Enhancing Effect of Centrifugation on Isolation of Influenza Virus from Clinical Specimens

MASATO SENO,†* YASUO KANAMOTO,† SHINICHI TAKAO,† NAOMI TAKEI,† SHINJI FUKUDA,‡ AND HIROYUKI UMISA†

Division of Microbiology, Hiroshima Prefectural Institute of Public Health, Ujinakanda 1-5-70,† and Department of Biometrics, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Kasumi 1-2-3,‡ Minami-ku, Hiroshima 734, Japan

Received 13 November 1989/Accepted 5 February 1990

The use of centrifugation (700 × g, 60 min) in a plaque assay markedly increased (mean, 2.9-fold) the infectivity of all 42 influenza virus strains tested, compared with no centrifugation. Of 13 influenza virus strains isolated from 390 clinical specimens, 9 (69%) were efficiently isolated by the centrifugation assay compared with conventional culture methods. The centrifugation assay may be useful for isolating the influenza virus from clinical specimens.

Rapid diagnostic assays have been developed for the detection of influenza virus antigens (1, 3, 6, 7, 10). Early detection of the influenza virus by these assays is important in order to confirm the clinical diagnosis of influenza and initiate subsequent antiviral chemotherapy and other preventive measures (17). Conventional cell culturing remains the most sensitive method for detecting the influenza virus (3, 8, 10). Direct detection of the influenza virus without isolation is a drawback because isolates provide reference antigens that are used to detect antigenic drift in the hemagglutinin and for antiviral drug susceptibility testing (15). Although a recent report has suggested that centrifugation of the inoculated cells might enhance the sensitivity of influenza virus cultures (3), further investigation on the effect of centrifugation is required (16). The present study was performed to determine whether centrifugation enhances the sensitivity of influenza cultures and improves the isolation rates.

In the initial experiments, influenza virus type A H1N1, type A H3N2, and type B were studied (14 isolates). The stock virus was grown in MDCK cell cultures and harvested by freeze-thawing the cultures. The supernatant virus was clarified by centrifugation (1,600 × g) and frozen at −80°C. For the centrifugation studies, MDCK cell cultures were grown in six-well plates (35-mm diameter; Costar, Cambridge, Mass.). Forty-eight-well cell cultures were inoculated with 1.0 ml of virus suspension in which the infectivity titer of the stock influenza virus was adjusted to 20 to 30 PFU/ml for each strain. The 48 wells inoculated with each strain were divided into groups of 12 and centrifuged for 0, 15, 30, and 60 min each at 700 × g at 23°C in a centrifuge with an RS-9A rotor (Kubota, Tokyo, Japan). The 12 wells that did not undergo centrifugation were incubated at 34°C for 60 min in a CO₂ incubator. After centrifugation, standard procedures were employed for determination of the PFU of the well cultures (13, 14). The results described here were analyzed statistically by the Wilcoxon matched-pairs signed-rank test within pairs or the Mann-Whitney U test without pairs (P < 0.05) (9).

For the centrifugation technique, another experiment using the same isolates was performed. The yields of hemagglutinin activity in serial 10-fold dilutions were determined with a 0.5% suspension of chicken erythrocytes (2). For each strain, 8 wells in 24-well plates (16-mm diameter; Costar) of MDCK cell cultures were inoculated with 0.2 ml of each dilution of virus. Half of the wells inoculated were centrifuged for 60 min at 700 × g, and the half that were not centrifuged were incubated at 34°C for 60 min. After centrifugation, 1.0 ml of serum-free medium supplemented with trypsin was added to each well, and the plates were incubated at 34°C for 5 days. After incubation, the fluids recovered from the wells after a single freeze-thaw cycle were tested for hemagglutinin activity.

A total of 390 throat swab specimens were collected (each in 5 ml of transport medium) from patients at participating hospitals in a pathogenic-microbe surveillance system in Hiroshima Prefecture. The specimens were centrifuged at 1,600 × g for 30 min at 4°C and frozen at −80°C to be processed for isolation. Eight wells of the 24-well plates of MDCK cell cultures were inoculated with 0.2 ml of the supernatant. The wells were divided into two groups. The procedures described above for the experiment with the serial 10-fold dilutions were followed to isolate influenza viruses after three blind passages.

With the use of centrifugation in the plaque assay, the number of plaques observed increased with the duration of centrifugation and without any difference between the serotypes (Table 1). With 15 min of centrifugation, the plaques of 35 strains (83%) significantly increased in comparison with those in cultures that were not centrifuged. In addition, with 30 and 60 min of centrifugation the number of plaques of all strains significantly increased. From these results, we found that 60 min of centrifugation was most useful for routine isolation, since the advantages of a centrifugation time longer than 60 min might be diminished.

In the centrifuged wells, the hemagglutinin activities of 32 strains (76%) were 10-fold-greater than those in wells without centrifugation, and there was no difference between the serotypes. There was a significant difference in the detection of the influenza virus between plates that had undergone centrifugation and those that had not undergone centrifugation. The results of two experiments using stock viruses were compatible with the growth cycle of influenza viruses (13), and it was suggested that centrifugal inoculation of influenza viruses onto monolayer cultures might be useful in

* Corresponding author.
isolating the virus from specimens containing low levels of the influenza virus.

Of the clinical specimens, three type A (H1N1) and nine type B specimens were positive for influenza viruses by the centrifugation assay; six, five, and one specimen were isolated at one, two, and three passages, respectively. Six type B specimens were positive by assay with no centrifugation; three, two, and one specimen were isolated at one, two, and three passages, respectively. Of 13 isolates, 1 was isolated with no centrifugation, but none were isolated with centrifugation, as the specimen had probably not been agitated thoroughly. In nine isolates (69%), the centrifugation assay was shown to be effective. There was no significant difference in the efficient isolation of the influenza virus between the two assays (0.05 < P < 0.06), although the number of positive specimens studied was relatively small.

Several studies have shown the advantages of centrifugation in viral diagnostic techniques (3–5, 11). Espy et al. (3), using laboratory strains of influenza virus type A (H3N2), found that centrifugation of the inoculated shell vials increased the number of fluorescent foci observed. However, centrifugation of clinical specimens onto a cell monolayer has not been clearly shown to increase sensitivity for the influenza virus. Tenser (11) reported that centrifugation (1,000 × g) results in a 10-fold increase in herpes simplex virus infectivity. The centrifugal enhancement was of a degree similar to that reported by Tenser. In positive clinical specimens, there probably are not only specimens containing low concentrations of influenza virus which are difficult to detect by centrifugation assay but also specimens containing virus that is easily detectable by conventional culturing. Therefore, in clinical specimens it may be difficult to statistically show that the centrifugation assay is significantly different from an assay with no centrifugation. However, the results in this study indicate the usefulness of centrifugation. In fact, the number of isolates obtained with centrifugation was twice as high as that obtained with no centrifugation, and the number of isolates obtained with centrifugation of the first passage was equal to that obtained with no centrifugation with three passages. Further studies with larger numbers of specimens may more clearly define the role of centrifugation in isolation. The possibility of enhanced herpes simplex virus penetration into cells by ultracentrifugal inoculation has been reported (12). With low-speed centrifugation, it is hypothesized that aggregates are brought into contact with the monolayers (5, 11).

In summary, the present study has demonstrated that the usefulness of centrifugation in the plaque assay enhances the infectivity of influenza viruses on monolayers and suggests that the 24-well plate centrifugation assay provides a sensitive method for the efficient isolation of the influenza virus from clinical specimens, especially those containing low levels of virus.

**TABLE 1. Effect of the length of centrifugation time on the infectivity of influenza viruses in a plaque assay**

<table>
<thead>
<tr>
<th>Influenza virus type</th>
<th>No. of isolates tested</th>
<th>Increase in no. of plaques (mean ± SD) after centrifugation for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15 min</td>
</tr>
<tr>
<td>A (H1N1)</td>
<td>14</td>
<td>1.83 ± 0.40</td>
</tr>
<tr>
<td>A (H3N2)</td>
<td>14</td>
<td>2.08 ± 0.52</td>
</tr>
<tr>
<td>B</td>
<td>14</td>
<td>1.74 ± 0.32</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>1.88 ± 0.44</td>
</tr>
</tbody>
</table>

* Relative to number of plaques in cultures with no centrifugation (1.00). Centrifugation was at 700 × g.

**LITERATURE CITED**