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Replication of Respiratory Viruses, Particularly Influenza Virus, Rhinovirus, and Coronavirus in HuH7 Hepatocarcinoma Cell Line

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Detection of viral antigens and isolation methods has long been used for the diagnosis of respiratory virus infections. The objective was to determine the ability of HuH7 cells to support the replication of prototype and wild strains of respiratory viruses. The cell culture-adapted strains of influenza viruses A and B, parainfluenza viruses 1–4, respiratory syncytial viruses A and B, both strains of the human metapneumoviruses, numerous rhinoviruses, most of the adenoviruses, coronaviruses 229E and OC43, and a number of enteroviruses (poliovirus type 3, coxsackie virus B1, echovirus type 30) replicate in HuH7. The kinetic study of the replication of influenza A and B viruses showed that there were infected cells in HuH7 and MDCK lines as early as 24 hr post-infection. However, the replication of influenza A and B viruses was more rapid and intense on MDCK cells than on HuH7 cells. During the three winters of 1999–2000, 2000–2001, and 2001–2002, of the 1,226 (23.3%) direct fluorescent assay-positive nasal aspirates from children admitted to hospital, 788 were positive for respiratory syncytial virus, 228 for influenza virus, 133 for parainfluenza virus, and 77 for adenovirus. Of the 4,032 direct fluorescent assay-negative nasal aspirates, 571 virus isolates were identified by using HuH7 cell culture; 272 rhinoviruses, 100 influenza viruses A and B, 85 enteroviruses, 40 adenoviruses, 35 coronaviruses, 31 parainfluenza viruses, and 10 respiratory syncytial viruses. Interestingly, 100/328 (30.5%) influenza viruses A and B, 40/189 (21.1%) adenoviruses, and 31/164 (19%) parainfluenza viruses type 1–3, not detected by direct fluorescent assay, were identified by isolation in HuH7 cell culture. ***J. Med. Virol. 77:295–301, 2005.***

KEY WORDS: HuH7; influenzavirus; rhinovirus; coronavirus; respiratory viruses

INTRODUCTION

Detection of viral antigens by immunological methods, first described by Gardner and McQuilllin [1980], has long been used for rapid diagnosis of influenza virus, respiratory syncytial virus, parainfluenza virus, and adenovirus infections of the respiratory tract. The sensitivities of these assays vary, often depending on the adequacy of the sample, but most are in the range of 70%–95% [Gardner et al., 1972; Fulton and Middleton, 1974; Freymuth, 1980; Takimoto et al., 1991]. Previous studies have reported that isolation in cell culture is more sensitive than antigen detection for influenza virus and adenovirus diagnosis [Matthey et al., 1992; Madeley et al., 1996; Ruuskanen et al., 1997; Freymuth et al., 2000; Steininger et al., 2002]. There is no serological reagent currently available for the detection of rhinoviruses, coronaviruses, and the human metapneumovirus, and isolation of virus from respiratory secretions is still required for diagnosis of respiratory virus infections. Primary monkey kidney cells have been used widely for isolation and propagation of most respiratory viruses [Frank et al., 1979]. In recent years, several cell lines have been found useful as substitutes for primary monkey kidney cells for respiratory viruses: the Madin–Darby canine kidney (MDCK) cells [Krug, 1972; Meguro

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et al., 1979], the A549 cells [Lieber et al., 1976; Woods and Young, 1988], the NCI-H292 cells [Carney et al., 1985; Hierholzer et al., 1993]. Vabret et al. [2001] and Pene et al. [2003] isolated coronaviruses in a continuous epithelial cell line derived from a human hepatocarcinoma, HuH7 cells [Nakabayashi et al., 1982]. These cells were examined for the isolation of respiratory viruses. The objectives of this study were to determine the ability of HuH7 cells to support the replication of prototype and wild strains of viruses likely to be encountered in respiratory viral infections and to assess, prospectively, their efficacy in the diagnosis of respiratory virus infections in children, particularly associated with influenza virus, rhinovirus, and coronavirus and when an immunofluorescence assay does not detect antigen directly.

MATERIAL AND METHODS

Viral Strains and Clinical Respiratory Samples

Prototype and wild strains from a large selection of respiratory viruses were obtained from our reference collection (Table I). Prototype strains originally came from the American Type Culture Collection or from the National Reference Center for Influenza in France. Wild strains were isolates from nasal aspirates that were recovered from monkey kidney cells, human embryonic lung diploid fibroblast (MRC5), or human lung carcinoma epitheloid (A549) cell cultures. These viruses were identified initially by neutralization (enterovirus) or sequencing (human metapneumovirus, rhinovirus, parainfluenza virus type 4). Prototype and wild strains were passaged in appropriate cell cultures for future use. Clinical samples were nasal aspirates obtained from pediatric patients admitted to the University Hospital of Caen for acute lower respiratory tract infections over a 3-year period, from October 2000 to June 2002. The collected samples were placed in 4 ml of viral transport medium. When received in the laboratory, samples were held at 2–8°C and processed immediately for direct fluorescent assay using monoclonal antibodies to influenza A and B viruses, respiratory syncytial virus, parainfluenzavirus types 1–3, adenovirus (IMAGEN®, Dako, UK). If the direct fluorescent assay was negative, then the clinical specimens were inoculated onto HuH7 shell vial cultures and MRC5 cell cultures.

Cell Cultures

HuH7 cells were grown in RPMI-1640 medium with glutamine (Gibco, Invitrogen, Cergy-Pontoise, France) supplemented with 5% fetal calf serum (Gibco), penicillin (100 U/ml), and gentamycin (50 μg/ml) and distributed in 48-well tissue culture microplates (5×10^4 cells/well). When HuH7 cells were 80% confluent, the growth medium was discarded and each well

TABLE I. Infection of HuH7 Cells by Prototypes or Wild Strains of Respiratory Viruses

Virus[a]	CPE[b]	Virus[a]	CPE[b]	Virus	CPE[b]
Influenza virus A H1N1		Adenovirus sp A		Rhinovirus	
A/PR8/1934 (p)	+	AdV 12 (p)	+	RV 1B (sq)	+
A/Singapore/6/1986 (p)	+	AdV 18 (p)	+	RV 2 (sq)	+
A/NewCaledonia/20/1999(p)	+	Adenovirus sp B		RV 5 (p)	+
Influenza virus A H3N2		AdV 3 (p)	+	RV 6 (p)	+
A/Sichuan/2/1987 (p)	+	AdV 7 (p)	+	RV 7 (sq)	+
A/Beijing/32/1992 (p)	+	AdV 11 (p)	+	RV 9 (p)	+
A/Sydney/5/1997 (p)	+	AdV 14 (p)	0 (f+)	RV 12–14 (sq)	+
A/Panama/2007/1999 (p)	+	AdV 21 (p)	+	RV 19–20 (sq)	+
Influenza virus B		AdV 34 (p)	+	RV 23–24 (sq)	+
B/Victoria/9/1987 (p)	+	Adenovirus sp C		RV 28–29 (sq)	+
B/Yamagata/16/1988 (p)	+	AdV 1 (p)	+	RV 31 (p)	+
B/Sichuan/379/1999 (p)	+	AdV 2 (p)	+	RV 34 (sq)	+
B/Hong-Kong/330/2001 (p)	+	AdV 5 (p)	+	RV 38–39 (sq)	+
Respiratory syncytial virus		AdV 6 (p)	+	RV 41 (sq)	+
A Long (p)	0 (f+)	AdV 8 (p)	+	RV 42–43 (sq)	+
B (p)	0 (f+)	Adenovirus sp D		RV 46–49 (sq)	+
Human metapneumovirus		AdV 13 (p)	+	RV 52–56 (sq)	+
Caen 45/01 (sq)	0 (PCR+)	AdV 20 (p)	+	RV 58–61 (sq)	+
Caen 78/02(sq)	0 (PCR+)	AdV 36 (p)	+	RV 63–68 (sq)	+
Parainfluenza virus		Adenovirus sp E		RV 70 (sq)	+
VPI 1 (f)	+	AdV 4 (p)	+	RV 73 (sq)	+
VPI 2 (f)	+	Adenovirus sp F		RV 75–79 (sq)	+
VPI 3 (f)	+	AdV 40 (p)	0	RV 82–83 (sq)	+
VPI 4 (sq)	+	Enterovirus		RV 89 (sq)	+
Coronavirus		Coxsackie B1 (n)	+	RV 90 (p)	+
CoV 229E (p)	+	Echovirus 30 (n)	+	RV 92 (sq)	+
CoV OC43 (p)	+	Poliovirus 3 (n)	+	RV 95 (sq)	+
				RV 97 (sq)	+

[a]Prototypes viral strains (p), or wild strains identified by neutralization (n), immunofluorescence assay (f) or sequencing (sq).
[b]+ = cytopathologisc effect (CPE) after fourth day of incubation and identification by immunofluorescence assay (f): influenza virus, parainfluenza virus, respiratory syncytial virus, human métapneumovirus, adenovirus, or by PCR: rhinovirus, coronavirus, enterovirus.

was inoculated with 50 μl of the prototype or wild viral strains or with 100 μl of the clinical samples. Microplates were centrifuged at 1,500 rpm for 30 min at 30°C. Samples were then removed and each well was filled with 1 ml of the same culture medium, supplemented with 2% fetal calf serum (Gibco), and 1% of porcine pancreatic trypsin at 150 μg/ml (Sigma, Saint Quentin Fallaviou, France). By day 4 post-infection, HuH7 cultures were examined for a cytopathologic effect (CPE) and the supernatants and cells were collected separately. The supernatants were stored at −80°C and used for amplification techniques. HuH7 cells were harvested by scraping the cell sheet and were used to prepare smears for the direct fluorescent assay. The replication of influenza A and B viruses, respiratory syncytial virus, parainfluenza virus types 1–3, adenovirus was detected by direct fluorescent assay with the reagents described below. Reverse transcription-PCR (RT-PCR) techniques were used for the detection of human metapneumovirus (hMPV), coronavirus 229E and 0C43, rhinovirus and enterovirus, according to procedures described previously [Freymuth et al., 1999; Vabret et al., 2001; Freymuth et al., 2003]. Briefly if there were CPE, RNAs were extracted from 100 μl of the supernatant using the Qiamp RNA Viral Minikit (QIAgen, Courtaboeuf, France). The RT-PCR assays were carried out using one step combined RT-PCR amplification kit (QIAgen, Courtaboeuf, France) and primers, and probes defined previously for the detection of rhinovirus and enterovirus, coronavirus 229E and OC43.

The replication of prototype strains of influenza A virus H3N2 (strain/Panama/2007/1999), influenza A virus H1N1 (strain/NewCaledonia/20/1999), and influenza B virus (strain/Victoria/1987) was compared in HuH7 and MDCK cells grown in 48-well microplates. The time necessary to detect influenza virus-infected cells was determined by using direct fluorescent assay at day 1–4 post-infection. The replication titers were assessed in the supernatants by a real-time RT-PCR assay using a LightCycler instrument (Roche Diagnostics, Meylan, France). RNAs were extracted from 100 μl of the supernatants of HuH7 and MDCK cell cultures using the Qiamp RNA Viral Minikit (QIAgen). The primers, probes, and the real-time RT-PCR procedures were fully adapted from those described by Schweiger et al. [2000].

RESULTS

Viruses for the Retrospective Study

The replication of prototype or wild strains of respiratory viruses inoculated onto HUH7 cells was detected by the presence of a CPE, while virus identification was by direct fluorescent assay or PCR techniques. These cell culture-adapted strains showed that influenza viruses A and B, parainfluenza viruses 1–4, very numerous rhinoviruses, most of the adenoviruses, coronaviruses 229E and OC43 as well as enteroviruses (poliovirus type 3, coxsackie virus B1, echovirus type 30) produced CPE when replicating in HuH7 cells (Table I). The CPE, which is characterized by small cells, almost non-refringent, scattered on the culture, and resulting in a progressive lysis of the cell sheet, cannot be used for identifying viruses or family of viruses. After 4 days in culture, the prototype strains of A and B respiratory syncytial viruses, both strains of the human metapneumoviruses, adenoviruses spF and Ad 14 replicated on HuH7 cells without producing any CPE (Fig. 1).

The replication of influenza A virus H1N1, influenza A virus H3N2, and influenza B virus was compared in HuH7 and MDCK cells. The kinetic study of infection showed that there were infected cells in both cell lines as early as 24 hr post-infection, for both influenza

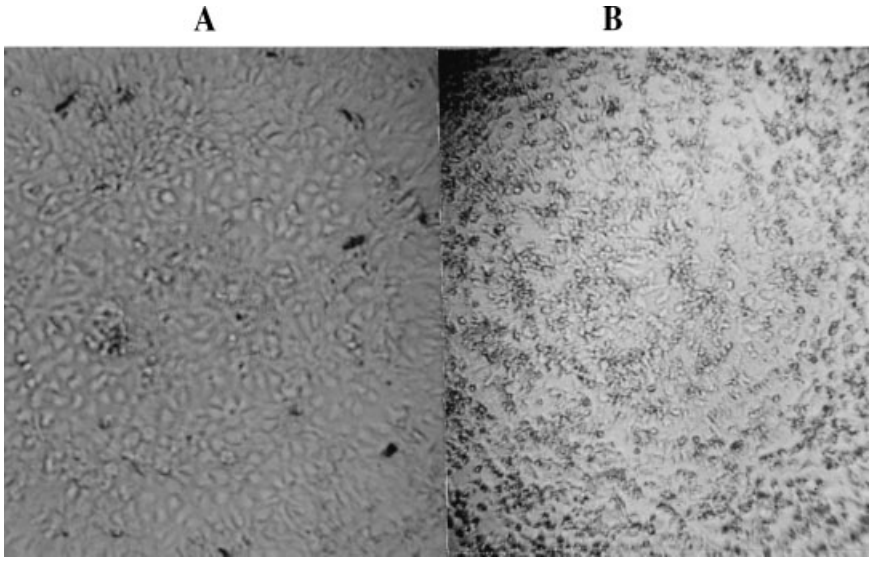

Fig. 1. Cytopathic effect caused by the replication of influenza A virus and rhinovirus in HuH7 cells on day 4 post-infection. **A**: Control HuH7 and (**B**) influenza A virus H3N2 (strain Panama/2007/199).

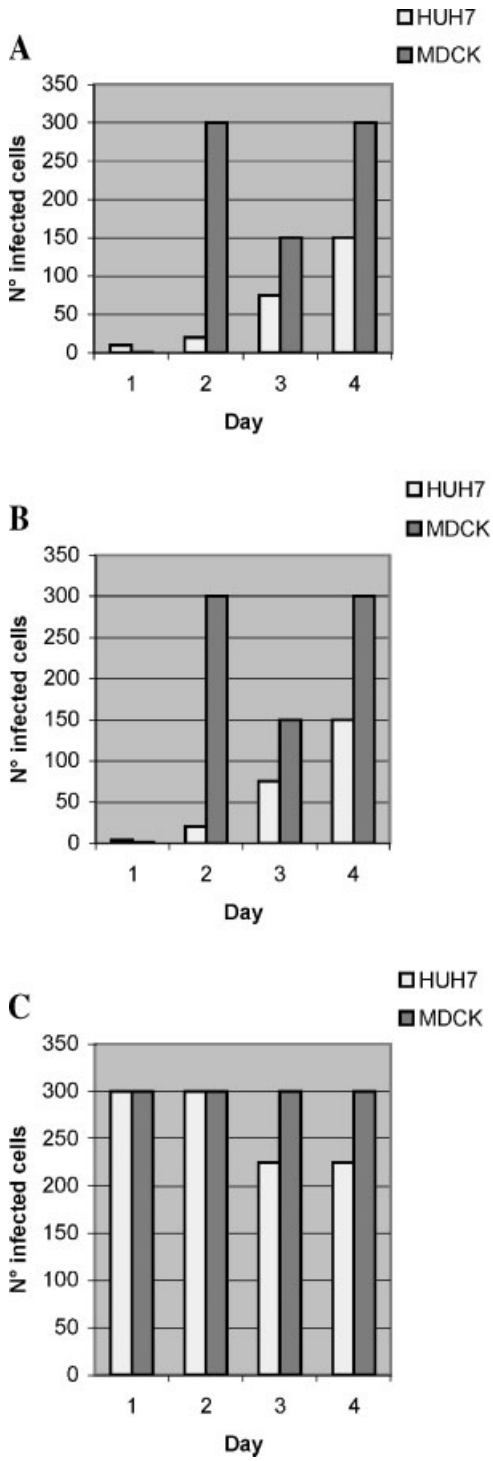

Fig. 2. Kinetic of replication of influenza virus in HuH7 and MDCK cells. Infected cells were quantified using a direct immunofluorescence assay (Imagen®, Dako, UK). **A**: Cells infected with 0.9×10^2 genome equivalents per ml (GE/ml) of influenza A H3N2 (strain Panama/2007/1999), (**B**) cells infected with 2×10^2 GE/ml of influenza A H1N1 (strain/NewCaledonia/20/1999), and (**C**) cells infected with 4.2×10^3 GE/ml of influenza B (strain /Victoria/1997).

virus type A or B (Fig. 2). However, the number of viral genomes found in culture supernatants showed (Fig. 3) that the replication of influenza A virus H3N2 (strain/Panama/2007/1999) and influenza A virus H1N1

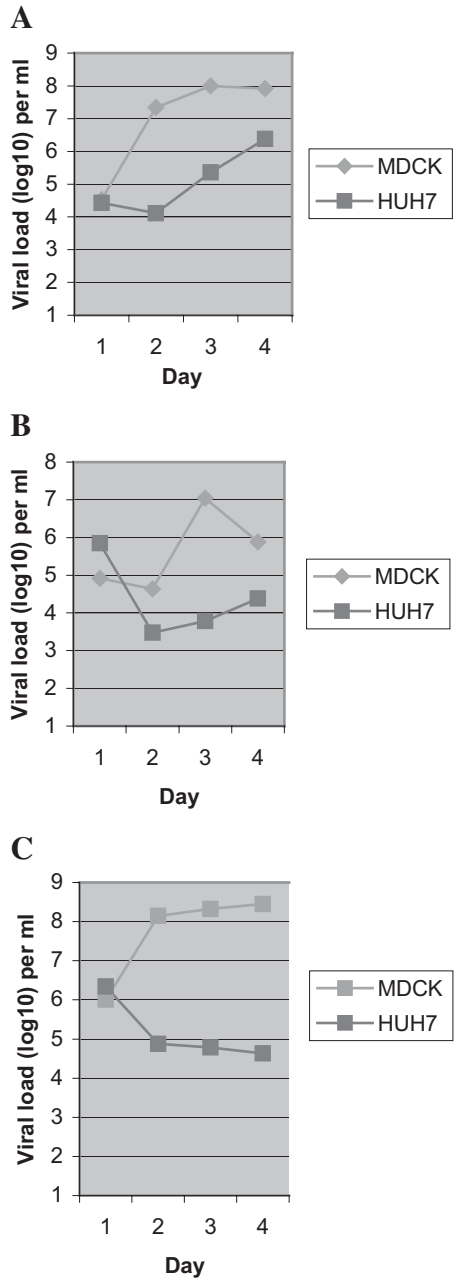

Fig. 3. Quantitative and comparative evaluation of influenza virus production in HuH7 and MDCK cells. Viral titers were evaluated using a real-time RT-PCR method. **A**: Cells infected with 0.93×10^2 genome equivalents per ml (GE/ml) of influenza A H3N2 (strain Panama/2007/1999), (**B**) cells infected with 2×10^2 GE/ml of influenza A H1N1 (strain/New Caledonia/20/1999) and (**C**) cells infected with 4.2×10^3 GE/ml of influenza B (strain/Victoria/1997).

(strain/New Caledonia/20/1999) was more rapid and intense in MDCK cells than in HuH7 cells, and that HuH7 cells were less permissive for the strain of influenza B virus (strain/Victoria/1987).

Prospective Study

During the three winters of 1999–2000, 2000–2001, and 2001–2002, 5,258 nasal aspirates obtained from

TABLE II. Comparative Efficiencies of Direct Fluorescence Assay (DFA) and HuH7 Cell Culture for the Detection of Virus in Nasal Aspirates From Children

	No. of positive samples[a]	No. of DFA positive samples	No. (%) of DFA-negative HuH7-positive samples
Respiratory syncytial virus[b]	825	788	10 (1.2)
Influenza virus A	224	155	69 (30.8)
Influenza virus B	104	73	31 (29.8)
Parainfluenza 1	41	28	13 (31.7)
Parainfluenza 2	9	4	5 (55.5)
Parainfluenza 3	114	101	13 (11.4)
Adenovirus[b]	189	77	40 (21.1)
Rhinovirus[b]	482	na	272 (56.4)
Enterovirus[b]	127	na	85 (66.9)
Coronavirus OC43	33	na	33 (100)
Coronavirus 229E	2	na	2 (100)

[a]No. of samples found to be positive by DFA, culture in HuH7 or MRC5 cells.
[b]Some strains isolated in MRC5 cell cultures only; na: reagent not available.

children admitted to the pediatric wards for a respiratory tract infection were examined using direct fluorescent assay for detecting influenza viruses A and B, respiratory syncytial virus, parainfluenza virus types 1–3, and adenoviruses. Of the 1,226 (23.3%) direct fluorescent assay-positive nasal aspirates, 788 were positive for respiratory syncytial virus, 228 for influenza virus, 133 for parainfluenza virus, and 77 for adenovirus (Table II). Of the 4,032 direct fluorescent assay-negative nasal aspirates, 571 viruses were identified by using the HuH7 cell culture. There were 272 rhinoviruses, 100 influenza viruses A and B, 85 enteroviruses, 40 adenoviruses, 35 coronaviruses, 31 parainfluenza viruses, and 10 respiratory syncytial viruses. Very few (1.2%) respiratory syncytial viruses were detected on direct fluorescent assay-negative samples using culture. In contrast, 100/328 (30.5%) influenza viruses A and B, 40/189 (21.1%) adenoviruses, and 31/164 (19%) parainfluenza viruses type 1–3 not detected by direct fluorescent assay were identified by HuH7 cell culture. Finally, viruses for which there were no direct fluorescent assay reagents available were identified using these cells, that is, 272 rhinoviruses, 35 coronaviruses, and 85 enteroviruses. Table II shows that the total number of respiratory syncytial viruses, rhinoviruses, adenoviruses, and enteroviruses was higher than the inclusion of the direct fluorescent assay and HuH7 cell culture positive results. These viral strains were detected by isolation in MRC5 cells.

DISCUSSION

HuH7 cells are a continuous cell line of epithelial cells derived from a human hepatocarcinoma [Nakabayashi et al., 1982]. They have been first used for the propagation of hepatitis C virus [Lohmann et al., 1999]. HuH7 cells thus appear to have a broad viral spectrum allowing the detection of influenza viruses, respiratory syncytial viruses, human metapneumovirus, parainfluenza viruses, adenoviruses, some rhinoviruses, and coronaviruses 229E and OC43. We, therefore, suggest HuH7 cells as a substitute of other cells used commonly for detection of human respiratory viruses. Primary monkey kidney cells have long been preferred for the isolation and the propagation of a large number of respiratory viruses but they are no longer available [Frank et al., 1979]. In recent years, several cell lines have been suggested as substitutes for primary monkey kidney cells but most had narrower spectrum sensitivity to viruses. MDCK and LLC-MK2 cells support the growth of a large number of influenza and parainfluenza viruses but the growth of other respiratory viruses is limited [Frank et al., 1979; Meguro et al., 1979]. Parainfluenza viruses and certain strains of rhinoviruses, respiratory syncytial viruses, and adenoviruses replicate in A549 and Vero cells [MacFarlane and Sommerville, 1969; Woods and Young, 1988]. The continuous cell line of NCI-H292 human lung mucoepidermoid cells appears to be the best substitute for primary monkey cells. This cell line is permissive for the replication of adenoviruses, parainfluenza viruses type 1–4, both groups of respiratory syncytial viruses, and many strains of enteroviruses and rhinoviruses [Hierholzer et al., 1993]. In contrast, they are not permissive for the isolation of coronaviruses and many strains of influenza A virus H1N1, A virus H3N2, and influenza B. The HuH7 cell line is of special interest, since it is permissive for all the viruses mentioned above, especially for influenza viruses, rhinoviruses, and coronaviruses. Another liver cell line, HepG2, has been suggested for the isolation of influenza viruses type A and B [Ollier et al., 2004]. In comparing MDCK cells, which is the most valuable cell system for use in laboratories for isolating influenza viruses [Zambon, 1999], it has been observed that influenza viruses A and B can be detected in HuH7 cells as rapidly as in MDCK cells but the replication of viral strains is more rapid and intense in MDCK cells than in HuH7 cells.

To date, rapid diagnosis of upper and lower respiratory tract infections is based on the direct detection of viral antigen in respiratory secretions. Due to the large number of reagents available "off-the-shelf," direct immunofluorescence is the method used most commonly for detecting influenza viruses type A and B, parainfluenza viruses type 1–4, respiratory syncytial virus, and adenoviruses. However, several studies have

demonstrated that this method is less reliable than isolation by culture for detecting influenza viruses and adenoviruses: as outlined by different authors, there are variations in permissivity from 65% to 100% for influenza viruses and from 28% to 75% for adenoviruses [Matthey et al., 1992; Madeley et al., 1996; Ruuskanen et al., 1997; Freymuth et al., 2000; Steininger et al., 2002]. In addition, this technique cannot be used to detect rhinoviruses and coronaviruses. According to the present study, it was of interest to use direct fluorescent assay-negative samples in order to attempt viral isolation in HuH7 cells. The detection of influenza viruses types A and B, parainfluenza viruses types 1–3, and adenoviruses in cultured HuH7 cells appeared to have increased by 30.5%, 19%, and 21.1%, respectively. This phenomenon has not yet been fully elucidated: it may be explained by a smaller number of infected cells in the respiratory samples taken from infected patients, or to a more rapid viral cytolysis of these cells not allowing detection when using direct fluorescence assay. Moreover, two important groups of respiratory viruses (rhinoviruses and coronaviruses) were detected using HuH7 cells. The isolation of rhinoviruses, long and difficult in most cases, usually requires the use of several cell lines due to cell changing permissivity in relation to serotypes: human embryonic fibroblasts MRC5, continuous human cell lines HeLa R and NCI-H292 [Hierholzer et al., 1993; Savolainen et al., 2003]. In our study, further detection of rhinoviruses, adenoviruses, and enteroviruses were obtained by using MRC5 cell culture. However, it was not possible to assess whether permissivity difference between HuH7 and MRC5 cell cultures was related to serotype of rhinoviruses, enteroviruses, and adenoviruses, or to particular strains of respiratory syncytial viruses. The phenomenon is likely to be related to the duration of incubation which, in the case of HuH7 cells, is limited to only 4 days. In the meantime, a large number of rhinoviruses and certain strains of adenoviruses, enteroviruses, or respiratory syncytial viruses with slow growth would require an incubation period from 10 to 21 days for replication in MRC5 cells. We have noted among the respiratory coronaviruses, a replication of coronavirus 229E in human diploid cell line MRC5, replication of coronavirus OC43 only in HRT18 cells [Vabret et al., 2001], and a difficult replication of the new coronavirus NL63 in LLC-MK2 cells [Van Der Hoek et al., 2004]. It has been observed that HuH7 cells are permissive for replication of a murine coronavirus called MHV [Koetters et al., 1999] and we have demonstrated in this study that they are also permissive for both human coronaviruses OC43 and 229E.

Thus, using HuH7 cell isolation technique as a supplement to a direct fluorescent assay-negative test is of value for increasing the detection of influenza viruses A and B, parainfluenza viruses 1–3, and adenoviruses. It is also value for the detection of rhinoviruses, enteroviruses, and coronaviruses. The multiplex-like molecular tools used directly on respiratory samples can also be considered a reliable and simpler technique than the combination of direct fluorescent assay and culture [Carman, 2001; Coiras et al., 2004]. The isolation by culture of respiratory viruses should, however, be maintained since the recent emerging respiratory viruses such as the human metapneumovirus, SARS, and NL63 coronaviruses have been identified using this method.

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