The Fluorescent Antibody Technique in the Diagnosis of Equine Rhinopneumonitis Virus Abortion

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SUMMARY

Using two known positive equine viral rhinopneumonitis (EVR) sera, conjugates were prepared with fluorescein isothiocyanate and tested for specificity using EVR infected tissue culture cells. The conjugate was then applied to selected tissues from 32 aborted fetuses and foals submitted during a natural outbreak of EVR. Antigen was detected in various tissues by immunofluorescence in 20 cases (62.5%). In 24 cases bovine fetal kidney cell monolayers were inoculated with a pool of lung and liver and EVR virus was isolated from 15 (62.5%). Histological examination of various tissues from 29 cases resulted in the diagnosis of EVR in 19 (65.5%), based upon the presence of focal areas of necrosis and intranuclear inclusion bodies. Correlation of results was not obtained in two cases. One was diagnosed positive histologically and negative on fluorescence, the other was negative histologically and by virus isolation but showed fluorescence. The distribution of fluorescence in various infected fetal tissues indicated that the combined examination of lung and thymus gland was most likely to provide a positive diagnosis.

RÉSUMÉ

Les auteurs ont conjugé deux sérums contenant des anticorps contre le virus de la rhinopneumonite équine (RPE) à de la fluorescéine, et ils ont éprouvé la spécificité de ce produit. Ils l'ont ensuite utilisé sur des tissus sélectionnés de 32 foetus avortés et poulains prématurés, morts en-dedans de quelques jours, et apportés au laboratoire lors d'une épizootie de RPE. Le virus de la RPE fut isolé de rein foetal bovin, inoculé en cultures monocellulaires à partir de divers tissus (poumons et foie), chez 15 animaux sur 24 (62.5%). A l'examen histopathologique de différents tissus, basé sur la présence de foyers nécrotiques et d'inclusions intranucléaires, on a pu diagnostiquer 19 cas de RPE sur un total de 29 (65.5%). Dans 20 cas sur 32 (62.5%), les auteurs ont décelé la présence d'antigène viral par l'immunofluorescence. Dans deux cas, on n'a pu obtenir de corrélation complète des résultats. Dans le premier cas, bien que l'examen histopathologique des tissus eût révélé des lésions pathognomoniques de la RPE, les anticorps fluorescents n'ont pas pu déceler le virus. Dans le second cas, on n'a pu poser un diagnostic de RPE que par l'immunofluorescence. Lorsqu'examinés ensemble, le poumon et le thymus se sont avérés les tissus de choix pour confirmer la RPE par la méthode des anticorps fluorescents.

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INTRODUCTION

Although abortion storms caused by equine viral rhinopneumonitis (EVR) were known in horses in the United States as early as 1936 (3), the agent was not isolated in Canada until 1963 (2,6). Diagnosis has depended largely on the clinical history supported by detection of typical macroscopic abnormalities and histological lesions in tissues of aborted fetuses and by retrospective demonstration of complement-fixing (CF) or virus-neutralizing (VN) antibodies in sera of aborting mares. Both pathological and serological examinations

have been used to support diagnosis by virus isolation from fetal tissues (6). CF antibodies are relatively transient after abortion (4) while VN antibodies persist for many months. Both the VN test and virus isolation require several days to complete. Since the tissues of EVR-infected fetuses contain virus and have been used as a source of antigen for CF tests (4), the fluorescent antibody technique (FAT) appeared to offer a means of rapid diagnosis. This paper records a comparison of FAT with histological examination and virus isolation from specimens submitted during an outbreak of EVR abortion.

MATERIALS AND METHODS

Animals

Abortions began in January 1969 in a herd maintained for the commercial production of estrogen from pregnant mares' urine. No clinical illness was reported among the mares during the course of the outbreak. A total of 32 aborted fetuses or premature foals that died within a few days of birth, were submitted for pathological, virological and immunofluorescent examinations. Each examination was conducted independently. As pasture breeding was practiced, the duration of gestation was not known and the relevant data in Table I are based either on measurements of crown-rump length or on rectal palpation findings conducted during the first four months of pregnancy.

Tissues

For routine immunofluorescent study, tissues were stored on receipt at 4°C overnight and then mounted and sectioned at 4 u in a cryostat at -20°C in a standard manner (10). After fixation with acetone, the sections were stained with conjugates for one hour by a method already described (10). A second sample was stored at -20°C for later virological examination, which was usually carried out within the following two weeks. A third sample was processed for histological examination (2). The tissues used for the three methods of examination were collected from the same general area of each specimen.

Stock Virus and Tissue Cultures

The EVR virus used in this laboratory, which is serologically indistinguishable from the prototype equine herpesvirus 1 (6), was stored at -40°C as the 40th tissue culture passage. This material, suspended in basal Eagle's medium (BME) with Earle's salt solution (ESS) was used to infect primary monolayers of bovine fetal kidney (BFK) cells. These cells were grown in a medium composed of BME, Hanks' salt solution and 10% fetal calf serum together with 200 iu penicillin and 100 ug streptomycin per ml. When monolayers were confluent, the cells were maintained in BME with ESS and antibiotics. Monolayers grown on 18 × 75 mm coverslips in Leighton tubes supplied infected and uninfected cells for investigations of the efficacy of the globulin conjugates from the sera of various horses. Coverslips were fixed and stained by a method already described (1).

Immunofluorescent Methods

Two sera, containing CF and VN antibody to EVR, from naturally infected mares which had aborted, were used. These sera were stored at -20°C. One serum (H-RS) had been used routinely for several years as a standard EVR antiserum in this laboratory. The other serum (H-52) was taken from a mare during the outbreak under study. A third serum (H-2) without CF or VN activity against EVR, was obtained from a horse kept in isolation and used as a negative control.

Precipitated globulins from each serum were conjugated with fluorescein isothiocyanate (10). Each conjugate was absorbed for one hour with buffer-moistened liver or spleen powder prepared from a clinically healthy horse. Conjugates absorbed with 100 mg of wet powder per ml were stored at 4°C in lyophilized form. The conjugates, when reconstituted with a phosphate buffer (10), were held at 4°C and used within one week.

Virus Isolation and Serum Neutralization Tests

The isolation technique followed standard methods. Portions of fetal tissues stored at -20°C were allowed to thaw at room temperature and a pool of lung and liver was homogenized in BME with ESS to a final concentration of approximately 10%. After

TABLE 1. Comparison of Immunofluorescent, Histological and Virological Examinations in 32 Foals During Rhinopneumonitis Outbreak

Foal No.	Fetal Age (days)	Virus Isolation	Histological (H) and Immunofluorescent (I)[a] Findings											
			Lung		Liver		Spleen		Thymus		Adrenal		Placenta	
			H	I	H	I	H	I	H	I	H	I	H	I
1	220	+		3		2		1		3				
2	275		+	3	+	1	+		–	–				
3	240		–	–	+	–	–		–	–				
4	250		–	–	–	–	–	–	–	–		–		
5	270		+	3	+	2	+	1	+	3				
6	265	+	+	3	+	3	+	3	+	3				
7	300	+	+	3	+	1	+	1		1				
8	290	+	+	3	+	1	+	2	+	–				
9	320	–	–	–	–	–	–	–	–	–		–		
10[b]	295	–	–	–	–	–	–	–	–	–				
11[b]	295			–		–		–		–				
12	250		–	–	–	–	–	–	–	–	–		–	
13	290		+	3	+	3	+	2	+	3				
14	270		+	3	+	1	+	3	+	3		–		
15	260	+	+	3	+	1	+	3	+	3	+	2		1
16	275	+	+	3	+	–	+	–	+	–	–	–		–
17	305	+	+	3	+	2	+	3	+	3	+	1		–
18	270	+	–	1	+	3	+	3	+	3	–	3		–
19	310[c]	–		–		–		–		–		–		–
20	300[c]	–	–	–	–	–	–	–	–	–	–	–		
21	300	–	–	–	–	–	–	–	–	–	–	–		
22	320	–	–	–	–	–	–	–		–	–	–		
23	290	+	+	2	+	–	+	–	+	3	+	–		
24	265	–	–	–	–	–	–	–	–	–	–	–		
25	300	+	+	3	+	2	+	3	+	3	–		–	–
26	310	+	+	–	+	1	+	1	+	3	+	2		
27	32C[c]	+	–	1	+	1	+	2	+	3				
28	315[c]	+	–	1	+	1	+	2	+	3				
29	31C[c]	–	–	–	–	–	–	–	–	–	–	–		
30	315[c]	–	–	1	–	–	–	–	–	1	–			
31	315[c]	+	–	1	+	–	+	–	+	3	–	–	–	–
32	300[c]	+	–	1	+	–	+	–	+	3	–	1	–	–

[a]1: Fluorescence in one focus or scattered cells per 10 microscopic fields
2: Fluorescence in one focus or scattered cells per 1 microscopic field
[b]3: Fluorescence in many foci and scattered cells per 1 microscopic field
Twins
[c]Foal born alive

a clarifying centrifugation at 500 × G for 15 minutes the supernates were further diluted to provide an approximate 1:100 final dilution, to which 200 iu penicillin, 200 ug streptomycin and 50 iu mycostatin per ml were added.

Inoculated BFK monolayers in ten 16 × 150 mm disposable glass tubes were observed for four to six days for evidence of cytopathic effects (CPE) as determined by comparison with uninoculated control tubes.

For VN tests, the sera H-RS and H-2 were inactivated at 56°C for 30 minutes. They were then diluted in BME with ESS in two-fold steps beginning with a 1:10 dilution. One ml of each serum dilution and 1 ml of virus dilution containing approximately 100 $TCID_{50}$ of the test and standard EVR viruses were held at 25°C for two hours. One ml of this mixture was added to each of two tubes containing monolayers of BFK cells from which the original growth medium was drained. These cul-

tures were incubated at 37°C on roller drums and inspected from time to time for CPE up to the sixth day after inoculation. The interpretation of results was based on the presence or absence of cytopathogenicity, complete neutralization of virus in both tubes designating the titration end point.

RESULTS

Virus Isolation

An agent causing CPE in BFK cells was isolated from the pooled lung and liver tissues of 15 of the 24 specimens examined by this technique. Four isolates were tested at random using a known positive EVR serum to confirm their identity.

Histolocical Examination

Of the 32 animals submitted (Table I), tissues from 29 were examined histologically. Nineteen showed lesions typical of EVR as previously described (2). Three were diagnosed as being caused by bacterial infection and no diagnosis could be made in seven. No attempt was made to determine the extent and severity of the lesions in each specimen. Virus isolations were obtained in 15 of the 16 histologically-positive cases examined.

Immunofluorescent Studies

Tissue culture — Preliminary investigations showed that viral antigen was detectable in BFK cells infected with the stock EVR virus strain after staining with dilution of up to 1:32 of conjugates H-RS and H-52. Both sera used for conjugation had a titre of 1:200 when checked for VN antibodies. Despite the fact that H-52 serum had a much higher CF antibody titre (1:160) than H-RS (1:40), observations failed to establish any difference in the titre of the two immunofluorescent conjugates. A 1:6 dilution of the H-2 (normal) conjugate did not produce staining of viral antigen in EVR infected BFK cells. No fluorescence was elicited by these three conjugates in uninfected BFK monolayers examined at the same time as infected monolayers. Twenty hours after inoculation of BFK cells with 1000 $TCID_{50}$ of virus, staining with H-RS and H-52 immunoconjugates showed the viral antigen as relatively large granular masses of brightly fluorescing material located mainly in the cytoplasm but appearing also at times in the nucleus of affected cells (Fig. 1). Affected cells occurred in foci scattered throughout the otherwise apparently normal sheet. This pattern of staining was evident about 15 hours before obvious CPE was detectable by routine microscopy.

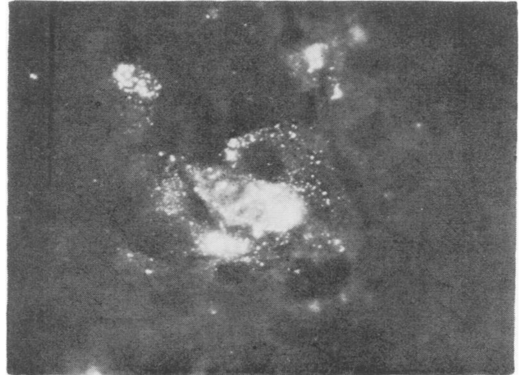

Fig. 1. BFK tissue culture infected with ERP virus showing location of viral antigen by FA technique.

Fetal tissues — The results are detailed in Table I. Of the 32 specimens examined, tissues from 20 animals showed fluorescence with H-RS (immune) conjugate but not with H-2 (normal) conjugate. In only one case (No. 30) was a diagnosis of EVR obtained by immunofluorescence which was not supported by either histological findings or virus isolation. As typical focal fluorescence was observed in two tissues (lung and thymus), it is felt that virus was in fact present in this specimen. In one other case (No. 3) no fluorescence was observed although lesions were detected histologically in the liver.

The distribution of EVR lesions in the foal infected *in utero* has been studied in detail (4, 2). Areas of focal necrosis bounded by cells that sometimes contain intranuclear inclusion bodies occur in EVR in various fetal tissues. In the 20 FAT-positive cases, portions of the lung, liver, spleen and thymus were examined except in No. 2, from which spleen was not available. In 12 of these, all tissues showed some reaction but, in general, the antigen detected by FAT was most abundant in the

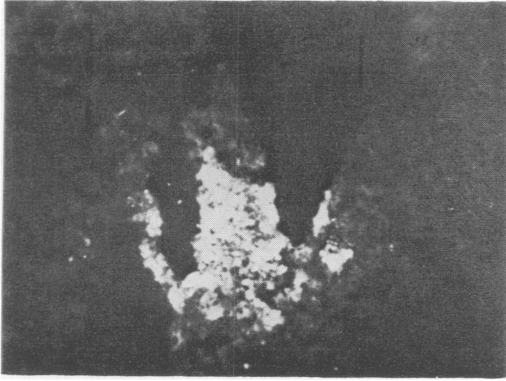

Fig. 2. Focal distribution of ERP viral antigen in liver of aborted foal as shown by immunofluorescence.

lung and thymus. The viral antigen in each organ examined was focally distributed (Fig. 2). There was only one case (No. 26) in which the lung specimen was negative when positive reactions were observed in other tissues, and six cases (No. 18, 23, 27, 28, 31 and 32) where the amount of viral antigen in the lung was less than in at least one of the other tissues examined. It therefore appears that routine diagnosis by FAT should succeed in detecting an infected fetus if only lung and thymus are examined. In most lungs, the antigen was distributed throughout the tissue and occurred in macrophage-like cells as well as in the epithelial cells of the terminal air passages. In the spleen, in addition to foci of infected cells in the follicles, individual positively-staining cells were often scattered throughout the parenchyma. The liver and adrenal glands of infected animals usually showed the least amount of viral antigen and in only one case (No. 18) was antigen as abundant in the adrenal gland as in the other tissues.

Only seven placentae were obtained. In sections from one specimen (No. 15) a single focus of fluorescing epithelial cells, typical of a positive reaction, was seen.

Persistence of detectable viral antigen in infected tissues — Tissues from two known infected fetuses (No. 1 and 13) were stored at 4°C and –20°C and sections from these specimens were examined from time to time. The results indicate that tissues may be stored for at least 28 days at 4°C before the amount of viral antigen detectable by the immunoconjugate diminishes noticeably. Likewise, in tissues maintained at –20°C, good FAT reactions were obtained with positive conjugates after three months storage but these reactions were not evident after six months. In addition sections from freshly harvested tissues stored in acetone at –20°C retained their staining intensity for at least three months. Storage of tissues in these various ways did not produce any non-specific fluorescence as determined by simultaneous staining of the preparations with normal (H-2) conjugate.

DISCUSSION

There was excellent correlation between the three methods used for the detection of EVR in aborted foals. When each foal is considered individually, diagnosis of the infection by FAT was apparently as reliable as either of the other techniques. In one case, no histological changes were observed. While the FA method seems likely to be useful in facilitating diagnosis of equine abortions caused by EVR virus, its usefulness in diagnosing the respiratory syndrome by this agent remains to be investigated.

Since only sera from naturally infected animals were available for conjugation, the precise specificity of the immunoconjugates used in this study cannot be stated. However, the close correlation between the results of the three methods studied is strong indirect proof that this requirement was met. Furthermore, in a limited study, neither immunoconjugate produced cross-fluorescence in monolayers infected with the equine coital exanthema virus (7). However, the relationship between equine coital exanthema and equine herpesvirus 2 which have been reported, (8, 9), is not known. In tissue cultures infected with the latter virus there were no cross reactions between immunoconjugates prepared in rabbits for strains of herpesvirus 1 and 2 (8). On the other hand, it is also known that strains of herpesvirus 1 associated with acute respiratory disease in horses are serologically closely similar to, but not identical with, the classic prototype (5). The abortifacient potential of these various, non-classical strains is apparently not known.

Of the six foals born alive (Nos. 19, 20, 27-30; Table I) only three were apparently infected with EVR. Viral antigen demonstrable by FAT was scanty in the lungs of these animals, although in two (Nos. 27,

28) it was plentiful in the spleen and thymus. This phenomenon might be associaaed with activation of the pulmonary clearance mechanisms at the onset of ventilation. Although no quantitative evaluation of virus or viral antigen was attempted, it appeared that larger amounts of virus were present in the lung and thymus than in the spleen and liver of younger fetuses. Final conclusion regarding the precise distribution of virus and the selection of particular tissues for FAT examination require more detailed experimental study.

REFERENCES

1. **BOULANGER, P., G. L. BANNISTER, A. S. GREIG, D. P. GRAY, G. M. RUCKERBAUER and N. G. WILLIS.** African Swine Fever IV. Demonstration of the viral antigen by means of immunofluorescence. Can. J. comp. Med. **31**: 16-23. 1967.
2. **CORNER, A. H., D. MITCHELL and E. B. MEADS.** Equine virus abortion in Canada. I. Pathological studies on aborted foetuses. Cornell Vet. **53**: 78-88. 1963.
3. **DIMOCK, W. W. and P. R. EDWARDS.** The differential diagnosis of equine abortion, with special reference to a hitherto undescribed form of epizootic abortion of mares. Cornell Vet. **26**: 231-240. 1936.
4. **DOLL, E. R., W. H. McCOLLUM, M. E. WALLACE, J. T. BRYANS and M. G. RICHARDS.** Complement-fixation reactions in equine virus abortion. Am. J. vet. Res. **14**: 40-45. 1953.
5. **DUXBURY, A. E. and D. T. OXER.** Isolation of equine rhinopneumonitis virus from an epidemic of acute respiratory disease in horses. Aust. vet. J. **44**: 58-63. 1968.
6. **GIRARD, A., A. S. GREIG and D. MITCHELL.** Equine virus abortion in Canada II. Isolation of viruses and detection of antibodies in tissue culture. Cornell Vet. **53**: 88-98. 1963.
7. **GIRARD, A., A. S. GREIG and D. MITCHELL.** A virus associated with a vulvitis and balanitis in the horse — a preliminary report. Can. J. comp. Med. **32**: 603-604. 1968.
8. **KEMENY, L. and J. E. PEARSON.** Isolation of herpes-like virus from equine leukocytes: comparison with equine rhinopneumonitis virus. Can. J. comp. Med. **34**: 59-65. 1970.
9. **PLUMMER, G. and A. P. WATERSON.** Equine herpesviruses. Virology **19**: 412-416. 1963.
10. **RUCKERBAUER, G. M., D. P. GRAY, A. GIRARD, G. L. BANNISTER and P. BOULANGER.** Studies on bluetongue V. Detection of the virus in infected materials by immunofluorescence. Can. J. comp. Med. **31**: 175-181. 1967.