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Application of Directigen FLU-A for the Detection of Influenza A Virus in Human and Nonhuman Specimens

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Directigen FLU-A, a new enzyme immunoassay membrane test, rapidly detects influenza A virus antigen in specimens from patients. Nasopharyngeal washes and pharyngeal gargles were used to determine the effectiveness of the assay as applied to different types of routinely collected clinical samples. All specimens had been previously shown to contain influenza A virus by virus isolation in tissue culture. Directigen FLU-A was 90% sensitive (95% confidence interval, 56 to 99.7%) with nasopharyngeal washes but only 39% sensitive (95% confidence interval, 17 to 64%) with pharyngeal gargles ($P = 0.018$) when used with samples containing similar amounts of infectious virus (50% tissue culture infective dose, 1.0 to 4.5). The intensity of the positive reaction with Directigen FLU-A did not correlate with the amount of virus in the specimens. Directigen FLU-A was found to detect cell-associated antigen more readily than free virus; only 20 infected cells were required to identify cell-associated influenza A virus antigen, whereas the limit of detection for free virus was 1.63×10^3 infectious virus particles. These findings suggest that Directigen FLU-A detects the cell-associated antigen present in clinical specimens rather than free virus. In addition, Directigen FLU-A detected avian and swine influenza A viruses in both cloacal swabs (75% sensitivity) and swine lung homogenates (86% sensitivity), indicating its potential usefulness in the surveillance of nonhuman influenza A viruses.

Influenza A virus infection in humans and other animal species continues to have a significant impact on world health (1, 6, 8). The rapid diagnosis of influenza A virus infection offers several important advantages. The early identification of influenza outbreaks could enable public health officials to hasten the initiation of appropriate infection control measures. Similarly, a rapid diagnosis in immunocompromised patients would allow the timely initiation of antiviral therapy with amantadine or rimantadine to treat or prevent influenza A virus infection. In addition, the epidemiology of influenza A virus infection compared with influenza-like illnesses caused by other viruses and with influenza B or C virus infection could be better defined by the use of rapid diagnostic techniques.

A newly developed enzyme immunoassay membrane test, Directigen FLU-A (Becton Dickinson Microbiology Systems, Cockeysville, Md.), has been shown to detect influenza A viruses in clinical specimens in less than 15 min and is simple to perform (10). In contrast, the commonly used tests, immunofluorescence and the enzyme-linked immunosorbent assay, which have been available to detect influenza viruses in clinical specimens, are relatively insensitive and require greater amounts of time and technical expertise to perform accurately (2, 3, 5, 9). Directigen FLU-A detects type-specific nucleoprotein antigen via enzyme-conjugated monoclonal antibodies that are specific for a conserved epitope of the nucleoprotein. Accordingly, all subtypes of influenza A (but not influenza B and influenza C) virus are detected.

The manufacturer reports a sensitivity of 91% and a specificity of 95% with the use of nasopharyngeal wash (NPW) or aspirate specimens. These specimens are the designated specimens of choice because they were found to be superior to other samples with respect to sensitivity and specificity. With NPWs as the primary clinical specimen (10), the new assay correlated favorably in sensitivity (100%) and specificity (91.6%) compared with testing by isolation in cell culture and direct immunofluorescence.

Little information is currently available on the clinical specimen type(s) that will be optimal with Directigen FLU-A, the viral concentration needed to obtain positive results, and the assay's potential usefulness in detecting avian or swine influenza A viruses. We, therefore, studied the assay to examine the issue of specimen of choice, to determine the test's limits of sensitivity, and to assess its ability to detect nonhuman influenza A viruses.

MATERIALS AND METHODS

Clinical specimens. NPWs and pharyngeal gargles (PGs) obtained from pediatric and adult patients during the 1985, 1988, 1989, and 1990 influenza seasons were used. NPWs, the standard specimens in the pediatric population, were placed in a viral transport medium (minimal essential medium with 0.5% gelatin and antibiotics) after collection. PGs were obtained from adult patients by using phosphate-buffered saline (PBS) with antibiotics because a PG is less irritating to the nasopharynx than an NPW and adults are able to provide this type of specimen easily. The specimens had been demonstrated to contain influenza A virus by isolation in either primary rhesus monkey kidney (PRMK) or Madin-Darby canine kidney (MDCK) cells prior to our study. The presence of influenza A virus was confirmed by direct immunofluorescence or by the hemagglutination inhibition assay as described previously (4, 7). The specimens were stored at −70°C immediately after an aliquot was inoculated into the tissue culture. NPWs were stored with-

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out the addition of other stabilizing agents; bovine serum albumin (final concentration, 1.0%) was added to the PGs. Duplicate samples were not obtained from the same patient. Repeat testing was not performed with individual samples.

Cloacal swabs from waterfowl and swine lung homogenates that had been stored at −70°C were used for the detection of animal viruses. Influenza A virus in these specimens had been demonstrated by inoculation into embryonated hens' eggs and confirmed by the hemagglutination inhibition assay. Cloacal swabs were transported and stored in a PBS solution containing 50% glycerol and antibiotics. Swine lung samples were prepared by homogenizing lung tissue in PBS with antibiotics and were stored without the addition of stabilizing agents. Each test sample was taken directly from the storage container and used without any processing of the sample to remove debris or tissue.

Directigen FLU-A. The Directigen FLU-A antigen test was performed according to the manufacturer's instructions immediately after thawing at 37°C. Briefly, the sample aliquot to be tested (125 μl) was mixed with detergent and a mucolytic agent and applied to the test membrane. After washing, the enzyme-conjugated monoclonal antibody was added and the reactivity was determined by visual color development. If the control dot (containing influenza A virus antigen) in the center of the indicator site turned purple, the test was considered interpretable. The appearance of a purple triangle on the test membrane signified a positive reaction. Reactive tests were graded 1+ to 4+ on the basis of the intensity of the color reaction. A test was judged negative if a purple triangle did not appear. Reactions were assessed independently by two of the investigators (K. A. Ryan-Poirier and Y. Kawaoka).

The assay's limit of sensitivity in the detection of free virions versus infected cells was determined by using A/Udorn/302/72 (H3N2) virus, which contained a known concentration of virus (1.6×10^7 PFU/ml). Serial 10-fold dilutions of the virus were made in PBS, and 125 μl of each viral dilution was used to perform the Directigen FLU-A assay. A confluent monolayer of MDCK cells was infected with virus at a multiplicity of infection of 5 to ensure each cell's infection. After 6 h of infection, the cells were removed by mild trypsin treatment, washed, and counted. Serial 10-fold dilutions of this infected cell suspension and an uninfected cell suspension (control) were then used to perform the Directigen FLU-A assay by using 125 μl of each dilution. The assay includes a step to lyse both the virus and cells with detergent prior to application to the membrane.

Viral culture. Samples of clinical specimens were inoculated onto PRMK and MDCK cells at the same time the antigen test was performed. The presence of virus was determined by the hemagglutination assay at days 3 to 4 and again on days 7 to 8. Hemagglutinating agents were further identified by conventional hemagglutination inhibition tests.

RESULTS

Comparison between NPWs and PGs. Twenty-four PGs and 17 NPWs, from which influenza A had been isolated (before storage at −70°C), were used. Seventeen (71%) of the PGs and 10 (59%) of the NPWs contained viable virus, as determined by reisolating the virus in MDCK or PRMK cells or both. Seven (39%) of the 17 PG specimens (95% confidence interval, 17 to 64%) with viable virus gave a positive result with the Directigen FLU-A assay (Table 1). PGs lacking viable virus did not give a positive reaction. Nine (90%) of the 10 NPW specimens (95% confidence interval, 56 to 99.7%) with viable virus tested positive by Directigen FLU-A. Two NPWs containing no viable virus gave positive reactions. The difference in the ability of Directigen FLU-A to detect influenza A virus in PGs versus NPWs was significant ($P = 0.018$ by the two-tailed Fisher exact test). No indeterminant reactions were obtained with any of the samples; however, the shape of the reactive purple triangle was slightly abnormal in one sample that gave a positive reaction.

TABLE 1. Comparison of Directigen FLU-A antigen detection in PGs and NPWs

Specimen (*n*)	No. (%) of specimens with viable virus	Directigen result (no. of specimens)[a]			
		With viable virus		Without viable virus	
		Positive	Negative	Positive	Negative
PG (24)	17 (71%)	7	10	0	7
NPW (17)	10 (59%)	9	1	2	5

[a] $P = 0.018$ for the difference in the ability of the Directigen FLU-A assay to detect antigen in PGs versus NPWs with the two-tailed Fisher exact test.

The intensity of the Directigen FLU-A reaction varied between the two types of specimens. The 7 Directigen-positive PGs produced less-intense readings (1+ or 2+) than did the 11 Directigen-positive NPWs (1+ to 4+) (Table 2). One-third of the NPW samples produced intensity readings of 3+ or 4+. The two samples that contained no viable virus but gave positive reactions had intensity readings of 2+ and 4+. Interestingly, the amount of virus in the clinical sample (50% tissue culture infective dose [$TCID_{50}$] per milliliter) did not correlate with the intensity of the Directigen FLU-A reaction. Samples containing more than 10^3 $TCID_{50}$/ml did not necessarily produce a stronger intensity reaction (Table 2). Five of the PGs were not titrated because of inadequate sample volumes. If the concentration of virus in these samples had been found to be greater upon titration (i.e., $>10^2$ $TCID_{50}$/ml), the negative or low-intensity Directigen FLU-A readings obtained with these samples would not have altered the observation described above.

Detection of cell-associated antigen versus free virus. Because the quantity of virus in the PGs and NPWs did not correlate with the Directigen FLU-A reaction, we thought that the test may preferentially detect cell-associated antigen rather than free virus. We therefore determined the ability of

TABLE 2. Intensity of Directigen FLU-A reaction compared with the concentration of virus in PGs and NPWs

Specimen and no. of samples with reactivity	Concn of virus (log_{10} $TCID_{50}$/ml)	No. of samples with the following Directigen reaction:				
		−	1+	2+	3+	4+
PG						
12[a]	1.0–2.0	7	3	2	0	0
5	3.0–4.5	3	1	1	0	0
NPW[b]						
2	<1.0	0	0	1	0	1
3	1.0–2.0	1	1	1	0	0
3	2.0–3.0	0	1	0	1	1
4	3.0–4.5	0	2	1	0	1

[a] Five samples in this group were not titrated.

[b] Includes the two samples that did not contain viable virus but produced a positive Directigen FLU-A reaction.

Directigen FLU-A to detect cell-associated antigen versus free viral particles in an in vitro assay. The limit of sensitivity with respect to cell-associated antigen was 20 influenza A virus-infected MDCK cells, whereas the limit of detection of free virus was 1.65×10^3 PFU on MDCK cells. Uninfected MDCK cells produced a negative Directigen reaction as expected. Similar results were obtained on repeat examination. This finding coupled with the lack of correlation between the amount of free virus in a specimen and the reaction intensity of the assay suggests that the results of Directigen FLU-A are determined by the amount of cell-associated antigen rather than that of free virus in clinical specimens.

Detection of animal influenza A viruses. Four cloacal swabs collected from Canadian ducks for influenza A virus surveillance purposes and shown to be positive for avian influenza A viruses of hemagglutinin subtypes H3 and H6 were tested by the Directigen FLU-A assay. Three of the four cloacal swabs (75%) gave positive reactions. Four cloacal swabs collected from ducks in the Memphis area that did not contain influenza A virus produced negative Directigen FLU-A reactions. Lung homogenates were prepared from lung tissue obtained at postmortem examinations from 14 swine during an outbreak of influenza-like illness, and a swine influenza A virus (H1N1) was isolated from these samples. When tested by the Directigen FLU-A assay, 12 (86%) were positive. No indeterminant reactions occurred in this group of samples, although three of the swine lung homogenates had to be diluted (1:4), in accordance with the manufacturer's instructions, because of the amount of cellular debris (lung tissue). These results indicate that the Directigen FLU-A assay may be effective for the rapid identification of influenza A virus infection in lower animals, including poultry.

DISCUSSION

The Directigen FLU-A assay offers a potentially useful tool for the direct detection of influenza A virus antigen in clinical specimens, but the effectiveness of the assay is affected by the type of clinical sample tested. When specimens other than those recommended by the manufacturer (NPW or aspirate) are used, the sensitivity of the assay is reduced. We demonstrate in this report that PGs are not optimal specimens for use with the Directigen FLU-A assay. PGs containing viable influenza A virus gave a negative Directigen reaction, producing false-negative results. The failure to detect influenza A antigen in PGs could not be correlated with the quantity of virus in the samples. Since the assay results seem to be determined by the amount of cell-associated antigen present in a specimen, the explanation may be that the amount of such antigen in a PG sample is below the assay's threshold for detection. If so, any specimen with insufficient cell-associated antigen would be inadequate for use with this new assay system and produce false-negative results. Although the volume of NPWs and PGs are not the same (NPW, ~3 ml; PG, ~20 ml), the amount of virus ($TCID_{50}$ per milliliter) in the PGs and NPWs did not differ significantly. However, the greater volume in a PG could result in the dilution of cell-associated antigen and therefore result in lack of detection by the Directigen FLU-A assay. Increasing the number of epithelial cells or cell debris via a commercially available microconcentration system might improve results with such specimens.

Another explanation for the lack of detection of influenza virus antigen in PGs could be the presence of a substance(s) that inhibits the Directigen FLU-A reaction. We examined this possibility by combining a Directigen-negative PG that contained viable virus with a Directigen-positive NPW that contained viable virus. Only one of four PGs examined reduced the intensity of the Directigen reaction (from 4+ to 3+) (data not shown). Thus, although in some instances, an inhibitory substance in a PG could reduce the sensitivity of the assay, this does not appear to be the major cause of the insensitivity of Directigen FLU-A with PGs. We are not able to discount the possibility that freeze-thawing of the specimens could have affected our results. However, since both PGs and NPWs underwent freeze-thawing in the same manner, such a possibility is unlikely. Further examination with freshly obtained samples would clarify this point.

Our findings illustrate that the value and the range of application of Directigen FLU-A may be limited by the choice of the clinical specimen(s). This will be less of a problem with younger pediatric patients in whom an NPW is routinely obtained. However, older children and adults are more likely to resist an NPW. Alternative specimens obtained from this population, such as PGs, may provide less reliable results. In addition, if specimens obtained during invasive diagnostic procedures, such as bronchial washes and tracheal aspirates, give unreliable results, the assay's usefulness with critically ill patients may be limited. Medical personnel will need to be aware of these limitations (as well as any limitations associated with the technical performance of the test or the interpretation of results) if accurate and reliable data are to be obtained with Directigen FLU-A.

Two of the NPW specimens that produced positive Directigen reactions did not contain viable virus. Since influenza A virus was initially isolated from these samples, the viruses probably did not survive the freeze-thawing process. These samples cannot be considered false positives since influenza A virus was known to be present in the sample on initial testing. The detection of viral antigen by using an analogous assay without recovery of the virus in tissue culture has also been demonstrated with respiratory syncytial virus (11). Waner reported a false-positive rate of 8.4% overall for Directigen FLU-A, on the basis of a positive Directigen reaction without recovery of the virus in tissue culture and by using isolation of the virus in culture as the standard for comparison (10). This type of result occurred with sputum and tracheal aspirates (not considered to be specimens of choice) and with NPW specimens. Some of these specimens could have been true positives (virus was not isolated but viral antigen was present), and some may represent false positives (no viable virus or antigen present).

Directigen FLU-A was able to detect influenza A virus antigen with reasonable sensitivity in specimens collected from ducks and pigs, indicating the assay's potential usefulness with material in the veterinary or agricultural industry, such as cloacal swabs or lung homogenates. It is noteworthy that influenza A virus antigen was detected in these samples despite the presence of a large amount of particulate matter (fecal material and lung tissue). These specimens may contain a significant amount of cell-associated antigen that facilitates antigen detection. In addition, it was possible to detect influenza A virus hemagglutinin subtype H6, which is not found in humans. Such rapid detection may be particularly useful in managing epidemics among livestock, allowing infected animals to be identified and isolated much more quickly than would be possible with conventional methods. Further study with larger numbers and a wider variety of specimens from different animal species is needed to assess Directigen FLU-A for this purpose.

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