

## FLUORESCENT ANTIBODY STUDIES ON EPHEMERAL FEVER VIRUS

A. THEODORIDIS, Veterinary Research Institute, Onderstepoort

### ABSTRACT

THEODORIDIS, A. Fluorescent antibody studies on ephemeral fever virus. *Onderstepoort J. vet. Res.*, 36 (2), 187-190, 1969.

The preparation and use of a highly specific fluorescein-conjugated antiserum against bovine ephemeral fever virus are described. The demonstration of fluorescent cytoplasmic inclusions is a dependable diagnostic test. The test also revealed cross reactions between the viruses of Japanese bovine epizootic fever and Australian ephemeral fever and that of South African ephemeral fever.

### INTRODUCTION

Bovine ephemeral fever virus was first isolated by Van der Westhuizen (1967) from natural and experimentally produced cases of the disease by intracerebral inoculation of suckling mice with leucocyte suspensions. He also demonstrated growth of this virus in the BHK-21 (clone 13) line of baby hamster kidneys cells of Macpherson & Stoker (1962). However, in spite of the successful cultivation of ephemeral fever virus in suckling mice and in BHK-21 cells, the primary isolation of the virus in these host systems is still difficult and cumbersome.

Various serological tests were therefore investigated as potential aids in the diagnosis of the disease and in further studies of the virus. A modification of the immunofluorescent technique developed by Coons (1958) proved to be most suitable for these purposes. It is the object of this paper to describe the details of the modified technique (B. J. Erasmus, Veterinary Research Institute, Onderstepoort, unpublished results) and some of the results obtained in the study of the ephemeral fever virus.

### MATERIALS AND METHODS

#### (a) Virus strain

The prototype E.F.I. strain (Van der Westhuizen, 1967) at the fourth mouse brain passage level was employed. Antigen for the immunization of mice was prepared by the intracerebral inoculation of suckling mice. Brains from moribund mice were harvested and a 10 per cent suspension was made in buffered lactose peptone\* and stored in a dry-ice cabinet. The infectivity titre of this antigen was  $10^{7.5}$ , measured as LD<sub>50</sub> units/ml in suckling mice.

#### (b) Mouse ascitic fluid

Immune globulin was prepared by the intraperitoneal inoculation of adult Swiss albino mice with infective mouse brain suspensions, according to the schedule described by Sommerville (1967). The ascitic fluid from several mice was collected by draining the distended abdomens with a large-bore sterile needle. The fluid collected from the first two harvests was allowed to clot overnight at 4°C and was then centrifuged at 2,000 rpm for 10 minutes to remove fibrins. The supernatant fluid was then removed aseptically and the yields from the two harvests were

pooled and stored at -20°C. A control ascitic fluid was similarly prepared using normal mouse brain as antigen.

#### (c) Extraction of gamma globulin

A 0.4 per cent solution of rivanol (2-ethoxy-6,9-diamino-acridine-lactate) was prepared in distilled water and 60 ml of this solution was added to 20 ml of ascitic fluid. This mixture was left at 4°C for 3 hours and then centrifuged at 1,000 rpm for 10 minutes. The supernatant fluid containing the partially purified gamma globulins was collected and after heating to 37°C, was mixed with an equal volume of 2.25 M Na<sub>2</sub>SO<sub>4</sub> previously warmed to 37°C. The mixture was kept at 37°C for 10 minutes and was then centrifuged at 3,000 rpm for 10 minutes. The precipitate consisted mainly of gamma globulin. This was resuspended in 0.01 M phosphate buffer solution (pH 7.5) to the original volume (of ascitic fluid) and was passed through a column of G.25 Sephadex, previously equilibrated with the same buffer.

Aliquots of 6 ml each were collected and their protein concentrations were determined by ultraviolet absorption at a wavelength of 280 μ.

#### (d) Conjugation of gamma globulin

The aliquots containing the highest protein concentration (usually Aliquots 4 to 12) were pooled, the protein concentration was adjusted to 1 per cent and the solution placed in a beaker immersed in ice. Crystalline fluorescein isothiocyanate\* was dissolved in 0.1 M Na<sub>2</sub>HPO<sub>4</sub> at a concentration of 200 μg/ml and gradually added to an equal volume of globulin with constant stirring on a magnetic stirrer. Conjugation was carried out for 2 hours at 4°C, the pH being maintained between 9.0 and 9.5 by the addition of 0.1 M Na<sub>3</sub>PO<sub>4</sub> when required. After this initial conjugation the fluorescein isothiocyanate-globulin mixture was left overnight at 4°C and then dialyzed against 0.01 M phosphate buffer (pH 7.5) for 2½ hours with regular changes of buffer.

Optimally-conjugated globulin molecules were separated from the mixture by a series of elutions from diethyl-amino-ethyl cellulose\*\* using the batch absorption method. The globulin solution was divided in two and each portion was added to about 12.5 g diethyl-amino-ethyl cellulose, which had been acti-

Received for publication on 21 August 1969. — Editor

(\*) M/50 phosphate buffer, pH 7.2 to 7.4, 1 per cent Difco peptone and 5 per cent lactose

(\*) Baltimore Biological Laboratories, U.S.A.

(\*\*) Cellex D- Bio-Rad Laboratories, California, U.S.A.

vated by the method of Levy & Sober (1960) and equilibrated with a 0.01 M phosphate buffer, pH 7.5. The globulin and diethyl-amino-ethyl cellulose was mixed thoroughly and left at room temperature for a period of 15 minutes with occasional shaking. The mixture was then centrifuged at 3,000 rpm for 10 minutes and the supernatant fluid was collected (Fraction A). Stepwise elution was carried out by repeating the above procedure with 50 ml of the following buffers in succession:—

- i) 0.01 M  $\text{PO}_4$ , pH 7.5 + 0.15 M NaCl (Fraction B)
- ii) 0.01 M  $\text{PO}_4$ , pH 7.0 + 0.5 M NaCl (Fraction C)
- iii) 0.03 M  $\text{PO}_4$ , pH 6.3 + 1.0 M NaCl (Fraction D)
- iv) 0.03 M  $\text{PO}_4$ , pH 6.3 + 2.0 M NaCl (Fraction E)

Fractions B, C, D, and E were dialyzed against 0.01 M phosphate buffer (pH 7.5) for 30 minutes. Each fraction was then concentrated to about a fifth of the original volume by dialyzing against polyethylene glycol (M.W. 6,000). Preliminary staining trials were carried out with each fraction to determine its specificity, the brightness of fluorescence and the presence or absence of background and non-specific staining. Fractions B and C gave optimal results and were subsequently used as conjugate for staining without prior absorption with tissue powders.

#### (e) Fluorescent antibody staining

(i) *Tissue Culture*: Fresh cultures of BHK-21 cells were infected with ephemeral fever virus and the cells harvested by low speed centrifugation after about 40 hours, when cytopathic effects were well advanced. Non-infected control cell cultures were similarly treated. The sedimented cells were washed once with phosphate buffered saline (PBS). Very thin smears were made on glass slides from the infected and the control cells and after drying in air the cells were fixed in chilled acetone ( $-20^\circ\text{C}$ ) for 10 minutes. The slides were placed in large petri dishes containing wet blotting paper. The smears were flooded with undiluted conjugate and left at room temperature for 1 hour before being washed twice in PBS and allowed to dry. Before examination under the ultraviolet microscope, coverslips were mounted on the slides using mounting fluid.

(ii) *Leucocytes*: Blood was collected in one per cent citrate from naturally infected bovines showing a febrile reaction. Leucocytes were separated by centrifugation and smears were made from the white cells after washing them with PBS. Control leucocyte smears were similarly prepared from uninfected susceptible bovines stabled under insect-free conditions. The procedure employed for staining the leucocyte smears was identical with that described for the staining of tissue culture cells. The smears were examined under a standard Zeiss ultraviolet microscope fitted with a darkfield condenser and a 0/44 barrier filter combination. An Osram HBO-200 light source was used in conjunction with a BG 12 exciter filter.

## RESULTS

Highly specific fluorescence was observed in the cytoplasm of virus infected BHK-21 cells as well as in the leucocytes from infected bovines, but never in uninfected control cells. The fluorescence tended to be rather diffuse in the BHK-21 cells compared to a more granular appearance in the leucocytes. In some cells fluorescence was limited to the outer layers of the cytoplasm [Plate 1 (1 to 4)].

The staining of BHK-21 cells at different intervals following infection revealed a diffuse cytoplasmic fluorescence as early as 24 hours after infection and a more granular appearance at about 40 hours. At later stages fluorescence was located peripherally, often appearing as an irregular band along the outer contour of the cell. The intensity of fluorescence was such that it could be detected even if only a small percentage of cells was infected at any time.

The results obtained with leucocyte smears from naturally infected bovines correlated extremely well with the subsequent demonstration of a rise in the neutralizing antibody titre in the convalescent phase serum samples. No false positive results were obtained, whereas very few cases, which proved positive serologically, gave negative results with the fluorescent antibody test. The failure to make a diagnosis by means of the fluorescent antibody test in most of the latter instances, can be explained either by the fact that the blood samples were collected late during the febrile reactions or after it.

## DISCUSSION

The development of the fluorescent-antibody test as a diagnostic aid in ephemeral fever is of considerable importance. Although success has been achieved with virus isolation, it still remains a tedious procedure which is successful only in a small percentage of cases. However, a definite diagnosis of ephemeral fever is often essential, because other viruses are known to produce similar clinical syndromes (K. E. Weiss, Veterinary Research Institute, Onderstepoort, unpublished observations). The fluorescent-antibody technique is fairly sensitive and very accurate and could be executed under field conditions. Furthermore, leucocyte smears prepared from citrated blood samples collected during the acute febrile stage from suspected cases in the field could be fixed and submitted to a laboratory for diagnosis. It is also anticipated that this technique will prove useful in the elucidation of the pathogenesis of ephemeral fever and the demonstration of the virus in insect vectors.

## ADDENDUM

Subsequent experiments with the fluorescent antibody technique indicated cross reactions between the E.F.I. strain of ephemeral fever virus and the Australian ephemeral fever virus isolate,\* as well as with the virus of Japanese bovine epizootic fever\*\* [Plate 1 (5 and 4)]. Similar serological cross-reactions were demonstrated in neutralization tests conducted in BHK<sub>21</sub> cell cultures.

\*) Strain 7721 kindly supplied by Dr. R. L. Doherty, Queensland Institute of Medical Research, Herston 4006, Brisbane

(\*\*) Virus kindly supplied by Dr J. Fujita, National Institute of Animal Health, Kodaira, Tokyo

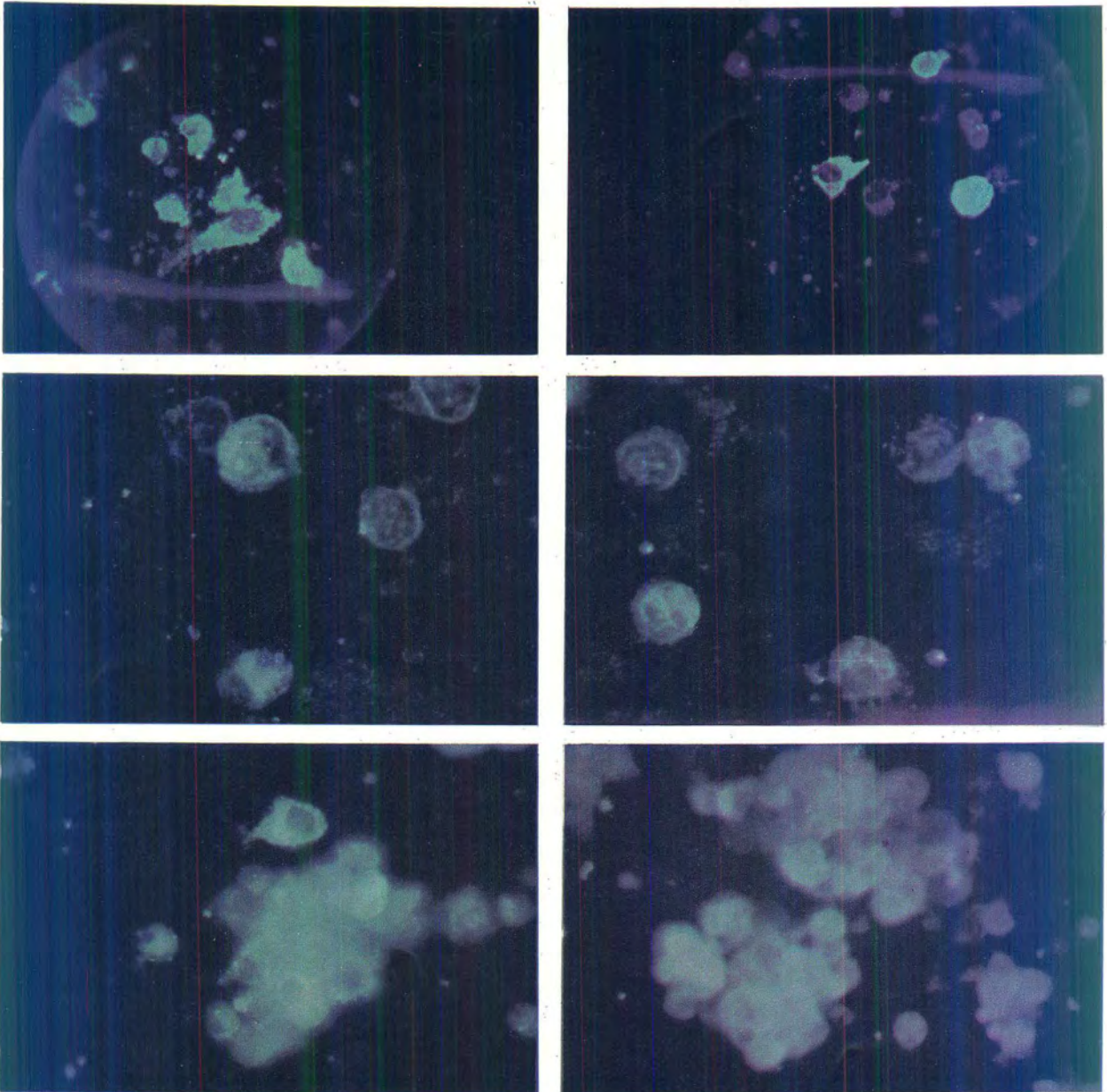


PLATE 1.—Bovine ephemeral fever-fluorescent antibody microscopy

- (1 and 2) BHK-21 cells infected with E.F.1 virus and stained with the fluorescent antibody conjugate ( $\times 1,000$ )  
 (3 and 4) Leucocyte smears from infected cattle stained with the fluorescent antibody conjugate ( $\times 1,000$ )

- (5) BHK cells infected with Japanese bovine epizootic fever virus and stained with the fluorescent antibody conjugate ( $\times 400$ )

- (6) BHK cells infected with the Australian 7721 isolate of ephemeral fever virus and stained with the fluorescent antibody conjugate ( $\times 400$ )

ONDERSTEPSOORT JOURNAL OF  
VETERINARY RESEARCH

Vol. 36, No. 2, 1969

---

*Page 189:* Colour Plate 1. The sections should be numbered as follows:

<b>1</b>	<b>2</b>
<b>3</b>	<b>4</b>
<b>5</b>	<b>6</b>

*Page 293:* The statistical formula on top of first column should read:

$$\sum_{i=0}^y \binom{n_t}{i} (1-p')^i (p')^{n_t-i}$$

SUMMARY

The application of a fluorescent-antibody technique in the study of bovine ephemeral fever virus infection is described. A highly specific conjugated antiserum was used to demonstrate that the site of viral synthesis is in the cytoplasm of infected cells. The technique is useful as a simple but dependable and fairly sensitive diagnostic test. Cross reactions between ephemeral fever virus and the viruses of both Japanese bovine epizootic fever and Australian ephemeral fever were found.

ACKNOWLEDGEMENTS

The author is grateful to Professor K. E. Weiss and Dr. B. J. Erasmus for advice and criticism. Sincere thanks are expressed to Mr. J. M. Botha and Mr. S. T. Boshoff for their invaluable assistance.

REFERENCES

- COONS, A. H., 1958. Fluorescent-antibody methods. *Gen. Cytochem. Meth.*, 1, 399-422.
- LEVY, H. B. & SOBER, H. A., 1960. A simple chromatographic method for preparation of gamma globulin. *Proc. Soc. exp. Biol. Med.*, 103, 250-252.
- MACPHERSON, I. A. & STOKER, M. G. P., 1962. Polyoma transformation of hamster cell clones — an investigation of genetic factors affecting cell competence. *Virology*, 16, 147-151.
- SOMMERVILLE, R. G., 1967. The production of fluorescent antibody reagents for virus diagnosis in the albino mouse. I. Hyperimmune antispecies serum. *Arch. ges. Virusforsch.*, 20, 445-451.
- VAN DER WESTHUIZEN, B., 1967. Studies on bovine ephemeral fever. I. Isolation and preliminary characterization of a virus from natural and experimentally produced cases of bovine ephemeral fever. *Onderstepoort J. vet. Res.*, 34, 29-40.