Hog Cholera I. Investigation 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 investigated to detect hog cholera virus in splenic and pancreatic tissues of swine. Special attention was given to the conditions influencing the sensitivity and specificity of the test. These studies emphasize the strict techniques and methods required in the test in order to detect specific antigen — antibody reaction. Absorption studies performed on serum fractions prepared by DEAE cellulose chromatography and studied electrophoretically, indicated the specific reaction was given by fractions, migrating in the gamma-globulin region and was absorbed by infected but not by normal spleens. The sensitivity of the test is dependent to a great extent on the successful liberation of the viral antigen from the tissue.

RESUME

L'épreuve de précipitation par diffusion dans l'agar fut employée pour déceler le virus de la peste porcine dans la rate et le pancréas de porcs infectés. Une attention spéciale fut apportée aux conditions qui influencent la sensibilité et la spécificité de l'épreuve. L'accent fut mis sur les exigences rigoureuses de l'épreuve en vue de démontrer la réaction spécifique antigène-anticorps. L'absorption des fractions de sérum préparées par chromatographie et étudiées par l'électrophorèse a indiqué que la réaction spécifique était due aux fractions dont la mobilité s'identifiait à celle des gamma-globulines. Ces réactions sont absorbées par les tissus de rate infectées mais non par les tissus normaux. La sensibilité de l'épreuve est dépendante en grande partie du succès obtenu dans la libération de l'antigène des tissus.

In Canada, hog cholera is controlled by the eradication of the disease through a slaughter policy. The field diagnosis is based on the herd history, the clinical and the post-mortem findings. For confirmation at the laboratory, known susceptible and immune swine are inoculated with suspected material. These experimental animals must be kept in well isolated units to prevent the spread of infection, and under observation for up to 6 weeks before final results are available. Such a procedure is costly and time consuming. Consequently, a reliable *in-vitro* test is desirable.

Various diagnostic tests, none of which have been generally adopted, have been described in the literature. Among these are the tissue culture methods described by Gillespie (1), Janowska (2) and Kumagai (3), the agglutination and the haemagglutination tests of Segre (4-5), the conglutination test investigated by Millian (6), the gel diffusion precipitation test recommended by Mansi (7) and Darbyshire (8), the amylase and haemolytic tests described by Taylor (9), and, more recently, the fluorescent antibody technique investigated by Mengeling (10), Stair (11) and Solarzano (12).

Preliminary work was conducted in this laboratory with the majority of the above techniques, the results of which led us to believe the agar gel double-diffusion precipitation test was among those most likely to serve as a practical diagnostic tool. The present report is an evaluation of Darbyshire's method (13) and points out our preliminary modifications made to increase

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the sensitivity of the test. Subsequent reports will deal with the specificity and applicability of the method to differentiate hog cholera infected tissues from normal ones and those derived from swine infected with diseases other than hog cholera.

Materials and Methods

In the present study, an attempt was made to follow as closely as possible the method described by Darbyshire (8, 13). The modifications considered were incorporated in the method only after they increased the sensitivity and specificity of the test.

Diffusion Medium

The medium described by Darbyshire is an isotonic, 0.01 M phosphate-buffered, 1.5 per cent agar of pH 7.0 - 7.2 containing 0.5 per cent phenol to prevent bacterial contamination. In our media, of the various concentrations of agar tried, 1.0 per cent proved best. The medium finally adopted contains the following: "Oxoid" ion agar 10 grams, sodium chloride 8.5 grams, sodium phosphate anhydrous (Na_2HPO_4) 1.42 grams, sodium phosphate monobasic ($NaH_2PO_4.H_2O$) 1.56 grams, melted phenol 5 ml., glass double distilled water sufficient to make up 1 liter volume. The salts are dissolved in farm water, the pH adjusted to 7.3 then the agar is added and the mixture sterilized at 10 pounds pressure for 15-20 minutes. The warm mixture is filtered through sterile, coarse filter paper, the warm melted phenol is then added and the pH readjusted to 7.3 if necessary. The medium is, while still warm, poured into flat-bottomed, disposable, plastic petri dishes, in 10 ml. amounts. It is important to avoid remelting of the agar. The plates are stored in a moist atmosphere at 9°C. in a closed container.

Immune and Normal Sera

Three hog cholera antisera (Nos. 10, 309 and 310) were prepared and a commercially prepared serum was imported from a United States manufacturer. A small amount of dessicated control antiserum was also received through the courtesy of Dr. J. H. Darbyshire, Central Veterinary Laboratory, New Hay, Weybridge, England. The normal sera were obtained either from "specific pathogen-free" swine or from normal swine killed at a local slaughter house.

No. 10 antiserum. A pre-inoculation blood sample was collected from an adult pig, No. 10, which was then given an intramuscular inoculation of 2 ml. of a commercial lapinized hog-cholera vaccine. It was challenged intramuscularly 4 times at 2 week intervals with 2 ml. of virulent blood. Two months later this animal was given 800 ml. of virulent blood subcutaneously. Two weeks after the last inoculation, it was exsanguinated and the serum stored frozen.

Nos. 309 and 310 antisera. Pre-inoculation bleedings were taken from two "specific pathogen-free" swine, Nos. 309 and 310, which were then vaccinated intramuscularly with lapinized hog cholera virus consisting of defibrinated virulent rabbit blood. Two and 4 weeks post-inoculation, both were challenged, intramuscularly, with 3 ml. of hog cholera virulent swine blood. On the 10th and 17th day post challenge a normal pig inoculated intramuscularly with 5 ml. of virulent blood was placed in contact with them as a means of further exposure. Twenty seven days after introduction of the second contact animal, serum was harvested from both swine 309 and 310 and stored in the frozen state.

Concentration of Globulins

Ammonium sulphate precipitation. The serum globulins were precipitated in the cold by the addition of saturated ammonium sulphate to give either a 30 or 50 per cent final concentration as described in a previous paper (14).

Fractionation with DEAE cellulose column. The serum was dialysed in the cold for a 24 hour period against 0.002 M phosphate buffer, pH 7.0, then for another 18 hour period against 0.0175 M phosphate buffer, pH 6.3. After each dialysis, any precipitate formed was removed by centrifugation. The column was prepared with a packed volume of at least 2.5 ml. of diethylaminoethyl cellulose (DEAE) per ml. of serum to be fractionated, then equilibrated with 0.0175 M phosphate buffer pH 6.3. The serum sample was applied to the top of the column followed by 0.0175 M phosphate buffer pH 6.3. The first effluents, equal to about half the bed volume of the column, were discarded; thereafter 10 ml. amounts were collected until about two bed volumes of effluent were obtained. The macroglobulins and non-gamma-globulin serum proteins remained in the column under these condi-

tions. Thirty six of the eluates collected were tested electrophoretically on a Shandon Universal Electrophoretic Apparatus with a Vokam power pack. Cellulose acetate strips 2.5 by 12 cm. were placed over an 8 cm. gap and "Tris" buffer pH 8.98 of 0.1 ionic strength was used. The serum sample, 2.5 to 3 lambda, was placed at an origin of 4 cm. from the cathode. The run was for a 2 hour period in a current kept constant at 6 mA per 6 strips. The strips were then dried in an oven and stained with 0.2 per cent Ponceau S in 3 per cent trichloroacetic acid. The strips were decolourized in a 5 per cent acetic acid solution. The chromatography conditions firstly, and the information gained from the electrophoresis, determined which fractions should be pooled to give the final fractions. The pooled fractions were stored either in the frozen state, lyophilized or in a 3 per cent sodium chloride solution at 9°C. The composition of the final pool was also studied electrophoretically in comparison with the original hog cholera immune serum and a normal serum control. In this instance, in order to facilitate the examination of the so-called gamma-globulin region, the test was made with veronal acetate buffer, pH 8.2 of 0.05 ionic strength. All other conditions were the same as before with the exception of an experimental shift in the position of the origin from 4 cm. to 6 cm. from the cathode.

Tissue Antigens

The spleen was the tissue of choice in most of our study, although pancreas was used at first because Darbyshire had shown it gave optimum reaction. The tissues investigated originated from the following sources: —

(a) *Swine inoculated intramuscularly with positive field specimens.* Of 68 animals in this group, tests were conducted on pancreas and spleen in 36 cases; on spleen only in 26 cases, on pancreas only in 6 cases.

(b) *Swine inoculated intramuscularly with laboratory strains of hog cholera virus.* Of 17 animals in this group, tests were conducted on spleen and pancreas in 5 cases, on spleen only in 4 cases and on pancreas only in 8 cases.

(c) *Field specimens positive on clinical, post-mortem observations and on contact history.* Tests were performed with the spleen alone from 93 swine in this group. The presence of hog cholera in these animals was established on the basis of the clinical manifestations supported by the evidence of contact with herds where hog cholera was diagnosed recently.

(d) *Experimental swine fed infected hog cholera tissue.* The spleen and pancreas from all 42 animals in this group were submitted to the test.

The tissues, unless otherwise specified, were kept in the frozen state until used in the test.

Procedure of the Test

On the day of a test, the basins or wells were cut in the agar with a cork borer, having under the petri dish a pattern consisting of seven holes spaced at equal distance. For the pancreatic tissue, the basins had a diameter of 5 mm. and were spaced 2 mm. apart. In the case of splenic tissue, due to the diffusing haemoglobin, 7 mm. basin were preferred and they were spaced 4 mm. apart. The cut agar plugs were removed by vacuum and the bottom of each basins sealed with a minute drop of melted agar. The unknown antigen, which consisted of pulp, cut or scraped by a scalpel blade from the soft part of the tissue, was packed in wells 2, 4 and 6. (Figure 1). The pulp from the known positive tissue was packed in the centre well. Two hours later, basins 1 and 3 were filled with immune serum or preferably its globulin concentrate. The normal serum control or its globulin concentrate was used to fill well 5. The plates were incubated at room temperature in a petri dish sterilizing box

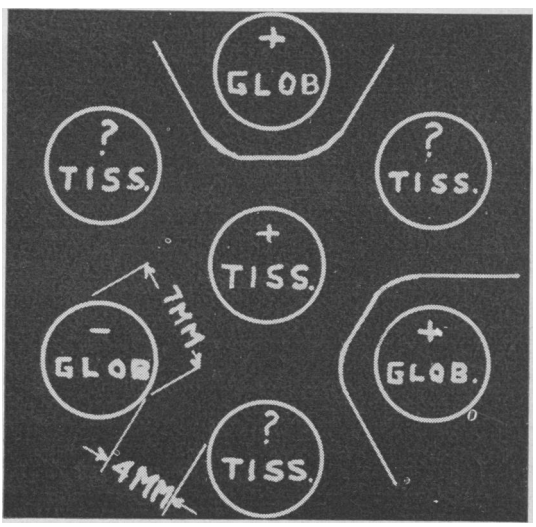

Fig. 1. Distribution of the reagents in the agar double diffusion precipitation test.

containing a small amount of water to maintain a moist atmosphere. After 18 hours, the plates were examined and, when the reactions were not sufficiently developed, the basins containing the sera were refilled and the test reincubated another 24 hours.

Before reading, the plates were washed with 10 per cent formalin to remove the tissues and residual serum globulins from the basins. The reading was made either over a box specially constructed to supply a good source of indirect illumination, or with the help of a Quebec Colony counter. A magnifying glass often was of assistance in viewing the fine precipitation lines. A typical reaction is shown in Figure 2. A sharp line of precipitation formed between the basin containing the known-positive control tissue and the corresponding immune sera. When the unknown tissue in the basins also contained the virus, a precipitation line formed between these tissues and the immune sera. These lines together with the one from the positive tissue control took a U-shaped appearance.

Modification of Procedure

In addition to the modifications referred to above such as 1 per cent agar, glass-double distilled water, flat bottomed petri dishes, immune serum concentrate, and the use of spleen as antigen instead of pancreas, the major changes were in the treatment of the antigen. Tissues were tested freshly harvested as well as after being frozen for various periods. In order to further liberate the antigen, they were also maintained at 37°C. for 18 hours or at 9°C., for 4 days. Tissues were not homogenized but in the case of the spleen, the pulp was scraped with a scalpel blade whereas in the case of pancreas the fat surrounding the glandular tissue was omitted from the sample.

Results

Results from the preliminary work performed 4 years ago with the agar double-diffusion precipitation method, made us believe that the test was not reliable for the diagnosis of hog cholera. As pointed out by various workers (15-19), it was observed that extracts from some normal pancreatic tissues as well as those from infected tissues produced bands of cloudiness limited by clear zones which simulated broad lines of precipitation.

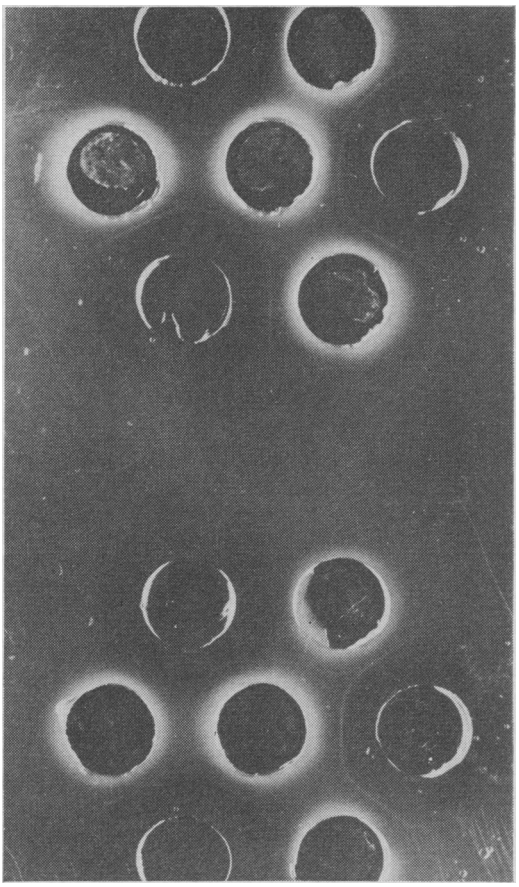

Fig. 2. Typical precipitation lines formed between hog cholera containing tissues and the corresponding immune serum.

Over a year ago we became aware of the results of the Weybridge workers. Darbyshire (13) in a monograph on the diagnosis of hog cholera, pointed out some conditions that must be observed to obtain a specific, typical, precipitation reaction. When our preliminary work was repeated using this information, we observed that strict requirements must be met for the test to behave as described by the British workers. For example, the extraction of tissue resulting in dilution should be avoided. Homogenization of the tissue resulting in a 1:5 or greater dilution, as is done by many workers, eliminates the reaction. Also, the space between the diffusion wells must be kept to a minimum. In addition, the wells containing the globulins should be refilled only once. The reading should be made after 18 hours or at most 42 hours after incubation at 22°C. When the test was kept for a longer period, bands

Table 1 — Reaction with tissues from field cases of hog cholera, and laboratory passage of field cases and standard strains of virus

Spleen		Pancreas	
No. tested	Reactions	No. tested	Reactions*
41	32	41	17
123	80	—	—
—	—	14	7
164	112(68.1%)	55	24(43.5%)

*No reaction was given by these tissues when tested with normal globulin.

Table II — Reactions with tissue from swine experimentally infected by feeding virulent hog cholera tissues

Treatment of tissue	Spleen		Pancreas	
	No.	Reactions	No.	Reactions
Fresh	42	4±(9.5%)*	42	0(0.0%)
18 hrs. at 37°C.	42	22(52.3%)	41	7(17.1%)
96 hrs. at 9°C.	42	3±(7.1%)	42	2(4.7%)
Frozen	42	22(52.4%)	42	9(21.4%)
18 hrs. at 37°C.	42	37(88.1%)	42	4(9.5%)
96 hrs. at 9°C.	42	32(76.2%)	41	17(40.5%)

*±: incomplete or weak reactions

of cloudiness made their appearance. These obscured the typical reaction and sometimes even simulated precipitation lines which could confuse inexperienced observers. Finally, a selected reactive tissue control must be incorporated in the test. In addition to serving as a control, this known reactive tissue sometimes enhanced and boosted the reaction of the unknown. It also permitted the observation of the continuity of the lines which indicated the homogeneity of the antigen in the unknown and the known positive control.

At first, a considerable number of difficulties were encountered in obtaining a reaction with pancreas from known infected animals. Of all the modifications attempted in an effort to overcome this difficulty, it seemed that a few, such as the concentration of the serum antibodies by ammonium sulphate precipitation, the decrease of agar in the diffusion medium to 1 per cent concentration, the addition of the antigen to the basins 1 or 2 hours prior to the serum and the use of splenic instead of pancreatic tissue as antigen, slightly improved the test. The appropriate treatment of the tissue leading to the release of the antigen had the greatest influence on the resulting reaction.

Table I gives the results obtained on tissues from field cases of hog cholera, from swine inoculated with positive field specimens, or from swine challenged with laboratory strains of hog cholera virus. All tissues were stored in the frozen state for various intervals and representative samples tested immediately on thawing. In 41 cases, both the spleen and the pancreas from the same animal were tested and 32 spleens and 17 pancreases gave typical reactions. In 123 cases, only the spleens were tested and 80 of these reacted, whereas in 14 cases only the pancreas was tested and 7 of these reacted typically. Out of the 164 spleens tested in this first trial, 112 or 68.1 per cent and 24 or 43.5 per cent of the 55 pancreases reacted. None of the tissues in this series gave a reaction with the globulin fraction of normal serum. Furthermore neither the 42 spleens nor 32 pancreases collected from normal swine at a local slaughter house reacted with the globulin fraction from normal or immune sera. During the course of this study, it was suspected that the failure of some tissues to give a reaction was due to an insufficient liberation of the antigen from the specimen.

In the next series, 42 swine were exposed to hog cholera by feeding infected tissues in order to simulate as closely as possible field conditions. The animals were killed from the fourth to the tenth day following rise in temperature. In general, the animals were killed when showing one or more of the following clinical manifestations: lacrymation, anorexia, trembling, crowding, incoordination, posterior paralysis, slight cyanosis and some diarrhea. The fresh tissues were tested on the day of collection. Representative samples were also allowed to autolyse for 18 hours at 37°C. and for 96 hours at 9°C. before being tested. Duplicate tissue samples were frozen on the day of collection and upon thawing they were tested as above, following autolysis. The results of this series are given in Table II. The test performed on fresh tissues on the day of collection from the animals gave only a few reactions indicating poor liberation of the antigen. If the tissues were allowed to autolyse for 18 hours at 37°C., 52.3 per cent of the spleens and 17.1 per cent of the pancreases

reacted. Only a few reactions occurred when the tissues were allowed to stand at 9°C. for 96 hours indicating either a poor liberation of the antigen or its denaturation. None of the tissues submitted to the various treatments reacted with the globulin from the normal control serum.

The frozen portion of the same spleens and pancreases when tested immediately on thawing gave 52.4 per cent and 21.4 per cent reaction respectively. This indicated that the freezing of tissues activated the release of the antigenic material. The frozen spleens, when allowed to autolyze for 18 hours at 37°C., or 96 hours at 9°C., gave 88.1 and 76.2 per cent reaction respectively. However, 11 spleens (26.6 per cent) and 4 pancreases (9.5 per cent) gave a reaction with normal serum globulin, which, even though often atypical could be confusing. These atypical reactions were not obtained when the tests were repeated with tissue from the same origin. From this experiment it became apparent that the breakdown of the cells is beneficial, but bacterial putrefaction should be avoided. With this in mind, the effect of repeated freezing and thawing on the release of antigen from tissue was investigated. It was observed that four consecutive freezings and thawings released the antigen from the majority of tissues. This procedure seemed the best method of antigen extraction for the agar gel diffusion test as will be reported in the next paper on specificity and sensitivity of the test.

In order to determine if the precipitation reaction observed was the result of a hog cholera specific antigen — antibody reaction, a series of absorption studies were conducted with known reagents. Small samples of normal pig serum and immune hog cholera serum concentrates and globulin fractions were absorbed with 10 known normal and 10 known hog cholera infected spleens. One gram of wet tissue was used to absorb 1 ml. of serum. This suspension was shaken overnight at 9°C. then centrifuged for 15 minutes at 2000 RPM in an International centrifuge Model SBV, size 1. The absorbed sera were then tested in parallel with non-absorbed portions as outlined previously. The precipitating activity was removed by the infected spleens but not by the normal, suggesting a specific reaction between hog cholera virus and its homologous antibody.

Figure 3 demonstrates the electrophoretic

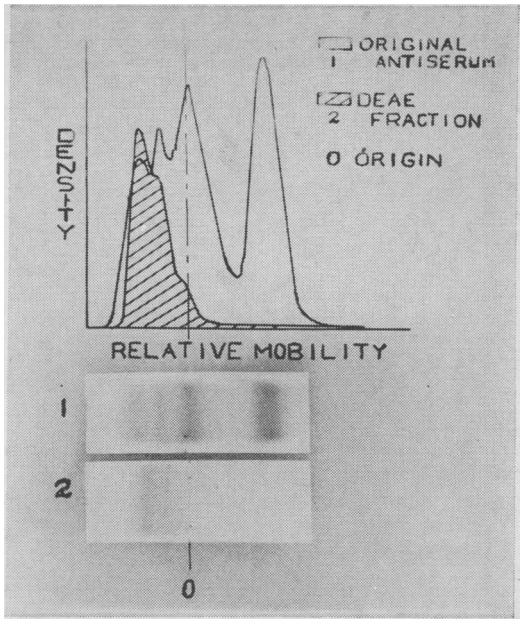

Fig. 3. Protein electrophoretic pattern and densitometer tracing of the original hog cholera antiserum and the corresponding DEAE cellulose fraction containing precipitating activity.

pattern of the original hog cholera antiserum and the corresponding DEAE cellulose pooled fraction containing the precipitating activity which was absorbed by known infected spleens. The electrophoretic pattern of the hog cholera antisera was found to be comparable to that of normal pig sera. The DEAE cellulose fraction of the hog cholera antisera showed upon electrophoresis only two bands which were located towards the cathode in the region corresponding to that of the gamma-globulins. The non-gamma-globulin fractions tested were devoid of precipitating reactivity.

Discussion

Conflicting results have been obtained by workers using the agar double-diffusion precipitation test for the detection of hog cholera viral antigen. Many failed to demonstrate a specific hog cholera antigen as observed by the British workers (7-8). The failures are in part attributable to the fact that most workers apparently did not recognize the necessity for complying with the strict requirements governing the test. Purification procedures resulting in dilutions of the extract are among the most common sources of failure. Homogenization resulting in a 1:5 or greater dilution, as is often done, eliminates the reaction. Hog

cholera virus is a lipovirus which is destroyed by extraction with ether, chloroform, etc. (20), consequently it is necessary to avoid chemical purifications which have been effective with other groups of viruses. The reading of the reaction should be made after 18 hours or at most 42 hours after incubation at 22°C. The wells containing the globulins should be refilled only once. When attempts are made to incubate the test for longer periods bands of cloudiness make their appearance. These bands are not associated with the viral antigen but are due to other diffusing material. They obscure the typical reaction sometimes even simulating precipitation lines which can confuse inexperienced observers. This was particularly noticeable with the pancreatic tissue.

The percentage of reactions obtained in this preliminary study may help to establish a diagnosis on a herd basis provided specimens from three or four animals from the same herd are submitted. Evidence was obtained which indicated that the sensitivity of the test, was among other factors, dependent on the release of the antigen from the tissue. Very little antigen was released by fresh, unfrozen tissue; one freezing liberated the antigen in a greater percentage of cases. However, putrefaction of the tissues should be avoided.

Under the conditions of the test in the present studies, the reactions observed were related to the interaction of a tissue antigen connected with the hog cholera virus and the gamma-globulin fraction of the immune sera. Typical reactions were blocked by absorption of the gamma-globulin fraction of the immune sera by infected spleens but not by spleens derived from normal swine.

The next paper will deal with the sensitivity and specificity of the test making use of an improved method of freezing and thawing to liberate the tissue antigen. Special attention will be given to the value of the test in differentiating between tissues derived from hog cholera infected pigs, and those collected from normal animals or animals suffering from various viral or bacterial infections.

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