JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1995, p. 318–321 Vol. 33, No. 2
0095-1137/95/$04.00+0

Type- and Subtype-Specific Detection of Influenza Viruses in Clinical Specimens by Rapid Culture Assay

THEDI ZIEGLER,[1,2]* HENRIETTA HALL,[1] ALICIA SÁNCHEZ-FAUQUIER,[3] WILLIAM C. GAMBLE,[1] AND NANCY J. COX[1]

Influenza Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[1]; Department of Virology, University of Turku, FIN-20520 Turku, Finland[2]; and Department of Molecular Biology, Centro Nacional de Microbiologia, Virologia e Immunologia Sanitarias, Majadahonda, E-28220 Madrid, Spain[3]

Received 25 April 1994/Returned for modification 12 July 1994/Accepted 27 October 1994

A rapid culture assay which allows for the simultaneous typing and subtyping of currently circulating influenza A(H1N1), A(H3N2), and B viruses in clinical specimens was developed. Pools of monoclonal antibodies (MAbs) against influenza A and B viruses and MAbs HA1-71 and HA2-76, obtained by immunizing mice with the denatured hemagglutinin subfragments HA1 and HA2 of influenza virus A/Victoria/3/75, were used for immunoperoxidase staining of antigens in infected MDCK cells. MAb HA1-71 reacted exclusively with influenza A viruses of the H3 subtype, while MAb HA2-76 reacted with subtypes H1, H3, H4, H6, H8, H9, H10, H11, and H12, as determined with 78 human, 4 swine, and 10 avian influenza virus reference strains subtyped by the hemagglutination inhibition test. To determine if the technique can be used as a rapid diagnostic test, 263 known influenza virus-positive frozen nasal or throat swabs were inoculated into MDCK cells. After an overnight incubation, the cells were fixed and viral antigens were detected by immunoperoxidase staining. Influenza A viruses of the H1 and H3 subtypes were detected in 31 and 113 specimens, respectively. The subtypes of 10 influenza A virus-positive specimens could not be determined because they contained too little virus. Influenza B viruses were detected in 84 specimens, and 25 specimens were negative. We conclude that this assay is a rapid, convenient, non-labor-intensive, and relatively inexpensive test for detecting, typing, and subtyping influenza viruses in clinical specimens.

A large number of laboratories participate in the worldwide surveillance of influenza virus activity and contribute to the early recognition of newly emerging epidemic strains. Differentiation between influenza A and B viruses and determination of the subtypes of influenza A virus isolates are the first steps in the characterization of influenza viruses. This analysis is traditionally done by hemagglutination inhibition (HI) tests with specific antisera raised in ferrets, chickens, or sheep. After primary isolation, one or more passages of the virus in embryonated hens' eggs or in cell cultures are often required to obtain a sufficiently high hemagglutination titer to allow performance of the HI test. Because this method is labor-intensive and time-consuming, many laboratories postpone the subtyping until after the influenza season, causing a considerable delay in the reporting of accurate epidemiologic data.

To facilitate improved analysis of influenza virus strains, laboratories need more rapid and less cumbersome methods for the type- and subtype-specific identification of influenza viruses directly from clinical specimens or at most after one passage. The adequate prophylactic and therapeutic use of the drugs amantadine and rimantadine also requires rapid laboratory identification of influenza A viruses in clinical specimens obtained from individuals with influenza-like symptoms.

Of the numerous techniques developed for the rapid diagnosis of viral infections during recent years, detection of viral antigens by immunofluorescence or immunoperoxidase staining in infected cell cultures after a short incubation has found wide application for the identification of a number of viruses. This technique combines the sensitivity of virus isolation with the speed and specificity of cell culture-independent methods such as direct detection of viral antigens in clinical specimens by immunofluorescence and immunoassays or the detection of viral nucleic acids by PCR. Using well-established pools of monoclonal antibodies (MAbs) against influenza A and B viruses and MAbs against the denatured HA1 and HA2 subfragments of the influenza A virus hemagglutinin, we developed a rapid culture assay for the type- and subtype-specific detection of currently circulating human influenza A (H1N1) and A (H3N2) and B viruses in clinical specimens.

MATERIALS AND METHODS

Cells and viruses. MDCK cells (ATCC CCL34; American Type Culture Collection, Rockville, Md.) were used in all experiments. Unless otherwise stated, they were grown in Dulbecco's modified Eagle medium supplemented with 5% fetal bovine serum (FBS), 0.2% bovine serum albumin, 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), penicillin, and streptomycin. Ten avian influenza viruses of different hemagglutinin subtypes, 4 swine, and 78 human influenza virus strains that had previously been subtyped by HI (7) were included in the study (Table 1). All isolates were grown in embryonated hens' eggs and were selected from the strain collection of the World Health Organization Collaborating Center for Influenza at the Centers for Disease Control and Prevention (Atlanta, Ga.). The influenza viruses A/Texas/36/91(H1N1), A/Beijing/352/89(H3N2), and B/Panama/45/90 were used as controls in all experiments.

Clinical specimens. A total of 263 influenza A or B virus-positive nasal and/or throat swabs collected by physicians participating in the sentinel physician network (1) during five influenza seasons between 1988 and 1993 were available for testing. All specimens had been divided into two parts after collection. One part was frozen at −70°C and was later used in the present study, and the other part was tested immediately for the presence of influenza viruses by a rapid culture assay (4).

MAbs. For typing influenza A and B viruses, two pools of MAbs were used (13). Pool A contained two MAbs against the nucleoprotein of influenza A virus, and pool B contained one MAb against the nucleoprotein and one against the

* Corresponding author. Mailing address: Influenza Branch, Mailstop G-16, DVRD, NCID, Centers for Disease Control and Prevention, 1600 Clifton Road, N.E., Atlanta, GA 30333. Phone: (404) 639-3506. Fax: (404) 639-2334. Electronic mail address: txz3@CIDDVD1.EM.CDC.GOV (Internet).

TABLE 1. Influenza A viruses used to determine the reactivities of MAbs HA1-71 and HA2-76

Virus strain	Virus strain	Virus strain
Human H1N1 viruses	2. A/Duck/Czechoslovakia(H4N6)	6. A/Washington/91
1. A/Texas/36/91	3. A/Tern/South Africa/61(H5N3)	7. A/Beijing/03/90
2. A/Arizona/36/91	4. A/Shearwater/Australia/73(H6N5)	8. A/Hong Kong/25/90
3. A/France/431/91	5. A/Turkey/Oregon/71(H7N3)	9. A/Beijing/337/90
4. A/Oklahoma/33/91	6. A/Turkey/Ontario/6118/68(H8N4)	10. A/Shanghai/06/90
5. A/Stockholm/26/90	7. A/Turkey/Wisconsin/1/66(H9N2)	11. A/South Africa/1000/90
6. A/Yamagata/32/89	8. A/Chicken/Germany"N"/49(H10N8)	12. A/Yokohama/18/90
7. A/Sichuan/32/89	9. A/Duck/Memphis/546/74(H11N9)	13. A/Czechoslovakia/19/89
8. A/Czechoslovakia/02/89	10. A/Duck/Alberta/60/76(H12N5)	14. A/England/648/89
9. A/Fiji/02/88		15. A/Guangdong/39/89
10. A/France/115/88	Human H2N2 viruses	16. A/Czechoslovakia/16/89
11. A/South Carolina/06/88	1. A/Leningrad/549/80	17. A/Guizhon/54/89
12. A/Victoria/36/88	2. A/Ann Arbor/7/67	18. A/Victoria/5/89
13. A/Pennsylvania/34/86	3. A/Tokyo/3/67	19. A/Beijing/352/89
14. A/Beijing/01/86	4. A/Thailand/394/65	20. A/England/427/88
15. A/Singapore/06/86	5. A/Pittsburgh/2/65	21. A/Los Angeles/2/87
16. A/Stockholm/19/86	6. A/New Jersey/3/65	22. A/Shanghai/11/87
17. A/Taiwan/01/86	7. A/Taiwan/1/64	23. A/Sydney/1/87
18. A/Trinidad/02/86	8. A/Georgia/1/63	24. A/Sichuan/2/87
19. A/Dunedin/27/83	9. A/Japan/170/62	25. A/Victoria/7/87
20. A/Victoria/7/83	10. A/Panama/1/61	26. A/Leningrad/188/86
21. A/Chile/1/83	11. A/England/1/61	27. A/Washington/1/86
22. A/India/6263/80	12. A/Chile/2/61	28. A/Sweden/100/86
23. A/England/333/80	13. A/Spain/1/61	29. A/Mississippi/1/85
	14. A/Chile/13/57	30. A/Parana/3/85
Porcine H1N1 viruses	15. A/Japan/305/57	31. A/Stockholm/8/85
1. A/Sw/USA/31	16. A/Okuda/57	32. A/USSR/26/85
2. A/Sw/Northern Ireland/38		33. A/Capetown/2/85
3. A/Sw/Cambridge/39	Human H3N2 viruses	34. A/Caen/1/84
	1. A/Beijing/32/92	35. A/Christchurch/1/84
Porcine H3N2 virus	2. A/Brazil/02/92	36. A/Rome/6/85
1. A/Sw/Hong Kong/77	3. A/England/261/91	37. A/Taiwan/16/83
Avian influenza viruses	4. A/Kawasaki/1/91	38. A/Panama/1/83
1. A/Duck/Hong Kong/273/78(H2N2)	5. A/Shanghai/14/91	39. A/Philippines/2/82

hemagglutinin of influenza B virus. In addition, two MAbs, HA1-71 and HA2-76, were used for subtyping influenza A viruses. These two antibodies were obtained by immunizing mice with the denatured subfragments HA1 and HA2 of influenza virus A/Victoria/3/75 (9, 10). Pools A and B were used at a dilution of 1:500, and MAbs HA1-71 and HA2-76 were used at a dilution of 1:100.

Typing and subtyping of influenza virus reference strains. Ninety-six-well cell culture plates (Costar, Cambridge, Mass.) were seeded with 10^4 cells per well in 100 μl of growth medium containing 1% FBS. After an overnight incubation at 37°C in 5% CO_2, 90 μl of medium without FBS, but 4 μg of tolylsulfonyl phenyalanyl chloromethyl ketone (TPCK)–trypsin (Sigma Chemical Co., St. Louis, Mo.) per ml was added without prior removal of the growth medium. To each of four wells 10 μl of an isolate was inoculated. On each plate, four wells were left empty and the three control viruses were inoculated in the same way as the isolates under study. The plates were incubated at 37°C for 10 min to equilibrate the pH and were then wrapped in plastic film and centrifuged at 700 × *g* at ambient temperature for 45 min. After an overnight incubation, usually for 16 to 20 h, the medium was removed, and the cells were washed twice with phosphate-buffered saline (PBS), fixed with absolute methanol at ambient temperature for 10 min, and again washed twice with PBS. The antibodies, diluted in PBS containing 5% nonfat dry milk, were then added so that one well for each isolate was incubated with one of the antibodies. The plates were kept at 37°C for 90 min, washed four times with PBS, and further incubated with horseradish peroxidase-labeled sheep antibodies to mouse immunoglobulins (Amersham Corporation, Arlington Heights, Ill.) for 60 min. The substrate solution was prepared by dissolving 20 mg of 3-amino-9-ethylcarbazole (Sigma) in 5 ml of dimethylformamide and diluting it to a final concentration of 200 μg/ml in sodium acetate buffer (pH 5.0). The solution was clarified by filtration and was stored frozen in aliquots at −20°C. Before use, 1 μl of 30% H_2O_2 per ml was added and 100 μl of substrate solution was dispensed to each well. The color reaction was developed for 30 to 60 min. The substrate solution was then replaced by 100 μl of PBS per well, and the plates were read on a light box and with an inverted microscope at a ×100 magnification. Intracellular red staining in the absence of background staining in uninfected cells was regarded as a positive result.

Type- and subtype-specific detection of influenza viruses in clinical specimens. Cells (5×10^4) in 500 μl of growth medium containing 5% FBS were added to each well of 24-well cell culture plates. On the next day, the growth medium was replaced by 450 μl of medium without FBS but containing 2 μg of TPCK-trypsin per ml. The specimens were quickly thawed in warm water and were then kept on ice until it was inoculated. After vigorous vortexing, 50 μl of specimen was inoculated into each of four wells. The control viruses were included in each batch, and the assay was performed as described above. Clinical specimens that showed five or fewer cells positively stained with pool A were considered positive but were not subtyped.

Subtyping of influenza A viruses isolated from clinical specimens by HI. Twenty influenza A virus-positive specimens were reinoculated into MDCK cells, and when the cultures showed evidence of an advanced cytopathic effect, the viruses were subtyped by HI with sheep hyperimmune sera against the influenza reference viruses A/Taiwan/01/86(H1N1) or A/Beijing/353/89(H3N2).

RESULTS

Intense cytoplasmic staining was seen in infected cells stained with pool A. Slightly less intensive cytoplasmic and membrane staining was observed with pool B. MAbs HA1-71 and HA2-76 gave a less strong but clear cytoplasmic staining, often with perinuclear accumulation. The reaction patterns of MAbs HA1-71 and HA2-76 with viruses of different hemagglutinin subtypes are shown in Table 2.

The assay was evaluated on human, avian, and swine influenza viruses previously subtyped by HI. HA1-71 reacted exclusively with human influenza viruses of the H3 subtype and with the swine influenza virus A/SW/Hong Kong/77(H3N2). HA2-76 gave a signal with all the human H1 and H3 isolates, all the swine viruses, and the avian influenza viruses of subtypes H4, H6, H8, H9, H10, H11, and H12. Neither HA1-71

TABLE 2. Reaction patterns of MAbs with different hemagglutinin subtypes

Virus	Subtype	Reaction pattern of MAb:			
		Pool A	HA1-71	HA2-76	Pool B
A/Texas/36/91	H1N1	+	−	+	−
A/Duck/Hong Kong/273/78	H2N2	+	−	−	−
A/Beijing/32/92	H3N2	+	+	+	−
A/Duck/Czechoslovakia	H4N6	+	−	+	−
A/Tern/South Africa/61	H5N3	+	−	−	−
A/Shearwater/Australia/73	H6N5	+	−	+	−
A/Turkey/Oregon/71	H7N3	+	−	−	−
A/Turkey/Ontario/6118/68	H8N4	+	−	+	−
A/Turkey/Wisconsin/1/66	H9N2	+	−	+	−
A/Chicken/Germany"N"/49	H10N8	+	−	+	−
A/Duck/Memphis/546/74	H11N9	+	−	+	−
A/Duck/Alberta/60/76	H12N5	+	−	+	−
B/Panama/45/90		−	−	−	+

nor HA2-76 reacted with the avian or any of the 16 human influenza viruses of subtype H2.

The results obtained from the 263 clinical specimens are summarized in Table 3. Influenza A viruses reacting with MAbs HA1-71 and HA2-76 were classified as subtype H3, and viruses giving a signal only with HA2-76 were regarded to be of subtype H1. Of the 263 specimens, 31 contained an influenza A virus of subtype H1 and 113 contained an influenza A virus of subtype H3. Ten influenza A virus-positive specimens could not be subtyped because there were too few infected cells. Eighty-four specimens contained influenza B virus, and 25 specimens were negative.

Eight of the 20 clinical isolates subtyped by rapid culture and HI were identified as H1 viruses, and 12 were identified as H3 viruses; identical results were obtained by the two methods.

All cultures were carefully examined microscopically. The staining with pool A and pool B was so intense that 52% of the clinical specimens containing influenza A viruses and 23% of those containing influenza B viruses, respectively, could be identified as positive on the light box without microscopic observation.

DISCUSSION

A major concern when using MAbs in diagnostic tests is the possibility of obtaining false-negative results because of the high degrees of specificity of these antibodies. With the frequent occurrence of new variants of epidemic influenza viruses, it is important to thoroughly evaluate new diagnostic reagents. For this reason we tested the antibodies used in the present study against a substantial number of human influenza virus strains of the currently circulating subtypes H1N1 and H3N2. These viruses originated from different parts of the world and were collected over a period of 12 years. Although no H1-specific antibody was available, the reaction patterns of MAbs HA1-71 and HA2-76 allowed the identification of all human isolates as either subtype H3 or "non-H3" (i.e., H1), indicating that these two MAbs recognize well-conserved epitopes on the hemagglutinins of influenza A viruses.

TABLE 3. Results from 263 clinical specimens tested by rapid culture assay

Influenza season	No. of specimens of the following type and subtype:				
	H1N1	H3N2	A	B	Negative
1992–1993	3	1	1	32	1
1991–1992	16	82	4	1	4
1990–1991	7	3	0	34	4
1989–1990	0	21	1	0	4
1988–1989	5	6	4	17	12

The subtypes in 10 influenza A virus positive-specimens could not be identified because there were too few infected cells in the wells stained with MAbs HA1-71 and HA2-76. Five of these specimens had one to four stained cells in the wells stained with pool A and MAb HA2-76 but no stained cells in the well stained with MAb HA1-71. This distribution might have been due to inappropriate homogenization of the specimen before inoculation, and the viruses were therefore not characterized as H1. Because of the small volume of specimen available, no attempt was made to regrow these viruses.

Although all clinical specimens were initially identified as influenza virus positive, 25 (9.5%) remained negative when they were tested in the present study. The specimens were stored for as long as 5 years before retesting, which may explain this drop in sensitivity. This explanation is further supported by the fact that only one specimen from the 1992–1993 influenza season was negative, while more than 25% of the specimens collected during the 1988–1989 season were negative. Another factor which may have contributed to the relatively high percentage of negative specimens is the small volume of material that was inoculated into each well. Under routine conditions with fresh clinical specimens, one would normally inoculate 100 to 200 μl into each well, which is two to four times the volume available for our experiments. The small volume of clinical material available for the present study did not allow us to study systematically whether a longer incubation, e.g., 36 h instead of only 18 h, would increase the sensitivity of the assay. With cell culture-adapted influenza viruses, the size but not necessarily the number of plaques increases if the incubation is extended to 36 h, which makes the identification of a weakly positive specimen easier. By careful microscopic examination, however, single infected cells are readily recognized.

Since all the specimens available for the present study were influenza virus positive, it was not possible to determine the diagnostic sensitivity of the newly developed rapid culture assay. Several other investigators, however, have used the same pool A and pool B in rapid culture assays, for which the sensitivity was between 56 and 100% (4, 8, 12, 14, 15). Five of our influenza A-positive specimens contained between one and three positive cells in the wells stained with MAb HA1-71 and/or MAb HA2-76, but no positive cells in the well stained with pool A. Again, unequal homogenization of the specimen rather than the lack of specificity of the MAbs included in pool A may have contributed to this finding. In a next step, the sensitivity of this rapid culture assay needs to be determined on fresh clinical specimens in a careful comparison with standard isolation in cell cultures or hens' eggs.

Only a few attempts have been made to determine the subtypes of influenza A viruses by methods other than HI either after passage in cell culture or directly from clinical specimens. Rabbit or chicken sera and egg yolk antibodies have been used in immunofluorescence staining, allowing the determination of the subtypes of influenza viruses in infected, cultured cells (3, 5, 6). These polyclonal antibodies have been used to subtype isolates collected during one influenza season; however, it is unclear whether these reagents would also recognize homologous viruses during subsequent influenza outbreaks. The rabbit hyperimmune sera obtained by immunization with purified hemagglutinin permitted the subtype-specific identification of

influenza A viruses by immunofluorescence staining of nasopharyngeal cells obtained from a few individuals with influenza virus infections (6). Type- and subtype-specific MAbs have been obtained by immunizing mice with virus concentrated from allantoic fluid, and these MAbs have been successfully used for the subtyping of influenza A isolates after cell culture passage (11). The H3N2-specific MAb, obtained by immunization with the virus A/Berkeley/1/80, reacted with the homologous virus, with the closely related virus A/Bangkok/1/79, and with nine H3N2 isolates collected during the 1980–1981 influenza season. However, the antibody failed to react with viruses of the homologous subtype collected during earlier seasons. More recently, the reverse transcriptase PCR method has been applied for the type-specific (2) and subtype-specific (16) detection of influenza virus RNA in clinical specimens. By choosing primer sequences that were conserved over many years, it was possible to amplify cDNA fragments which allowed discrimination between H1N1 and H3N2 viruses and to achieve an assay sensitivity comparable to that of standard virus isolation (16).

The rapid culture assay described here allows for the type-specific identification of influenza viruses and the discrimination between the currently circulating subtypes of human influenza A viruses. In addition, the reaction patterns of pool A and MAbs HA1-71 and HA2-76 also allow for the rapid identification of H2N2 viruses if they reemerge in the human population. However, the subtype of any isolate which gives a signal with pool A but which does not react with MAbs HA1-71 and HA2-76 should be determined by other techniques.

Although the assay as such does not provide a cell culture isolate for further antigenic and genetic analyses, the culture supernatant can be stored for reinoculation in standard cultures. The rapid culture assay is easy to perform and the results are easy to interpret, it allows for the cost-effective processing of a large number of samples, and the result is available within 24 h of arrival of the specimen in the laboratory.

ACKNOWLEDGMENTS

We thank J. A. Melero for MAbs HA1-71 and HA2-76 as well as for the critical revision of the manuscript. We also thank Mike Williams (U.S. Food and Drug Administration, Washington, D.C.) for providing the sheep sera and John P. O'Connor for revising the manuscript. We gratefully acknowledge the physicians participating in the Centers for Disease Control and Prevention's sentinel physicians network.

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