African Swine Fever III. The Use of the Agar Double-Diffusion Precipitation Test For the Detection of the Virus in Swine Tissue

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SUMMARY

The agar double-diffusion precipitation test was applied successfully in the demonstration of ASF viral antigen in spleen and liver from swine experimentally infected by the oral route.

Positive reactions were obtained with tissues collected as early as 24 hours after the onset of pyrexia and before other clinical manifestation of the disease. Cross-reactions were observed between the various ASF strains used in the study, making the test practical for routine diagnosis in which different strains may be encountered.

RESUME

L'épreuve de précipitation par diffusion dans l'agar fut employée avec succès pour démontrer l'antigène du virus de la peste porcine africaine dans le foie et la rate de porcs infectés expérimentalement par voie buccale.

Des réactions positives furent obtenues avec des tissus prélevés aussitôt que 24 heures après apparition de la fièvre. Des réactions croisées ont été données par les diverses souches de virus étudiées, ce qui rend l'épreuve pratique pour fin de diagnostic.

Specific antigen-antibody reactions were obtained with hog cholera reagents (1-2) using a modification of the agar double-diffusion precipitation test of Darbyshire (3). Even though Canada has always been free of African swine fever (ASF) it seemed desirable to determine the value of this test as a means of differentiating the two infections.

Malmquist (4) in 1963, working with strains of ASF virus, observed a group reaction in the agar gel test between antigens derived from infected cell culture debris and sera from swine surviving the infection. In 1965, Hess *et al* (5) obtained a precipitation reaction between ultrasonic-disrupted tissue culture antigen and sera of swine infected with a tissue culture modified ASF virus.

In our study we proposed to determine if the serum of swine recovered from or im-

TABLE I. Results of the agar double-diffusion precipitation test on liver and spleen of swine collected at various intervals after exposure to the Spencer strain of African swine fever virus in tests with antiserum prepared against the Portuguese strain

Pig Number	Exposure	Duration of pyrexia	Precipitation	
			Spleen	Liver
138	6 days	1 day	Pos.	Pos.
162	5 days	1 day	N.S.	Pos.
142	3 days	1 day	Ques.	Ques.
140	5 days	2 days	Pos.	Pos.
141	4 days	2 days	Pos.	Pos.
148*	4 days	2 days	Pos.	Pos.
139	6 days	3 days	Pos.	Pos.
147*	6 days	3 days	Pos.	Pos.
145	8 days	4 days	Pos.	Pos.
161	7 days	4 days	Pos.	Pos.
149*	6 days	4 days	Pos.	Pos.
146	8 days	5 days	Pos.	Pos.
125	7 days	5 days	Pos.	Pos.
130	6 days	5 days	Pos.	Pos.
Sow 160*	7 days	0 day	N.S.	Pos.
Sow 156	7 days	2 days	Pos.	Pos.
Sow 159	7 days	3 days	Pos.	Pos.
Normal 1-9	none	none	Neg.	Neg.

* : These swine died during the night before collection of tissue.

N.S.: Not satisfactory; the tissue contained excessive amounts of haemoglobin which interfered with reading the test.

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TABLE II. Reactivity in the agar double-diffusion precipitation test of spleen and liver of swine infected with various strains of African swine fever virus in tests with an anti-serum prepared with the Portuguese strain

Strain	Pig number	Exposure	Duration of pyrexia	Precipitation	
				Spleen	Liver
Portuguese	129*	9 days	3 days	Pos.	Pos.
Portuguese	131*	4 days	2 days	Pos.	Pos.
Gasson	156	5 days	2 days	Pos.	Pos.
Gasson	157	5 days	1 day	S.Q.	S.Q.
Spencer	125	7 days	5 days	Pos.	Pos.
Spencer	130	6 days	5 days	N.S.	Pos.
Madrid 1	166	5 days	3 days	Pos.	Pos.
Madrid 1	167	5 days	3 days	Pos.	Pos.
Madrid 2	176	5 days	1 day	Pos.	Pos.
Madrid 2	175	5 days	2 days	Pos.	Pos.
Portuguese vaccine strain	172	3 days	1 day	Ques.	Neg.
	174	4 days	0 day	Ques.	Neg.
	173	5 days	1 day	Tr.	Neg.

* These swine died during the night before collection of tissues.
N.S. Not satisfactory; the tissue contained excessive amounts of haemoglobin which interfered with reading the test.
S.Q. Slightly questionable.
Ques. Questionable.
Tr. Trace.

munized against ASF virus would detect in the precipitation test, the virus in tissues collected from diseased swine as it did in infected tissue culture cells. There seemed to be a good opportunity to verify the accuracy of the test since the presence of viral antigen in the tissues under study had already been determined by the modified direct complement-fixation test as reported previously. (6).

During the course of our investigation, we became aware of the studies of Coggins *et al* (7) in which 70 percent of the tissues from 23 swine dying with acute ASF showed precipitinogen. Positive reactions were observed in one or more of the tissues from all 23 swine that they tested.

Materials and Methods

INFECTIOUS AGENTS AND EXPERIMENTAL ANIMALS

The origin and source of the ASF virus strains included in this study were described previously (8). The five virulent isolates used were the Spencer, the Gasson, the Portuguese, the Madrid 1 (1960) and the Madrid 2 (1965) strains. The vaccine strain was the Portuguese tissue culture-modified virus.

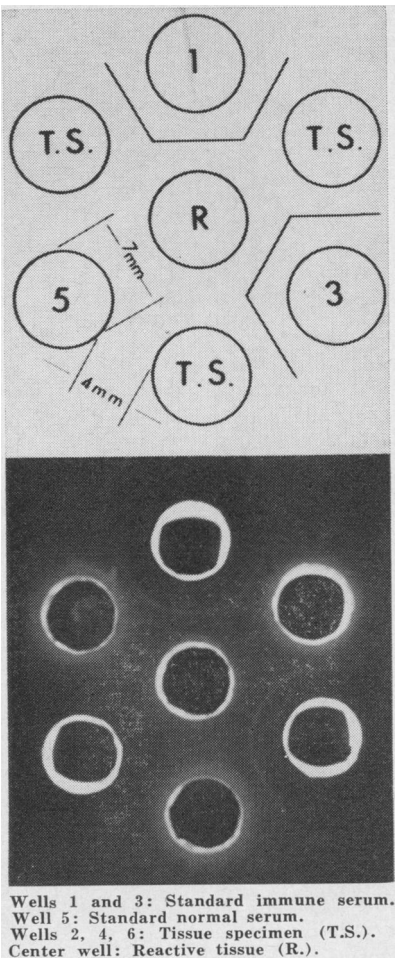
In the first part of the experiment, 14 swine weighing 50 to 100 pounds and 3 breeding sows were exposed by feeding tissues infected with the Spencer virus. Temperatures of all animals were recorded daily, and examination for clinical manifestations was conducted. The swine were killed or died at various intervals after exposure following a pyrexia of 1 to 5 days, as outlined in Table 1. Specimens of liver and spleen were collected from these swine.

In the second part of the experiment five groups of 2, and one group of 3, swine weighing 50 to 100 pounds were exposed by feeding virulent tissue infected with one of the 6 strains of virus listed above. Swine were observed daily and died or were killed at various intervals after exposure as recorded in Table II.

IMMUNE SERA

Various methods were tried in attempts to produce immune sera as described in a previous publication (8). Most of the tests were performed with serum from pig 117 although a limited number of trials were made with serum 115. The swine supplying these sera were vaccinated with the 81st tissue culture passage of the Portuguese attenuated strain, followed at 71 days post-vaccination (d.p.v.) by feeding

Fig. 1. Disposition of the reagents in the agar double-diffusion precipitation test and location of typical precipitation lines.



Wells 1 and 3: Standard immune serum.
Well 5: Standard normal serum.
Wells 2, 4, 6: Tissue specimen (T.S.).
Center well: Reactive tissue (R.).

tissues infected with the Portuguese virulent strain. A second challenge, in the form of 3.0 ml. of virulent blood was given intramuscularly at 87 d.p.v.

AGAR DOUBLE-DIFFUSION PRECIPITATION TEST

The technique, with the exception of a few minor modifications, was the same as described previously in our studies with hog cholera virus (1-2). The diffusion medium consisted of 1 percent No. 2 Oxoid Ionagar, pH 7.2, in disposable petri dishes. Six wells, 7 mm. in diameter, were punched in the agar at a 4 mm. distance from each other and from a central well. Tissue pulp of spleen or liver was placed in three of the alternate outside wells (Nos. 2, 4, 6). Two intermediate wells (Nos. 1 & 3) were filled with a standard immune serum. The last well (No. 6) was filled with a standard normal serum to serve as a control. The central well in each test was filled with a known reactive infected tissue. Before setting up the test the spleen or liver tissues were often frozen and thawed one to three times, but in certain cases tests were performed with fresh unfrozen tissues. Wells containing the tissue pulp were filled one to two hours before those containing the sera to allow time for diffusion of the antigen. Sera were heat-inactivated at 56°C for 30 minutes before setting up the test. The plates were incubated at room temperature for 24 hours in a closed container with a high relative humidity. At this time a preliminary examination was made for the development of characteristic precipitation lines (Fig. 1). The wells containing normal or immune sera were refilled once, and the tests were incubated for an additional 24 hours, after which time final readings were made before discarding the plates.

Results

Much less difficulty was encountered in adapting the agar double-diffusion precipitation test to the detection of ASF virus in tissue than had been experienced with hog cholera virus (1-2). The aging of tissues in the frozen state followed by alternative freezing and thawing to release the virus from infected cells, was found to be unnecessary. In the case of ASF, good reactions were obtained with tissues frozen only once or even with freshly collected unfrozen tissues. With spleen tissue, many alternate freezings and thawings had the disadvantage of liberating much haemoglobin which can interfere with reading of the test.

Reactivity of control immune sera. The two immune sera used in the test were diluted so as to provide a large excess of antibodies. Serum 117, with an end-point titre in this test of 1:32, was used in 1:2 dilution. Serum 115 was diluted 1:4 although its end-point titre was 1:128.

Influence of exposure intervals on the degree of reaction. The reactions obtained in the agar double-diffusion precipitation test with spleen and liver from 17 swine infected by oral exposure to the Spencer strain of ASF virus are listed in Table I. With the exception of two spleens (Nos. 160 &162) where an excessive amount of haemoglobin interfered with reading of the

test, the viral antigen was detected in spleen and liver harvested from these swine after one to five days of pyrexia. Good reactions were also obtained with tissues collected after the animals died of the infection. With tissue from animals killed in the early stage of infection, a greater amount of antigen was present in spleen than in liver. However, the liver, because it contained less haemoglobin, was preferred when the tissues were from animals dead or dying.

Cross-reactivity of the various ASF strains. As shown in Table II, cross-reactivity was obtained in the agar double-diffusion precipitation test between immune serum 117 *(Portuguese attenuated and virulent)* and the spleen and liver from swine inoculated with one or other of the 5 virulent strains. However, pig 157 inoculated with the Gasson strain gave only a slightly questionable reaction. Serum from this pig was also less reactive in the complement-fixation test (6) than that of its counterpart, 156, inoculated with the same strain.

Tests with liver from the 3 swine inoculated with the Portuguese vaccine strain were negative, but the spleen from two of these gave a questionable precipitation reaction. Spleen from the 3rd animal, killed the 5th day after inoculation, was negative. This observation suggests the transient nature of the infection produced by the vaccine strain.

Reaction of tissue from chronically infected swine. No reaction was observed in the agar double-diffusion precipitation test with the spleen and liver from pig 117 which was used in the production of immune serum. However, as will be reported in another publication (9), the viral antigen was demonstrated by immunofluorescence in the lung, spleen and lymph node of this animal. Viral antigen was also seen by immunofluorescence in the lung of pig 115 which had a high serum antibody titre when destroyed for examination. Its spleen and liver, instead of reacting as an antigen in the agar double-diffusion precipitation test, produced precipitation with the known positive antigen as if they contained antibodies. This observation suggests that this test may be of little value in detecting viral antigen in chronically infected animals at a time when the organs have both a high antibody and viral content.

Discussion

Much less difficulty was encountered in the demonstration of viral antigen in ASF infected tissue than was the case with hog cholera (1-2). No special treatment of the tissue was needed to liberate the antigen from infected cells. Tissue collected from infected animals after only 24 hours of pyrexia were reactive. It was not necessary to wait until swine were moribund to obtain a positive reaction. Since all the ASF strains studied were found to be cross-reactive, the test can apparently be performed effectively with a single antiserum, which greatly facilitates diagnosis. Tissue from swine chronically infected did not react, possibly due to their high antibody content. In such cases, however, the complement-fixation test (6) and immunofluorescence (9) could be successfully used.

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