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Demonstration of Type-specific Influenza Antibody in Mammalian and Avian Sera by Immunodiffusion

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The detection of antibody against the ribonucleoprotein antigen of influenza virus is useful because its type-specificity allows the use of serological surveys to detect evidence of recent infections. Antigenic differences between strains limit the usefulness of the techniques, such as the haemagglutination-inhibition test, that detect antibody against surface antigens.

This paper describes an agar-gel precipitation (AGP) test that will detect type-specific antibody in avian or mammalian sera. Convalescent levels of antibody against either type A or B influenza virus were demonstrated in human sera. Positive but inconsistent results were obtained with swine sera. The antigens used in the AGP test are non-infectious and stable. The test is easy and economical to perform. Its sensitivity compares favourably with that of the complement-fixation test using human and equine sera.

While not a replacement for any of the serological tests at present in current use, the AGP test should prove useful in a variety of diagnostic and research situations.

Influenza infections result in the production of antibody against the type-specific ribonucleoprotein (RNP) or soluble (S) antigen and the strain-specific surface or envelope antigens of the virus. The detection of the antibody against the RNP antigen is useful because its type-specificity allows the use of serological surveys to detect evidence of recent infections. Antigenic differences between strains limit the usefulness of the techniques that detect antibody against surface antigens such as the haemagglutination-inhibition (HI), virus neutralization and strain-specific complement-fixation (CF) tests.

Examination of sera for evidence of avian influenza antibody by the HI test requires numerous strains for antigenic coverage broad enough to give meaning to negative results. Although all non-human influenza viruses have been of type A, the possibility that the antibodies are due to a new strain within that type cannot be excluded by negative HI results, regardless of the number of antigens used (Pereira et al., 1966). The use of a type-specific serological test would reduce the number of antigens needed to no more than three (type A, B, and C) with human serum and to one (type A) for lower animals.

To utilize the benefits of type-specific antibody identification, workers have generally relied on the complement-fixation test and only with mammalian sera. This report describes the use of an agar-gel precipitation (AGP) test on a glass slide for the demonstration of antibody against the RNP antigen of types A and B influenza viruses in mammalian and avian sera.

Jensen & Francis (1953) were the first to apply the immunodiffusion technique to demonstrate virus–serum antibody reactions. They used influenza virus to show strain specificity with antigens prepared from infected allantoic fluids. Immunodiffusion was also used (Styk & Hana, 1966; Hana & Hoyle, 1966) with disrupted influenza A and B viruses to demonstrate numerous antigens. Schild & Pereira (1969) recently reported the use of disrupted influenza A viruses with rabbit antiserum to demonstrate that NP antigen is composed of a single antigenic component.

MATERIALS AND METHODS

Viruses

The influenza viruses originally obtained from Dr B. C. Easterday, University of Wisconsin, USA, and Dr Gerhard Lang, University of Guelph, Ontario, Canada, were prepared in 10-day embryonated eggs. The strains used were as follows:

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Turkey/Wisconsin/66, Turkey/Wisconsin/68, A2/Japan/305/57, A/Swine/S15/31, A/Equi-1/Prague/56, B/Johannesburg/59, A/PR8/34, Duck/Canada/53, Duck/Czechoslovakia/56, and Duck/England/62.

Immunodiffusion antigens

Ten-day embryonated chicken eggs were inoculated with 0.1 ml of 10^{-2} dilutions of stock virus and incubated at 37°C for 24 hours. The eggs were removed from the incubator, the small end of the egg shell was cut off with scissors and the embryo and its attachments were discarded. The chorio-allantoic membrane (CAM) was left adhering to the shell wall. The CAM was then removed with forceps. Sterile phosphate-buffered saline (PBS) (0.8% NaCl, pH 7.2) was used to wash the membranes, which were re-collected by pouring into a gauze funnel. The membranes were then homogenized in a blender (Virtis) operated at approximately 15 000 rev/min for 1–2 minutes. After homogenization, the suspension was put through 3 freeze–thaw cycles before being centrifuged at approximately 700 *g*. The liquid phase was carefully withdrawn and placed in screw-capped vials. Approximately 0.5 ml of antigen resulted from each membrane used.

The antigen was then treated with 0.1% formalin at 37°C for 36 hours to destroy any infectivity. If the antigens formed a gel during the inactivation, they were shaken vigorously and a small amount of PBS was added. The antigen was centrifuged again at 700 *g* after the formalin treatment to remove aggregates of cellular debris and stored at −20°C.

Immune sera

The sera used in these studies were obtained from different laboratories. They will be described at the same time as the test results.

Immunodiffusion medium

The medium consisted of 0.7% Ionagar No. 2 (Colabs), 8.0% NaCl, and 1 : 10 000 thiomersal in distilled water buffered to pH 7.2 by the addition of 10 ml per 100 ml of 0.1 M phosphate buffer. The medium was melted in the autoclave at 121°C for 5 minutes, stored at room temperature, and melted again as needed.

Slide preparation

Ordinary microscope slides, frosted on one end, were cleaned with methanol. They were positioned on a level surface with the frosted side upwards and 1 ml of the melted agar was placed on the clear section with a pipette.

Wells were cut in the agar after it was cool, using a cutter made from 7 reamed copper or brass tubes soldered to a brass plate. Six wells were evenly positioned around a centre well. The wells were 4 mm apart and 4 mm in diameter. After cutting, the agar plugs were removed with a Pasteur pipette attached with rubber tubing to a vacuum flask.

When cutting the wells, care was required to prevent disturbing the union between the agar and the surface of the slide. The agar came off during the washing and staining process and reagents leaked under the agar when this attachment was broken.

Slides were held in a humid container until the wells were cut and the reagents added. The slides were then returned to a level position in the container and kept at room temperature (23°C) for 20–48 hours before examination over indirect light.

The reagents were added using disposable Pasteur pipettes approximately 10 cm long fitted with a rubber dropper-bulb. Slight pressure on the bulb would cause reagent to accumulate on the tip of the pipette. It would fill the well when carefully touched to the bottom. Reagent identification was made on the frosted portion of the slide.

Slide staining

After 20–48 hours at room temperature, slides were examined and discarded or placed in PBS for 24 hours at room temperature to wash out unprecipitated antigen and serum protein prior to staining. The frequence of changing the PBS depended on the volume of PBS and the number of slides. The slides were placed in an "on-edge" position in a glass slide-rack. On removal from the PBS the slides were placed in a solution of 0.1% thiazine red in 1% acetic acid for 15–20 minutes (Crowle, 1961). They were then removed and decolorized in 1% acetic acid for several hours until the lines could be readily distinguished from the background agar. Slides were then left in the slide-racks to dry at room temperature under a dust cover. Cover-slips were mounted on the slides with Permount (Fisher), providing a permanent record of the reaction.

RESULTS

Equine sera

Influenza occurred in a stable of horses at the University of Wisconsin in December 1968 after they had been assembled over a period of 30 days from several areas of the USA.[1] Both A/Equi-1 and

[1] B. Tumova, to be published.

TABLE 1

RESULTS OF AGAR-GEL PRECIPITATION (AGP) AND COMPLEMENT-FIXATION (CF) TESTS ON SERA FROM HORSES DURING AND AFTER A NATURALLY OCCURRING INFECTION WITH EQUINE INFLUENZA VIRUSES

Horse	Date of bleeding											
	12 Dec. 1968 [a]		20 Dec. 1968		2 Jan. 1969		27 Jan. 1969		20 March 1969		11 June 1969	
	AGP [b]	CF [c]	AGP [b]	CF [c]	AGP [b]	CF [c]	AGP [b]	CF [c]	AGP [b]	CF [c]	AGP [b]	CF [c]
Gyp	−	0	+	40	+	20	+	0	+	0	+	0
Cha	+	20	+	20	+	20	+	80	+	20	+	<10
Blaze	−	0	+	320	+	40	+	40	+	40	−	0
Pride	−	0	NT	320	+	80	+	40	NT	40	−	10
Dolly	−	10	+	20	+	20	+	20	NT	20	+	20
Windy	−	<10	+	160	+	40	+	20	NT	NT	+	20
Dancer	+	20	+	20	+	20	+	80	+	80	+	80
Babe	+	160	+	40	+	40	+	30	NT	15	−	10

[a] Acute phase bleeding.

[b] − = absence of precipitin line; + = presence of precipitin line; NT = not tested.

[c] CF titres represent the reciprocals of serum dilution at the 50 % end-point according to the method of Pereira et al. (1964); NT = not tested. Sera and CF titres provided by Dr B. Tumova.

A/Equi-2 viruses were isolated. Sera were obtained at intervals after the illness, tested by CF and provided by Dr Tumova. Results of the AGP test with type A NP antigen prepared with Turkey/Wisconsin/66 virus are shown in Table 1 together with the CF titres. The presence of antibody against the A influenza NP antigen could be demonstrated as long as 6 months after the illness with the AGP test (Fig. 1). The intensity of the precipitation lines in the last samples was considerably less than that of those observed with earlier sera. The lines also formed closer to the serum wells with the later samples. The results indicated that 3 or 4 of the 8 horses had experienced an influenza infection within the previous 6 months as evidenced by the results of the AGP and CF tests on the acute-phase sera.

Human sera

Paired human sera together with their CF titres were furnished by Dr Marion Coleman, WHO International Influenza Center for the Americas, Atlanta, Ga., and by Mrs Julia Eubanks, Virology Section, Georgia Department of Public Health. The sera were obtained from patients during the acute phase of their illnesses and 12–21 days later. AGP tests were performed with type A antigen prepared with Turkey/Wisconsin/66 virus and with type B antigen prepared with B/Johannesburg/59 virus. Results indicate good correlation between the AGP and CF tests with both type A and type B antigens (Table 2) when precipitin lines are graded as to intensity and location. Some patients who experienced a rise in type B antibody titres had constant levels of type A antibody activity (Fig. 2).

Avian sera

Chicken and turkey antisera furnished by Dr Gerhard Lang and Dr B. C. Easterday were tested by the AGP test with antigens prepared from several different avian influenza isolates. Some of the sera, together with a fowl-plague antiserum from the US Department of Agriculture, Plum Island Animal Disease Laboratory, were placed in the outer wells with a type A NP antigen in the centre well. Lines of precipitation formed between the antigen well and sera representing 5 of the avian subtypes. The lines were continuous, with no spurs between sera, indicating antigenic identity (Fig. 3). No line formed where normal chicken serum was used.

NP antigens prepared with 5 viruses that represented 4 avian influenza subtypes were placed in the outer wells and tested against convalescent serum, in the centre well, prepared in chickens, with

TABLE 2

RESULTS OF AGAR-GEL PRECIPITATION (AGP) AND COMPLEMENT-FIXATION (CF) TESTS ON ACUTE AND CONVALESCENT HUMAN SERA WITH TYPE A AND TYPE B INFLUENZA ANTIGENS

Patient	Acute sera				Convalescent sera			
	Type A antigen		Type B antigen		Type A antigen		Type B antigen	
	AGP [a]	CF [b]	AGP [a]	CF [b]	AGP [a]	CF [b]	AGP [a]	CF [b]
R	+	8	−	NT	++++	128	−	NT
Si	+	16	−	8	++++	128	−	8
Sh	+	16	−	16	++++	256	−	8
M	+	8	−	8	++++	128	−	8
P	++	NT	−	8	++	NT	++	64
Sm	++	16	−	16	++	8	++	32
Ma	+	8	−	8	+	8	++	64
B	+++	32	++	32	+++	16	+++	128

[a] AGP results + to ++++ based on intensity of precipitin lines and their proximity to serum or antigen wells; − = absence of precipitin line.

[b] CF values presented as reciprocals of titre with the microprocedure; NT = not tested.

Turkey/Wisconsin/66 virus. Well-defined precipitin lines of identity indicate specific type A antigen–antibody reactions (Fig. 4). No line formed between normal CAM suspension (control antigen) and the serum.

Antigens and immune sera prepared with avian influenza isolates were tested against antigens or immune sera prepared with human, equine or porcine influenza strains. Lines of precipitation formed that were continuous between either antigens or sera when they were in the peripheral wells, regardless of the source of type A influenza virus used to prepare them. A NP antigen prepared from B/Johannesburg/59 influenza virus and a type B immune serum prepared in rabbits was included in another test as a type-specificity control (Fig. 5).

Swine sera

Sera obtained from pigs at intervals after aerosol exposure to A/Swine/S15/31 influenza virus were furnished, together with the HI titres, by Dr B. C. Easterday.

Generally, very poor results were obtained with swine sera when the AGP test was used. There were some exceptions, as shown in Fig. 6, where a pig was exposed to S15 virus and blood samples were taken frequently. These sera had high HI titres and produced clear precipitation by 7 days after exposure. Sera with high HI titres usually gave negative results with the AGP test, regardless of the source of type A antigen or the percentage of agar or NaCl in the medium.

Antigen stability

Formalin-treated antigens prepared from 6 influenza strains were placed at 23°C and tested at intervals for reactivity with a known positive serum. There was no apparent loss in activity after 300 days at this temperature. Antigens have been stored at −20°C and repeatedly frozen and thawed with no adverse effects on their activity.

The formalin treatment of the antigen was sufficient to inactivate the virus as no infectivity was demonstrated in embryonating eggs.

Antibody stability

Sera from the different species that gave positive results with the AGP test were stored at +4°C. The sera remained positive by the AGP test after the maximum test-period of 28 days. Reference avian and mammalian sera were repeatedly frozen and thawed with no apparent reduction in AGP activity.

DISCUSSION

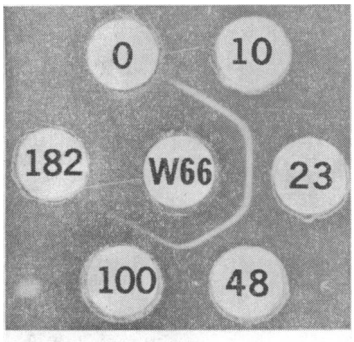
While it is not a replacement for any of the presently used serological tests, the AGP test should prove useful in a variety of diagnostic and research situations.

Sera from naturally occurring field outbreaks of influenza in turkeys have been examined with the AGP technique. Results will be reported in detail elsewhere (Beard, 1970). No difficulty was experienced in demonstrating clear precipitin lines with sera from any of the flocks that had been infected, while uninfected flocks remained negative.

The non-infectious formalin-treated antigen preparations have adequate stability without refrigeration to permit shipment and use in many locations.

FIG. 1

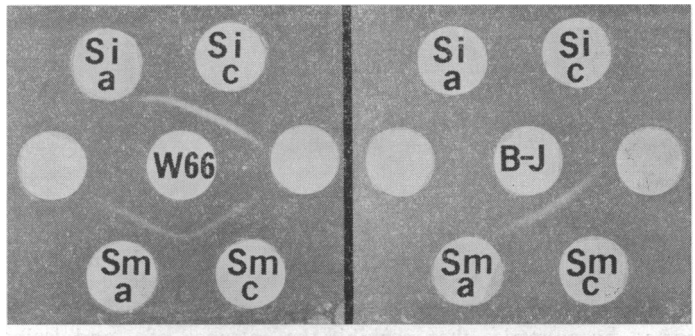
AGP TEST WITH EQUINE SERA (HORSE "GYP") TAKEN AT INDICATED DAYS AFTER A NATURAL ILLNESS OF EQUINE INFLUENZA [a]



[a] Type A antigen in centre well is Turkey/Wisconsin/66. Figures represent day of blood sampling.

FIG. 2

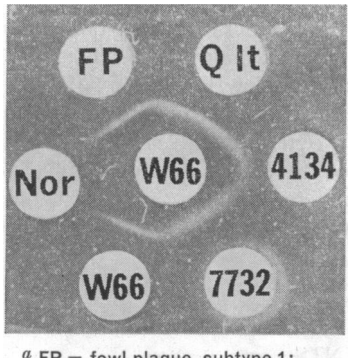
AGP TEST WITH ACUTE (a) AND CONVALESCENT (c) HUMAN SERA AFTER AN ILLNESS WITH THE HONG KONG STRAIN (Si) AND A 1969 TYPE B ILLNESS (Sm) [a]



[a] Type A antigen in centre well is Turkey/Wisconsin/66; type B antigen is B/Johannesburg/59.

FIG. 3

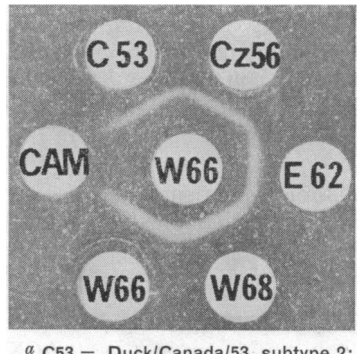
AGP TEST WITH AVIAN SERA REPRESENTING DIFFERENT AVIAN SUBTYPES AGAINST Turkey/Wisconsin/66 ANTIGEN [a]



[a] FP = fowl plague, subtype 1;
Q It = Quail/Italy/544/66, subtype 2;
4134 = Duck/Ontario/4134/67, subtype 4;
7732 = Turkey/Ontario/7732/66, subtype 5;
W66 = Turkey/Wisconsin/66, subtype 6;
NOR = serum from non-infected chickens.

FIG. 4

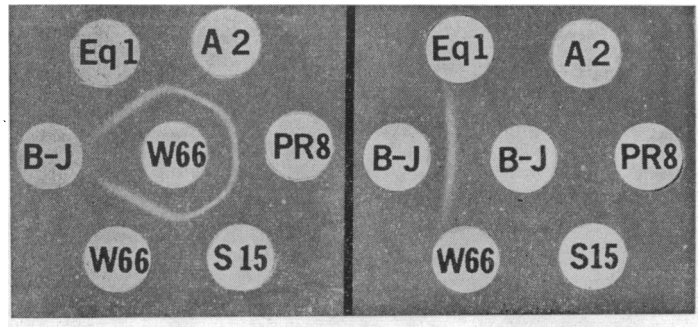
AGP TEST WITH ANTIGENS PREPARED FROM AVIAN INFLUENZA VIRUSES REPRESENTING 4 DIFFERENT SUBTYPES AGAINST Turkey/Wisconsin/66 CHICKEN ANTISERUM [a]



[a] C53 = Duck/Canada/53, subtype 2;
Cz56 = Duck/Czechoslovakia/56, subtype 4;
E62 = Duck/England/62, subtype 4;
W68 = Turkey/Wisconsin/68, subtype 5;
W66 = Turkey/Wisconsin/66, subtype 6;
CAM = antigen prepared from non-infected eggs.

FIG. 5

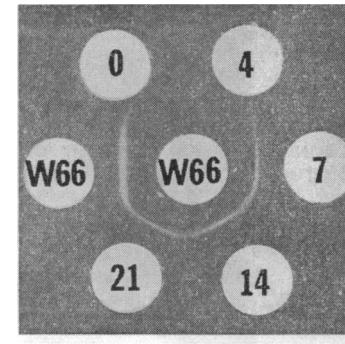
AGP TEST WITH ANTIGENS PREPARED WITH INFLUENZA VIRUSES FROM DIFFERENT AVIAN AND MAMMALIAN HOSTS IN THE OUTER WELLS TESTED AGAINST TYPE A CHICKEN ANTISERUM OR TYPE B RABBIT ANTISERUM [a]



[a] W66 = Turkey/Wisconsin/66; B-J = B/Johannesburg/59; Eq 1 = A/Equi-1/Prague/56; A2 = A2/Japan/305/57; PR8 = A/PR8/34; S15 = A/Swine/S15/31.

FIG. 6

AGP TEST WITH SWINE SERA TAKEN FROM A PIG AT INDICATED DAYS AFTER EXPOSURE TO A/Swine/S15/31 VIRUS TESTED AGAINST Turkey/Wisconsin/66 TYPE A ANTIGEN [a]



[a] One outer well (W66) contains chicken antiserum against Turkey/Wisconsin/66 as a positive control.

Perhaps the formalin-treated antigens may be imported by countries that prohibit the entrance of viable virus preparations.

Judging from the data acquired with human and equine sera, the sensitivity of the AGP compares favourably with that of the CF test. The horses described in this report were not isolated from each other; therefore, a second exposure cannot be completely excluded as a cause of the persistent antibody. The ease with which the AGP test is performed is a distinct advantage over the sometimes tedious CF test.

The use of a high NaCl concentration in the AGP media was necessary because of the avian sera (Grabar, 1959). Mammalian sera produced clearly visible lines of precipitation in the medium containing 8.0% NaCl, resulting in the need for a single medium for all AGP tests regardless of the species involved.

It is the author's experience that lines could be easily observed or photographed with indirect light with the medium in its fresh state. Washing, staining and drying of the slides were desirable only in order to preserve the actual reaction; they were not essential in order to observe results.

The precipitin lines were usually very sharp and well defined. When more diffuse lines did occur, they were with sera prepared by repeated antigen injections rather than by a single exposure to influenza virus. Perhaps the hyperimmunizing procedures resulted in antibody against minor antigenic determinants that are not present in convalescent serum. Fig. 3 shows 3 sera prepared by hyperimmunization (Quail/Italy, Duck/Ontario/4134/67 and Turkey/Ontario/7732/66) and 2 convalescent sera (fowl plague and Turkey/Wisconsin/66).

No explanation is offered for the poor results generally obtained with swine sera. Further study is needed to determine the peculiarities of swine antiserum responsible for the inconsistent AGP reactions.

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RÉSUMÉ

MISE EN ÉVIDENCE, PAR IMMUNODIFFUSION, D'UN ANTICORPS ANTIGRIPPAL SPÉCIFIQUE DE TYPE DANS DES SÉRUMS DE MAMMIFÈRES ET D'OISEAUX

L'auteur décrit une épreuve de précipitation en gel de gélose capable de déceler les anticorps spécifiques de type (dirigés contre l'antigène nucléoprotéique des virus grippaux) dans des sérums d'oiseaux ou de mammifères. L'intérêt de cette méthode est de n'utiliser que trois antigènes (types A, B et C) pour les sérums humains et un seul (type A) pour les sérums d'animaux, alors que pour les tests révélant la présence d'anticorps spécifiques de souche on doit recourir à une vaste gamme d'antigènes.

On a prélevé des sérums chez des chevaux à divers intervalles après une épidémie de grippe due aux virus A/Equi-1 et A/Equi-2. Soumis à l'épreuve de précipitation en présence d'un antigène nucléoprotéique A (souche Turkey/Wisconsin/66), les antisérums des animaux atteints ont donné des lignes de précipitation indiquant l'existence d'anticorps spécifiques de type A et leur persistance, dans certains cas, pendant les 6 mois suivant l'infection.

On a également étudié par immunodiffusion des sérums humains couplés, prélevés chez des patients atteints de grippe à la phase active de l'infection et en période de convalescence, en présence d'un antigène de type A (souche Turkey/Wisconsin/66) et d'un antigène de type B (souche B/Johannesburg/59). Les résultats ont montré une concordance satisfaisante avec les résultats des réactions de fixation du complément.

Des antisérums représentant cinq sous-types de virus aviaire ont donné des lignes de précipitation en présence d'un antigène nucléoprotéique de type A. De même, des arcs de précipitation sont apparus entre antigènes ou antisérums d'origine aviaire et des antigènes ou antisérums d'origine humaine, équine et porcine.

Par contre, on a obtenu en général des résultats moins concluants lorsque les épreuves d'immunodiffusion ont été pratiquées sur des sérums de porcs.

Selon l'auteur, les résultats de l'épreuve de précipitation appliquée à des sérums de convalescents ou à des sérums équins peuvent être avantageusement comparés avec les résultats des réactions de fixation du complément spécifiques de type sous le rapport de la sensibilité.

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