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TECHNICAL MANUSCRIPT 243

# HEMAGGLUTINATION-INHIBITION METHOD AND IMMUNOFLUORESCENCE STAINING WITH VEE VIRUS



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AUGUST 1965

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# HEMAGGLUTINATION-INHIBITION METHOD AND IMMUNOFLUORESCENCE STAINING WITH VEE VIRUS

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In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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## ABSTRACT

Hemagglutination and fluorescent antibody are compared for the direct detection of virus devoid of host cells. The minimum number of tissue plaque-forming units of Venezuelan equine encephalomyelitis virus that could be detected by the hemagglutination technique was determined. Similar concentrations of the virus in bovine albumin borate saline, brain-heart infusion broth, and demineralized water were tested by the fluorescent antibody technique. Somewhat higher concentrations of the virus in bovine albumin borate saline were used in the hemagglutination-inhibition test. The quantitative hemagglutination procedure employed for these studies was carried out at 37 C for 75 minutes with variations in concentration of goose red cells. As a result of lowering the red cell concentration, smaller concentrations of virus were detected. The direct fluorescent antibody staining procedure applied to slide preparations containing known numbers of tissue culture plaque-forming units of virus was negative. Adsorbed viral antigen on agglutinated goose erythrocytes was visualized by direct and indirect FA techniques.

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## I. INTRODUCTION

The purpose of this investigation was to compare the hemagglutination-inhibition (HAI) test with fluorescent antibody (FA) in the detection of Venezuelan equine encephalomyelitis (VEE) virus relatively free of host cells. Data relating to FA staining of VEE virus adsorbed on goose erythrocytes are also presented.

The existence of hemagglutinins in a number of arthropod-borne viruses (arboviruses) was initially shown by [illegible]n and his associates.[1-5] Since Porterfield[7] first demonstrated that [illegible]e erythrocytes could be agglutinated by arboviruses, goose cells were thereafter routinely used by investigators[8,9] in hemagglutination (HA) and hemagglutination-inhibition (HAI) tests. The use of the HAI reaction in classifying arboviruses has been reported.[6,10] The use of immunological characteristics for classification of arboviruses has also been reported.[7,11-13]

The present study, directed toward utilizing the HA technique for detecting arboviruses, was influenced by the fact that the HA test is simple and rapid, and it is best performed in the absence of host cells. The FA procedure up to the present time has been applied successfully only to tissues infected with viruses.[14-16] There is a lack of data in the literature concerning the use of FA techniques in the detection of arboviruses free of host cells. In this study, the sensitivity of the HA reaction is shown to be related to the quantity of red cells employed.

## II. MATERIALS AND METHODS

### A. VIRUS

The origin of the Trinidad strain of VEE virus employed in this study was previously described.[17] The plaque technique on chick embryo monolayers described by Dulbecco[18] with Western equine encephalomyelitis virus was used for quantitative assay of plaque-forming units (pfu) in the stock virus. Pooled infectious culture fluids were centrifuged at 3000 rpm for 30 minutes, and the supernatant fluid was then filtered through a membrane filter* to insure the removal of all particulate matter. This material served as a source of virus for this investigation.

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* HA Millipore Filter, Millipore Filter Corp., Waltham, Mass.

## B. CHEMICAL REAGENTS AND ERYTHROCYTES EMPLOYED FOR HEMAGGLUTINATION AND HEMAGGLUTINATION-INHIBITION TEST

A full description of the source and preparation of goose erythrocytes and preparation of all reagents has been given by Clarke and Casals.[9]

## C. DIRECT COUNTING OF ERYTHROCYTES

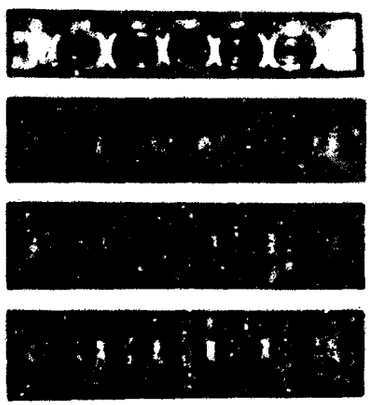
The total number of cells used for conducting HA and HAI tests was counted directly with the Levy-Hausser, double ruled Neubauer blood cell counting chamber.

## D. IMMUNE SERA FOR THE HEMAGGLUTINATION-INHIBITION TEST

Specific rabbit VEE antiserum was prepared according to the method of Shepard and Flugerman.[16] The serum gave a hemagglutination-inhibition titer of 1:10,240. Before using the serum, it was treated with kaolin and adsorbed with erythrocytes to remove nonspecific inhibitors and agglutinins by the method of Clarke and Casals.[9]

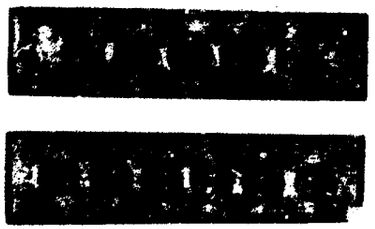
## E. DETECTION EXPERIMENTS WITH HEMAGGLUTINATION TEST

Initial experiments were designed to examine the effect of red blood cell concentration on the number of virus pfu that could be detected. The hemagglutination test with goose red cell suspensions, which varied in concentration from 7,500,000 to 1,500,000 cells per milliliter, was a modification of that described elsewhere.[9] Twofold dilutions of tissue culture fluid containing VEE virus were prepared in 0.2% bovine albumin borate saline (BABS), pH 9.0. A total of four dilutions were utilized (0.5 ml per dilution). Each dilution of virus was combined with different concentrations of goose red cells (0.5 ml volume). The resulting mixtures (pH 6.4) were shaken and the reaction was allowed to proceed at 37 C for approximately 75 minutes. The final tube showing complete agglutination, manifested by a uniform, thin, translucent shield of cells covering the lower surface of the tube, was taken as the endpoint. An incomplete shield combined with a dark ring of cells indicated partial hemagglutination. Ordinarily, the transition from complete agglutination to the absence of agglutination was rather abrupt, with one tube showing partial agglutination between these extremes (Fig. 1 and 2). The test was not considered valid unless the erythrocyte cell controls were completely negative.



| Row Number | Total RBC Conc. | Tissue Culture Plaque-Forming Units | | | | |
|---|---|---|---|---|---|---|
| 1 | 7,500,000 | 28,500<br>+ | 14,250<br>+ | 7,125<br>± | 3,562<br>0 | Endpoint<br>14,250 |
| 2 | 6,000,000 | 28,500<br>+ | 14,250<br>+ | 7,125<br>± | 3,562<br>0 | Endpoint<br>14,250 |
| 3 | 3,000,000 | 14,250<br>+ | 7,125<br>+ | 3,562<br>± | 1,781<br>0 | Endpoint<br>7,125 |
| 4 | 1,500,000 | 7,125<br>+ | 3,562<br>+ | 1,781<br>± | 890<br>0 | Endpoint<br>3,562 |

Figure 1. Top View of Tubes Showing Patterns Formed by Sedimented Cells. Tubes at the extreme right are 0.2% bovine albumin borate saline cell controls. The characteristic pattern of complete agglutination is seen in the first two tubes of row 1. A partial reaction is illustrated in the third tube in row 2.



| | Total RBC Conc. | Tissue Culture Plaque-Forming Units | | | | Controls | |
|---|---|---|---|---|---|---|---|
| | | | | | | Saline Cell | Serum Cell |
| Virus-cell | 3,000,000 | 14,250<br>+ | 7,125<br>+ | 3,562<br>± | 1,781<br>0 | 0 | |
| Virus-cell-specific antiserum | 3,000,000 | 1,825,000<br>0 | 912,500<br>0 | 456,250<br>0 | 228,125<br>0 | 0 | 0 |

Figure 2. Top View of Tubes Showing Patterns of Sedimented Cells. Virus without the specific antiserum is in the top row and the specific antiserum plus virus in the bottom row. The tube in the upper row at the extreme right is the 0.2% bovine albumin borate saline cell control. The two tubes in the right side of the lower row represent the saline cell control and serum cell control, respectively.

The hemagglutination-inhibition test was conducted according to the method of Clarke and Casals.[9] The homologous immune rabbit serum was used in a dilution of 1:64 in 0.2% BABS, pH 9.0. An appropriate volume (0.5 ml) of goose red cell suspension was added to each serum-virus mixture. A serum control was prepared by combining equal volumes of serum and BABS. The serum control assured that nonspecific agglutinins were absent. Specific identification of virus was indicated by a complete HAI reaction, with red cells accumulating as sharply demarcated discs. In the virus control, the cells agglutinated and settled as a thin, uniform translucent shield covering the entire bottom of the tube.

## F. DETECTION EXPERIMENTS WITH THE DIRECT FLUORESCENT ANTIBODY TECHNIQUE

For comparative studies, concentrations of virus previously detected by the HA test were selected for fluorescent antibody staining with labeled VEE antibody globulin.

### 1. Chemical Reagents

Fluorescein isothiocyanate (FITC)* was used to label globulins in the direct method and lissamine rhodamine RB 200* for counterstaining.

### 2. Fluorescein Isothiocyanate-Labeled Antibody

Rabbit antiserum used was the same as that mentioned previously in the HA test. The immune globulin was precipitated from whole serum by the methanol method of Dubert et al.,[20] and reconstituted to five-eights of the original volume. This globulin solution yielded an HAI titer of 1:5120. Total protein was determined by the micro-Kjeldahl procedure.

The conjugated globulin fraction was prepared according to a modification** of the method of Marshall, Eveland, and Smith.[21] The powdered FITC was dissolved in a mixture of unbuffered saline and 0.5 M carbonate-bicarbonate buffer (pH 9.0) prior to adding globulin solution (preadjusted to pH 9.0 with 0.1 M NaOH). The serum-dye mixture contained 10% buffer and 0.04 mg FITC per mg of protein. The final pH of the mixture was 9.0. The mixture was allowed to react at 4 C for 18 hours with gentle agitation. Untagged dye was removed by passage through a Sephadex column (G-25, medium grade), and eluted with physiological saline. The antibody titer of the final conjugate was 1:1280 as determined by HAI test. The concentration of bound FITC was 28.2 micrograms per mg of globulin. This was determined from standard calibration curves relating FITC concentrations in 0.1 N NaOH to absorbance at 490 mμ, and protein content (biuret reaction) to absorbance at 560 mμ.

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* Secured from Baltimore Biological Laboratories (BBL).

** Suggested by Maxwell R. Klugerman, personal communication.

3. Preparation, Fixation, Staining and Examination of Smears

The stock VEE-virus preparation used in the HA test was also used in the preparation of smears for FA staining. A quantity of virus pfu in 0.05 ml was deposited within a 1-cm square on a 3- x 1-inch glass slide. The slides were dried and kept in the refrigerator at 4 C until processed. Smears were fixed in 10% formalin[16,22] for 10 minutes at room temperature and washed twice in buffered saline, pH 7.2. Preparations were dried at 37 C for 20 minutes. Four slide preparations were used with each concentration of virus tested. Two of the preparations were counterstained with lissamine rhodamine RB 200. Smears were stained directly with a drop of VEE fluorescein-labeled antibody solution for 30 minutes in a moist chamber at room temperature. Following this, the slides were rinsed and washed for 10 minutes in carbonate-bicarbonate buffer (0.5 M, pH 9.0) and mounted in glycerol adjusted to pH 9.0 with carbonate-bicarbonate buffer.[23] Labeled globulin was used undiluted and diluted 1:2 with neutral buffered saline. Specifications for the fluorescence lamp and microscope used have been described previously.[23] Slides were examined for specific staining of viral particles and fluorescent reactions graded on the basis of lowest to highest estimated intensity (1+ to 4+).

G. IMMUNOFLUORESCENCE TECHNIQUES IN STAINING OF VEE VIRUS ADSORBED ON GOOSE ERYTHROCYTES

The agglutination of erythrocytes by VEE virus suggested exploration of the possibility of using fluorescent antibody to visualize virus particles attached to the surface of goose erythrocytes and to compare direct and indirect fluorescent antibody staining methods.

Prior to use of fluorescent antibody staining, the appearance of erythrocytes agglutinated by the viral antigen was observed. The HA test was conducted as follows: A quantity of $6 \times 10^6$ goose erythrocytes was added to $35 \times 10^5$ tissue culture plaque-forming units (tcpfu) of virus in RABS. Virus-erythrocyte suspensions were shaken continuously in a flat-bottomed plastic petri dish (50 mm) for 10 minutes. The effect of virus adsorption on goose erythrocytes was examined both macroscopically and microscopically (Fig. 3). Photomicrographs were taken on Panatomic X film at an exposure of 1/125 second through a Zeiss microscope equipped with a 12X ocular and 10X objective of N.A. 0.25.

Smears were prepared from the agglutinated erythrocyte-virus mixture, allowed to air-dry, and then fixed in formalin. Specific whole rabbit anti-VEE serum was used for direct and indirect staining of the erythrocyte-virus complex. A portion of the unlabeled globulin fraction was also used. A fluorescein-conjugated goat anti-rabbit serum used for indirect staining was obtained commercially (BBL). The direct staining technique used in this study was that described previously for smears made from infectious

fluids. The indirect technique consisted of covering the smears with one or two drops of appropriate dilutions of either immune whole serum or globulin. After incubation for 30 minutes at room temperature, the smears were washed by immersion in carbonate-bicarbonate buffer, pH 9.0, for 10 minutes. Preparations were then stained with FITC-labeled goat anti-rabbit globulin for one hour at room temperature. Following staining, slides were again washed for 10 minutes and smears were mounted in buffered glycerol. Reactions were viewed by fluorescence microscopy. Photographs were taken on 35 mm Tri-X film with a Leitz fluorescent microscope equipped with OSRAM HBO-200-W fluorescent lamp and 10X photo ocular and 40X objective. Filters used were Schott BG-12 exciter filter (3 mm) in combination with barrier filter OG-1 (2 mm). Controls consisted of examing (i) unstained smears of erythrocytes treated with VEE virus; (ii) erythrocytes without virus treated with normal rabbit globulin and stained with fluorescent-labeled, goat anti-rabbit gamma globulin; and (iii) infected erythrocytes used for inhibition of fluorescence in the one-step blocking test.[24] In this test, smears were stained in the usual manner with mixtures of equal parts of labeled antibody and unlabeled antiserum. Proper dilutions of each reagent were used.

## III. RESULTS

Figure 1 shows that as the concentration of red cells decreases, the HA test becomes more sensitive, and fewer infective particles are detected. The highest level of sensitivity (least number of viral particles detected) was obtained with approximately 1,500,000 RBC. Small concentrations of virus could be detected with an equally small concentration of RBC. Larger numbers of RBC tended to mask the presence of virus (Fig. 1). A mixture containing $6 \times 10^6$ RBC and $3.5 \times 10^3$ tcpfu did not produce hemagglutination (row 2), but a RBC suspension of $1.5 \times 10^6$ resulted in complete agglutination (row 4). Experimental results tend to indicate a direct relationship between the lowest virus concentration that can be detected and the number of indicator cells used in the test. When the RBC concentration was increased from $6 \times 10^6$ to $7.5 \times 10^6$, the smallest detectable concentration of virus was 14,250 tcpfu (row 1). The results show that low concentrations of virus can be detected with small numbers of RBC. However, the use of very low concentrations of RBC was precluded by limits of visibility. When RBC were utilized in concentrations below $1.5 \times 10^6$, reactions could not be accurately assessed because button formation was not easily observed. In consequence of these findings, red cell concentrations of $1.5 \times 10^6$ to $3 \times 10^6$ were selected for routine dilution tests.

As seen in Figure 2, the quantities of the test virus used were completely inhibited as indicated by the cells' settling as sharply demarcated red discs. Agglutination was observed in tubes without serum. The third tube in the virus cell control showed an incomplete shield combined with a dark ring of cells indicating partial hemagglutination.

The comparative assays of fluorescent antibody and agglutination-inhibition procedures are shown in Table 1. The direct fluorescent antibody method failed to show any evidence of specific fluorescence with virus deposited on slides. On the other hand, virus concentrations of 28,500, 14,200, 7,100, and 3,500 were detected with the HA test.

TABLE 1. COMPARISON OF HEMAGGLUTINATION-INHIBITION AND DIRECT FLUORESCENT ANTIBODY PROCEDURE IN DETECTION AND IDENTIFICATION OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS

| Type of Holding Fluid | TCPFU[a] | HAI[b] | Fluorescent Antibody Procedure |
|---|---|---|---|
| 0.2% bovine albumin borate-saline, pH 9.0 | 28,500 | +[c] | -[c] |
| | 14,250 | + | - |
| | 7,125 | + | - |
| | 3,562 | + | - |

a. Tissue culture plaque-forming units.
b. Hemagglutination-inhibition.
c. +, Hemagglutination-inhibition (positive with rabbit anti-VEE serum)
-, Fluorescent antibody reaction negative with rabbit anti-VEE conjugate.

Figure 3 shows goose erythrocytes with and without adsorbed VEE virus. Treated cells in photograph B characteristically display aggregated of red cells forming large dark masses of agglomerated cells; untreated cells in A show absence of aggregates. Photomicrographs $A_1$ and $B_1$ demonstrate the agglutination effect more clearly.

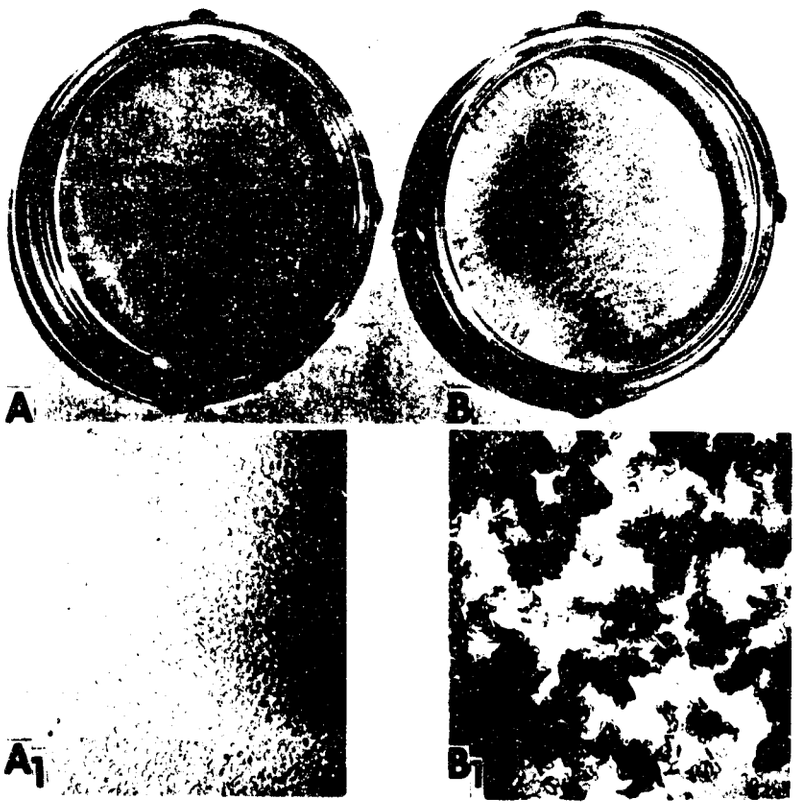


Figure 3. Photographs of Goose Erythrocytes Treated and Untreated with VEE Virus.

A. Untreated red cells observed macroscopically demonstrating absence of cell aggregates. (X 1.7)
B. Treated red cells examined macroscopically showing the presence of aggregates of cells. (X 1.7)
$A_1$ and $B_1$. Higher magnification of the agglutination effect. (X 110)

A comparison of the direct and indirect fluorescent antibody staining method for visualization of VEE virus adsorbed on goose erythrocytes can be seen in Figure 4. Photograph A shows positive fluorescence at the periphery of red cells. Brilliant staining of viral aggregates was obtained by indirect staining (photographs B and C). The fluorescent reagent, applied after covering the smears with whole serum and serum fraction, stained virus-agglutinated cells with equal intensity. Areas of autofluorescence are seen in photograph D but the specific reactions appeared brighter, with color definitely yellow-green and more distinct.

## IV. DISCUSSION

The results presented in Figures 1 and 2 show that lower concentrations of VEE virus can be detected by reducing the number of goose erythrocytes. The HA test can detect the presence of VEE virus in 75 minutes. The settling patterns of cells served as the index of detection. Positive detection was evident by a thin shield of cells covering the entire lower surface of the tube. In no detection, the cells settled as dark, red central buttons.

An attempt to demonstrate the presence of naked virus in smear preparations by direct immunofluorescence failed with all virus concentrations tested (28,500 to 3,562 tcpfu). This result could be expected, since the virus is not sufficiently large for visualization under the ordinary light microscope. The average particle size of arboviruses in group A is approximately 50 mμ.[28-32] To visualize particles of this size it is necessary to use the electron microscope.

The amount of fluorescent antibody reacting with an individual virus particle is undoubtedly too low to visualize in the fluorescent microscope. In experiments with radioiodinated sera, Boursnell, Coombs and Rizk[33] showed that measurement of antibody was difficult when the amount of antibody combined with antigen was small. In addition, viral particles in smear preparations were scattered and not confined to a localized area. In contrast, infected tissue cells contain large amounts of antigen in discrete foci as a result of virus multiplication.[34] Therefore, these foci can be readily observed when stained with specific fluorescein-labeled antibody.

In theory, fluorescent antibody seems to be a sensitive technique for detecting virus. However, some investigators have reported a lack of correlation between fluorescent antibody reactions in tissue culture and other standard tests. It is known from the work of Hatch, Kalter, and Ajello[34] that a positive neutralization test was not always correlated with a positive FA reaction. They compared neutralization and fluorescent

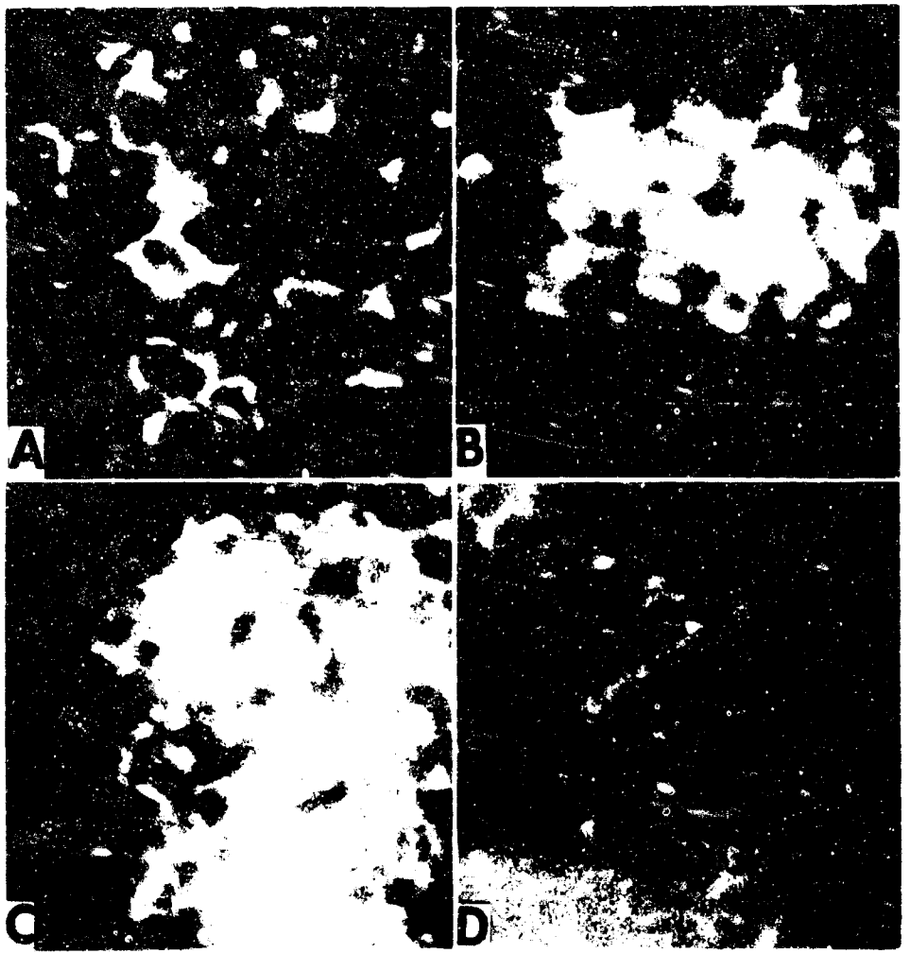


Figure 4. Photographs of Goose Erythrocytes ($6 \times 10^6$) - VEE Virus ($35 \times 10^5$ TCPFU) Complexes Stained by the Direct and Indirect Methods of Immunofluorescence.

A. Direct staining with fluorescein-tagged whole anti-VEE rabbit serum.
B. Virus agglutinated cells treated with whole anti-VEE rabbit serum and counterstained with fluorescein-labeled goat anti-rabbit globulin (indirect method).
C. Virus agglutinated cells treated with rabbit anti-VEE globulin fraction and counterstained with fluorescein-labeled goat anti-rabbit globulin (indirect method).
D. Erythrocytes (without virus) treated with normal rabbit globulin and counterstained with fluorescein-labeled goat anti-rabbit globulin.

antibody reactions for identification of poliovirus in tissue cells. Unsuccessful attempts to detect rubella virus in monkey kidney cells with direct[85] and indirect[86] staining have been reported. These failures to demonstrate virus may have resulted from a low rate of virus multiplication.

The employment of goose erythrocytes provides a convenient method for concentrating and visualizing VEE virus aggregates by fluorescent microscopy. A similar procedure has been used by Ewy and Lui[87] and involved fluorescent staining of influenza virus adsorbed on chick red blood cells. In the current study, approximately $35 \times 10^5$ tcpfu of VEE virus and $6 \times 10^6$ goose red blood cells were employed. This was the limit of virus quantity examined. The indirect fluorescent antibody method appeared to produce better visualization of adsorbed virus. Reactions were less discernible with the direct technique.

Data presented in this report show the possibility of detecting certain arboviruses belonging to group A with antisera containing specific antibodies. In addition, the results tend to indicate that the HA test is more sensitive than fluorescent antibody in the detection of VEE virus. This may possibly be explained on the basis that small scattered virus particles (50 mμ or less) free of tissue cells are beyond visualization in the fluorescent microscope. The use of goose erythrocytes for concentrating virus may be a valuable adjunct to fluorescent antibody staining procedures.

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13 ABSTRACT

Hemagglutination and fluorescent antibody are compared for the direct detection of virus devoid of host cells. The minimum number of tissue plaque-forming units of Venezuelan equine encephalomyelitis virus that could be detected by the hemagglutination technique was determined. Similar concentrations of the virus in bovine albumin borate saline, brain-heart infusion broth, and demineralized water were tested by the fluorescent antibody technique. Somewhat higher concentrations of the virus in bovine albumin borate saline were used in the hemagglutination-inhibition test. The quantitative hemagglutination procedure employed for these studies was carried out at 5° C for 75 minutes with variations in concentration of goose red cells. As a result of lowering the red cell concentration, smaller concentrations of virus were detected. The direct fluorescent antibody staining procedure applied to slide preparations containing known numbers of tissue culture plaque-forming units of virus was negative. Adsorbed viral antigen on agglutinated goose erythrocytes was visualized by direct and indirect FA techniques.

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