African Swine Fever II. Detection of the Virus In Swine Tissues by Means of the Modified Direct Complement-Fixation Test

by P. Boulanger, G. L. Bannister, D. P. Gray, G. M. Ruckerbauer and N. G. Willis*

SUMMARY

The modified direct complement-fixation test, supplemented with unheated normal calf serum, was used to demonstrate antibodies in sera of swine immunized to African swine fever virus. These antibodies did not react in the ordinary direct non-supplemented complement-fixation test.

African swine fever complement-fixing antigen in infected swine tissue is not denatured by extraction with fat solvents. Consequently, good antigens devoid of non-specific reactivity were obtained by extraction with a mixture of acetone and ether.

The virus was detected in infected swine tissue harvested one day after beginning of pyrexia. The modified direct complement-fixation test demonstrated cross-reactions between the six strains of virus studied.

RESUME

L'épreuve directe modifiée de la fixation du complément fut employée pour démontrer les anticorps dans les sérums de porcs immunisés avec le virus de la peste porcine africaine. Ces anticorps ne réagissaient pas dans l'épreuve directe ordinaire de la fixation du complément.

L'antigène du virus de la peste porcine africaine n'est pas dénaturé durant l'extraction des tissus infectés par les solvents lipides. En conséquence de bons antigènes, dépourvus de réactions non-spécifiques, furent obtenus par extraction avec un mélange acetone-ether.

Le virus fut décelé dans les tissus infectés prélevés un jour après le début de la pyrexie. L'épreuve directe modifiée de la fixation du complément a donné des réactions croisées avec les six souches de virus étudiés.

In 1963 it was reported that non-complement-fixing swine serum antibodies (1) could be detected by an indirect complement-fixation test developed by Rice in 1947 for the testing of avian serum antibodies (2). This observation indicated that the procomplementary activity of swine antiserum (3-5) is not the full answer to the poor fixability of swine antiserum-antigen mixtures with guinea-pig complement. In a study with hog cholera virus (6) it was observed that swine serum antibodies could also be demonstrated by the modified direct complement-fixation technique employed for the detection of cattle and avian viral antibodies (7-9). However, since hog cholera tissue antigen cannot be extracted with fat solvents without loss of activity, these crude extract antigens tend to show non-specific properties in complement-fixation tests.

A modified direct complement-fixation test, incorporating normal bovine serum as supplementing factor, was utilized by Cowan (10) for the detection of African swine fever (ASF) serum *antibodies* using

*Animal Pathology Division, Health of Animals Branch, Canada Department of Agriculture, Animal Diseaase Research Institute, Hull, Quebec.

a methanol-precipitated antigen prepared from tissue cultures infected with this virus. Our present study deals with the detection of viral *antigen* directly in extracts of tissues of infected swine.

Materials and Methods

MODIFIED DIRECT COMPLEMENT-FIXATION TEST

The general procedure described in the Standard Methods of the New York State Department of Health (11), which is based on a 50 per cent haemolytic unit, was followed closely for the routine titration of complement and amboceptor. All dilutions of reagents, unless otherwise indicated, were made in veronal buffered saline containing magnesium and calcium ions (12). Each reagent — diluted pig serum, antigen and modified complement — was added in 0.1 ml. amounts. The complement was diluted in 5 per cent pretested unheated normal calf serum to contain 3 fifty per cent haemolytic units in 0.1 ml. The tests were held overnight at 7-9°C to allow time for fixation. After the addition of 0.2 ml. of a 2.5 per cent suspension of maximally sensitized sheep red blood cells, the tests were further incubated for 30 min. at 37°C to allow time for haemolysis. For the antigen titration, two dilutions of the standard serum and serial two-fold dilutions of the tissue extract antigen were tested against each other in the presence of guinea-pig complement. Titres of the antigen were expressed in terms of the highest dilution with which 50 per cent haemolysis or less occurred with the standard antiserum. All swine sera used as normal or immune standard in the test, were inactivated by heating for 30 min. at 60°C on the day of the test. For the testing of serum antibodies, the antigen was kept constant at a predetermined concentration and the serum was added to the test in two-fold dilutions from 1:5 to 1:10,240.

IMMUNE SERA

The three standard immune sera (Nos. 117,114,115) used were prepared as described in a previous publication (13). These sera showed no interfering procomplementary properties. They appeared completely devoid of activity when tested by the regular direct complement-fixation test. Strong fixation was obtained with infected tissue extract when these sera were tested by the modified direct complement-fixation test. The majority of the tests were performed with serum 117, end-point 1:80, used in 1:20 and 1:40 dilutions to provide an antibody excess. The preliminary tests were carried out with serum 114, end-point titre 1:20, used in 1:5 and 1:10 dilutions. Occasionally a third serum 115, end-point 1:5120, was used in dilutions of 1:640 and 1:1280.

TISSUE ANTIGEN

Spleen and liver were the tissues of choice for extraction of viral antigen. Generally fresh tissues were extracted on the day of collection from swine, but equally good extracts were prepared from infected tissues kept frozen for various periods. In preliminary trials, a long method of extraction based on the acetone-ether extraction method of Casals (14) was used (15). This method was gradually shortened as described below. This short method was mainly used in the tests reported in this paper.

Long extraction procedure — Ten grams of tissue were blended in a Virtis homogenizer with 10 volumes of cold acetone. The suspension was shaken for 15 min. at 9°C, and centrifuged at 2500 r.p.m. for 10 min. The supernatant was discarded. The extraction was repeated once more with acetone, once with a 1:1 mixture of acetone-ether, then twice with ether alone. The residual ether was evaporated at room temperature by periodic stirring of the tissue powder with a glass rod. For elution of the viral antigen, 20 ml. of saline was added to the dry tissue powder and the suspension shaken overnight at 9°C. The next morning the coarse tissue was removed by filtering through a piece of absorbent cotton, and the filtrate centrifuged at 500 r.p.m. for 15 min. The supernatant fluid was tested immediately for viral antigen activity or kept frozen until ready for use.

Short extraction procedure — Ten grams of tissue were blended with 20 volumes of 1:1 cold acetone-ether mixture. The suspension was shaken for 2 hours or overnight at 9°C. After centrifugation at 2500 r.p.m. for 15 min., the supernate was discarded and the residual acetone-ether mixture evaporated at room temperature as above. Elution of the viral antigen from the dry tissue powder was accomplished by shaking with 20 ml. of saline for 1.5 hours or overnight at 9°C.

TABLE I. Results of the modified direct complement-fixation test on extracts of tissues from swine collected at various intervals after exposure to the Spencer African swine fever virus tested with an antiserum prepared with the Portuguese strain

Pig Number	Exposure	Duration of pyrexia	C.F. Titres spleen	liver
138	6 days	1 day	1:32	1:16
162	5 days	1 day	1:4	1:16
142	3 days	1 day	1:8	1:2
140	5 days	2 days	1:32	1:16
141	4 days	2 days	1:16	1:8
148**	4 days	2 days	1:2	1:16
139	6 days	3 days	1:32	1:8
147**	6 days	3 days	1:8	1:32
145	8 days	4 days	1:8	1:32
161	7 days	4 days	1:32	1:64
149**	6 days	4 days	1:32	1:32
146	8 days	5 days	1:8	1:32
125	7 days	5 days	1:16	1:16
130	6 days	5 days	1:32	1:64
Sow 160**	7 days	0 day	1:2	1:16 ac 1:2*
Sow 158	7 days	2 days	1:32	1:16
Sow 159	7 days	3 days	1:32 ac 1:8*	1:32
Normal 1-9	none	none	Neg.	Neg.

*: ac means anticomplementary in the dilution indicated.
**: These swine died during the night before collection of tissues.

INFECTIOUS AGENTS

Six strains of African swine fever virus were included in this study. The Spencer and Gasson strains were obtained from a collection made by Colonel Fred D. Maurer, College of Veterinary Medicine, Texas A. & M. University, College Station, Texas, U.S.A. The Portuguese vaccine and the challenge virus strains were received from Dr. W. Ribeiro, National Veterinary Research Laboratory, Lisbonne, Portugal. The Madrid 1 (1960) and the Madrid 2 (1965) strains were furnished by Dr. S. Botija, Service of Animal Pathology and Biology, Madrid, Spain. The last four strains mentioned were obtained through the kind offices of Dr. A. Heflin, United States Department of Agriculture. The various strains were passaged in swine and at height of infection, blood and tissue were collected as infective material for further experiments.

INFECTION OF SWINE

With the exception of three breeding sows, the swine used were in the weight range of 50 to 100 pounds. Infection with the Portuguese vaccine strain was achieved by the intra-muscular (I.M.) inoculation of 3.0 ml. of virulent blood. The five virulent strains were administered by feeding infected tissues. Temperatures of all animals were recorded daily, and examination for clinical manifestations conducted. The swine were killed at various intervals after exposure. Specimens of tissue were also collected from swine which died of the infection.

Results

The extraction of tissues by the long or short acetone-ether procedures resulted in an antigen devoid of interfering anticomplementary or non-specific reactivities. Generally the long procedure of extraction resulted in antigens less non-specific than those prepared by the short method. However with tissues containing large amounts of haemoglobin, such as those collected from animals which died of the disease, more interfering haemoglobin color was present in the final product prepared by the long method.

The results of antigen titration of extracts of spleen and liver collected from 17 swine at various intervals after exposure to the Spencer strain of ASF virus are recorded in Table I, together with those from nine normal swine. It can be seen that significant reactions were obtained with an extract of tissues collected even after only one day of pyrexia. In general the titres of the antigens increased with the advance of clinical manifestations. In advanced cases or in animals found dead, the liver antigen was generally more reactive than the one prepared from spleen. Liver antigen also contained less haemoglobin which made the reading of the test less difficult.

An exception to the good fixability of the tissue antigen with standard ASF antiserum was noted with the spleen and liver extracts of the vaccinated-challenged pig 115 which had a serum antibody titre of 1:5120 when killed for examination. No reaction was obtained in the complement-fixation test with its tissues. This pig had been showing intermittent pyrexia and the virus was demonstrated in its lung by immuno-fluorescence (16). The spleen and liver extracts instead of acting as antigen in the test, showed the presence of antibodies to a titre of 1:32 and 1:64 respectively when they were tested with a known positive antigen. Furthermore these extracts did not react with the known nega-

TABLE II. Reactivity of the modified direct complement-fixation test with extracts of tissues from swine infected with 6 strains of African swine fever virus tested with an antiserum prepared with the Portuguese strain

Strains	Pig Number	Exposure	Duration of pyrexia	C.F. Titres	
				spleen	liver
Portuguese	129*	9 days	3 days	ND	1:32
"	131*	4 days	2 days	Pool 1:32	
Gasson	156	5 days	2 days	1:16	1:8
"	157	5 days	1 day	1:4	1:8
Spencer	125	7 days	5 days	1:16	1:16
"	130	6 days	5 days	1:32	1:64
Madrid I	166	5 days	3 days	1:64	1:64
"	167	5 days	3 days	1:64	1:64
Madrid II	176	5 days	1 day	1:8	1:16
"	175	5 days	2 days	1:32	1:32
Portuguese vaccine strain	172	3 days	1 day	1:32	Neg.
	174	4 days	0 day	1:16	tr 1:32
	173	5 days	1 day	1:8	Neg.

* = These swine died during the night before collection of tissues.
ND = Not done
tr = Trace or partial reaction up to the titre indicated.

tive antigen. This observation suggests that in chronic infection the complement-fixation test, designed to detect viral antigen in tissue, might fail to reveal the disease. This could happen in areas in which the infection is endemic and where swine may apparently recover and become chronic carriers at a time when they have a high serum antibody titre. Such cases could be detected by including a complement-fixation test for the detection of antibodies in serum in addition to the one for the detection of virus in tissue generally done at the acute stage of the disease.

The cross-reactivity observed in the modified direct complement-fixation test with tissue extracts from swine infected with one or other of the six strains used in the study is shown in Table II. Strong cross reactions were obtained between the standard Portuguese antiserum and the antigens derived from the five virulent strains. However, in the case of the Gasson strain particularly with pig 157, the reactivity was slightly lower than with the other strains. This might be explained by the fact that this viral strain had not been passaged in swine for a number of years. No viral complement-fixing antigen was detected in the liver extracts from the three swine 172, 173 and 174 inoculated with the Portuguese vaccine strain, but their spleen extracts were reactive. The antigen titres obtained decreased in proportion to the interval between inoculation of this vaccine strain and the time at which the animal was killed. This pointed to the possible transient nature of the infection caused by inoculation of the vaccine strain.

Discussion

The present study emphasizes the usefulness of a modified direct complement-fixation test supplemented with non-heated normal bovine serum, to demonstrate viral antibodies in swine serum. No fixation was obtained with the ordinary direct complement-fixation test even when applied to serum with an exceptionally high antibody content.

Contrary to the observation made in hog cholera, the ASF antigen is not denatured by extraction with an acetone-ether mixture. This makes possible the preparation of infected tissue antigen devoid of non-specific reactivity. The modified direct complement-fixation test is highly sensitive in the detection of virus in tissue collected from swine at the acute stage of the disease; positive reactions are obtained as early as the second day of pyrexia. In chronic infection, as for example in apparently recovered swine or with immune-challenged ones containing high serum antibody titres, the test may fail to demonstrate the virus even though its presence in the lung is demonstrable by immunofluorescence (16). Such cases could be detected by orienting the complement-fixation test towards the detection of antibodies in serum in addition to the detection of virus in tissue as it is generally done at the acute stage of the disease.

The modified direct complement-fixation

test is cross-reactive with the various ASF virus strains studied. However, with the Portuguese vaccine strain the antigenicity in tissue from inoculated swine decreases as the interval after inoculation increases.

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Fellowships Awarded to O.V.C. Students

The Ayerst Fellowship of $500 given by Ayerst, McKenna and Harrison of Montreal has been awarded to Dr. W. D. Black, a graduate student in the Department of Physiological Sciences. Dr. Black is registered for a Master of Science degree in pharmacology.

A Cyanamid of Canada Fellowship of $600 has been awarded to Dr. J. R. Long, a graduate student in the Department of Avian Pathology, Wildlife Diseases, and Virology. Dr. Long is registered for a Ph.D. degree with a research program in bovine respiratory diseases.

Following graduation in 1965, Dr. Black spent a year in general practice at Wellesley, Ontario. Dr. Long engaged in mixed veterinary practice in Prince Edward Island from graduation in 1962 until 1964 when he enrolled at Cornell University for a Master of Science degree in nutrition. Immediately after receiving the Master's degree he returned to OBC this year for the Ph.D. degree.

The 1966 Borden Fellowship for 1966 has been awarded to Dr. G. P. Searcy. Dr. Searcy is registered at the University of Guelph for a Master of Science degree, and is studying in the Department of Pathology, Ontario Veterinary College. His present program involves investigations in bovine streptotrichosis. The disease, which was considered rare in Ontario cattle, has been found to be quite common.

Dr. R. W. Putnam, a graduate student in the Department of Clinical Studies of the Ontario Veterinary College, has been awarded the Ballard Fellowship of $500 provided by Standard Brands Limited of Montreal. He is now registered for a Master of Science degree in orthopaedic surgery, and it is expected that his thesis will deal with investigations of patella subluxation in the dog.

Dr. O. Narayan received $500 from K-Vet Laboratories, Galt, to assist in his program leading to the Master of Science degree. He is now enrolled in the Department of Avian Pathology, Wildlife Diseases and Virology for

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