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The use of a radial haemolysis test for neuraminidase antibodies in the diagnosis of influenza A infection

By E. A. GRILLI and A. J. SMITH

Influenza Research Unit, Public Health Laboratory, St Luke's Hospital, Guildford, Surrey GU1 3NT

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SUMMARY

A radial haemolysis test designed to detect antibody specifically directed against the neuraminidase is described and compared with currently available techniques for the routine diagnosis of infection with influenza viruses.

Paired sera were available from two outbreaks of influenza A, one with the N1N1 subtype and the other with the H3N2 subtype. This technique confirmed diagnosis in 92% of cases diagnosed by other laboratory methods.

INTRODUCTION

The routine laboratory diagnosis of clinical cases of influenza is still, in the main, performed by isolation of the virus or by demonstrating a rise in antibody in paired sera by the complement fixation (CF) test. The antigen in this test is the nucleoprotein and as such can only distinguish between types of influenza – A or B. It cannot distinguish between infections caused by the currently circulating A/H1N1 and A/H3N2 subtypes.

Other tests are available which are dependent on the surface antigens of influenza viruses and these can more closely define the infecting strain. Such tests as the haemagglutination inhibition (HI) test are usually performed using naturally occurring strains of virus as antigen. It is possible to use recombinant viruses where only the haemagglutinin is from a human strain to restrict the function of the test to the measurement of anti-haemagglutinin antibodies. To date the only accepted methods for estimating antibodies purely to the neuraminidase are the neuraminidase inhibition (NI) enzyme test (Aymard-Henry *et al.* 1973) and the neuraminidase haemagglutination inhibition (NHI) test described by Kendal, Minuse & Davenport (1972) and Smith & Davies (1976). The NI test is cumbersome, and although useful as a research tool, has no role as a routine diagnostic test. The NHI test although easier to perform is still complex and requires meticulous standardisation to produce reliable, reproducible results.

Over recent years radial haemolysis (RH) has become a recognised diagnostic procedure and serological surveillance technique. For influenza B viruses RH has been shown to be superior to HI both in the diagnosis of infection and in serological surveys (Chakraverty, 1980; Grilli & Davies, 1981). RH has also become an alternative to the HI test for strains of influenza A virus.

In 1976 Callow and Beare described the detection of anti-neuraminidase antibodies by RH. Their study showed the technique to be reliable, specific and quantitative. They did, however, stress the need for care in the preparation of test plates to ensure adequate adsorption of virus to red cells. The technique described here for the detection of neuraminidase antibodies uses a modification of the RH technique described for influenza A virus by Oxford *et al.* (1979) using chromic chloride as an agent to aid the adsorption of virus to red cells. For strains of both influenza A and B viruses this agent has been shown to improve the quality of RH plates: zones of lysis are clearer and the results more reproducible than plates prepared without the use of chromic chloride.

The techniques of virus isolation (VI), CF, HI and RH for naturally occurring strains of influenza virus (RH-whole) and neuraminidase using recombinant strains where only the neuraminidase is from human strains (RH-N) are compared as diagnostic procedures.

MATERIALS AND METHODS

Paired sera and throat swabs were available from 215 clinically diagnosed cases of influenza A. These patients comprised 121 cases in 1980 from an outbreak caused by a recent influenza A H3N2 strain, intermediate between A/Texas/1/77 and A/Bangkok/1/79 and 94 cases in 1978 from an outbreak caused by a strain of influenza A H1N1 similar to A/USSR/92/77.

Viruses

Three strains of influenza A H3N2 which were available at the time of the outbreak in 1980 were used in the HI test and the RH-whole virus test. These were A/Victoria/3/75 (A/Vic), A/Texas/1/77 (A/Tex) and A/Bangkok/1/79 (A/BK). Three recombinant viruses containing equine haemagglutinin (H7) and human neuraminidase (N2) were used for the RH-N test. These were A/equine/Prague/1/56 (H7) × A/Hong Kong/1/68 (N2), X 15, A/equine/Prague/ 1/56 (H7) × A/Victoria/3/75 (N2), Vic N and A/equine/Prague/1/56 (H7) × A/Texas/1/77 (N2), Tex N.

Infections in 1978 with the H1N1 subtype were investigated using the A/USSR/92/77 virus for the HI and RH-whole virus tests and the recombinant virus A/equine/Prague/1/56 (H7) × A/USSR/92/77 (NI) for the RH-N test.

An equine virus, A/equine/Prague/1/56 (H7N7), was used to prepare control plates in the RH-N test to ensure that there were no reactions between the equine virus and human sera. All these viruses were obtained from Dr G. C. Schild, National Institute for Biological Standards and Control, Hampstead, U.K.

Stock viruses were grown in the allantoic cavity of 10-day embryonated hens' eggs and the harvests clarified by centrifugation at 1500 rev./min in an MSE super minor and concentrated by ultracentrifugation at 20000 rev./min for 90 min in a Beckman L 5–50 ultracentrifuge using a type 50 fixed angle rotor. The pellet was resuspended in saline, sonicated at 8 Kc for 1 min in an MSE sonicator and kept in the gas phase of a liquid nitrogen storage container. The concentration of virus in these preparations was assayed by haemagglutination.

The antigen used in the CF test was supplied by the Public Health Laboratory Service, Colindale, London, U.K.

Virus isolation

Throat swabs were collected at the onset of illness into virus transport medium containing no antibiotics. These were transported to the laboratory within 24 h of collection and were immediately inoculated onto cell cultures of baboon kidney, Madin-Darby canine kidney and human fibroblasts (MRC-5) as available. Virus was detected by cytopathic effect, haemadsorption of the cell sheet and by haemagglutination (HA) of the culture fluid using human group O red blood cells (rbc). Identification was by HI using post-infection ferret sera. Fluid from the throat swabs collected in 1980 was also inoculated, with the addition of antibiotics, into the amniotic sac of 10-day embryonated hens' eggs. These were harvested after 5 days and examined for specific HA activity.

Complement fixation test

This test was performed by the method of Bradstreet & Taylor (1962) modified to use 0·025 ml volumes in microtitre plates, using complement at $3HD_{50}$ and sheep rbc sensitized with rabbit-anti-sheep serum (Wellcome Reagents Ltd.) to give a final dilution of 1 % rbc.

Haemagglutination inhibition test

For H3N2 strains the test was performed by the method of Smith & Davies (1976) using human group 0 rbc at 60000 cells/mm^3 and virus at 6 HA units. The receptor destroying enzyme (RDE) used for the treatment of sera to remove non-specific inhibitors of agglutination was made from *Vibrio cholerae* after the method of Palmer *et al.* (1975) and diluted to give 100 units activity for use.

For the A/USSR/92/77 (H1N1) strain 3 HA units of virus were used and the red cell agglutination patterns read after 1 h at 4 °C. It was important to read the plates promptly as agglutination patterns tended to collapse with this virus dose.

Controls

Controls for each serum to test for non-specific haemagglutinating activity were included on a separate plate at the initial 1 in 10 dilution. The effective removal of non-specific inhibitors of agglutination from test sera following treatment with RDE was checked by the use of an equine recombinant of influenza A, A/equine/Prague/1/56 × A/Port Chalmers/1/73 (H7N2), at a 1 in 10 dilution of serum.

Known positive and negative control sera were included in each batch of HI tests.

Radial haemolysis

The basic RH technique was as described for influenza B viruses by Grilli & Davies (1981) with the following modifications:

H3N2 viruses. The method was as described for influenza B viruses. Sheep rbc were obtained fresh from a local abbatoir and used at an 8 % concentration. The amounts of virus used for sensitization were: A/Victoria/3/75, 1000 units, A/Texas/1/77, 1500 units and A/Bangkok/1/79, 1000 units. For all viruses the virus dose was selected to give the best zone definition and maximum sensitivity. The unitage of a virus was defined as the amount of virus in 1·0 ml causing 50 %

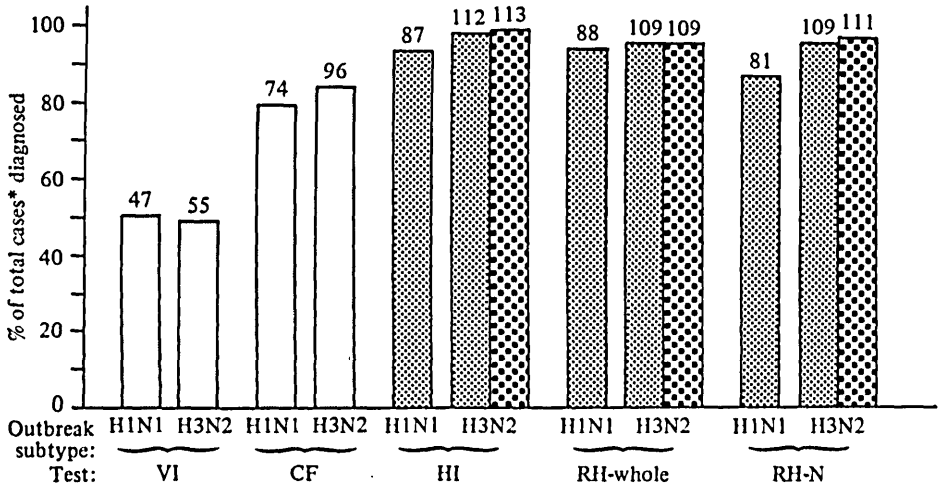

Fig. 1. Comparative efficiency of laboratory tests in the diagnosis of influenza A infection. The tests for antibody to surface antigens used only strain(s) representative of the outbreak strains (⊡) or multiple strains (▩). *A total of 93 cases from the H1N1 outbreak and 114 cases from the H3N2 outbreak were investigated. The figures above the columns indicate the number of cases diagnosed. Serological tests used were as defined in Materials and Methods.

agglutination of 0·25 ml of 0·5 % chick rbc (i.e. four times the titre using an equal volume test).

H1N1 virus. The maximum sensitivity for strains of this subtype was achieved when the concentration of sheep rbc was reduced from 8 % to 3 %. The cells were sensitized and stored overnight at 4 °C. The concentration of virus used was 1500 units. Plates were prepared on the day of use. This procedure reduced non-specific lysis.

Neuraminidase recombinants. An 8 % suspension of oxalated horse erythrocytes (Difco) was used in the preparation of plates for both N1 and N2 recombinant viruses. The cells were sensitized and stored overnight at 4 °C. Horse rbc gave superior results to those obtained when the plates were prepared with either sheep or chick cells, the zones of lysis were clearer and the sensitivity was improved. The amounts of virus used were X15, 1000 units, Vic N, 1500 units, Tex N, 2000 units and H7N1, 500 units. Plates were prepared on the day of use.

Controls

To demonstrate the absence of non-specific reactions with erythrocytes sera were examined on control plates prepared in a similar manner to the test plates but containing no virus.

For the RH-N test an additional control system was included using cells sensitized with influenza A/equine/Prague/1/56 (H7N7). A zone of lysis was produced in such plates by pony serum obtained from Dr J. Wood, National Institute of Biological Standards and Control, which contained antibody to both H7 and N7. Zones of lysis were not observed with any of the human sera tested.

Appropriate known positive and negative human control sera were included on every plate.

Table 1. *Influenza A H1N1: comparison of tests for the detection of antibody to surface antigens in 93 confirmed cases*

Number (%) diagnosed by						Total (%) positive by surface antigen tests
All three tests*	HI and RH-whole	RH-whole and RH-N	HI only	RH-whole only	None	
79 (85)	5	2	3	2	2†	91 (98)

* HI, RH-whole and RH-N.
† diagnosed by CF test only.

Table 2. *Influenza A H3N2: comparison of tests for the detection of antibody to surface antigens in 114 confirmed cases*

Number of diagnostic antigens	Number (%) diagnosed by					Total (%) positive by surface antigen tests
	All three tests	HI and RH-whole	HI and RH-N	RH-whole and RH-N	None	
6*	107 (94)	2	4	—	1‡	113 (99)
3†	104 (91)	4	4	1	1‡	113 (99)

* A/Vic, A/Tex, A/BK, X 15, Vic N, Tex N.
† A/Tex, A/BK, Tex N.
‡ Diagnosed by CF test only.

Evidence of infection in serological tests was a four-fold or greater change in titre between sera for the CF and HI tests and an increase in zone diameter of 1·0 mm or more for the RH tests. This size increase was regarded as significant from the results of earlier studies on the reproducibility of zone size (Grilli & Davies, 1981).

RESULTS

Specificity of the RH-N test

Monoclonal antibodies raised against the neuraminidase of the H3N2 and H1N1 subtypes were obtained from Dr J. Oxford, National Institute for Biological Standards and Control. The anti-N2 monoclone was raised against a Texas-like strain and the anti-N1 monoclone was raised against A/USSR/92/77.

Each monoclone was examined on all test and control plates. Zones of lysis were not detected on any of the control plates. With the anti-N2 monoclone zones of lysis were detected on all plates which contained the N2 neuraminidase independent of strain. Similarly zones were produced with the anti-N1 monoclone on all plates which contained this neuraminidase. There were no cross-reactions between subtypes.

Efficiency of all techniques in diagnosis of infection

There were 94 cases examined from the H1N1 outbreak and 121 from the H3N2 outbreak. For one case which occurred during the H1N1 outbreak and seven which occurred during the H3N2 outbreak there was no laboratory evidence of infection. These have been excluded from all the analyses.

The rate of diagnosis by each of the laboratory techniques assessed is shown in Fig. 1. Approximately 50% of cases, in both outbreaks, were diagnosed by virus isolation. All of these were confirmed by at least one serological test. The CF test

Table 3. *Influenza A H3N2: comparison of diagnosis rates for A/Tex and A/BK in HI and RH-whole tests on 114 confirmed cases*

Test	Number diagnosed by				Total (%) confirmed by test
	Both strains	A/Tex only	A/BK only	None	
HI	110	—	2	2	112 (98)
RH-whole	101	1	7	5	109 (96)

Table 4. *Influenza A H3N2: diagnosis of infection in 114 confirmed cases by multiple strains using three serological tests*

Test	Number (%) diagnosed by						Total (%) confirmed by test
	A/Vic	A/Tex	A/BK	X 15	Vic N	Tex N	
HI	105 (92)	110 (96)	112 (98)	—	—	—	113 (99)
RH-whole	97 (85)	102 (89)	109 (96)	—	—	—	109 (96)
RH-N	—	—	—	67 (59)	108 (95)	109 (96)	111 (97)

diagnosed about 80% of infections. There were two cases in 1978 and one in 1980 which were diagnosed by the CF test alone.

For the H1N1 virus the efficiencies of the tests for antibodies to the surface antigens were similar, ranging from 87% to 95% of the total confirmed cases (Fig. 1). Eighty-five per cent of the confirmed cases in the H1N1 outbreak were diagnosed by all these serological tests (Table 1). Two cases, on the other hand, were missed by all these same tests. Although the total cases diagnosed by HI and RH-whole were similar (87 for HI and 88 for RH-whole) it can be seen from the table that there were seven that were diagnosed by one test and not the other – four by RH-whole and not HI and three by HI and not RH-whole. No cases were diagnosed by the RH-N test alone.

The H3N2 outbreak was investigated using three strains of influenza virus and three different neuraminidase recombinants all of which were available at the time of the outbreak. In these circumstances 94% of the cases were diagnosed by all the serological tests for antibody to the surface antigens (Table 2). All the cases confirmed by these techniques were diagnosed by the HI test.

The H1N1 outbreak occurred during the first year of circulation of the H1N1 subtype and was investigated using the only strain then available, A/USSR/92/77. It is therefore relevant in a study of the comparative efficiency of the laboratory techniques for the two subtypes to consider investigations using only representative strains for both outbreaks. Since the H3N2 outbreak strain was intermediate between A/Texas/1/77 and A/Bangkok/1/79 the efficiency of the procedures was determined with both these strains. The neuraminidases of both A/Texas/1/77 and A/Bangkok/1/79 are similar (Schild, personal communication) and the Tex-N recombinant was therefore representative of the outbreak strain. If only these strains are considered it can be seen from Table 2 that 91% of the confirmed cases in the H3N2 outbreak were diagnosed by all the serological techniques for antibody to the surface antigens. There was one case that was diagnosed by RH-whole and RH-N but not HI. The overall diagnosis rate for these tests was unchanged by the reduction in the number of antigens.

The relative efficiency of each of the antigens (A/Tex, A/BK and Tex-N) in these tests was also considered. In the HI test (Table 3) 110 of the 112 diagnosed by this method were diagnosed by the A/Tex strain and all 112 by the A/BK strain. With the RH-whole test 102 of the 109 positive were diagnosed by A/Tex and 108 by A/BK. The Tex-N recombinant diagnosed 109 infections, 96% of the total confirmed cases.

Diagnosis of infection using only strains remote from the infecting strain was also considered (Table 4). In the HI test A/Vic diagnosed 105 of the 113 that were positive using all three strains, i.e. 92% of the total 114 confirmed cases. One case was confirmed by the A/Vic strain alone. In the RH-whole test this strain diagnosed 97 of the 109 cases that were diagnosed using three strains, i.e. 85% of the total cases. In this test there were no cases diagnosed by A/Vic alone. In the RH-N test the Vic-N recombinant diagnosed 108 infections (94%) including two confirmed by this strain alone. The X 15 recombinant diagnosed 67 infections (59% of the total) and there were no cases diagnosed only by this strain.

DISCUSSION

It has been shown that radial haemolysis can be used successfully to detect antibody specifically directed against the neuraminidase of influenza viruses by the use of recombinant strains (Callow & Beare, 1976). Further evidence to support these findings was obtained from this study. The use of monoclones raised against the neuraminidases of the H3N2 and H1N1 subtypes showed that antibody directed specifically against this antigen did result in zones of lysis. The reaction was specific for the subtype, although not for the strain against which the monoclones were raised, and there were no cross-reactions between subtypes. It is interesting to note that these monoclones gave zones of lysis on plates containing whole human virus of the appropriate subtype. It follows that zones produced by whole sera on such plates probably result from antibody both to the haemagglutinin and the neuraminidase. There were however seven sera collected convalescent to the H1N1 outbreak which only produced zones on the whole virus plate. This may represent a response to the haemagglutinin alone or merely reflect a difference in sensitivity between the RH-whole and RH-N plates in the detection of antibodies to the neuraminidase of this virus.

In addition to this evidence from monoclonal antibodies there was circumstantial evidence of specificity obtained from the test sera. The sera available included acute and convalescent pairs from an outbreak with a strain similar to A/USSR/92/77 where the challenged population were all experiencing primary infection with this subtype. However all the patients had been shown in earlier studies to have evidence of infection with strains of the H3N2 subtype. The absence of zones of lysis in the acute sera when tested against the H1N1 virus is further evidence of the subtype specificity of the reaction in the RH-N test. Where infection was demonstrated by other laboratory methods 81 out of 93 (87%) showed a seroconversion by the RH-N test (see Table 1). Comparable results were obtained from 121 paired sera available from an outbreak caused by a strain intermediate between the A/Texas/1/77 and A/Bangkok/1/79 strains of influenza A H3N2: 111 of 114 (97%) cases which were confirmed by other techniques were diagnosed by the RH-N test. Since human sera failed to produce zones of lysis on

control plates containing whole equine virus (H7N7) it can be inferred that the observed reaction in RH-N plates was due to antibodies reacting with neuraminidase.

When considering the diagnosis of infection it is interesting to note that previous infection did not affect the virus isolation rate. In 1978 all cases were primary infections with the H1N1 subtype but in 1980 all but one of those investigated showed evidence of infection with earlier strains of the H3N2 subtype. In both outbreaks virus was isolated from approximately 50% of cases. In fact this figure rather underestimates the efficiency of virus isolation. It is the practice of this laboratory in the investigation of clinical cases from outbreaks to obtain paired sera from all cases where there is failure to isolate virus. Where a virus is isolated a convalescent serum may not always be collected. Thus patients from whom throat swabs and paired sera were available include a disproportionate number of cases from whom virus was not isolated. If all cases where throat swabs were collected are included the overall isolation rate was 58% for the H1N1 and 59% for the H3N2 outbreak. Virus isolation alone, without the additional benefit of serology, would not provide a sufficiently sensitive diagnostic method.

The CF test diagnosed about 80% of infections. There was only one case in 1980 which was diagnosed by this test alone. As high concentrations of antibody were detected in the acute serum by all the other tests this probably represented a slightly earlier asymptomatic infection. Two further cases in 1978 were diagnosed by the CF test alone and both represented failures of the other techniques. The CF test does not distinguish between subtypes of influenza A and therefore is inadequate as a diagnostic test, particularly in the area of epidemiological investigations.

In the present situation where there are two subtypes of influenza A in circulation simultaneously there is a greater need to identify the subtype responsible for infections. All the tests described here to detect infection using the surface antigens of the virus were efficient. The RH-N test described was comparable in sensitivity to the other tests which are designed to detect antibody to the haemagglutinin. The results obtained using monoclonal antibodies directed solely against the neuraminidase suggest that the RH test using whole human influenza virus will detect antibodies to both the haemagglutinin and neuraminidase. Other workers have produced evidence that the reaction is specific for the haemagglutinin (Schild, Pereira & Chakraverty, 1975). Whilst it may be assumed that the use of monoclones would produce unequivocal evidence for the nature of the reaction the limitations of these new tools must not be forgotten. For example there may be cross-reactions between proteins which have an epitypic relationship (Oxford, 1982). In the HI test the H7N2 control was selected as an inhibitor-sensitive strain containing an equine haemagglutinin to which the sera tested had no antibodies. It showed not only that the non-specific inhibitors were removed effectively but also that antibodies to the N2 component alone did not lead to false positive results. A comparison of naturally occurring strains and recombinants which contained only the relevant haemagglutinin showed that some enhancement of titres was possible if natural strains were used with human sera containing both haemagglutinin and neuraminidase antibody (Smith, 1977).

The RH-N test is the first test for detection of antibody to the neuraminidase

which is suitable for routine diagnostic use. It has been shown to be specific for neuraminidase antibodies and will certainly be a valuable test for surveillance programmes. Ninety-two per cent of the 207 confirmed cases from both outbreaks made a response to the homotypic neuraminidase. Ninety-five per cent of the H3N2 cases made a response to the Vic-N and 59% to the very remote X 15 strain. The neuraminidase of this latter strain was characteristic of strains circulating at the end of the Asian (H2N2) era and still present in early strains of the Hong Kong (H3N2) era. This breadth of response may be useful in diagnosis where the current strain may not be available.

For a diagnostic laboratory interested in identifying the subtype of influenza responsible for clinical cases the HI test using just the currently circulating strain is an effective test. Eighty-seven of the 93 cases (94%) caused by H1N1 and 112 of the 114 cases (98%) caused by H3N2 were diagnosed using just one strain to represent each subtype, A/USSR/92/77 (H1N1) and A/Bangkok/1/79 (H3N2). The HI test does have the disadvantage of the necessity to remove non-specific inhibitors of agglutination. However, once removed the test gives consistent results for influenza A. The limitations of a test involving serial dilutions are well known, but it is clear from the results that in diagnosis of clinical illness where paired sera are available the necessity of a four-fold change in titre does not reduce the sensitivity of the test.

For radial haemolysis the only pre-treatment of sera necessary is heat inactivation. The test is however influenced markedly by the reagents used, in particular complement.

With both the subtypes of influenza investigated there were cases diagnosed only by RH-whole and not by HI when only strains representative of the outbreak strain were used. However the reverse situation also occurred to a similar extent. There were no cases diagnosed by RH-N alone where all three serological tests for antibodies to surface antigens were used. However, if only RH was to be used there were four cases in 1980 diagnosed by RH-N and not by RH-whole.

The presence of zones on control plates for the RH test was not a problem in this study. Not one of the total 430 serum samples was excluded because of control zones.

Since the tests described to detect antibody to surface antigens had a similar efficiency any of them could be used. The RH-N technique described represents a simple, reliable method for diagnosing infections with influenza viruses using the neuraminidase antigen. A diagnostic technique based on neuraminidase would not now be the method of choice. However since there are currently two subtypes of influenza A in circulation which have no shared surface antigens the potential exists, at least theoretically, for any combination of the four antigens to circulate, i.e. H1N1, H3N2, H1N2 and H3N1. In the event that these were all circulating simultaneously tests for both the haemagglutinin and the neuraminidase would be necessary to identify the infecting subtype.

The technique may be useful in the assessment of previous experience with influenza virus. A study of the nature of the response to infection with different strains and the persistence of antibody is under investigation.

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