Isolation of respiratory syncytial virus from nasopharyngeal aspirates stored at –20ºC from one to fifteen months after collection

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Cell culture isolation is used for recovering respiratory syncytial virus (RSV) from respiratory specimens. As RSV is a thermolabile virus, specimens destined for inoculation into cell culture require special transport, handling, and storage. The isolation rate of RSV from nasopharyngeal aspirates (NPA) stored at –20ºC for one to 15 months after collection was investigated. A total of 126 samples considered positive for RSV by indirect fluorescence-antibody were tested by virus isolation in HEp-2 cell culture. RSV was isolated from 47/126 specimens (37.3%). These results show that RSV may be recovered from NPA stored at –20ºC by cell culture.

Key words: respiratory syncytial virus - HEp-2 cell culture - nasopharyngeal aspirates

Several diagnostic methods have been developed since respiratory syncytial virus (RSV) was defined as an important respiratory human pathogen. During the 1980s and early 1990s, viral isolation in tissue culture was widely used in the diagnosis of RSV infections (Arens et al. 1986, Waner et al. 1990). Although this method is considered the gold standard for laboratory diagnosis of RSV, results are not available quickly enough to be the basis for initiating antiviral therapy or infection control measures. Primary isolation of RSV in conventional cell culture generally takes three to seven days, but can range from two to ten days (Piedra et al. 2002).

For faster diagnosis, the isolation of RSV in cell culture has been replaced by antigen detection-based assays and new sensitive molecular techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) (WHO 2000, Henrickson 2004, Perkins et al. 2005). However, the cell culture method continues to be valuable because it allows the amplification of small amounts of virus that are present in a specimen, providing isolates for subtyping and further analysis. Moreover, it is less likely to diagnose false epidemics and permits the recovery of several additional agents that may be present in a specimen (Halstead et al. 1990, Piedra et al. 2002, Hall & McCarthy 2004).

RSV is a highly thermolabile virus requiring stringent adherence to transport and storage guidelines, and immediate inoculation of specimens in permissive cell lines for optimal recovery of the virus (Piedra et al. 2002). When this is not possible, specimens need be stored carefully. RSV does not tolerate slow freezing and thawing. Approximately 50% of RSV infectivity is lost when samples are submitted to a single freezer-thaw cycle, with a complete loss of viability when they are frozen slowly at –20ºC and then thawed. The residual viral titer can be maintained for several years when the clinical samples with RSV are stored at –70ºC (Tristram & Welliver 1995, Piedra et al. 2002).

Since 2001, the identification of RSV infections at the Virology Laboratory of the Pathology and Legal Medicine Department of the Federal University of Ceará (UFC) has been performed using indirect fluorescence-antibody (IFA). Due to the lack of a –70ºC freezer, the respiratory specimens collected from children with acute respiratory infections (ARI) were stored at –20ºC.

The purpose of this study was to establish the rate of recovery of RSV in cell culture from nasopharyngeal aspirates (NPA) stored at –20ºC from one to 15 months after collection.

NPA were obtained from children with ARI within seven days of onset, treated at the Albert Sabin Pediatric Hospital, located in Fortaleza, Ceará, Brazil, from January 2003 to August 2004. They were obtained by manual suction through a nasal catheter as described previously (Gardner & McQuillin 1980). Samples were transported to the laboratory on wet ice. An aliquot of each sample was processed immediately and then tested by IFA for antigen detection of RSV and other respiratory viruses (adenovirus, influenza A and B, parainfluenza types 1, 2, and 3) according to manufacturer instructions (Respiratory Viruses Panel I Viral Screening & Identification Kit, Chemicon Light Diagnostics, Temecula, CA, US). A second aliquot of each NPA was placed in a tube with 1 ml of fresh Eagle's minimal essential medium (MEM-E) containing L-glutamine (2 mM), penicillin (1000 U/ml), and streptomycin (1000 µg/ml) and stored at –20ºC for further analysis.

One hundred and twenty-six NPA, positive for RSV by IFA, were stored for one to 15 months after collection before being used in this study. NPA were cultured for the presence of RSV in fresh monolayers of human laryngeal carcinoma cells (HEp-2 cells), as described by Moura et al. (2002). HEp-2 cells were maintained in the medium de-

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scribed above supplemented with 10% fetal bovine serum (FBS). When the cells in monolayer presented 70% of confluence, the medium was discarded and 0.2 ml of NPA was added to each tube. Specimens were allowed to adsorb for 30 min at 35ºC before adding 1.5 ml of fresh MEM-E plus 2% FBS to the tubes. The cultures were incubated at 35ºC and observed daily for cytopathic effect (CPE) for 21 days. RSV was identified by IFA on the cell culture as described above. HEp-2 cells monolayers not exhibiting CPE were similarly examined by IFA.

RSV was identified in 47 out of 126 inoculated samples, resulting in an isolation rate of 37.3% (see Table). Approximately 55.3% (26/47) of these specimens presented syncytial formation and this CPE was observed between the third and sixth day after inoculation of NPA. The majority of samples inoculated in HEp-2 cells (77.8% - 98/126) had been stored for up to six months. The isolation rate was 37.8% (37/98) for samples that were frozen for up to six months and 41.7% (10/24) for those stored between six and 12 months. The number of inoculated samples that were frozen for up to six months was four times the number of those stored at the same temperature for six to 12 months. RSV was not recovered from samples frozen for more than 12 months. The results indicate that, for samples frozen for up to 12 months, the isolation of RSV is influenced more by the early collection than the length of time stored. A total of 95.7% (45/47) of the positive NPA were collected during the first four days of the disease and 64.6% (51/79) of the negative samples were obtained between the fifth and seventh days of the disease. All of the positive samples that were stored for more than six months were collected during the first four days of the disease. In addition, the only two samples isolated from NPA collected after this period of the disease were frozen for up to six months.

The main findings of this study show that RSV can be isolated even after storing the specimens for up to 12 months at –20ºC, showing that the viability of the virus is in part maintained. This contrasts with the report that complete loss of viability occurs with slow freezing at –20ºC followed by thawing (Piedra et al. 2002). The rate of RSV isolation observed in this study was surprising. Rates of 11.5% and 35.7% for isolation of RSV from NPA have been cited in two Brazilian studies (Siqueira et al. 1986, Moura et al. 2002). In one them, the samples were processed immediately for isolation in three different cells lines (Siqueira et al. 1986). In the other, HEp-2 cells were used but the samples were stored at –70ºC before being inoculated (Moura et al. 2002).

TABLE

Isolation rate of respiratory syncytial virus from nasopharyngeal aspirates according to the period that samples remained stored at –20º C and days of disease

Nasopharyngeal aspirates	Number of inoculated samples (%)	Number of positive samples (%)
Period of freezing (months)		
1 –| 6	98 (77.8)	37 (78.7)
7 –| 12	24 (19.0)	10 (21.3)
>12	4 (3.2)	0 (0.
Total	126 (100.0)	47 (100.0)
Days of disease		
0 –| 4	73 (57.9)	45 (95.7)
5 –| 7	53 (42.1)	2 (4.3)
Total	126 (100.0)	47 (100.0)

The recovery rate of RSV reached in this study was surprising in several aspects. The samples were stored in unsuitable temperature conditions and only HEp-2 cells were used. A combination of human epithelial cell lines, primary monkey kidney cells lines and human fibroblast has been recommended for optimal RSV recovery (Tristram & Welliver 1995). Another guideline for this same objective is the immediate inoculation of specimens into cell culture (Piedra et al. 2002). The utilization of cell culture in the diagnosis of viral ARI in Virology Laboratory of UFC only became possible in 2004. For this reason the samples were placed in virus transport medium and frozen.

Among the aspects that explain these results were the type and quantity of the samples collected. It has been reported that specimens collected by aspiration and nasal washes provide a superior specimen for RSV diagnosis (Ahluwalia et al. 1987, Heikkinen et al. 2002). About 1 ml of NPA was collected from each patient. In children, the amount of RSV present in these samples is very high, ranging from 10^3 to 10^8 plaque forming units/ml (Piedra et al. 2002).

About 44.7% of NPA were considered positive by IFA on the cell culture, since monolayers did not show CPE. This percentage is higher than the values described in the literature, which report that, on average, 26% of isolates of RSV grow without syncytial formation (Tristram & Welliver 1995). The degree of syncytial formation depends on the type of cell culture, the weight of the cell sheet, the medium, the strain of virus, the multiplicity of infection, and its laboratory adaptation (Hall & McCarthy 2004). The CPE was detected between three and six days after inoculation of samples, agreeing with other studies (Halstead et al. 1990, Smith et al. 1991).

On the other hand, all samples used in this study were obtained early in the course of infection, during the first week of the disease, in an attempt to guarantee high viral titers. More than 95% of positive NPA were collected during the first four days of the illness, demonstrating the high virus shedding during the first days after onset of the disease. Moreover, NPA obtained after four days of the disease were responsible for 64.6% of negative samples by cell culture. According to Piedra et al. (2002), infants and young children shed RSV for a mean nine days and adults shed for one to two days. Infants excrete RSV for over three weeks.

In Brazil, as in other countries, viral isolation in cell culture has been replaced by rapid tests, such as IFA or molecular techniques, due to the importance of the time element in clinical virology. Moreover, the cost of running a cell culture has limited the number of laboratories that provide this service in developed countries (Ogilvie 2001). However, cell culture will remain important to maintain a source for genetic analysis and antigenic change in

virus populations and for discovering new viruses. Even so, laboratories with the skills and resources for cell culture are becoming scarcer (Henrickson 2004).

This study shows that more than 40% of RSV isolates remain infectious when stored at –20ºC for up to 12 months. The infectivity seems to be lost for samples stored at this temperature for more than 12 months. Nevertheless, only four samples were tested in this condition. The results presented in this study indicate that RSV may be recovered by cell culture from NPA specimens stored at –20ºC. It may be useful for epidemiological studies especially in those places where it is not possible to store clinical specimens at –70ºC for viral diagnosis.

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