Hog Cholera II. Reliability of the Agar Double Diffusion Precipitation Test for the Differentiation of H. C. Virus from Other Infectious Agents in Swine Tissue

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SUMMARY

The specificity in the agar diffusion precipitation test of the reaction between the antigen of hog cholera virus diffusing from infected tissues and its homologous antibody was verified.

Alternate freezing and thawing of infected tissues was found to give optimum release of the antigen from fresh tissue frozen for 18 hours. A study of the effect of the size and age of pigs upon the diffusion of the antigen from tissues showed that tissues from pigs of less than 250 lbs. gave good results provided the tissues were from animals showing gross clinical manifestations. Specimens from infected breeding sows and dead animals usually did not give a reaction.

RÉSUMÉ

On a étudié par diffusion dans l'agar, la spécificité de l'épreuve de précipitation entre l'antigène provenant de tissue infectés avec le virus de la peste porcine et les anticorps correspondents.

Il a été constaté que des congélations et dégeles consécutifs des tissus infectés permettent la libération optimum de l'antigène des tissus fraichement prélevés et congelés depuis 18 heures. Une étude de l'influence du poids et de l'âge des porcs sur la libération de l'antigène des tissus, a montré que les tissus des sujets de moins de 250 livres donnent de bons résultats dans l'épreuve pourvu qu'ils proviennent d'animaux montrant des signes avancés de la maladie. Les tissus des truies de reproduction et des sujets morts doivent être évités parce que généralement ils ne donnent pas de reactions.

Preliminary studies (1) of the agar double diffusion precipitation test confirmed the observation of Darbyshire (2) that a reaction takes place in the agar medium between antigens from hog cholera (HC) infected tissues and antiserum for this virus. Absorption and chromatographic studies showed that the reaction occurred between the gamma-globulin component of the hyperimmune serum and the viral antigen diffusing from the infected tissues. Modifications of Darbyshire's method incorporated into the earlier tests such as the use of 1 per cent No. 2 Oxoid Ionagar instead of 1.5 per cent, immune serum globulin fractions instead of whole serum and spleen as antigen in place of pancreas improved the sensitivity of the test. The method of liberation of antigen from the tissues had the greatest influence on the results. There appeared to be a slight improvement with aging of the tissues, while chemical extraction with ether and alcohol or sonic vibration to disrupt the cells eliminated the reactivity. Preliminary work on alternate freezing and thawing of the tissues appeared to improve their reactivity in the test. Depending upon the particular treatment of the tissues used for the liberation

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TABLE I

Results of the agar double-diffusion precipitation test and of precipitation-inhibition on random samples of spleens from normal swine and swine experimentally infected with hog cholera virus and other infectious agents

Tissue source	Number of spleens	Direct precipitation			Precipitation-inhibition		
		Pos.	Ques.	Neg.	Pos.	Ques.	Neg.
Hog cholera virus							
H. C. Canadian isolates	69	52	9	8	52	7	10
American 1030 isolate	8	6	1	1	5	2	1
Total	77	58	10	9	57	9	11
Per cent		75.3	13.0	11.7	74.0	11.7	14.3
Teschen virus							
T. vetesucci	4			4			4
T. konratice	5			5			5
T. french	3			3			3
Total	12			12			12
HEV virus	3			3			3
VE virus	3			3			3
ASF virus	5			5			5
Erysipelothrix insidiosa	7			7			7
Normal Tissues	45			45			45

of the antigen, from 52.3 to 68.1 per cent reactions were observed with the same group of specimens.

The studies were continued to verify the effects of the incorporated refinements on the sensitivity and specificity of the test, when applied to the differentiation of the HC virus from other infectious agents in swine tissues. Two main series of studies were conducted. In the first series, field conditions were simulated as closely as possible by random distribution of tissues harvested from HC infected swine, normal swine and swine infected with other diseases. In the second series, attention was given to the minimum storage time necessary for best release of the antigen, the optimum time for harvest during the course of the disease and the influence of the age of the animal on the diffusion of the antigen from infected tissue.

Materials and Methods

Experimental Animals and Infectious Agents

Eight hog cholera virus isolates were used in this study: standard ADRI-1, Green, Windsor 53, U5516, U5674, TC1 and American 1030. Other infectious agents such as Teschen virus (Vetesucci, Konratice, French), HEV (the porcine haemagglutinating encephalomyelitis virus of Greig), vesicular exanthema virus B-1 and *Erysipelothrix insidiosa*, some of which produce symptoms that might be confused clinically with those of hog cholera, were also included to establish the specificity of the test. Each viral inoculum contained 20,000 I.U. penicillin and 3 mg. streptomycin per ml. of infective material. The animals were kept in small groups in strict isolation and each group was exposed to only one particular infectious agent. Temperatures of the animals were taken daily.

Series 1: The swine in this series were minimal disease pigs of 30 to 40 pounds weight each. Sixty-nine swine, as indicated in Table 1, were inoculated intramuscularly with 5.0 ml. of hog cholera virulent blood derived from passages of infectious material collected in previous Canadian outbreaks. In addition eight swine were similarly inoculated with 3.0 ml. of virulent blood from the American 1030 isolate.

Twelve pigs were inoculated intracranially with 0.2 ml. of Teschen virulent brain suspension, three received 1.0 ml. of HEV tissue culture propagated virus, three were inoculated intradermalingually with 1.0 ml. of vesicular exanthema epithelium suspension virus, five intramuscularly with 5.0 ml. of African swine fever virulent blood and seven with 10.0 ml. of a virulent *Ery-*

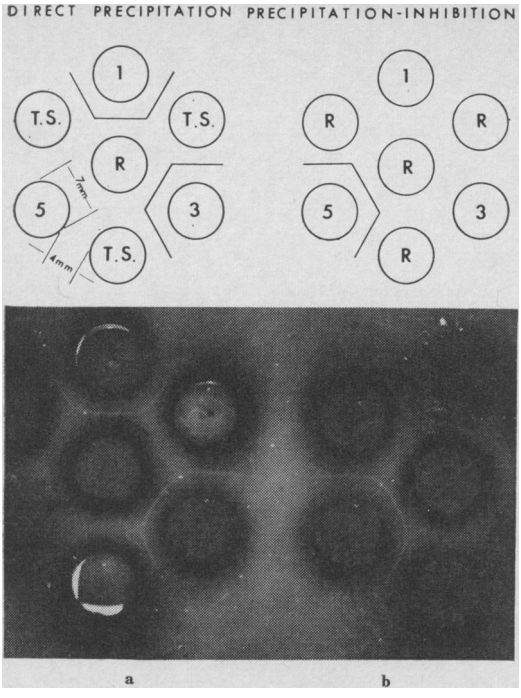

Fig. 1. Disposition of reagents and patterns of reactions in the direct and precipitation-inhibition agar tests.
(a) T.S. — Unknown test spleen

R — Known reactor spleen
Wells 1, 3 — HC Immune swine serum globulins
Well 5 — Normal swine serum globulins
(b) R — Known reactor spleen
Wells 1, 3 and 5: HC positive swine serum globulins absorbed by spleen tissue from 1: known hog cholera reactor,
3: unknown test swine and
5: known normal control swine

sipelothrix insidiosa culture intravenously. Forty-five specimens were collected from normal animals at the slaughter house or from a minimum disease herd.

Series 2: In this series all the swine were inoculated intramuscularly with 5.0 ml. of hog cholera virulent blood derived from the standard ADRI-1 isolate. One group of 24 weighing between 30 and 65 pounds were killed at various intervals post inoculation, that is, on the third, fifth, seventh and tenth day. Another group of 24 swine including four weighing 100 to 150 pounds, eight of 160 to 200 pounds, four of 210 to 250 pounds and eight sows weighing over 300 pounds were killed when showing manifestations of the disease.

Tissue Antigens

Spleens only were used in both series because previous work had indicated that this tissue was a better source of antigen.

Series 1: The spleens were harvested from the 3rd to the 17th day post inoculation. They were assigned a code number unknown to the person setting up the test and stored in small amounts in the frozen state. This storage period varied from 2 to 109 days. The spleens were frozen and thawed four times in groups of 18 to 20 samples and held in the freezer for three to ten days before testing.

Series 2: The spleens when harvested were divided into four small portions and handled as follows: portion number 1 was refrigerated overnight at 9°C, frozen and thawed four times in a glass container submerged in a dry ice-alcohol bath and tested on the same day; portion number 2 was kept frozen overnight, thawed the next day and tested following four alternate freezings and thawings; portion 3 was frozen and thawed four times, then incubated for 18 hours at 37°C with 3000 I.U. penicillin and 4.5 mg. streptomycin per gram of tissue to prevent bacterial multiplication; portion 4 was frozen and thawed four times, then stored in the freezer from four to seven days before being thawed and tested. As normal controls, tissues were obtained from a local slaughter house and processed in the same manner as the infected tissues.

Normal and Immune Serum Globulins

The globulin fractions from normal and HC hyperimmune pig sera were precipitated at 8°C by dialysing one volume of serum against nine volumes of ammonium sulphate adjusted to give a final concentration of 30 per cent in the sera and the dialysate. In earlier tests, no difference was observed in the reactions obtained with globulin fractions from three locally produced hyperimmune sera and a commercial serum; therefore, the commercial serum globulins were used because of their availability in large volume. The normal serum globulins were obtained from a specific pathogen free (SPF) sow and from blood collected at a local slaughter house. In the latter the globulins were derived by precipitation of one volume of serum with an equal quantity of saturated ammonium sulphate (1).

Technique of the Test

The procedures were similar to those described in a previous paper (1) for the testing of splenic tissue. The diameter of the diffusion wells and their respective distances were also the same as described pre-

TABLE II

Influence of storage in the frozen state on the liberation of the precipitating antigen from infected tissues

Period of storage days	Number of spleens	Reactions	
		Pos. or ques.	Negative
0 — 9	10	7	3
10 — 19	4	0	4
20 — 29	26	26	0
30 — 39	9	9	0
40 — 49	15	15	0
50 — 59	—	—	—
60 — 69	—	—	—
70 — 79	3	3	0
80 — 89	2	1	1
90 — 99	4	4	0
100 — 109	4	3	1

TABLE III

Influence of the duration of pyrexia at the time of collection of tissues on detection of hog cholera viral antigen

Duration of pyrexia days	Number of samples	Reactions	
		Pos. or ques.	Negative
0	2	1	1
1	2	2	0
2	2	1	1
3	9	5	4
4	9	6	3
5	5	5	0
6	16	16	0
7	9	9	0
8	9	9	0
9	6	6	0
10 — 15	8	8	0

viously. The diffusion medium consisted of 1 per cent No. 2 Oxoid Ionagar, pH 7.2, in disposable petri dishes. To compensate for a slower rate of diffusion of antigen from the tissue material, wells containing the tissues were filled two hours before the addition of the serum globulins. The incubation period and the method were not changed. The tests were read after 24 and 48 hours incubation at room temperature.

The first series of tissues were also tested by a precipitation-inhibition test. Both tests were conducted in parallel in the same petri dish and the reagents were disposed as indicated in Fig. 1 which also exemplifies typical reactions. On the day prior to the test, three 0.5 ml. portions of immune globulin concentrate were absorbed respectively with 1.0 gram of splenic pulp from a known normal, a known positive and the unknown spleen tissue. Absorptions were carried out for 18 hours, with continuous agitation, in a cold room. The suspensions were then centrifuged in an international centrifuge model SVB 1 for 30 minutes to remove tissue. The remainder of the procedures, including reading, were the same as for the ordinary precipitation method.

Results

SERIES 1

In this series the strict requirements of the test, outlined in a previous publication (1) were rigidly followed and the spleen tissues were subjected to four alternate freezings and thawings to improve the release of the antigen. Of 77 spleens collected from hog cholera infected swine, 68 or 88.3 per cent gave either positive or questionable reactions in the agar double-diffusion precipitation test (Table 1). The precipitation-inhibition test was positive or questionable in 65 of the 68 specimens which reacted in the ordinary test. One spleen reacted in the inhibition and not in the ordinary test. This apparent discrepancy between the two tests is probably due to the fact that for practical reasons the inhibition test was conducted with only concentrated antibody. Consequently, the spleen with a low content of virus did not release enough antigen to completely neutralize the antibody. This discrepancy was not noted in repeat tests when adjustments were made between the volumes of globulin concentrates and absorbing tissues. No reaction was given by spleens collected from swine inoculated with Teschen, HEV, vesicular exanthema and African swine fever viruses nor by those collected from the

TABLE IV

Influence of the duration of the disease at the time of collection of tissues on the detection of the presence of hog cholera virus antigen

Days post-inoculation	Number of Spleens	Reactions	
		Pos. or Ques.	Negative
3 — 5	11	6	5
6 — 8	38	34	4
9 — 11	21	21	0
12 — 14	3	3	0
15 — 17	4	4	0

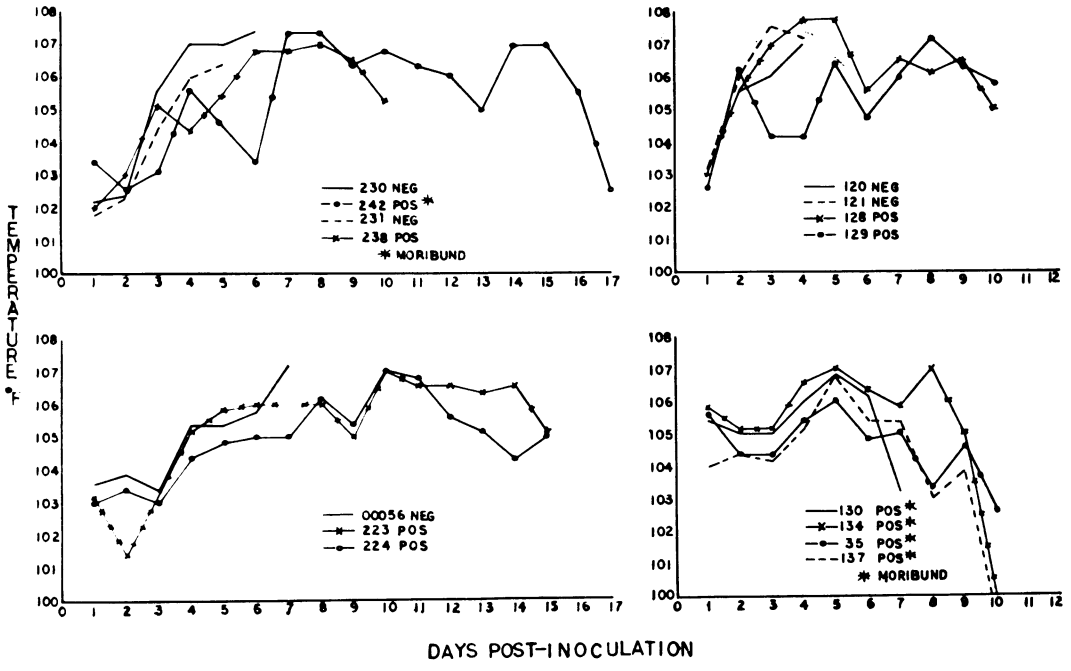

Fig. 2. Temperature curves and time of harvest of reactor and non-reacting spleens from hog cholera infected swine.

swine infected with *Erysipelothrix insidiosa* or from normal swine.

The storage period of the tissues was considered as a possible influence on the sensitivity of the test since it seemed in preliminary trials that the reactivity of some tissue increased with aging. However, the distribution of positive and negative results among the specimens grouped according to storage time (Table II) suggested that this influence was only apparent because two of the spleens stored for more than 80 days remained non-reactive.

Examination of the results (Table III) indicated that the reactivity was dependent on the duration of pyrexia at the time of collection of tissues. This is also exemplified in Table IV which indicates that 62 of the 66 spleens, collected from swine six days or more after inoculation, reacted in the test. Among the 62 were 10 swine in the early moribund stage, with a long period of pyrexia, symptoms of inco-ordination and paralysis, as well as a normal or subnormal temperature at the time of collection of the spleens. The temperature curves of five of the ten strongly reactive moribund swine, (nos. 242, 130, 134, 135 and 137), are given in Fig. 2. For contrast the temperature curves are also given for five non-reactor swine (nos. 230, 231, 00056, 120 and 121) the spleens of which were collected at the height of temperature but soon after infection.

The observations made in series I indicate that the height of fever at the time of collection of specimens, the strain of HC virus, the route of inoculation or the period of storage of tissue in the frozen state have little influence. Four alternate freezings and thawings of the tissue and the collection of the specimens from swine with advanced clinical manifestations, presumably with a higher content of virus in the spleen tissue, is of prime importance.

Series 2

Comparison of the four treatments described above showed that freezing the sample for 18 hours, rather than refrigerating for 18 hours before freezing and thawing four times released much more antigen. Storage of the frozen sample for longer periods did not materially increase or reduce the amount of antigen released. The fresh specimens, frozen and thawed four times, then incubated at 37°C for 18 hours (with penicillin and streptomycin) often gave a much stronger reaction in a shorter period. However, the heavy diffuse cloudiness usually accompanying the reaction obscured the precipitation lines and

TABLE V

Effect of post-inoculation interval and duration of pyrexia on efficiency of the agar precipitation test for detection of hog cholera virus antigen in infected tissues

Days post-inoculation	Mean days' duration of pyrexia	Number of pigs	Reactions Pos.	Negative
3	2	6	0	6 (100%)
4*	2	1	0	1
5	3	5	3 (60%)	2 (40%)
7	6	6	5 (83.4%)	1 (16.6%)
8*	4	1	1	0
10	8	5	5 (100%)	0

*Found dead.

made interpretation very difficult. Since the only difference between the results of the four treatments was in the intensity of the reactions only a single result was recorded for each spleen tested.

This series of experiments was divided into two sections, the first of which dealt with the optimum time of harvest of tissues during the course of the disease (Table V). Of the twenty-four 30 to 65 pound pigs, 22 were killed at regular intervals from three to ten days post infection, one died at four days and another died at eight days post inoculation and gave very weak or negative reactions. In the results obtained, there was a definite correlation between the time of harvest post inoculation, duration of pyrexia, and advanced clinical manifestations of the disease. The five spleens harvested after only three days of infection and two days pyrexia were negative. Positive reactions were seen with all of the spleens from pigs with at least eight days pyrexia and ten days of infection. This proportion decreased to five of six pigs at seven days post inoculation with six days pyrexia, three of five at five days post inoculation and three days pyrexia. All 12 of the corresponding normal control tissues obtained from a slaughter house were negative.

In the second section of series 2 the possible influence of age and size of pigs, on the diffusion of antigen from infected tissue was studied. As indicated in Table VI, there were four negative results in the group of eight 160 to 200 pound pigs, but all four represented spleens collected from dead animals in which the viral antigen may have been destroyed during tissue autolysis. There was one negative sample in the 210 to 250 pound group but this animal had only five days of low grade fever so that as discussed before the virus titer may have been too low to be detectable. The negative results obtained from specimens of these samples can be accounted for on the same basis as those in the 30 to 65 pound group collected under similar conditions. There were no other differences in the reactions produced by tissues from the remaining animals in the 100 to 250 pound

TABLE VI

Influence of the size of pigs on the diffusion of antigen from hog cholera infected tissues

Size of pig	Number of pigs	Days post-inoculation	Days of pyrexia	Reactions Pos.	Ques.	Negative
pounds		range	range			
100-150	4	7 — 8	6 — 8	4	0	0
160-200	8	5 — 8	4 — 8	4		4*
210-250	4	6 — 8	5 — 8	3		1**
300 + Adult sows	8(a)	5 — 11	2 — 7	1	2†	4***

*All four pigs died.
**5 days low grade fever.
***One sow died 5 days post inoculation.
(a)Tissues from one sow unsatisfactory for test.
†Very weak questionables.

range. However, there was a striking difference in the results obtained with tissues from adult sows. Of this group, only one positive result was observed with a frozen stored sample harvested nine days post inoculation from a sow with seven days high fever and showing post-mortem lesions typical of hog cholera. Of the other six specimens two showed very weak questionable reactions and four gave negative results.

The observations made from series 2 confirmed the indications of series 1. The most important factors for obtaining consistent positive results in the immunodiffusion test were the alternate freezing and thawing of fresh spleen tissue from pigs of less than 250 pounds weight with advanced clinical manifestations of hog cholera.

Discussion

The results of the present study support the conclusions drawn previously (1) that the precipitation reaction obtained in the agar diffusion test was due to the hog cholera virus in the infected tissue. The specificity of the reaction was further substantiated by the absence of reaction with all the tissues from normal animals and those infected with the five other agents. This observation was also supported by the precipitation-inhibition test. The immune globulins absorbed with hog cholera infected tissue did not react with the positive control spleen antigen, whereas those absorbed with tissues from the five other diseases and the tissues from normal swine exhibited no inhibition and reacted in the test with the control viral antigen.

In addition to the conditions outlined in the previous paper, the sensitivity of the test was found to be dependent on the proper treatment of the tissues for the release of antigen. While little antigen was released by fresh, unfrozen tissues, slightly more was obtained from tissues frozen and thawed once. Repeated experiments indicated that four alternate freezings and thawings of specimens frozen for 18 hours gave the optimum diffusion.

The clinical state of the animal from which tissues were collected greatly influenced the results of the tests. It was found to be important that animals show gross clinical manifestations of the disease in order to obtain an optimum amount of antigen. The best results were observed when pyrexia and sickness had been present for six to eight days before the harvest of material. However, since tissues from infected breeding sows, pigs of over 250 lbs. or dead animals tended to give negative results, these should be avoided in the test.

The 75 per cent or more positive reactions obtained indicated that the test was a valuable diagnostic tool for hog cholera when used on a herd basis. It was most reliable when several samples were collected from each group of infected pigs.

ACKNOWLEDGMENTS

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An Outbreak of Visceral and Cerebral historiosis in a Flock of Sheep in South East England

Sixty out of 360 lambs and 3 out of 280 ewes died in an outbreak of listeriosis. The ewes had nervous symptoms and the lambs, 2 to 7 days old, appeared dull, some were scouring and all were losing condition. The outstanding post-mortem lesion in the lambs was "sawdust" liver and histeria monocytogenes was regularly isolated from all internal organs but never from a brain. Histological examination revealed typical lesions of cerebral listeriosis in one ewe while in the lambs numerous foci of necrosis were found in the liver, spleen and gastro-hepatic lymph nodes.

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