Two types of positivity were considered: qualitative (presence in the shell vial of cells with specific fluorescence, positive) and quantitative (number of infectious foci, IFs, present in each shell-vial positive).

Table 1
A comparison of hMPV growth in different cell lines

Cell lines				No. (%)
LLC-MK2	Hep-2	Vero	MDCK	
+	+	+	+	1 (3.1)
+	+	+	–	8 (25.0)
+	+	–	–	13 (40.6)
+	–	–	–	10 (31.2)
32 (100) ^a	22 (68.7)	9 (28.1)	1 (3.1)	32

^a Percentage of hMPV positive cultures are shown in brackets.

Table 2
A comparison of different incubation times in the growth of hMPV

Cell line	Incubation times		Total
	3 days	5 days	
LLC-MK2	11 (34.3) ^a	21 (65.6)	32
Hep-2	3 (13.6)	19 (86.4)	22
Vero	0	9 (100)	9
MDCK	0	1 (100)	1
MRC-5	0	0	0

^a Percentage of hMPV positive cultures are shown in brackets.

3. Results

Over the study period 2029 nasopharyngeal aspirate samples were analyzed, of which 483 (23.8%) were considered positive (isolation of a respiratory virus). In 32 cases the hMPV (6.6% of positive samples and 1.5% of total samples) was isolated. Only one sample per patient was evaluated.

The overall sensitivity (qualitative study) of the cell lines studied for the isolation of hMPV gave the following results: 100% for the LLC-MK2, 68.7% for the Hep-2, 28.1% for the Vero, 3.1% for the MDCK and 0% for the MRC-5. Only one strain (3.1%) showed growth in the four cell lines studied and 10 (31.2%) strains only grew in the LLC-MK2 cell line (Table 1).

The analysis of incubation times showed that only 14 strains (43.7%) were able to grow after 3 days of incubation, while all strains (100%) showed growth after 5 days. Only the LLC-MK2 and Hep-2 cell lines showed growth at 3 days, while the rest of cell lines required 5 days of incubation for the isolation of the hMPV (Table 2).

With reference to quantitative sensitivity in the LLC-MK2 line (Table 3), at 3 days of incubation 63.6% of the strains showed less than 6 infectious foci in the monolayer, while at 5 days 50% of hMPV strains presented more than 16 IFs in the cell culture.

Table 3
Quantitative analysis of hMPV growth in the LLC-MK2 cell line

No. of infectious foci	Incubation time	
	3 days	5 days
1–5	7 (63.6) ^a	4 (12.5)
6–15	3 (27.2)	12 (37.5)
>16	1 (9.2)	16 (50.0)

^a Number of positive samples (%).

4. Discussion

To date the method of genetic amplification (RT-PCR) is that has shown the best efficacy in hMPV detection from respiratory samples with respect to both sensitivity and specificity, so that it is considered the gold standard or reference method for definitive diagnosis of respiratory infections caused by this virus (Boivin et al., 2002; Cote et al., 2003; Kahn, 2006; Peiris et al., 2003). The development of specific monoclonal antibodies against hMPV (Mab-8) enables us to detect the presence of viral antigens directly in the sample, by means of immunofluorescence or EIA assays (Barry-Murphy et al., 2006; Ebihara et al., 2005; Ingram et al., 2006), and to detect positivity in both classic and shell vial cell cultures (Barry-Murphy et al., 2006; Ingram et al., 2006; Landry et al., 2005).

The replication of hMPV *in vitro* is restricted to a limited number of cell lines and requires supplementation of the medium with trypsin for propagation (Boivin et al., 2002; Van den Hoogen et al., 2001). Various initial studies have shown that hMPV grows poorly in the Vero and A-549 (human lung adenocarcinoma) cell lines and that it replicates with difficulty in the MDCK and MRC-5 cell lines, routinely used for the routine isolation of other respiratory viruses (Peret et al., 2002; Van den Hoogen et al., 2001). Nevertheless, Boivin et al. (2002, 2003) showed that hMPV grows very well in the LLC-MK2 cell line, routinely used for the isolation of the parainfluenza viruses, with a cytopathic effect of cellular rounding but without the formation of syncytia. The incubation period for the cell culture is variable. Originally a minimum of 14–17 days of incubation were recommended when classic culture is used (Peret et al., 2002; Van den Hoogen et al., 2001), but later studies seem to demonstrate that a more prolonged incubation would provide a higher number of positive results (Chan et al., 2003).

Our study confirms that the commercial LLC-MK2 cell line is that which permits the detection and isolation of all the hMPV strains detected from pediatric respiratory samples. In the study of Schirm et al. (2004) with the tMKC cell line (LLC-MK2-like) in the shell vial assay, this cell line permitted the isolation of hMPV in 63% of respiratory samples with respect to positive detection in the RT-PCR (100% sensitivity). Of the rest of the cell lines analyzed in our study, only the Hep-2 (68.7%) displayed an acceptable isolation capacity although it should never be used as the only cell line for isolation of hMPV (10 strains did not grow in this cell line). The experimental studies by Landry et al. (2005) also showed high sensitivity in the Hep-2 cell line, together with the A549 line, in the isolation of hMPV. Chan et al. (2003) have reported that when RT-PCR was used to follow all cell cultures, the sensitivity of Hep-2 cells was higher than LLC-MK2 cells, but when LLC-MK2 cells were incubated for 28 days, hMPV was only isolated in 12% from this cell line.

With respect to the optimal incubation time for the isolation of hMPV, Schirm et al. (2004) observed that 72% of shell

vial cultures (tMKC cell line) with positive results required 48 h of incubation. However, Landry et al. (2005) obtained a higher yield after 2–3 days of incubation both in the LLC-MK2 and the Hep-2 cell lines. The experimental study of Percivalle et al. (2005) showed that the majority of hMPV strains display 100% cytopathic effect 5 days postinfection at titers of $>10^7$ 50% tissue culture infective doses/ml. In the classical culture, Boivin et al. (2002) reported that the cytopathic effect of hMPV took a mean incubation time of 17.3 days to develop. By prolonging the incubation time to 20–28 days, more hMPV infections could be detected (Chan et al., 2003; Kahn, 2006). In our study only 34.3% of hMPV strains grew at 3 days of incubation against 65.6% which grew at 5 days. Even clearer results were obtained with the other cell lines. Only 13.6% of the strains that grew in the Hep-2 cell line did so at 3 days of incubation. These data confirm the practical difficulties inherent in the routine isolation of hMPV from clinical samples and the need for prolonged incubation periods before designating a sample as definitely negative (Boivin et al., 2003; Chan et al., 2003; Kahn, 2006; Landry et al., 2005).

In the quantitative study carried out only in the LLC-MK2 cell line, in relation with incubation times for shell vial cultures, we observed that the number of infectious foci detected at 3 days of incubation were considerably less than those observed at 5 days. Thus, the majority of strains which grew at 3 days (63.6%) presented less than 6 IFs in the monolayer, making it difficult to read and to consider as a positive culture. On the contrary, at 5 days of incubation the majority of the monolayers (50%) presented more than 16 IFs, which permits to reach a rapid and specific diagnosis with no interpretative difficulties. We agree with Landry et al. (2005) in that the staining was brighter in the LLC-MK2 than in the Hep-2 cell line, which facilitates the detection and identification of the infectious focus.

In summary, the commercialization of specific monoclonal antibodies against hMPV permits its detection and isolation in cell cultures. The use of the LLC-MK2 cell line in the shell vial assay has displayed utility and efficacy in the isolation of hMPV from pediatric respiratory samples. The difficulties involved in its isolation are compensated by prolonging the incubation time of cell cultures.

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