The Application of the Soluble Antigen Fluorescent Antibody Test for the Diagnosis of Avian Influenza

M. Al-Attar, K. Nielsen and W.R. Mitchell*

ABSTRACT

The application of the soluble antigen fluorescent test as a tool for serological investigation of influenza type A infection in wild birds was studied. The soluble antigen fluorescent antibody test is basically an indirect fluorescent antibody test except that an artificial matrix of cellulose acetate discs is used as a substrate for antigen and the test results are scanned and recorded by a fluorometer. The influenza type A soluble antigen fluorescent antibody antigen was obtained from concentrated and detergent disrupted virus particles, absorbed onto cellulose acetate discs. Anti-influenza sera were prepared in pheasants and ducks to A/turkey/Ontario/6118/67 and in pigeons to A/turkey/Ontario/6213/68. The antigen-antibody complex was detected by specific staining with monovalent or polyvalent fluorescein isothiocyanate conjugated rabbit anti-avian immunoglobulins.

The soluble antigen fluorescent antibody test is a sensitive technique for the detetection of specific influenza A antibodies in several avian species, and could be adapted for use in large scale surveys.

RÉSUMÉ

Cette expérience consistait à vérifier la possibilité d'utiliser l'épreuve d'immunofluorescence avec un antigène soluble, comme moyen d'effectuer une enquête sérologique sur l'infection des oiseaux sauvages par le virus du type A de l'influenza. Il s'agit fondamentalement d'une épreuve indirecte d'immunofluorescence, sauf qu'on utilise une matrice artificielle de disques d'acétate de cellulose, comme substrat pour l'antigène, et que les résultats sont scrutés et enregistrés par un fluoromètre. La source de l'antigène provenait de particules virales concentrées, rupturées à l'aide de détergent et absorbées sur disques d'acétate de cellulose; celle des anticorps provenait de faisans et de canards infectés avec la souche A/turkey/Ontario/6118/67, ainsi que de pigeons infectés avec la souche A/turkey/Ontario/6213/68 du virus de l'influenza. On détecta le complexe antigène-anticorps par une coloration spécifique avec de l'isothiocyante de fluorescéine mono ou polyvalent et conjugué avec des immunoglobulines anti-aviaires, obtenues chez le lapin.

Cette épreuve d'immunofluorescence représente une technique sensible pour la détection d'anticorps à l'endroit du virus du type A de l'influenza, chez plusieurs espèces aviaires; on pourrait l'adapter de façon à pouvoir l'utiliser sur une grande échelle, dans des enquêtes sérologiques.

INTRODUCTION

The demonstration of influenza antibodies and isolation of influenza A viruses have been reported from a variety of animal species including several avian species in different parts of the world (8). Many influenza type A viruses have been isolated from chickens (12, 15), turkeys (13, 14), ducks (17), quail, pheasants (16), terns (3), shearwater (7) and from different species of exotic birds (21). The close antigenic relationship between the avian influenza isolates have suggested that migratory birds might play an important role in the dissemination of these viruses over vast areas of the world (8). It was also suggested that free-flying wild birds might provide optimum conditions for genetic recombination between avian strains of influenza A virus and those prevalent in man and result in new hybrid strains (20, 25).

Antigenic variation is one of the most striking and unique features of the influenza type A virus (24). As a result of this remarkable ability, immunity developed in response to infection by one strain may give little or no protection against viruses which may arise subsequently (18). An understanding of the epidemiological behavior of influenza can only be achieved by intensive and continued international surveillance (18).

Seroepidemiological surveys can provide some evidence on the

*Department of Pathology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1 (Al-Attar), Animal Pathology Division, Food Production and Inspection Branch, Agriculture Canada, Box 11300, Station H, Nepean, Ontario K2H 8P9 (Nielsen) and Department of Veterinary Microbiology and Immunology, Ontario Veterinary College, University of Guelph, Guelph, Ontario N1G 2W1 (Mitchell).

Submitted February 4, 1980.

epidemiological behavior of past and current influenza viruses and immune status of the population under investigation.

Hemagglutination-inhibition tests (HI), complement fixation tests (CF) and double immunodiffusion (DID) tests have been most widely used for such surveys. The HI test is useful in species naturally infected by a limited number of influenza virus serotypes, such as pigs, horses or humans, but it is too laborious and complicated in avian influenza where many different serotypes circulate simultaneously in the birds. Furthermore, the HI tests are very sensitive but also very susceptible to erroneous results because of nonspecific hemagglutinin-inhibitors in many sera (18).

The CF test, widely used in the past with human and equine sera, is not suitable for assay of all animal sera because of high anticomplementary activity and cannot be applied to avian sera in general because avian antibodies fix complement very poorly (18). The DID test like the CF test is capable of detecting the influenza type A antigens but requires high antigen and antibody concentration (18). Moreover, the test gives an inaccurate estimate of the titre of the unknown antibody or antigen. Hence a reliable, simple, inexpensive and rapid screening test for the influenza type A antibody would be of value for seroepidemiological investigation of avian influenza.

In the present article, the application of the soluble antigen fluroescent antibody (SAFA) test (23) is investigated as a screening test of influenza antibodies in some wild bird species and results are compared to HI test.

MATERIAL AND METHODS

PREPARATION OF VIRUS ANTIGENS

The following virus strains of influenza type A were used in this study:

A/duck/Manitoba/53
(Hav 2 Neq I)
A/duck/England/56
(Hav 3 Nav I)
A/duck/Czech/56
(Hav 4 Nav I)
A/turkey/Ontario/6213/68
(Hav 5 NI)
A/turkey/Washington/67
(Hav 6 N2)
A/turkey/Ontario/6118/67
(Hav 8 Nav 4)

The reference virus strains which were cross-checked by HI test were passed twice or more in the allantoic cavity of nine to ten days embryonated hen's eggs incubated at 38°C. The infected extraembryonic fluids were collected, pooled, and the hemagglutination titres (HI) were determined. Samples with HI titres of 1/32 or less were not used for the preparation of the SAFA antigen or for antibody production. The harvested extraembryonic fluids were either stored at -20°C or chilled and used immediately for the preparation of the SAFA antigen.

AVIAN SERUM SAMPLES

Serum samples were obtained from ring-necked pheasants (*Phasianus colchicus*), mallard ducks (*Anas platyrhynchos*) and pigeons (*Columba livia*).

Two mallard ducks and two pheasants were infected by intranasal and intraocular routes with a few drops of embryonic fluid containing A/turkey/Ontario/6118/67. The same virus was given again seven days later and the two ducks received a third administration 14 days after the primary infection. All animals were bled one week after the last exposure. Similarly four pigeons were infected with allantoic fluid containing A/turkey/Ontario/6213/68, twice at seven day intervals and bled at day 14. These sera were examined by the SAFA test without preliminary heat inactivation.

RABBIT ANTISERA TO AVIAN IMMUNOGLOBULINS

New Zealand white rabbits were inoculated subcutaneously and in the foot pads with 2.0 mg of immunoglobulin (19) fractions emulsified in Freund's complete adjuvant. The Ig's fractions were obtained using saturated ammonium sulphate precipitation (11) of sera from mallard ducks, pheasants or pigeons. Two additional intramuscular and subcutaneous injections of 2.0 mg Ig's were administered on day 30 and 37 and sera were collected on day 44 after the first inoculation. In an attempt to produce a polyvalent anti-Ig of these species a pool of Ig's from each species was given to another group of rabbits using the above schedule for immunization.

FLUORESCEIN-ISOTHIOCYANATE CONJUGATION

The Ig protein solution (15.0-20.0 mg/mL) was brought to pH 9.0 by adding a carbonate bicarbonate buffer (pH 9.0) at the ratio of one volume of buffer to two volumes of Ig-protein solution. For every 60 mg of protein in the solution 1.0 mg fluorescein isothiocyanate (FITC)isomer I[1] was added. The FITC, dissolved in carbonate bicarbonate buffer, was added dropwise to the antispecies Ig. The mixture was stirred for four hours at room temperature. Unconjugated FITC was removed by gel filtration through a column (30 x 1.5 cm) of Sephadex G25[2] medium packed and operated with Tris-HCl buffer (0.1 M, pH 8.6). Elution was observed visually, and those green coloured fractions which appeared first were collected and chromatographed a second time at room temperature on a similar column packed with DEAE-cellulose (DE-52)[3] and equilibrated with 0.1 M Tris-HCl pH 8.7 buffer, followed by elution with the same buffer containing increasing molarities of sodium chloride. The green coloured

[1]Baltimore Biological Laboratory (BBL), Baltimore, Maryland.
[2]Pharmacia, Fine Chemicals AB, Uppsala, Sweden.
[3]Whatman Biochemicals Ltd., dist. by Mandel Scientific Co., Montreal, Quebec.

eluted fractions of the 0.1 M, 0.15 M and 0.2 M NaCl were collected and their fluorescein/protein (F/P) ratios were determined. Those fractions with fluorescein/protein ratios between one and four (26) were pooled and concentrated to half the original volume by dialysis against polyvinylphrolidone (PVP)[4] powder. Conjugates were stored at -20°C.

SAFA TEST PROCEDURE

The procedure used for the soluble antigen fluorescent antibody (SAFA) test was that of Toussaint (23) and as modified by Garnham *et al* (9).

PREPARATION OF INFLUENZA TYPE A SAFA ANTIGEN

The method described by Dowdel *et al* (6) for concentrating influenza virus from infected allantoic fluid was followed. Cooled allantoic fluid was brought to pH 4.0 (±0.5) by adding 3.0 mL of HCl (1N) to 100.0 mL of fluid. A heavy precipitate developed which was removed by centrifugation. The sediment was redissolved in glycine sarcosyl buffer pH 9.0 (6) at a volume reduction of 1/100 of the original volume. The antigen concentrate was brought to pH 8.0 by overnight dialysis at 4°C against the corresponding Tris-HCl buffer. The control SAFA antigen was processed in the same way but using allantoic fluid from uninfected embryos.

The presence of type A influenza antigen was ascertained by double immunodiffusion against pigeon antisera to heterologous influenza virus (A/turkey/ Ontario/6213/67, Hav 5).

PREPARATION OF THE SAFA ANTIGEN DISCS

The concentrated SAFA antigen was poured into small plastic Petri dishes in 1.5 to 2.0 mL volumes. This volume was sufficient to individually soak eight cellulose acetate filter membranes of 47 mm diameter.[5] After overnight drying at room temperature, the antigen-impregnated membranes were perforated with a paper punch to obtain discs of 6.5 mm diameter.

SAFA ANTIGEN INSOLUBILIZATION AND SERUM ABSORPTION

Ten mL of SAFA antigens (positive and negative control) were dialyzed against two changes of 0.15 M NaCl at 4°C overnight. One mL of 1.0 M pH 5.0 acetate buffer was added to each of 10.0 mL of antigen followed by three mL of 8.5% gluteraldehyde solution added dropwise. The pH of the mixture was adjusted to 5.0 with acetic acid. The mixture was left at room temperature until insolubilization was completed, usually three hours.

The insolubilized proteins were washed twice in water and twice in 0.1 M glycine HCl buffer pH 2.8 and twice in PBS pH 7.3.

About 2.0 mL of the wet insolubilized proteins were mixed with 0.5 mL of each positive avian antiserum sample. The mixture was left standing at room temperature for 15 min and for an additional 60 min in a rotary shaker. Absorbed sera were recovered by centrifugation.

STATISTICAL METHODS

The Student t-test described by Sokal and Rohlf (22) was used to test the level of significance between differences in the mean of fluorometer dial reading (FDR) values of the triple positive serum discs and the mean FDR values of the triple negative serum discs.

RESULTS

To interpret the results of each SAFA test the titre of a serum sample was arbitrarily set at the highest serum dilution at which there was a significant difference between it and the negative serum control of the same serum dilution.

It was commonly observed that low serum dilutions gave lower fluorometer dial readings (FDR) than when diluted. These differences were such that readings did not differ statistically from controls at low serum dilution but were statistically significant at higher dilution. The cause of this "prozone" is not known (Fig. 1).

EFFECT OF CONJUGATE DILUTION

The data presented in Table I list the SAFA test results obtained with one in eight dilution of pheasant and duck immune sera to A/turkey/Ontario/6118/67 (Hav 8) and the pigeon serum to A/turkey/Ontario/6213/67 (Hav 5) with the antigen discs prepared from A/turkey/Ontario/ 6118/67 ribonucleoprotein (RNP) concentrate. The specific serological reaction between RNP antigen and the antibody was detected with conjugated rabbit antispecies Ig's diluted from 1/1 to 1/10. The results show that the FDR values of both influenza antibody positive and negative sera vary inversely with the conjugate dilutions. The higher the conjugate dilution the lower were the FDR values of pheasant, duck and pigeon sera. The standard deviation of the FDR values of replicate serum dilutions were

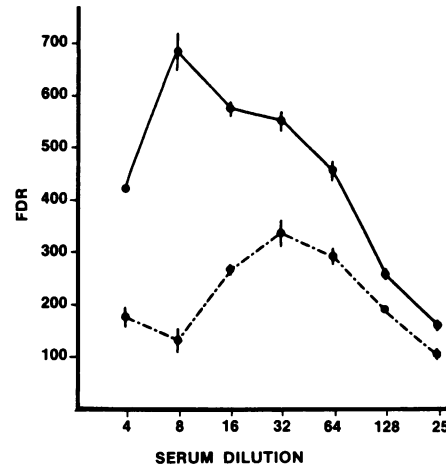

Fig. 1. Effect of serum dilution on FDR of positive (___), (HI titre 1/64) and negative (_._._) pheasant serum. Bars represent standard deviation. Serum dilution is expressed as the reciprocal of the dilution. Low FDR value at 1/4 positive serum dilution due to "prozone effects".

[4]Arthur H. Thomas Co., Philadelphia, Pennsylvania.
[5]Millipore Ltd., 55 Montpellier Blvd., Montreal, Quebec.

TABLE I. Effect of Conjugate Dilution on FDR* of Experimentally Produced Anti-Influenza Sera from Pheasant, Duck and Pigeon

Serum Sample	Conjugate Dilution	Mean FDR	Standard Deviation	Level of Significance for difference in (+)ve and (-)ve FDR
Pheasant				
(+)ve	undiluted	1030.00	165.22	P≤0.01
(-)ve	undiluted	403.33	55.07	
(+)ve	1/5	403.33	37.85	P≤0.01
(-)ve	1/5	233.33	49.32	
(+)ve	1/10	230.00	36.05	N.S.
(-)ve	1/10	176.66	37.85	
Duck				
(+)ve	2/3	223.33	20.81	P≤0.01
(-)ve	2/3	56.66	11.54	
(+)ve	1/5	103.33	5.77	P≤0.01
(-)ve	1/5	26.66	5.77	
(+)ve	1/10	56.66	11.54	N.S.
(-)ve	1/10	33.33	5.77	
Pigeon				
(+)ve	2/3	160.00	45.82	P≤0.01
(-)ve	2/3	10.00	17.32	
(+)ve	1/5	76.66	11.54	P≤0.01
(-)ve	1/5	13.33	5.77	
(+)ve	1/10	73.33	5.77	P≤0.01
(-)ve	1/10	26.66	5.77	

*Fluorometer dial reading

generally diminished as the conjugate was diluted.

EFFECT OF SERUM DILUTION

In general, differences between the FDR values of influenza antibody negative and positive sera were reduced as serum dilution were increased. However, serum dilution decreased the FDR values of influenza positive sera more than those of the negative serum controls (Figs. 2, 3, 4).

SPECIFICITY OF THE SAFA TEST

The reaction with the influenza RNP antigen in the SAFA test is demonstrable in the studies with

TABLE II. Comparison between SAFA Titres and HI Titres of Sera from Pigeons Infected with A/turkey/ONT/-6213/68 Influenza Virus

Pigeon Serum	HI Titres	SAFA Titres
#1	1/8	1/4
#2	1/16	1/128
#3	1/32	1/4
#4	1/64	1/64

the pigeon antisera to influenza virus Hav 5 (A/turkey/ Ontario/6213/67) and the SAFA antigen prepared from an influenza virus of Hav 8 serotype (Table II).

The data show the nonrelatedness of the two serological procedures because they are dealing with different antigens of the influenza virion. These SAFA titres were determined with the conjugated monovalent rabbit antipigeon Ig serum. The pigeon serum #4 was also tested with the conjugated polyvalent rabbit anti-Igs yield in a SAFA titre of 1/32 (Table III) which is within the fourfold range of titre variation generally accepted in diagnostic serology. These results indicate an immune reaction with the influenza type A RNP.

A further attempt was made to prove the type A specificity of the SAFA test by absorption experiments of the specific antibody with polymerized antigens. Polymerized concentrate of allantoic fluids

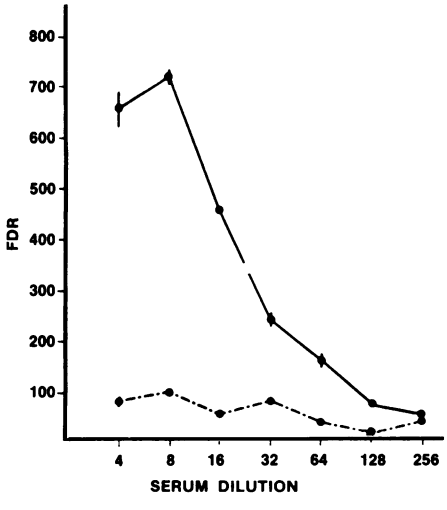

Fig. 2. Effect of serum dilution on FDR of positive (___), (HI titre 1/16) and negative (_._._.) pigeon serum. Serum dilution expressed as the reciprocal of the dilution. Bars represent standard deviation. SAFA titre 1/128.

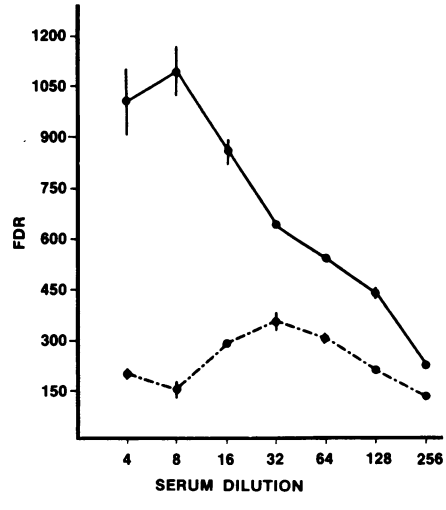

Fig. 3. Effect of serum dilution on FDR of positive (___) (HI titre 1/128) and negative (_._._.) pheasant serum. Serum dilution is expressed as the reciprocal of the dilution. Bars represent standard deviation. SAFA titre 1/256.

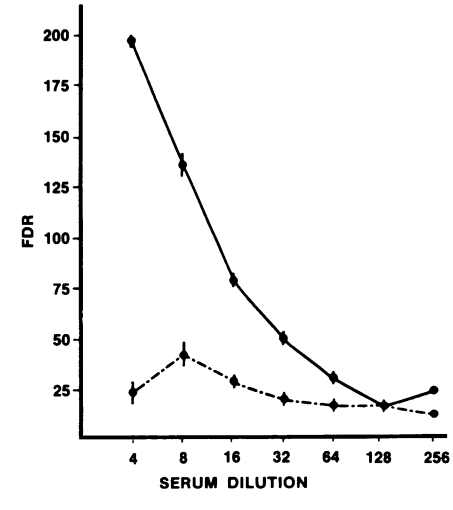

Fig. 4. Effect of serum dilution on FDR of positive (___),(HI titre 1/16) and negative (_._._.) duck serum. Serum dilution is expressed as the reciprocal of the dilution. Bars represent standard deviation. SAFA titre 1.32.

TABLE III. Effect of Serum Dilution on FDR[a] Values of Pigeon Anti-Influenza Prepared Against A/turkey/ONT/6213/68 Positive (+)ve and Negative (-)ve Serum Tested With Conjugated Polyvalent Anti-Igs

Positive Serum HI Titre 1/64					
Serum Sample	Serum Dilution	Number of Replicates	Mean FDR	Standard Deviation	Level of Significance for Difference in (+)ve and (-)ve FDR
(+)ve	4	3	210.00	40.00	
(-)ve	4	3	0.00	0.00	P≤0.01
(+)ve	8	3	180.00	10.00	
(-)ve	8	3	0.00	0.00	P≤0.01
(+)ve	16	3	120.00	10.00	
(-)ve	16	3	13.33	11.54	P≤0.01
(+)ve	32	3	80.00	10.00	
(-)ve	32	3	0.00	0.00	P≤0.01
(+)ve	64	3	43.33	20.81	
(-)ve	64	3	0.00	0.00	N.S.[b]
(+)ve	128	3	23.33	15.27	
(-)ve	128	3	0.00	0.00	N.S.
(+)ve	256	3	20.00	10.00	
(-)ve	256	3	0.00	0.00	N.S.

[a]FDR — Fluorometer dial reading
[b]N.S. — Not significant

TABLE IV. Comparison between SAFA Titres Obtained using Monovalent and Polyvalent FITC[a] Conjugated Antisera in Three Avian Species

Serum Sample	Monovalent Conjugate	Polyvalent Conjugate
Pheasant	1/256	1/64
Pigeon	1/64	1/32
Duck	1/16	1/16

[a]Fluoresceinisothiocyanate

from virus (Hav 8) infected and normal embryonated eggs were used for absorbing pheasant (anti-Hav 8 strain) and pigeon (anti-Hav 5 strain) antisera (Figs. 5, 6). Contrary to expectations, the FDR values were reduced to the same extent whether absorption was made with the specific RNP or the control isoluble antigens. Thus the specificity of the SAFA reaction could not be evaluated by this method.

When these absorbed sera (pheasant and pigeon) were examined by the HI test, it was found that both absorbed positive serum samples had lower HI antibody titres of one or two dilutions less than the HI titre of the unabsorbed portions. The unabsorbed and the two absorbed serum portions of pheasant sera were examined also by the DID test. The unabsorbed pheasant serum gave two distinct lines of precipitation when tested, while both absorbed serum portions demonstrated only one line of precipitation against the same SAFA antigen.

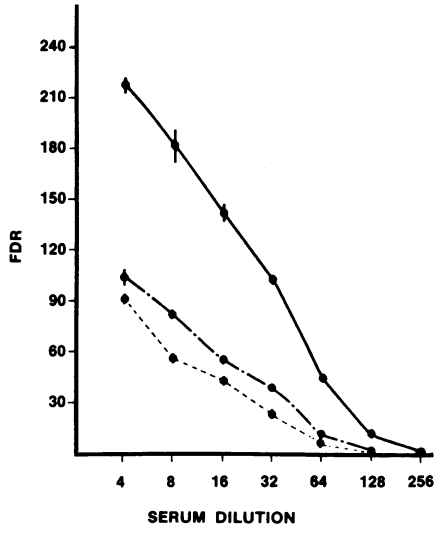

Fig. 5. Effect of absorption with polymerized positive and negative SAFA antigen on FDR of positive pheasant serum. Serum dilution is expressed as the reciprocal of serum dilution. Bars represent standard deviation. (___) unabsorbed, (_._._.) absorbed with polymerized negative SAFA antigen and (----) absorbed with polymerized positive SAFA antigen.

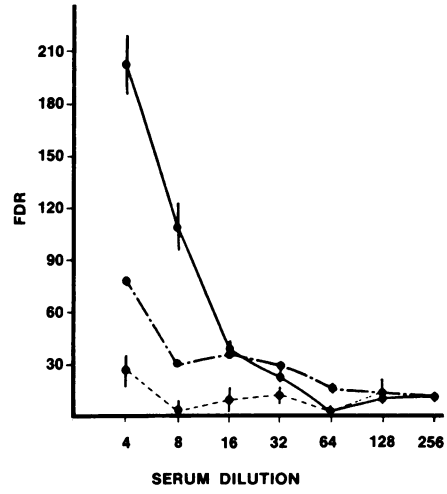

Fig. 6. Effect of absorption with polymerized positive and negative SAFA antigen on FDR of positive pigeon serum. Serum dilution is expressed as the reciprocal of serum dilution. Bars represent standard deviation. (___) unabsorbed), (_._._.) absorbed with polymerized negative SAFA antigen and (----) absorbed with polymerized positive SAFA antigen.

APPLICATION OF THE POLYVALENT RABBIT ANTI-IGS

The conjugated rabbit anti-polyvalent Igs was prepared as indicated. The conjugate was tested in the SAFA test in order to study the possibility of reducing the number of conjugates to be used for detecting anti-influenza antibodies in different avian species. Results of these experiments are presented in Table IV. Soluble antigen fluorescent antibody titres obtained by using the conjugated polyvalent antispecies Ig were comparable to those obtained by using the conjugated monovalent anti-Ig.

The level of nonspecific staining observed with negative sera of the species tested was very low when the polyvalent conjugate was used and the FDR values of the serum samples were in general lower than those obtained with the monovalent conjugates.

DISCUSSION

Seroepidemiological screening tests require the manipulation of large numbers of samples, thus they should be simple, rapid and inexpensive while maintaining satisfactory sensitivity and speci-

ficity. In the case of avian influenza, a serological test detecting antibodies to the type A virus antigens has definite advantages in this respect over tests detecting strain specific antibodies such as the HI and the SN tests. The most common technique used for detecting antibodies to the internal type specific antigens or typing of influenza virus is the double immunodiffusion test (DID) (2, 6). The DID test is highly specific but its sensitivity is low. More study is needed to compare the sensitivity of the DID test and SAFA for detecting type specific antigens.

The indirect fluorescent antibody test has been applied as a quantitative diagnostic test for the detection of influenza antibodies in postinfection human sera (5). The test has not been applied routinely because the procedure was technically exacting and difficult to control (18).

The SAFA test is basically an indirect fluorescent antibody test which possesses the sensitivity associated with the primary binding assays. In addition, the mechanical interpretation of the test results by a fluorometer eliminates the subjectivity of the conventional fluorescent antibody test. The replacement of tissue smears with antigen(s) absorbed to an artificial matrix (cellulose acetate filter discs) may have reduced the level of nonspecific fluorescence associated with the reaction between conjugated anti-Igs and tissue proteins and standardizes the substrate.

Dowdle *et al* (6) used the acidification method as a procedure for concentrating influenza virus and as a source of internal type A specific antigens (RNP and M protein) after treating the precipitate with detergent to disrupt the virus particles. The efficiency and simplicity of the acidification procedure for concentrating influenza virus from large volumes of infected allantoic fluid made it the choice for this study.

Conjugate dilution is one of the methods used to reduce the nonspecific staining in the conventional fluorescent antibody test. The level of nonspecificity has been shown in this and other studies (9) to decrease as the conjugate dilution was increased in the SAFA test as well. Similarly the FDR values of the negative sera decrease as the conjugate dilution was increased (Table I). The levels of nonspecific staining were different from species to species. Differences existing in the F/P molar ratios and the heterogenicity of the various conjugates possibly resulted in differences in the degree of nonspecific staining demonstrated with each species (10).

The presence of contaminating proteins (possibly serum proteins from chick embryo's blood) in the concentrated influenza antigens may also contribute to nonspecific staining. The use of purified influenza antigens may eliminate this kind of nonspecific staining.

Sera designated as negative in this study were tested with six different avian influenza hemagglutinins. This does not exclude the possibility of the presence of antibodies to other influenza type A hemagglutinins as well as the presence of low levels of antibodies to the type specific antigens which could not be detected by the DID test. With these doubtful negative sera in hand, it was possible, in many instances, to detect significant differences between the mean FDR values of the known positive sera from different avian species and those negative sera.

The conjugated polyvalent anti-Igs was shown to be specific for detecting antibodies to influenza internal antigens in the SAFA test when used with three different avian species sera (pheasant, pigeon, duck). These results, using broad-spectrum antispecies immunoglobulin, simplify the SAFA test. In addition to its practical application as an indirect fluorescent antibody test, the detection of antibodies by one conjugate from different related avian species may be extended for use in other serological tests.

The serological demonstration of antibodies to the influenza type A antigen by the SAFA test is evident in the experiments with the four pigeons immunized with a virus strain (Hav 5) differing from the virus strain (Hav 8) from which the SAFA antigen discs were prepared by the virus envelope antigens.

The methods used to remove nonspecific fluorescence demonstrated that absorption of conjugate with tissue powders resulted in an overall loss of serum proteins as well as a drop in the fluorescent staining titre of the conjugate (10). Treatment of known positive, pheasant and pigeon sera with gluteraldehyde polymerized positive and negative SAFA antigens was presumably responsible for the nonspecific absorption of serum-Igs and consequently reducing the FDR values of these sera.

Disagreement between the SAFA test serum titres and other tests such as the serum neutralization test and indirect fluorescent antibody test have been reported (9). In the present study the same inconsistency was observed between the SAFA test and the HI test titres (Table II). As described earlier, the HI test detected only antibodies directed to the hemagglutinin, while the SAFA test would be expected to detect antibodies in the internal type specific antigens of influenza virus type A. Accordingly, the inconsistency between serum titres of the HI and the SAFA test are probably due to the differences in specificity of the antibodies detected by both tests.

A variety of detergents have been used to disrupt influenza viruses for isolation and purification of influenza hemagglutinin and neuraminidase. The sensitivity of these two surface antigens to different detergents varies from strain to strain of influenza viruses (4, 19). Therefore, the hemagglutinin and neuraminidase of some influenza strains may remain active and undenatured after detergent treatment. In this study, no tests were performed to demonstrate the effect of sodium lauroylsarcosinate on the activity or the antigenicity of the haemagglutinin and the neuraminidase of the A/-turkey/Ontario/6118/67. As a con-

sequence, the contribution of these two surface antigens in detecting antibodies in the SAFA test remains unknown.

By choosing the type specific antigens of influenza virus A/turkey/Ontario/6118/67 as the antigen of the SAFA test, it gave the test the feature of type specificity rather than strain specificity. This was demonstrated by the finding that the SAFA test was equally effective in detecting antibodies to the type specific antigen in two antisera prepared against two different influenza type A virus strains, namely A/turkey/Ontario/6118/67 (Hav 8) and A/turkey/Ontario/6213/68 (Hav 5).

The DID test, in addition to its low sensitivity, gives a qualitative rather than true quantitative value of antibody measurement (18). The CF test is capable of detecting only IgM and certain subclasses of IgG antibodies, so the absence of a complement fixation reaction does not necessarily exclude the presence of antibody to the antigen used in the test (18). The SAFA test like the indirect fluorescent antibody test is a primary binding assay, and theoretically it should detect all antigen-antibody interaction (15). Accordingly, we should expect the SAFA test to be more sensitive than the DID and CF tests for detecting antibodies to the type specific antigen of influenza virus. However, studies comparing this test to established methods are required before this test can be considered as a seroepidemiological screening test for influenza antibodies.

The examination of one serum dilution by the SAFA test has been reported by Artenstein and Dandridge (1) for diagnosis of adenovirus infection in man. For simplifying the application of the SAFA test for use in large-scale surveys, the present studies show that for avian influenza it might be necessary (because of the occasional observation of prozone effects and to detect low serum antibody level) to test two serum dilutions of 1/8 and 1/16 to increase the reliability of the method.

REFERENCES

1. **ARTENSTEIN, M.A.** and **O.W. DANDRIDGE.** A new serologic test for adenovirus infection. J. Immun. 100: 831-834. 1968.
2. **BEARD, C.W.** Demonstrationof type-specific influenza antibody in mammalian and avian sera by immunodiffusion. Bull. Wld Hlth Org. 42: 779-785. 1970.
3. **BECKER, W.B.** The isolation and classification of tern virus: Influenza virus A/tern/South Africa/1961. J. Hyg. ,Camb. 64: 309-319. 1966.
4. **BUCHER, D.** and **P. PALESE.** Neuraminidase. *In* The Influenza Viruses and Influenza. Edwin D. Kilbourne, Ed. pp. 53-82-123. Academic Press Inc. 1975.
5. **DE LILVA, L.M.** and **J. O'H. TOBIN.** The post-mortem diagnosis of influenza infection by IgG, IgA and IgM antibody estimation. J. Med. Microbiol. 6: 15. 1973.
6. **DOWDLE, W.R., J.C. GALPHIN, M.T. COLEMAN** and **G.C. SCHILD.** A simple double immunodiffusion test for typing influenza viruses. Bull. Wld Hlth Org. 51: 213-218. 1974.
7. **DOWNIE, J.C.** and **W.G. LAVER.** Isolation of a type A influenza virus from an Australian Pelagic bird. Virology 51: 259-269. 1973.
8. **EASTERDAY, B.C.** and **D.O. TRAINER.** Evidence of infection with influenza viruses in migratory water fowl. Nature 219: 523-524. 1968.
9. **GARNHAM, J.A., B.N. WILKIE, K. NIELSEN** and **J. THORSEN.** Application of the soluble antigen fluorescent antibody (SAFA) test to the serodiagnosis of rabies. J. Immun. Meth. 14: 147-162. 1977.
10. **GOLDMAN, M.** Fluorescent Antibody Methods. Academic Press. 1968.
11. **HEBERT, G.A.** Ammonium sulphate, fractionation of sera: mouse, hamster, guinea pig, monkey, chimpanzee, swine, chicken and cattle. Appl. Microbiol. 27: 389-393. 1974.
12. **JOHNSON, D.C.** and **B.G. MAXFIELD.** An occurrence of avian influenza virus infection in laying chickens. Avian Dis. 20: 422-424. 1976.
13. **LONG, G., A.E. FERGUSON, M.C. CONNELL** and **C. G. WILLS.** Isolation of an unidentified hemagglutinating virus from the respiratory tract of turkeys. Avian Dis. 9: 495-504. 1965.
14. **LANG, G., O. NARAYAN, B.T. ROUSE, A.E. FERGUSON** and **M.C. CONNELL.** A new influenza A virus infection in turkeys I. Isolation and characterization of virus 6213. Can. vet. J. 9: 22-29. 1968.
15. **MINDEN, P., R.T. REID** and **R.S. FARR.** A comparison of seven commonly used methods for detecting antibodies to bovine albumin in human serum. J. Immun. 96: 180-187. 1966.
16. **PEREIRA, H.G., A. RINALDI** and **L. NARDELLI.** Antigenic variation amongst avian influenza A viruses. Bull. Wld Hlth Org. 37: 553-558. 1967.
17. **ROBERTS, D.H.** The isolation of an influenza A virus and a mycoplasma associated with duck sinusitis. Vet. Rec. 76: 470-473. 1964.
18. **SCHILD, G.C.** and **W.R. DOWDLE.** Characterization and diagnostic serology. *In* The Influenza Viruses and Influenza. Edwin D. Kilbourne, Ed. pp. 315-372. Academic Press. 1975.
19. **SCHULZE, I.T.** The biologically active proteins of influenza virus. The hemagglutinin. *In* The Influenza Viruses and Influenza. Edwin D. Kilbourne, Ed. pp. 53-79. Academic Press. 1975.
20. **SLEMONS, R.D., D.C. JOHNSON, J.S. OSBORN** and **F. HAYES.** Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Dis. 18: 119-124. 1974.
21. **SLEMONS, R.D., R.S. COOPER** and **J.S. OSBORN.** Isolation of type-A influenza viruses from imported exotic birds. Avain Dis. 17: 746-751. 1973.
22. **SOKAL, R.R.** and **F.J. ROHLF.** Introduction to Biostatistics. pp. 171-172. San Francisco: W.H. Freeman and Company. 1973.
23. **TOUSSAINT, A.J.** and **R.I. ANDERSON.** Soluble antigen fluorescent-antibody technique. Appl. Microbiol. 13: 552-558. 1965.
24. **WEBSTER, R.G.** and **W.G. LAVER.** Antigenic variation of influenza viruses. *In* The Influenza Viruses and Influenza. Edwin D. Kilbourne, Ed. pp. 269-314. Academic Press. 1975.
25. **WEBSTER, R.G., M. MORITA, C. MIDGEN** and **B. TUMOVA.** Ortho- and paramyzoviruses from migrating feral ducks. Characterization of a new group of influenza A viruses. J. gen. Virol. 32: 217-225. 1976.
26. **WELLS, A.F., C.E. MILLER** and **M.K. NADEL.** Rapid fluorescence of protein assay for fluorescent-antibody conjugates. Appl. Microbiol. 14: 271-275. 1966.