EVALUATION OF FLUORESCENT ANTIBODY TECHNIQUE FOR THE DIAGNOSIS OF HOG CHOLERA

by

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INTRODUCTION

Hog cholera (HC) was first recognized in the United States in 1833. However, it was not until 1903 that its viral etiology was discovered. Since that time there has been a definite need for a readily acceptable *in vitro* diagnostic test. Although a presumptive diagnosis can often be made on clinical signs and lesions, these are occasionally too indefinite to be of value. Additional confusion arises because the lesions of several other swine diseases may closely resemble those of HC. Moreover, when HC is caused by a virus of relatively low virulence, the signs and lesions are often atypical. These difficulties emphasize the desirability for laboratory confirmation of all tentative clinical diagnoses.

Two events have added considerable impetus to investigations of HC diagnostic tests. In 1957, African swine fever (ASF) spread from Africa to Southern Europe. Although ASF is presently exotic to the United States, its spread increases the threat of its introduction here. Since the lesions of ASF closely resemble those of HC, a method for a rapid differential diagnosis is imperative. In 1962, a HC-eradication program was implemented in the United States. A reliable means of diagnosis is essential for the program to be carried out most effectively.

Numerous *in vitro* tests have been proposed for the diagnosis of HC; however, it is apparent from reviewing published reports that these techniques are either inherently complex or have been subjected to criticism in regard to their reliability or have not been sufficiently investigated as to their practical diagnostic efficacy.

Since the fluorescent antibody technique (FAT) is in the latter category above, it was obvious that a critical evaluation of the test was required before recommendations could be made for its routine use to confirm clinical diagnoses of HC. Consequently, the following study was initiated to determine the reliability of FAT and the technical considerations necessary for its optimum performance when tissue samples from suspected naturally-occurring cases of HC are submitted to the diagnostic laboratory.

REVIEW OF LITERATURE

Three general types of *in vitro* tests, which depend on the identification of HCV or related antigens, are presently being used in various laboratories to confirm clinical diagnoses of naturally-occurring HC. These are agar gel diffusion (AGD), viral interaction (VI), and the FAT.

Agar gel diffusion is a serological test in which antigen and antibody are initially separated by agar gel. The reactants subsequently diffuse toward one another. At a place where their optimum relative concentration is reached, a visible precipitate is formed. The AGD method of Ouchterlony (1) was first used to identify HC-viral antigens by Molnar (2). He observed that precipitation lines were formed when tissues from HCV-infected swine were allowed to react with HC-immune serum; whereas, lines were not formed when tissues from uninfected swine were reacted against HC-immune serum. Subsequently, numerous other investigators (3-16) generally concurred with Molnar's findings; however, alterations in the technique were proposed and there was disagreement in some instances as to the most satisfactory tissue to be used for the test. In contrast, Janowski and Truszczynski (17), Janowski (18), and Pirtle (19) questioned the reliability of AGD. They observed that precipitation occurred when either HCV-infected or uninfected tissues were allowed to react with either HC-immune or nonimmune serum. Moreover, Pirtle (19) reported that precipitation lines were formed more frequently when uninfected tissues were allowed to react with either HC-immune or nonimmune serum than when HCV-infected tissues were reacted with the same sera. Based on the above

evidence, it was his opinion that AGD was not specific and therefore unsatisfactory as a diagnostic test. Pirtle (20,21) suggested that the nonspecific reactions which he observed could have been caused by tissue proteins which were not associated with HCV infection and that AGD might find application if a specific antigen could be obtained. As a consequence, he isolated and semi-purified a HCV-associated soluble antigen produced in infected swine kidney cell cultures. He was then able to employ this soluble antigen in AGD to identify homologous antibody in sera of swine previously exposed to HCV. When the semi-purified antigen was used, nonspecific reactions were not observed. A comprehensive review of the application of AGD to HC diagnosis has been published (22).

Many viruses have a characteristic cytopathic effect (CPE) in cell cultures. The presence of a particular virus can be confirmed by neutralization with homologous immune serum. Unfortunately, HCV, with one exception (23), is not cytopathogenic. Therefore, several indirect methods involving a second, i.e., an indicator virus, have been investigated for the detection of HCV infected cell cultures, and thus HCV itself. Viral interaction is evidenced by the alteration of a characteristic cellular response to infection with one virus caused by prior infection of the cell with another virus.

An ingenious technique was developed by Kumagai et al. (24). They found that the CPE of the Miyadera strain of Newcastle disease virus (NDV) on swine testicular cell cultures was exalted, in regard to the time it was first observed, if the cells were already infected with HCV.

The technique has been called the exaltation of Newcastle disease virus (END) method. Since its inception, the END method has been thoroughly investigated and, notwithstanding some minor technical difficulties that might be initially encountered in its application, has been proven reliable for the detection of HCV and homologous antibody (25-31). Matumoto et al. (26) applied the END method to the diagnosis of HC. Thirteen of 17 naturally occurring cases of HC, diagnosed by clinical and histopathologic examinations, were confirmed by the END method. Two additional cases were confirmed by a 2-step procedure wherein tissue suspensions are first inoculated onto swine spleen cell cultures. After allowing HCV to propagate in the cell cultures, the growth medium is examined by the END method. Hog cholera virus was not identified from tissues from the remaining 2 cases even though 1 of these was subsequently confirmed by swine inoculation.

Loan (32,33) suggested certain alterations in the basic technique for conducting the END method. The application of these changes would both simplify the method and increase its sensitivity 25-100 fold.

The importance of the strain of NDV employed as the indicator virus and the kind of cell cultures used is emphasized by the research conducted by Nishimura et al. (34). When HCV-infected swine kidney cell cultures were superinfected with the Sato strain of NDV, a different phenomenon was observed. The CPE of the Sato strain of NDV was not exalted, but suppressed; however, the yield of NDV associated hemagglutinin from dually infected cells was increased.

In addition to NDV, several other viruses such as Teschen disease virus (35), influenza virus (36), bovine viral diarrhea virus (37), poliovirus, and foot-and-mouth disease virus (38,39) have been used as indicators of HCV-infected cell cultures.

The FAT is a serological test wherein antibody is conjugated with a fluorescent dye. After allowing antigen and antibody to form a stable union with each other, the antigen is indirectly identified by microscopically examining the specimen with ultraviolet or near-ultraviolet light. An area of concentrated fluorescence indicates the presence of antigen.

This technique, developed by Coons et al. (40,41), was first described in 1941 and has since been successfully employed for the diagnosis of several viral diseases (42). Only recently, however, has the FAT been successfully used to identify HC-viral antigens. Two approaches have been made to apply this technique to the diagnosis of HC. Tissues from swine suspected of having HC have been examined directly for the presence of HCV-infected cells. A second approach has been to inoculate blood, serum, urine or tissue suspensions onto cell cultures and after an appropriate period of time to allow intracellular viral replication to occur, to examine the cultures for the presence of infected cells. This latter approach will subsequently be referred to as the fluorescent antibody-tissue culture test (FATCT) to distinguish it from the former which will be designated as the direct fluorescent antibody test (DFAT).

Sirbu et al. (43) used the DFAT to identify HC-viral antigens in cells of impression smears prepared from lymph nodes of HCV-infected swine. Infected cells were observed in lymph node impression smears of 24 of 27 (91.1%) experimentally infected swine and of 5 of 5 naturally infected swine.

Stair et al. (44) and Aiken et al. (45,46) reported that the DFAT could be used in conjunction with either tissue impression smears or tissue sections (cryostat technique) to identify HCV-infected cells. Results obtained with both methods compared favorably under most conditions. Limited experiments indicated that antigens associated with attenuated HCV could only be identified in tonsillar tissue. In addition, antigens associated with virulent HCV were identified in tonsillar tissue earlier after HCV infection than in other tissues. Therefore, tonsils were considered the tissue of choice. Since impression smears prepared from tonsils were not consistently satisfactory, the cryostat technique was suggested as the method of choice.

Solarzano (47) successfully applied the FATCT to identify cell cultures infected with HCV present in urine, blood and serum from HCV-infected swine. Mengeling et al. (48,49) used the FATCT to demonstrate HCV-virus infected cells of a chronically infected cell line and cells infected with HCV isolated from blood and spleen from swine affected with HC. Robertson et al. (50) investigated the FATCT and reported that it appeared to be a highly reliable method for the diagnosis of HC.

Karasszon and Bodon (51) found the indirect FATCT suitable for the identification of HC-viral antigens. The indirect method is a 2-step procedure. It requires labeled (fluorescent) antibody prepared for globulin of the species used to produce antibody for the antigen in question. In the first step, antigen and its homologous unlabeled antibody are allowed to react. In the second step, labeled anti-globulin is added. Consequently, the unlabeled antibody functions as antibody in the first reaction and antigen in the second reaction. Korasszon and Bodon used unlabeled rabbit anti-hog cholera sera and fluorescein labeled goat anti-rabbit globulin.

It would be advantageous if the reliability of the preceding 3 general types of in vitro tests could be compared, but such a comparison is difficult for several reasons. For example, Darbyshire (22) found that 36% of pancreases from HCV-infected pigs gave positive AGD reactions, whereas no reactions were observed with tissues from healthy pigs or with tissues from 1,032 pigs affected with a variety of other diseases. Therefore, one would conclude that AGD is not highly sensitive but nevertheless reliable from the standpoint that no false positive reactions were observed. On the other hand, Pirtle (19) obtained more reactions with pancreases from healthy swine than with those from HC-affected swine. Moreover, neither the FAT nor VI have been sufficiently investigated in relation to HC diagnosis to confidently state their reliability, but it is significant that, as yet, no false positive reactions have been reported for either type of test.

Several other *in vitro* tests have been proposed for the identification of HC-viral antigens and homologous antibodies. Segre (52,53) coated both resins and erythrocytes with HC antibody. Agglutination of the antibody-coated particles occurred in the presence of viral antigen. Segre (54) also demonstrated that HC virus could be identified by its CPE on cell cultures incubated under increased oxygen pressure. Boulanger *et al.* (55) were able to detect HC-viral antigens by complement fixation. Hog cholera antibody has been identified by conglutination complement adsorption, Engelhard and Millian (56), and Millian and Engelhard (57), and by serum neutralization, Coggins and Baker (58). None of these tests have as yet received general application for HC diagnosis. However, the identification of HC antibody may become increasingly important in the HC eradication program in the United States, particularly in epidemiology.

Three procedures have been developed for demonstrating HCV-infected tissues based on biochemical alterations of the tissues. These are the amylase and hemolytic tests of Taylor (59) and the chemical color test of Goncharov *et al.* (60). Results of investigations by Gray *et al.* (61) indicate that neither the amylase nor the hemolytic test is sufficiently reliable for HC diagnosis.

Histopathological changes in the brain and the occurrence of a leukopenia in swine affected with HC have been used as diagnostic aids. Neither can be considered as definitive proof of HC, but in practice both have been generally reliable, particularly when several swine are examined. The application of these methods has been reviewed by Dunne (62).

GENERAL APPROACH TO THE EVALUATION OF THE FLUORESCENT ANTIBODY-TISSUE CULTURE TEST

Tissues from suspected naturally occurring cases of HC were examined by the FATCT. Results thus obtained were compared to those obtained by the inoculation of HC-susceptible swine with a portion of the same tissues. Although swine inoculation is too expensive for routine use, it is the most reliable method for HC diagnosis and was therefore used to determine the reliability of the FATCT. The inoculation of HC-susceptible swine with field isolates of HCV also allowed for the determination of the relative virulence of these isolates.

For the purpose of clarity, the methods and results of the FATCT and swine inoculation are first presented separately and then the results of each are compared.

PART I. FLUORESCENT ANTIBODY-TISSUE CULTURE TEST

MATERIALS AND METHODS

Preparation of Fluorescent Antibody

Swine anti-hog cholera serum (anti-HC serum)

Three anti-HC sera were used to prepare fluorescent antibody. Anti-HC serum 9197 was produced in a farm-raised pig of mixed breeding. Initially, the pig was vaccinated on the farm of origin with crystal violet hog cholera vaccine. Eight months later it was purchased and brought to the National Animal Disease Laboratory (NADL) where its immunity was challenged by subcutaneously injecting 2 ml of swine blood containing the virulent Ames strain of HCV* (Ames HCV). Following a slight reaction to challenge, a transient leukopenia and temperature rise, the pig recovered and 1 month later was injected intravenously with 875 ml of swine blood containing Ames HCV. Exsanguination was performed 2 weeks later. The whole blood was allowed to clot and the serum obtained was clarified by centrifugation for 30 min at 2,000 x g**, bacteriologically sterilized by filtration and stored at 4 C.

A commercial anti-HC serum was made available by Dr. C. E. Phillips.*** The serum had been specially prepared from clotted whole blood rather

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*Lethal strain of HCV maintained at the NADL by serial passage in swine.

**All centrifugations unless otherwise specified were made with an International PR-2 refrigerated centrifuge.

***Dr. C. E. Phillips, NADL, Ames, Iowa.

than the usual commercial method of mechanical defibrination, followed by the addition of bean extract and NaCl. Swine anti-HC serum 7185 was kindly supplied by Dr. J. P. Torrey.*

Serum fractionation

Globulins, primarily gamma, were obtained from swine serum by the dropwise addition of 1/2 volume of a saturated solution of ammonium sulfate** at room temperature to 1 volume of swine serum at 4 C. During the addition of ammonium sulfate the serum was kept chilled by an ice bath and was constantly agitated with an electric stirrer. Stirring was continued for 10 min after ammonium sulfate had been added. Globulins were then sedimented by centrifugation for 15 min at 2000 x g. Supernatant fluid was carefully decanted from the sedimented globulins and the precipitate restored to the original serum volume by the addition of cold distilled water.

The fractionation procedure was repeated two additional times, with the exception that in the final step sufficient distilled water was added to bring the solution of globulins to only 1/2 the original serum volume.

Redissolved globulins were freed of residual ammonium sulfate by dialysis for 1 hr at 4 C against 0.85% NaCl (100 volumes saline/volume globulin solution), followed by 2 additional 2 hr dialyses against fresh saline. Fluid outside the dialysis tubing was constantly agitated with an

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*Dr. J. P. Torrey, NADL, Ames, Iowa.

**542 grams ammonium sulfate/liter.

electric stirrer. Insoluble material present after dialysis was removed by centrifugation at 2000 x g for 15 min.

Protein determination

Protein concentrations were determined spectrophotometrically by the biuret method (63) at a wave-length of 540 mμ utilizing a Coleman Jr. spectrophotometer.* Two ml of a 1:6 dilution of a globulin solution in 0.85% NaCl was mixed with 8 ml of biuret reagent and incubated at room temperature for 30 min before determining its optical density. A blank was prepared with 2 ml 0.85% NaCl and 8 ml of biuret reagent. The optical density of the unknown was compared to that of known quantities of purified bovine albumin** for which a standard curve had previously been prepared.***

Conjugation

The protein concentration of the globulin solution was adjusted to 10 mg/ml by dilution with 0.85% NaCl. Five-hundredths mg of fluorescein isothiocyanate (FITC)****/mg of protein was dissolved in a sufficient volume of carbonate-bicarbonate buffer, pH 9***** to comprise 10% of the combined volume of the reactants. The FITC solution was slowly added to the globulin solution at 4 C. During this procedure the globulin solution

*Coleman Instruments, Inc., Maywood, Ill.

**Armour Pharmaceutical Co., Kankakee, Illinois.

***Prepared by Dr. E. C. Pirtle, NADL, Ames, Iowa.

****Nutritional Biochemicals Co., Cleveland, Ohio.

*****See Appendix A.

was kept chilled by an ice bath and continuously agitated with an electric stirrer. The mixture was then reacted overnight (approximately 16 hrs) at 4 C with constant stirring (64).

Gel filtration

Separation of free FITC from the FITC-protein complex (labeled antibody) was accomplished by gravity passage of the mixture through a Sephadex gel* column (65). The sephadex (G-25, coarse) had previously been washed with phosphate buffered saline pH 7.2-7.4 (PBS) and was maintained as a stock preparation 1:4 in PBS. A poured column of Sephadex measuring approximately 2 by 20 cm was used for separation of 20-30 ml volumes, with correspondingly larger columns employed for greater volumes. After the gel had settled in the column, the fluid level was adjusted to its surface and the sample carefully added. As the last of the sample entered the gel, it was followed by a sufficient volume of PBS to elute the FITC-protein complex, leaving the unconjugated FITC in the upper portion of the column. Progress of the labeled protein through the column was easily followed by its yellow color.

Sorption

Elements of the labeled antibody preparation responsible for non-specific staining were essentially removed by "sorption" with 20-30 mg of rabbit liver powder (RLP)**/mg of protein. To determine the total

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*Pharmacia, Ltd., Uppsala, Sweden.

**See Appendix A.

quantity of RLP required, it was assumed that the preparation contained the same amount of protein as had been calculated prior to the conjugation procedure. A slurry was prepared by adding 2.5 ml of PBS to each gm of RLP. Phosphate buffered saline was added for its buffering capacity and to hydrate the powder, thus preventing a marked reduction in the volume of labeled antibody. Sorption was performed overnight with constant stirring at 4 C.

The bulk of the RLP was removed from the labeled antibody solution by centrifugation at 2000 x g for 30 min. Remaining RLP was sedimented by centrifugation at 78,000 x g for 1 hr using a #30 rotor in a Spinco Model L preparative ultracentrifuge.*

Standardization

A titration of each preparation of fluorescent antibody was performed to determine an appropriate dilution for routine use.

Cultures of PK-15 swine kidney line cells** grown on coverslips in Leighton tubes were each infected with approximately 500 tissue culture infecting doses of HCV and incubated for 24 hrs to allow circumscribed areas of infection (plaques) to develop from each initially infected cell. Plaques surrounded by uninfected cells provided the test system whereby the degree of nonspecific and specific staining could be graded and compared at various dilutions of fluorescent antibody.

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*Beckman Instruments, Inc., Spinco Division, Palo Alto, California.

**The original culture of these cells was kindly supplied by Cutter Laboratories, Berkeley, California.

The working dilution was selected in the following manner. Undiluted fluorescent antibody gave bright specific staining and a variable degree of nonspecificity, usually more than desired. As progressive twofold dilutions were made, the intensity of specific staining decreased slowly, whereas nonspecificity decreased rapidly. Therefore, a dilution was selected that allowed bright specific staining and a minimum of nonspecific staining. Usually a 1:8 to 1:16 dilution was satisfactory.

Storage

Fluorescent antibody was diluted with PBS to the working dilution and Merthiolate* was added to a final concentration of 1:10,000. Aliquots were distributed to screw-cap vials and maintained at -60 C in a mechanical freezer** until needed.

If an entire vial of fluorescent antibody was not used on the day it was thawed, it was maintained at 4 C and used as required. The fluorescent antibody was discarded if a precipitate formed during storage at 4 C.

Tissue (Cell) Cultures

The PK-15 swine kidney cell line was propagated and maintained by twice weekly subculture in milk dilution bottles and was nourished with Earle's balanced salt solution supplemented with 0.5% lactalbumin

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*Eli Lilly Co., Indianapolis, Indiana.

**Revco, Inc., Deerfield, Michigan.

hydrolysate and 10% serum from specific pathogen free (SPF-1)* or second generation SPF (SPF-2)** swine. Penicillin and streptomycin were added to final concentrations of 100 units/ml and 100 ug/ml respectively.

Twice weekly, PK-15 cells were implanted on coverslips contained in Leighton tubes to provide a continuous supply of young cultures for the FATCT. Cultures were used for the FATCT during an interval of 3-4 days after the cell layer had become confluent.

Procurement and Processing of Field Samples

Spleen and blood samples from swine herds in which HC had been tentatively diagnosed were submitted by either veterinary practitioners, or field representatives of the Animal Health Division, Agricultural Research Service, United States Department of Agriculture, or the Iowa State University Veterinary Diagnostic Laboratory. All samples received on the same day and from the same source from a suspected case of HC were referred to collectively as a specimen. Specimens were identified by number and were numbered in the sequence in which they were received at the NADL. Usually, only 1 specimen was submitted from each case and therefore the specimen and case number correspond. Occasionally more than 1 specimen was submitted. In these instances, the case number corresponds to a combination of the specimen numbers, e.g., specimens

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*Hysterectomy-derived, colostrum-deprived swine raised in an environment in which there was a minimal chance of exposure to swine pathogens.

**First generation progeny from SPF-1 swine.

18, 19 and 20, case number 18-19-20. Field isolates of HCV were given the same number as the respective case from which they were obtained.

Each blood sample was transferred from the container in which it was received to a sterile centrifuge tube and centrifuged for 15 min at 2000 x g. The clear serum or plasma was used as inoculum for both cell cultures and swine.*

Each spleen was freed of omentum and approximately 10 gm of splenic tissue was then transferred to a chilled blender jar containing 25-30 ml of cold cell culture medium (CCM). The type of culture medium used in this step was identical to that employed for propagating the PK-15 swine kidney cell line. The spleen-CCM mixture was blended for approximately 5 min. The mixture was then transferred to a sterile centrifuge tube and centrifuged for 15 min at 2000 x g. Clarified supernatant fluid was used for both cell culture and swine inoculation.

The portion of each sample which was not used for inoculum or in the preparation of inoculum was stored at -60 C for future reference.

Inoculation of Cell Cultures

Cell culture medium was decanted from an appropriate number of PK-15 cell cultures on coverslips in Leighton tubes. Each of 2 cell cultures was inoculated with 2 ml** of test material prepared from each field sample.

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*See PART II. SWINE INOCULATION TEST.

**If a sufficient amount of the field sample was available to prepare this volume of inoculum.

After 1 hr's incubation at 37 C to allow adsorption of HCV to occur, the test material was decanted. Cell cultures were carefully washed several times with CCM to remove any remaining debris. After the addition of 1 ml of fresh CCM to each culture, they were incubated at 37 C.

Staining and Examination of Cell Cultures

After 16-24 hrs' incubation, 1 of the 2 cell cultures inoculated with each sample was processed for fluorescent microscopy as follows: coverslip cultures were washed in PBS, dried, fixed in acetone at room temperature for approximately 10 min, dried, placed in a humidity chamber and stained with fluorescent antibody for a minimum of 10 min at 37 C, washed in PBS, dried and mounted in PBS-glycerine (1:1) on a microscope slide. Examinations were made using a Leitz ortholux microscope equipped with a darkfield condenser, BG-12 primary filter, OG-1 barrier filter and an Osram HBO-200 mercury vapor burner.

If 1 or more of the cell cultures inoculated with samples of a particular specimen was FATCT positive at the 16-24 hr examination, a diagnosis of HC was recorded and the duplicate cultures were discarded. If none of the cultures inoculated with samples of a particular specimen were FATCT positive at the 16-24 hr examination, the duplicate culture inoculated with each sample was examined after an additional 24 hrs' incubation.

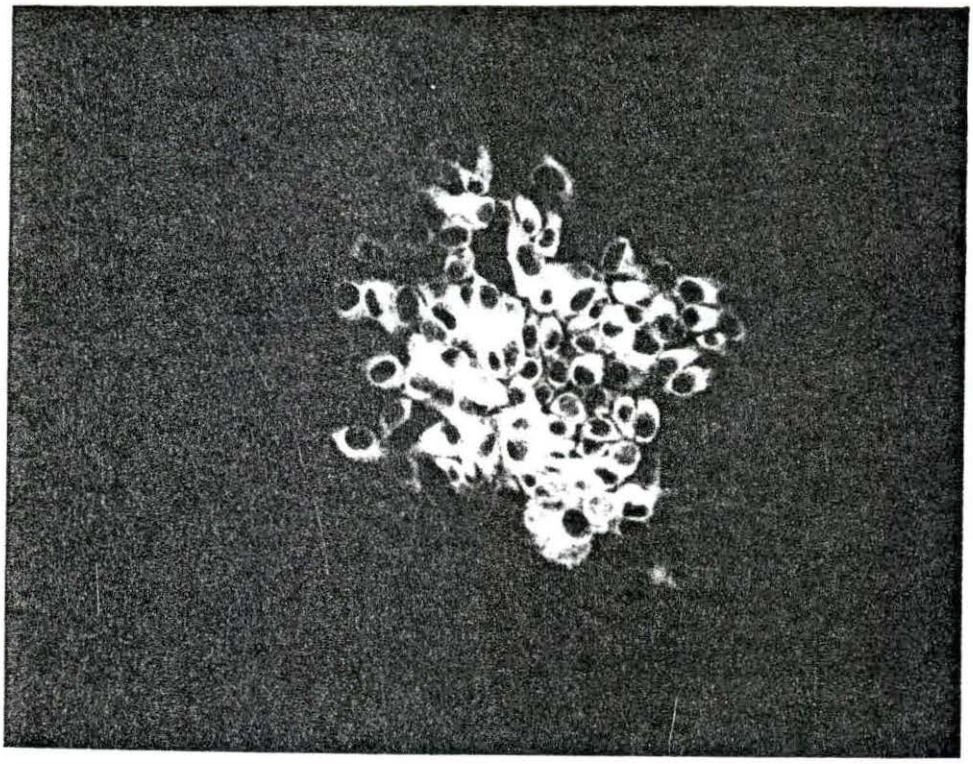
Estimation of the Virus Content of Field Samples

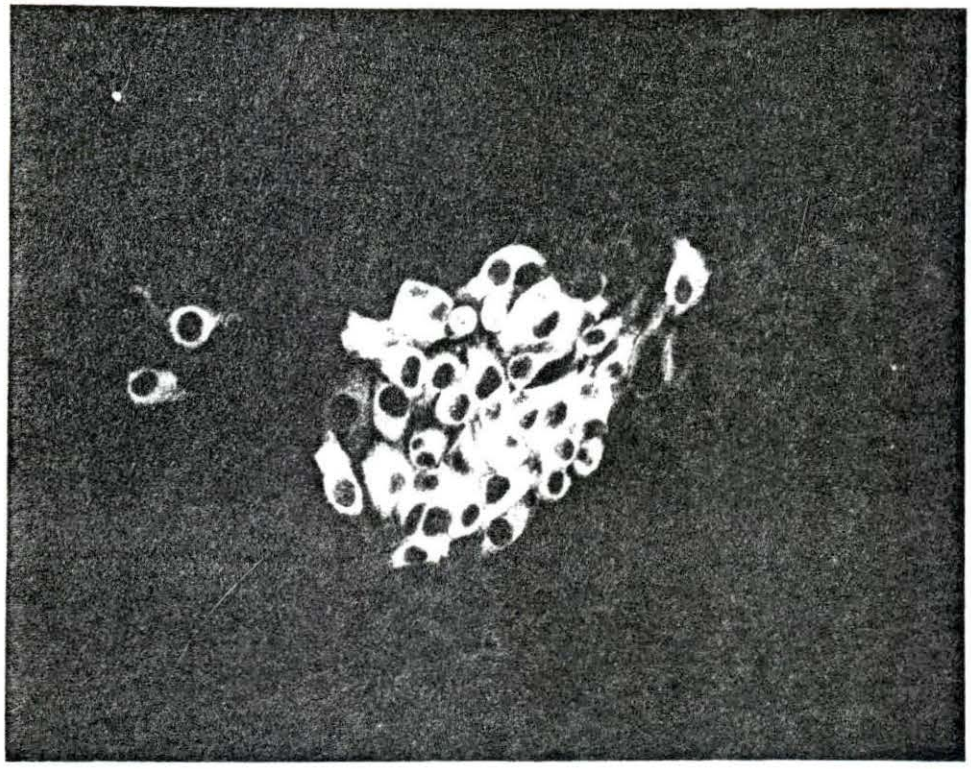
Early in this study, samples were recorded only as FATCT positive or negative. However, starting with the eighty-eighth field specimen, an estimation was made of the HCV titer of each FATCT positive sample. The titer was determined either by counting fluorescent foci of infection (plaques) or by estimating the % of infected cells of the total number of cells of the culture infected with the respective sample. When the virus titer was 10^3 or less, plaque counts were made. It had previously been demonstrated, that within 16-24 hrs after infection, there was only a minimal amount of virus spread from the initial foci of infection. If there were few plaques, the entire cell culture was examined. If the plaques were numerous, i.e., 100-1000, 20% of the culture was examined and the resulting plaque count multiplied by 5. The counting of plaques became increasingly difficult as titers approached 10^3. In fact, when titers were much greater than 10^3, plaque counting was impossible since plaques were so numerous that they formed confluent areas of infection. Titers greater than 10^3 were therefore estimated by the % infection method, i.e., the % of infected cells of the total number of cells of a culture. It was experimentally demonstrated that 50% infection of a culture, infected 16-24 hrs previously, corresponded to a virus titer of approximately 10^4. Consequently more than 50% infection corresponded to a titer greater than 10^4 (Figs. 1, 2, 3 and 4).

The method of titration just described was employed primarily because of its simplicity. The same cell culture used to determine if a sample

Fig. 1. Plaque, 24 hrs after cell culture infection

Fig. 2. Plaque, 24 hrs after cell culture infection





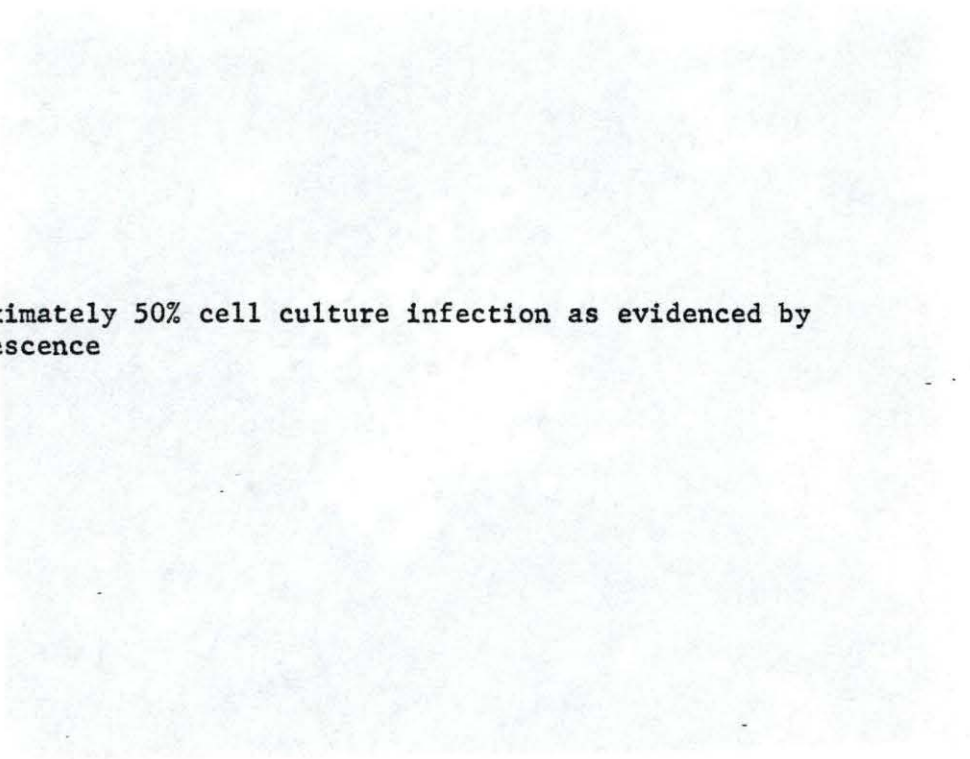


Fig. 3. Approximately 50% cell culture infection as evidenced by fluorescence

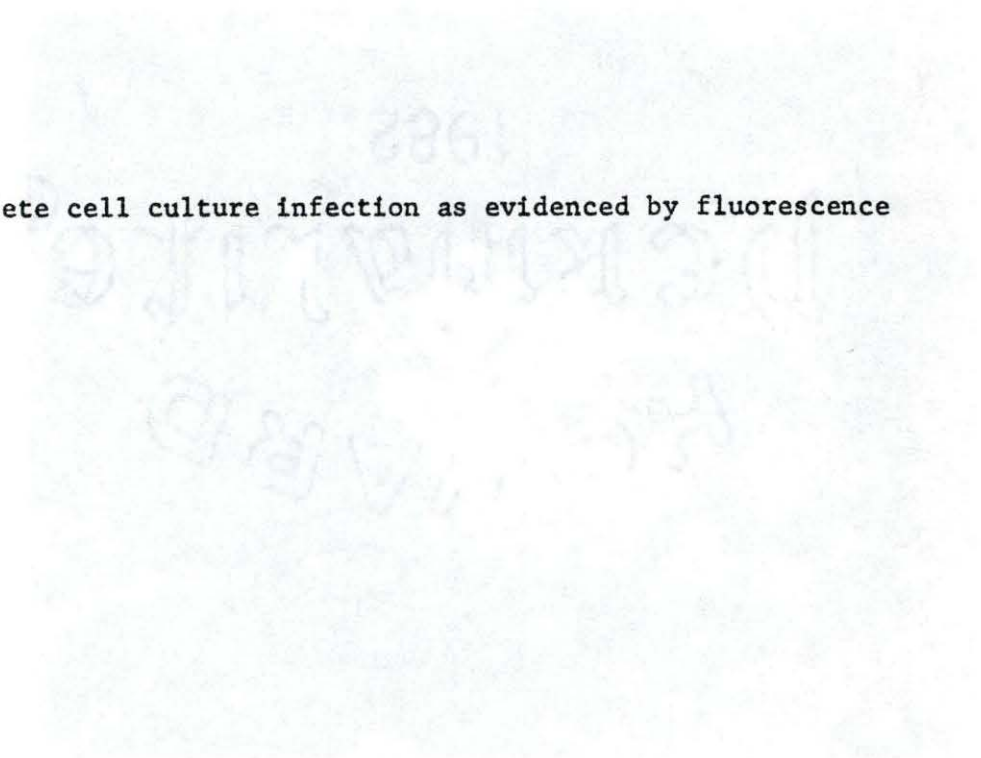
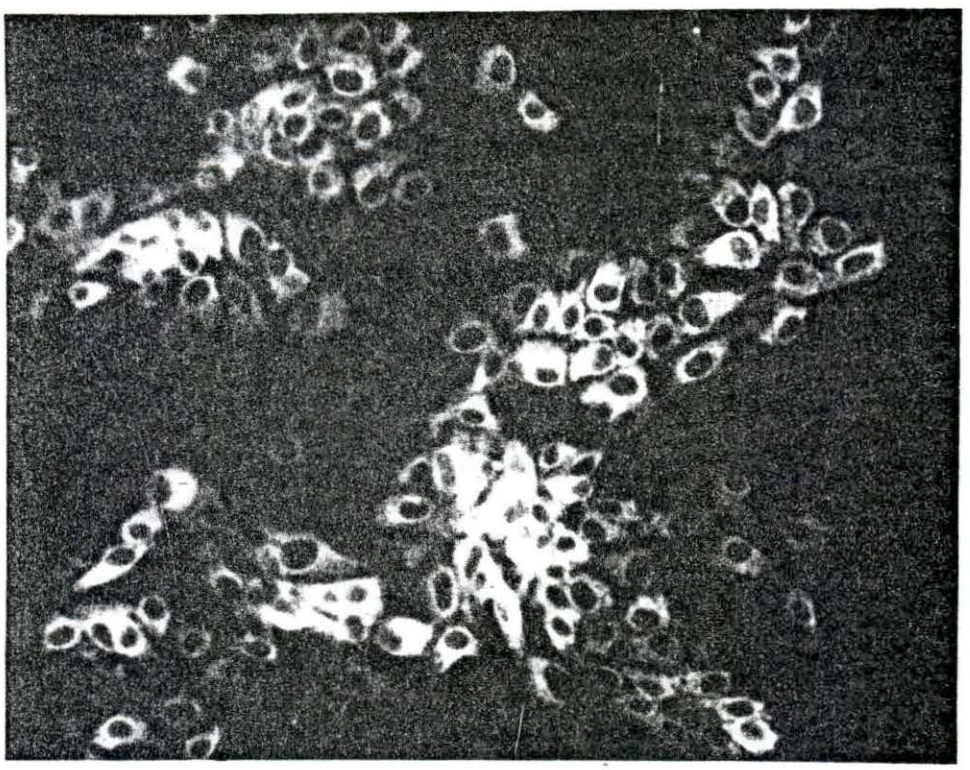
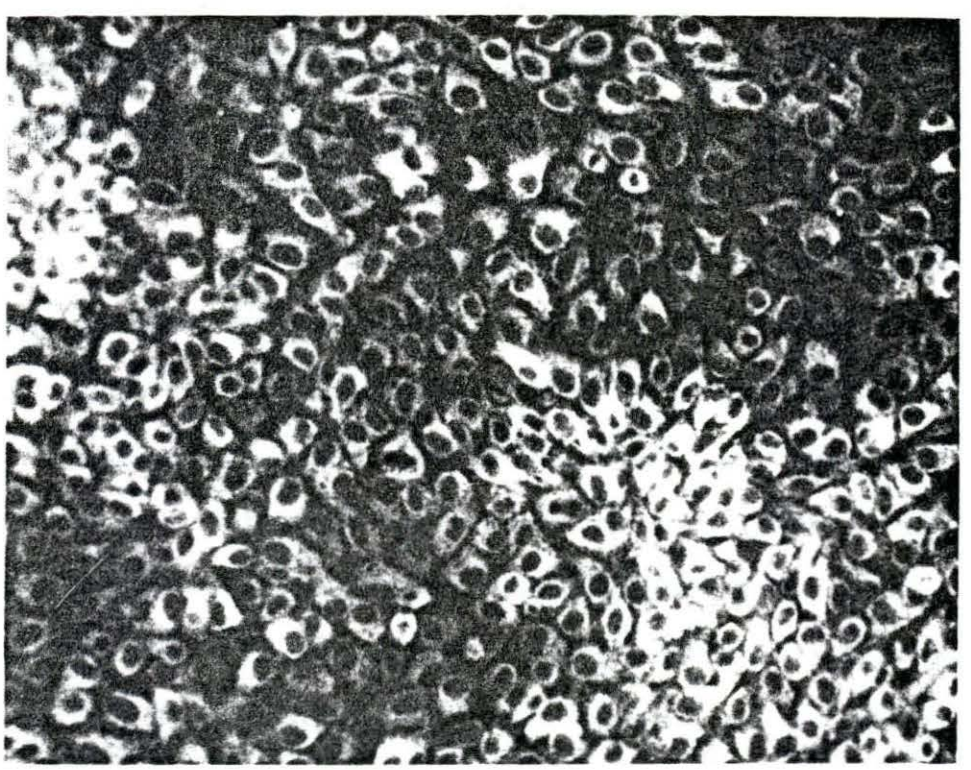


Fig. 4. Complete cell culture infection as evidenced by fluorescence





contained HCV, was also used for titration. The method was not extremely precise and therefore an exact titer was not assigned to any individual sample. Rather, samples were placed in 1 of 5 broad categories as follows: (1) 1-10 plaques, (2) 11-100 plaques, (3) 101-1000 plaques, (4) 1001 plaques - 50% cell culture infection, and (5) greater than 50% cell culture infection. For purposes of comparison, samples were given either an average titer, e.g., each sample in category 1 was assigned a titer of 5 infectious units, or were given the number of their category, e.g., each sample in category 1 was assigned the value of 1.

A few additional statements of explanation are necessary for the former method of comparison. Category 5, as defined, has no upper limit. Samples placed in this category simply had HCV titers which exceeded 10^4. Based on prior experience with HCV titrations by more conventional and also more accurate means, a working limit was placed on this category. The virus titer of tissues from HC affected swine usually does not exceed 10^6. Therefore, the upper limit was set at 10^6 and the average titer assigned to samples within this category was 10^5.

All titrations were made at the 16-24 hr examination of cell cultures except in the few instances when samples were found positive only at the 40-48 hr examination. These samples were placed in category 1.

Titers are expressed for the 2 ml of sample inoculum used to infect cell cultures. Even though spleen tissue was diluted approximately 1:4 in the preparation of cell culture inoculum, no adjustment was made for this dilution, i.e., the observed titer was not multiplied by 4.

RESULTS

From May 31, 1963 to June 2, 1964, samples were received from 338 suspected cases of HC occurring in 22 states (Table 1). A diagnosis of HC was confirmed by the FATCT on 225 (67%) of the suspected cases. Two

Table 1. State of origin of 338 suspected cases of HC from which samples were received

State of origin	Number of cases[a]	State of origin	Number of cases
Iowa	197	Florida	2
Missouri	80	Massachusetts	2
Indiana	23	California	2
Tennessee	5	North Dakota	1
South Dakota	3	Washington	1
Illinois	3	Minnesota	1
Arizona	3	New Jersey	1
Alabama	3	Virginia	1
Ohio	3	Colorado	1
Wisconsin	2	Georgia	1
Kansas	2	Rhode Island	1
		Total	338

[a]Samples from most of the cases occurring in Iowa were submitted by Iowa veterinary practitioners and personnel of the Iowa State University Veterinary Diagnostic Laboratory; most of those from other states were submitted by personnel of the Animal Health Division, Agricultural Research Service, U. S. Department of Agriculture.

hundred and twenty (98%) confirmations were made at the first, i.e. 16-24 hr, examination of infected coverslip cultures, whereas 5 (3%) confirmations required the examination of infected cell cultures that had been incubated 40-48 hrs.

From the 338 cases, a total of 918 samples were received, of which 490 were spleen and 428 were blood. The FATCT was performed with only 850 of the samples since 68 (7%) were either markedly decomposed or adulterated. Hog cholera virus was isolated and identified by the FATCT from 502 (59%) of the 850 samples examined (Table 2).

Table 2. Results of FATCT with samples from suspected field cases of HC

Diagnosis of HC based on FATCT	Number of cases	% of total cases	FATCT results with individual field samples: Spleen Number	Spleen % positive	Blood Number	Blood % positive
Positive	225	66.6	324[a]/348	93.1	178/286	62.2
Negative	97	28.7	0/123	....	0/93	....
None[b]	16	4.7	....	....	....	....
Total	338	100				

[a]Numerator = number FATCT positive; denominator = number examined by FATCT.

[b]All the samples from 16 suspected cases of HC were too decomposed or adulterated to examine by FATCT.

All 3 anti-HC sera used to prepare fluorescent antibody were found satisfactory. No significant difference was observed between any of these sera.

No particular difficulties were encountered in conducting the FATCT except those directly related to the condition of samples. These difficulties, however, caused an alteration in the method of performing the test. A preliminary study (49), indicated that a satisfactory method of testing samples was to inoculate 0.2 ml of a tissue suspension, plasma or serum directly into the nutrient medium of a cell culture. The inoculum was allowed to remain in the medium during the subsequent incubation of the culture. For 2 reasons, this technique did not suffice for the testing of field samples. Field samples were often heavily contaminated with bacteria; therefore, vast numbers of these bacteria were introduced into the cell culture medium with the tissue inoculum. In spite of the antibiotics in the medium, the bacteria occasionally multiplied to sufficient numbers to destroy the culture. Secondly, some of the decomposed samples were "borderline" toxic. Cell cultures could withstand the toxic effect of these samples only if the exposure to them was for a relatively short time. For the above reasons, an alternate method of testing was followed. This procedure is described in detail in MATERIALS AND METHODS. Briefly, it involved the inoculation of the test material directly onto the cell culture. After exposing the cells to the inoculum for 1 hr, the cultures were carefully washed. Fresh medium was then added. In this manner most of the bacteria and toxic

materials were removed from the culture. One added advantage of the latter method was that most of the cell debris, present in tissue suspensions, was also removed from the culture. Without washing, some of the debris remained affixed to the cell monolayer throughout subsequent processing for FATCT examination. Although the autofluorescence of these tissue fragments did not resemble specific fluorescence of infected cells, it nevertheless was undesirable.

Comparison of Spleen and Blood Samples

As a diagnostic sample, spleen was superior to blood in 2 ways. The most important was that spleen samples from swine herds, wherein a FATCT diagnosis of HC was made, were more frequently positive than were blood samples from these same herds (Table 2). Of less importance, but yet interesting, is the fact that generally more infective HCV was isolated from spleen than blood (Tables, 3, 4, 5, 6, and 7). It will be noted that all FATCT-positive spleen and blood samples represented in Table 2 are not found in Tables 3, 4, 5, 6 and 7 since the estimate of the HCV content of samples was not initiated until sometime after this evaluation study had been started. It is obvious from the examination of Table 3, that spleens generally contained more infective HCV than did blood samples. However, to more adequately compare these samples, the average virus titer of all spleen samples (Table 4) was compared to that of blood samples (Table 5). The comparison was made by dividing the average HCV titer of spleen samples, 31,306, by the average HCV titer of

Table 3. Estimated HCV content of field samples[a]

Type of sample		Infective units of HCV/2 ml of sample 1-10	11-100	101-1000	1001-10,000	>10,000	Total
Spleen	Number	25	18	49	103	81	276
	%[b]	9.1	6.5	17.8	37.3	29.3	
Blood	Number	38	30	20	25	33	146
	%	26.0	20.6	13.7	17.1	22.6	
Total	Number	63	48	69	128	114	422
	%[c]	14.9	11.4	16.4	30.3	27.0	100

[a]The method of estimation is described in MATERIALS AND METHODS.

[b]% of total spleen samples represented in the table.

[c]% of all samples represented in the table.

blood samples, 23,539. The ratio thus obtained was 1.33, demonstrating that approximately 33% more HCV was identified from spleen than from blood.

This type of comparison could be misleading. For example, if 50 spleens (each of 49 containing 1 infective unit and 1 containing 100,000 infective units) were compared with 50 blood samples (each containing 1,000 infective units) the following results would be obtained: average HCV titer spleen = 2001, average HCV titer blood = 1000, ratio spleen: blood = 2. Although mathematically correct, the single spleen sample containing 100,000 infective units biased the results; therefore, another method was also used for comparison. Samples, spleen or blood, were placed into 1 of

Table 4. Average HCV titer of spleen samples[a]

Category number	Infective units of HCV/2 ml of sample	Average number of infective units/2 ml of sample	Number of samples/ category	Total infective units of HCV/ category (samples x average titer)
1	1-10	5	25	125
2	11-100	50	18	900
3	101-1000	500	49	24,500
4	1,001-10,000	5,000	103	515,000
5	>10,000	100,000[b]	81	8,100,000
		Total	276	8,640,525
Average HCV titer of spleen samples = 8,640,525 ÷ 276 =				31,306

[a]Based on data presented in Table 3.

[b]The method by which this average titer was determined is described in MATERIALS AND METHODS.

5 categories depending on the number of infective units of HCV in a 2 ml portion of the sample. A sample was assigned a value corresponding to the number of the category in which it was placed. Samples with the least amount of virus were placed in category 1 and each was assigned a value of 1. Samples with the most virus were placed in category 5 and each was assigned a value of 5. Samples containing virus concentrations between these 2 extremes were appropriately placed in either categories 2, 3 or 4 and were assigned the corresponding values (Tables 6 and 7).

Table 5. Average HCV titer of blood samples[a]

Category number	Infective units of HCV/2 ml of sample	Average number of infective units/2 ml of sample	Number of samples/ category	Total infective units of HCV/ category (samples x average titer)
1	1-10	5	38	190
2	11-100	50	30	1,500
3	101-1000	500	20	10,000
4	1,001-10,000	5,000	25	125,000
5	>10,000	100,000[b]	33	3,300,000
		Total	146	3,436,690
Average HCV titer of blood samples = 3,436,690 ÷ 146 =				23,539

[a]Based on data presented in Table 3.

[b]The method by which this average titer was determined is described in MATERIALS AND METHODS.

This method, which will be referred to as the assigned value method, prevented bias by a few samples containing a large number of infective units of HCV. The average assigned value of spleen is 3.7 (Table 6) and the average assigned value of blood is 2.9 (Table 7). The comparative ratio (3.7 : 2.9) is 1.28. In this case, because of the distribution of samples, the ratio of the 2 types of samples is similar by either method of comparison.

Table 6. Average numerical value of spleen samples based on their relative HCV content[a] (assigned value method)

Category number	Number of samples/category	Numerical value assigned to each sample of a category	Total numerical value/category (samples x numerical value/sample)
1	25	1	25
2	18	2	36
3	49	3	147
4	103	4	412
5	81	5	405
Total	276		1,025
Average numerical value of spleen samples = 1025 ÷ 276 = 3.7			

[a]Based on data presented in Table 3.

Laboratory Samples

Two hundred and ninety-five blood samples and 4 spleen samples were obtained from pigs either injected with field specimens or injected with blood obtained from pigs injected with field specimens or from pigs exposed by contact to the pigs mentioned above. All except 1 of these samples, which will be referred to hereafter as laboratory samples, were examined by the FATCT. The results of these tests are more meaningfully presented in relation to the reaction of the pigs from which the samples were obtained. Consequently they are presented in detail in the following sections on the SWINE INOCULATION TEST (SIT) results and on the correlation

Table 7. Average numerical value of blood samples based on their relative HCV content[a] (assigned value method)

Category number	Number of samples/category	Numerical value assigned to each sample of a category	Total numerical value/category (samples x numerical value/sample)
1	38	1	38
2	30	2	60
3	20	3	60
4	25	4	100
5	33	5	165
Total	146		423

Average numerical value of blood samples = 423 ÷ 146 = 2.9

[a]Based on data presented in Table 3.

of FATCT and SIT results. It suffices to state here that of the 294 blood samples examined by the FATCT, 238 were positive and of the 4 spleens examined, 2 were positive.

PART II. SWINE INOCULATION TEST

MATERIALS AND METHODS

Hog Cholera Susceptible Swine

Thirty to 60 lb SPF-1 and SPF-2 swine of various breeds and Yorkshire (York) swine from the NADL purebred, inbred herd were used for the majority of the SIT's. These types of swine were selected because it was believed that their susceptibility to HCV was more uniform than that of average farm-raised swine.

Inoculation, Bleeding and Subsequent Challenge of Swine

A composite or pool of inoculae prepared from the individual samples of a specimen was used for swine inoculation. Routinely, 4 ml of the pool was injected subcutaneously; however, in some instances the inoculum that a pig received was either more or less than the 4 ml amount. Occasionally, there was an insufficient amount of the field sample(s) to provide the 4 ml volume. After injection, the pig was placed in an isolation cage. Controlled experiments had been conducted to demonstrate that these isolation facilities were reliable.*

At least one, 100 ml blood sample was subsequently obtained from most of the pigs which received FATCT-positive specimens. Blood samples were also taken from pigs which had an obvious clinical reaction after receiving FATCT-negative specimens or specimens not examined by the FATCT. Occasionally blood samples were taken from pigs which received FATCT-negative

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*Conducted by J. P. Torrey, NADL, Ames, Iowa.

specimens and which did not have a clinical reaction. In addition, 4 spleen samples were taken at autopsy. These laboratory samples were processed and examined by the FATCT in a manner identical to that employed for those from the field except that only 1 coverslip cell culture was inoculated with each sample. The cultures were examined 16-24 hrs later. The portion of each laboratory sample which was not used for the FATCT was stored at -60 C for future reference.

The majority of the blood samples were obtained 6-8 days after the pigs were injected with field specimens, but the time of bleeding depended primarily on the reaction of the pig. Pigs were usually bled during a period of marked clinical illness and consequently, during a period when a viremia was most likely to occur. Obviously, the time of bleeding could not be so advantageously selected when pigs received field isolates of HCV which were of low virulence.

Pigs which did not die or were not killed in a moribund condition after receiving a field specimen were subsequently moved to an isolation room and challenged with 2 ml of swine blood containing Ames HCV. If a pig had had no clinical reaction, it was challenged approximately 2 weeks after receiving the field specimen. If a pig had a reaction but recovered, it was challenged after it had been clinically normal for approximately 1 week, providing the date of challenge was approximately 2 weeks or more after the pig had been injected with the field specimen. The above intervals of time were increased in some instances when SIT's were repeated with several of the field specimens.

Usually a pig was removed from its isolation cage only for bleeding and/or challenge. Exceptions were made when it was desirable to study the reactions of pigs in greater detail. For this purpose, pigs were placed in isolation rooms.

Interpretation of Swine Inoculation Tests

A SIT was recorded as positive if after injection with a specimen, the pig either died or was killed in a moribund condition and had lesions suggestive of HC. A SIT was also recorded as positive if a pig survived after injection with a specimen and was found immune when subsequently challenged with Ames HCV.

A SIT was recorded as negative if the pig survived after injection with a field specimen but died or was killed in a moribund condition after challenge with Ames HCV.

The few exceptions to the above general statements will be pointed out in RESULTS.

Quantitation of the Reaction of Pigs Confined to Isolation Cages

The severity of the illness of pigs confined to isolation cages was evaluated on the basis of a daily point system. One point was recorded for a pig which was slightly anorectic and had slight or no definite depression. A pig having moderate anorexia and depression was given 2 points and a pig having complete anorexia and severe depression was given 3 points. If the daily point accumulation was 11 or less, the overall reaction was defined as slight. If the daily point accumulation was more than 11, the

overall reaction was defined as moderate to severe. The general confinement of pigs to isolation cages precluded more definitive measures of their reactions.

Classification of Hog Cholera Viruses

Each field isolate of HCV was classified as either lethal or immunizing depending on the reaction of the pig infected with the respective isolate. To more adequately indicate their relative virulence, lethal viruses were subclassified according to the duration of illness of the infected pig prior to death. This excluded the incubation period and any temporary recovery periods. Immunizing viruses were subclassified according to the severity of the pig's reaction prior to recovery and subsequent challenge. The classification scheme is outlined below.

I. Lethal Viruses (L)

L^1-----10 or less days of illness preceding death.

L^2-----11 to 21 days of illness preceding death.

L^3-----21 to 31 days of illness preceding death.

L^4-----31 or more days of illness preceding death.

II. Immunizing Viruses (I)

I^1-----causing a moderate to severe reaction prior to recovery.

I^2-----causing no reaction or a slight reaction prior to recovery.

Repeated Swine Inoculation Tests

Routinely, each field specimen was injected into 1 pig. However, for several reasons additional SIT's were conducted. If a pig which had been injected with a field specimen died within 24 hrs after a blood sample had been taken from the anterior vena cava, usually another pig was injected with the same specimen. The possibility existed that the cause of death was mechanical injury due to bleeding. In most instances when the results of the FATCT and the SIT did not agree, the SIT was repeated. Occasionally, 2 or more pigs were injected with the same specimen to compare the reactions of these pigs to the same field isolate of HCV.

Definition of Terms

Several terms will be used repeatedly in the following text and tables. Their meanings may be obvious but to avoid possible confusion they are defined here.

Incubation period -- The number of days from injection or other means of exposure of a pig to HCV to the first clinical signs of illness.

Temporary recovery period -- A period, following a clinical reaction, during which a pig had a normal appetite and was alert, irrespective of its temperature. However, usually the temperature corresponded to the degree of clinical illness that was observed.

Duration (or days) of illness preceding death -- The number of days of observed clinical illness of a pig. This excludes the incubation period and any temporary recovery periods.

Total days, exposure to death -- The number of days from injection or other means of exposure of a pig to HCV to the death of that pig. In some instances this interval cannot be obtained by simply adding the incubation period to the days of illness preceding death since 1 or more temporary recovery periods occurred, e.g., see Table 17.

RESULTS

Two hundred and thirty-eight (70%) of 338 suspected cases of HC were confirmed by SIT's. Six cases were recorded as questionable based on the contradictory results of several SIT's done with the corresponding specimens. Eighty-six cases were SIT negative. Specimens from the remaining 8 cases were excessively decomposed and were not injected into pigs (Table 8).

Table 8. Results of SIT's with specimens from suspected field cases of HC

Diagnosis of HC based on SIT's	Number of cases	% of total cases
Positive	238	70.4
Negative	86	25.4
Questionable	6	1.8
None[a]	8	2.4
Total	338	100

[a]All the samples from these 8 cases were severely decomposed and were not injected into pigs.

Classification of Hog Cholera Viruses

It was possible to determine the relative virulence of 222 field isolates of HCV (Table 9). Although a diagnosis of HC was confirmed on 238 cases, pigs injected with 16 of the field isolates (specimens

Table 9. Classification of virulence of 222 field isolates of HCV

	Classification of virulence[a]					
	Lethal (L)				Immunizing (I)	
	L^1	L^2	L^3	L^4	I^1	I^2
Number of isolates	72	86	11	8	16	29
% of total isolates classified	32.4	38.7	5.0	3.6	7.2	13.1
Number of isolates	177				45	
% of total isolates classified	79.7				20.3	

[a]Classification scheme repeated in part from MATERIALS AND METHODS for convenience of reference:

Lethal (L) -- Killed the experimentally infected pig, subclassified on the basis of the duration of illness of the pig preceding death.

L_2^1 -- 10 or less days of illness preceding death.
L_3^2 -- 10 - 21 days of illness preceding death.
L^3 -- 21 - 31 days of illness preceding death.
L^4 -- 31 or more days of illness preceding death.

Immunizing (I) -- Immunized experimentally infected pig against subsequent challenge with Ames HCV, subclassified on the basis of the severity of reaction prior to recovery and subsequent challenge.

I_2^1 -- moderate to severe reaction prior to recovery.
I^2 -- no reaction or slight reaction prior to recovery.

containing HCV) were killed in a moribund condition. These 16 isolates, therefore, could not be accurately subclassified, but there is little doubt that they may be considered as lethal viruses. Virus classification was based on the first positive SIT conducted with the respective isolate.

It will be noted from Table 9 that 177 field isolates of HCV were classified as lethal. If the 16 isolates mentioned above are added to the 177 from Table 9, it is found that only 81% of the HCV's from naturally occurring cases of HC killed experimentally infected pigs.

The relative virulence of Ames HCV was also determined. Since many pigs were injected with field specimens which apparently did not contain HCV, the virus classification scheme was applied to the reactions of these pigs after challenge with Ames HCV. Pigs which died following challenge but which previously had any reaction after injection with the field specimen were excluded from consideration. Although these pigs were clinically normal at the time of challenge, it was possible that they still harbored a pathogenic agent other than HCV which would contribute to their death following challenge. In addition, many of the challenged pigs were killed in a moribund condition to provide space for other SIT's. The reactions of 37 pigs remained for consideration after exclusion of many SIT's for various reasons, primarily those mentioned above. Ames HCV was classified as L^1 in 33 instances and L^2 in 4 instances.

Swine Inoculation Tests with Specimens Obtained from Various Sources

The percentage of SIT confirmed cases of HC was greatest for those cases investigated by personnel of the Animal Health Division (Table 10). Furthermore, generally more virulent HCV's were present in specimens submitted by this source (Table 11).

Table 10. Results of SIT's with specimens obtained from various sources

Diagnosis of HC based on SIT's	Source of specimens: Veterinary practitioner		Iowa State University Veterinary Diagnostic Laboratory		Animal Health Division	
	Number[a]	%[b]	Number	%	Number	%
Positive	76	63.9	35	54.7	127	90.1
Negative	43	36.1	29	45.3	14	9.9
Questionable	4	....	....	....	2	....
None[c]	6	....	....	....	2	....
Subtotal	129		64		145	
Total			338			

[a]Number of cases.

[b]% of total cases from respective source, excluding the questionable cases and those for which specimens were not injected into pigs.

[c]All samples from these cases were excessively decomposed and were not injected into pigs.

Repeated Swine Inoculation Tests

Several specimens containing HCV were injected into 2 or more pigs to determine if a particular isolate would cause a similar reaction in different pigs; thus, in effect, ascertaining the reliability of the virus classification data. The first test conducted with each isolate was used for its virulence classification. It was necessary to

Table 11. Relative virulence of 222 field isolates of HCV from various sources[a]

Virulence classification of field isolates		Source of field isolates: Veterinary practitioner	Iowa State University Veterinary Diagnostic Laboratory	Animal Health Division
Highly virulent (L^1 and L^2)	Number	44	17	97
	%[b]	61.1	53.1	82.2
Moderately virulent (L^3 and L^4)	Number	7	3	9
	%	9.7	9.4	7.6
Immunizing (I^1 and I^2)	Number	21	12	12
	%	29.2	37.5	10.2

[a]The source of the additional 16 lethal field isolates that were not subclassified is as follows. Veterinary practitioner 4, Iowa State University Diagnostic Laboratory 3, and Animal Health Division 9.

[b]% of total isolates from respective source.

arbitrarily select SIT 8472 to classify field isolate 346 since both SIT's were conducted on the same day (Table 12). With the exception of viruses classified as L^4, multiple tests with field isolates classified as lethal are presented in Table 12. Multiple tests with field isolates classified as immunizing are presented in Table 13. Some of the tests were repeated for purposes other than to compare the reactions of several

Table 12. Multiple SIT's with field isolates of HCV which were classified as lethal

HCV isolate number	SIT (pig) number	Test pig description: Type	Test pig description: Weight (lbs)	Date SIT conducted	Duration of illness preceding death of infected pig (days)	Virus classification based on the respective SIT
21	7411	SPF-1	40	7-31-63	23	L_1^3
	7461	SPF-1	50	8-9-63	....[a]	I^1
85	7550	SPF-1	40	11-18-63	3	L_1^1
	7769[b]	SPF-1	55	12-2-63	6	L^1
208	7960	SPF-2	35	3-17-64	5	L_1^1
	8478	SPF-1	45	11-13-64	8	L_1^1
	8479	SPF-1	45	11-13-64	10	L^1
213	7956	SPF-2	50	3-17-64	5	L_2^1
	8324	SPF-2	39	8-26-64	18	L^2
233	....	SPF-2	90	3-27-64	13	L_2^2
	8375	SPF-2	47	9-21-64	16	L^2
237	7906	SPF-2	90	3-27-64	10	L_2^1
	8466	SPF-1	37	11-13-64	11	L_1^1
	8470	SPF-1	37	11-13-64	9	L^1
243	8009	SPF-2	50	4-2-64	7	L_1^1
	8463	SPF-1	46	11-13-64	4	L_1^1
	8468	SPF-1	36	11-13-64	10	L^1
282	8081	SPF-2	25	4-20-64	10	L_2^1
	8465	SPF-1	42	11-13-64	17	L_1^2
	8476	SPF-1	43	11-13-64	7	L^1

[a] Recovered after 16 days of illness and was found immune when subsequently challenged.

[b] Injected with a blood sample from pig 7550.

Table 12 (Continued)

HCV isolate number	SIT (pig) number	Test pig description Type	Weight (lbs)	Date SIT conducted	Duration of illness preceding death of infected pig (days)	Virus classification based on the respective SIT
292	8099	SPF-1	50	4-29-64	15	L_2^2
	8387	SPF-2	32	9-21-64	13	L^2
336	8166	SPF-2	50	5-30-64	25	L_2^3
	8475	SPF-1	47	11-13-64	19	L_1
	8477	SPF-1	46	11-13-64	6	L^1
345	8168	SPF-2	47	5-30-64	28	L_2^3
	8391	SPF-2	26	9-21-64	16	L^2
346	8472	SPF-1	36	11-13-64	10	L_2^1
	8473	SPF-1	30	11-13-64	11	L^2

pigs, nevertheless, they are appropriately included here. Apparently there was no general tendency for isolates to lose virulence during storage nor to cause a more or less severe reaction in SPF-1 than in SPF-2 pigs. Except for lethal viruses 21 and 336, the pig reactions were fairly constant to a particular isolate.

Swine inoculation tests were repeated with specimens from 11 suspected cases of HC because the FATCT's had been negative whereas the corresponding SIT's had been positive. Five of the repeated tests were positive and 6 were negative. The latter 6 cases comprise the previously mentioned questionable group of SIT results (Table 14). Specimens from 3 other cases were FATCT negative and SIT positive but were not injected into additional pigs.

Table 13. Multiple SIT's with field isolates of HCV which were classified as immunizing

HCV isolate number	SIT (pig) number	Test pig description: Type	Test pig description: Weight (lbs)	Date SIT conducted	Severity of illness prior to recovery and subsequent challenge	Virus classification based on the respective SIT
18-19-20	7407	SPF-1	60	7-30-63	No reaction	I_2^2
	7526	York	42	8-21-63	Slight	I_2^2
	8474[a]	SPF-1	36	11-13-64	Slight	I^2
22-44	7462	SPF-1	55	8-2-63	Slight	I_2^2
	7548	SPF-1	45	9-13-63	Slight	I_2^2
	8460	SPF-1	33	11-13-64	Slight	I^2
158	9431[b]	SPF-1	50	2-26-64	Slight	I_2^2
	8327[b]	SPF-2	38	8-26-64	Slight	I^2

[a]Injected with blood from pig 7526.

[b]Injected with a FATCT positive blood sample from pig 7882 (not represented in the table) which, in turn, had been injected with FATCT positive field specimen 158. Pig 7882 had no reaction after receiving the field specimen but died following challenge with Ames HCV.

Table 14. SIT results with specimens from 6 suspected cases of HC on which a SIT diagnosis of HC was questionable

Case number	SIT (pig) number	Date SIT conducted	SIT results[a]	FATCT results[b]	Remarks
47	7552	9-20-63	+	-, +	The pig had no definite reaction until 10 days post field specimen inoculation (PFSI). Blood samples were taken on the 7th and 15th day PFSI. The 7th day blood sample was FATCT negative. The 15th day blood sample was positive.
	8332	8-26-64	-	....	....
53	9612	9-25-63	....	....	The pig died 4 days PFSI. Erysipelothrix insidiosa was isolated from its visceral organs. No samples were obtained for FATCT examination.
	9616	10-2-63	+	+	Inoculum filtered before injection.
	8382	9-21-64	-	....	Inoculum not filtered.
55	7600	9-27-63	+	+	....
	8462	11-13-64	-	....	....
167	7887	2-10-64	+	....	....
	8461	11-13-64	-	....	....
	8467	11-13-64	....	-	The pig was killed 6 days PFSI. Spleen and blood were examined by FATCT. Both were negative.

[a] + = positive; - = negative. Also applies to FATCT results.

[b] Blood sample from test pig.

Table 14 (Continued)

Case number	SIT (pig) number	Date SIT conducted	SIT results[a]	FATCT results[b]	Remarks
279	7915	4-17-64	+	+	No microscopic lesions of HC were observed in a brain submitted from this herd.[c]
	8100	4-29-64	....	-	The pig died shortly after a blood sample was taken on the day the pig was to be challenged. Neither HCV nor homologous antibody was detected in the sample.
	8386	9-21-64	-	....	....
296	8098	4-29-64	+	+	No microscopic lesions of HC were observed in a brain submitted from the herd. Leukocyte counts were elevated.
	8374	9-21-64	-	....	....

[c]Brain examinations done by H. A. McDaniel, NADL, Ames, Iowa.

Swine inoculation tests were repeated with specimens from 6 field cases because the FATCT's had been positive whereas the corresponding SIT's had been negative. Repeated tests were all positive (Table 15).

Table 15. Repeated SIT's with specimens from 6 suspected cases of HC. The first SIT was negative, whereas the corresponding FATCT was positive

Case number	SIT (pig) number	Date SIT conducted	Approximate HCV titer of inoculum	SIT results[a]	FATCT results[b]
109	7776	12-17-63	10^3_3	-	+
	8331	8-26-64	10^3	+	....
158	7882	2-4-64	$>10^4_3$	-	+
	9431[c]	2-26-64	10^3	+	-
	8327[c]	8-26-64	10^3	+	....
219	7964	3-21-64	10^4	-	+
	8326	8-26-64	10^4	+	....
300	8105	4-29-64	$<10^1_1$	-	+
	8376	9-21-64	$<10^1$	+	+
331	8148	5-21-64	10^3_3	-	+
	8385	9-21-64	10^3	+	+
342	8163	5-30-64	10^3_3	-	-
	8379	9-21-64	10^3	+	....

[a] + = positive; - = negative. Also applies to FATCT results.

[b] Blood sample from test pig.

[c] Injected with blood from pig 7882.

The negative results of the first SIT's were based on the failure of the test pig to survive subsequent challenge with Ames HCV. However, it is apparent that the first pigs injected with isolates 109, 158, 219, 300, and 331 had been infected with HCV prior to challenge since blood samples taken from them were FATCT positive. Although the blood sample obtained from the pig injected with isolate 342 was negative, it is probable that this pig was also infected prior to challenge. Therefore, the negative results were apparently not caused by the failure of the pigs to become actively infected following injection of the field specimen. The HCV titer of the total inoculum each pig received was approximated by averaging the titers of the respective samples from which the inoculum was prepared. It was necessary to classify these isolates by the results of the second SIT since the first test had been negative. Isolates 109 and 158 were classified as I^2; isolate 342 as I^1; isolates 300 and 331 as L^4; and isolate 219 as L^2.

Investigations with Field Isolates of Hog Cholera Virus Which Caused Prolonged Reactions in Experimentally Infected Pigs

Field isolates 7, 12, 92, 138, 300, 310, 331 and 348 were classified as L^4. By definition of L^4, the infected pigs were ill for a minimum of 30 days preceding death (Table 16). The SIT's which are found in the table for isolates 300 and 331 are the second tests conducted with them. As previously pointed out in the text and in Table 15, the first test with each of these isolates was negative.

Table 16. Swine inoculation test results with field isolates of HCV classified as L^4

Isolate number	SIT (pig) number	Test pig description Type	Weight (lbs)	Clinical reactions of infected pigs: Days of illness preceding death	Number of temporary recovery periods	Duration of longest recovery period (days)	Average points per day of illness[a]	Total days exposure to death	FATCT results[b]
7	7361	SPF-2	40	54	1	4	1.61	60	7+, 44+, 50+
12	7366	SPF-2	30	46	1	6	1.76	54	6+, 43+
92	7765	SPF-1	50	36	2	5	1.97	50	14+, 48+
138	7816	York	130	42	1	5	2.67	51	6+, 50+
300	8376	SPF-2	44	91	3	9	1.13	104	73+, 85+
310	8124	SPF-2	60	36	1	2	2.11	42	7-
331	8385	SPF-2	33	46	7	34	1.26	100	73+, 85+
348	8161	SPF-2	53	34	None	....	2.76	35	7+

[a]Accumulated daily points ÷ days of illness preceding death. The point system is described in MATERIALS AND METHODS.

[b]Blood samples from experimentally infected pigs. Number indicates on which day after injection of the field isolate that the blood sample was taken, + = positive; - = negative.

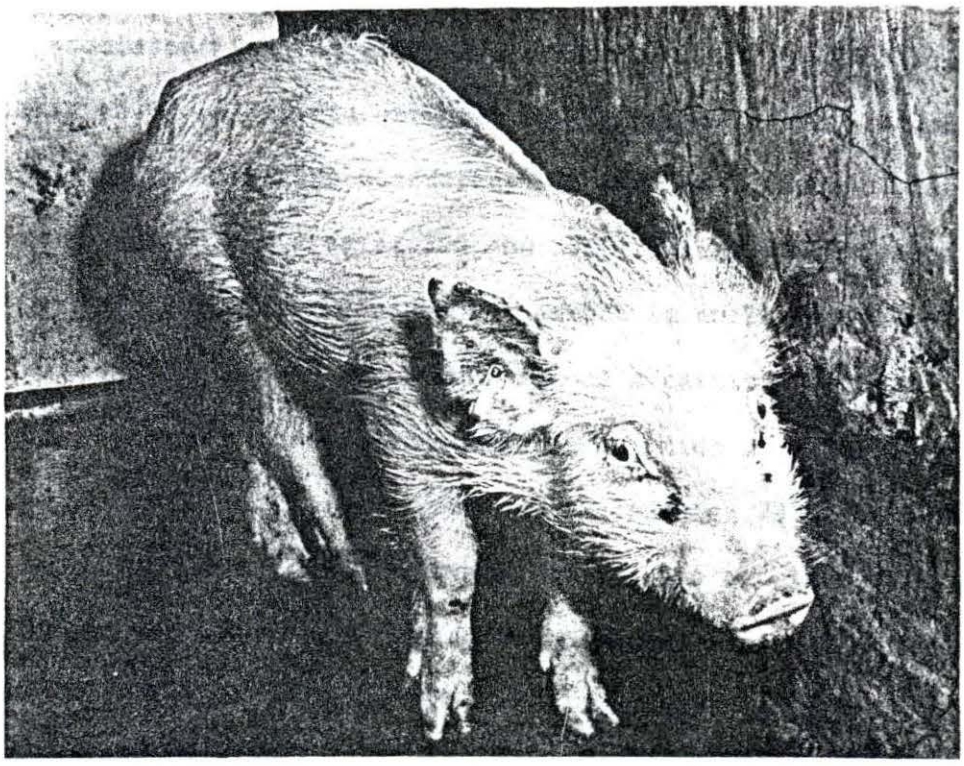
With 1 exception, all pigs had temporary recovery periods lasting from 1-34 days. Even though several pigs were moved to isolation rooms during their illness, the criteria used to determine if they were clinically normal was the same as had been used during isolation cage confinement. That is, if a pig had a normal appetite and was alert, it was recorded as clinically normal. This was done to interpret clinical reactions on a uniform basis. However, other criteria made it obvious that the recovery periods were only apparent. The most definitive was that the pigs eventually relapsed and died as a consequence of HCV infection. In some instances temperatures were elevated on days when a pig appeared normal. For example, the temperatures of pig 8385, infected with isolate 331, ranged from 102.8 - 104.8 F during its 34 day temporary recovery period. Moreover, the FATCT results indicated a persistent viremia.

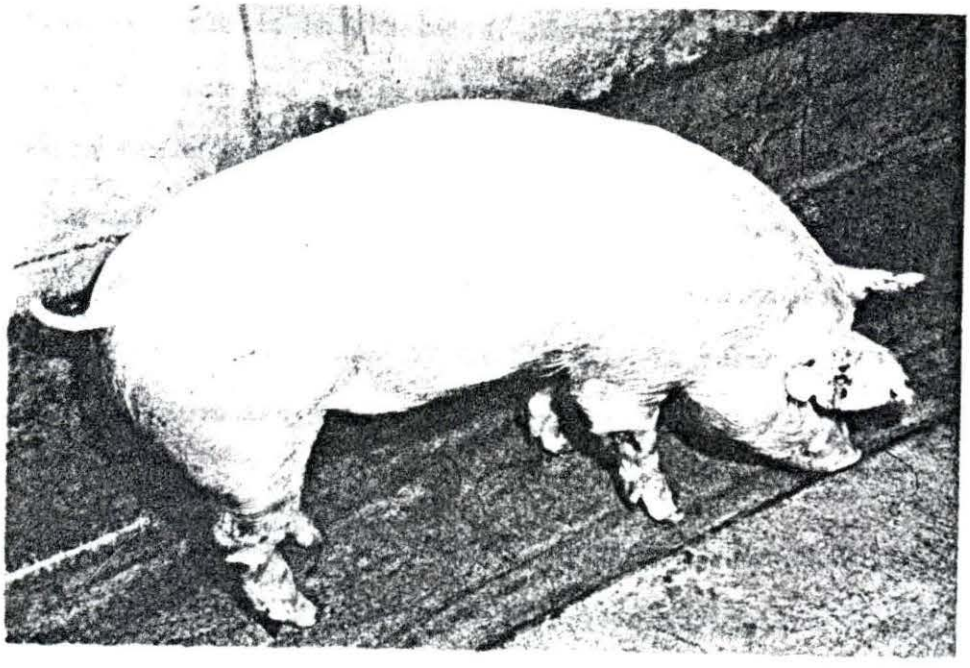
The overall severity of a pig's reaction was quantitated by the daily point system defined in MATERIALS AND METHODS. Although all these isolates were classified as L^4, some caused a generally severe reaction and some a rather mild reaction.

The long period of time which elapsed between exposure and death of pigs 8376 and 8385 stimulated considerable interest in them. Pig 8376 survived for 104 days following infection but during this time became extremely emaciated (Fig. 5). Pig 8385 gained weight during the 100-day interval prior to death (Fig. 6). There was also a significant difference in the gross lesions observed at autopsy. Lesions of pig 8376 were

Fig. 5. Pig 8376, 80 days after injection with field isolate 300

Fig. 6. Pig 8385, 80 days after injection with field isolate 331





primarily associated with the intestinal tract (Figs. 7, 9 and 11), whereas those of pig 8385 were primarily pneumonic (Figs. 8, 10 and 12).

Several experiments were conducted by exposing HC susceptible pigs to pigs infected with isolates 7, 300 and 331 (Table 17). Exposure was accomplished by placing the susceptible contact in an isolation room with the infected pig. They were separated by a partition which allowed only minimal physical contact. An exception was made in 1 instance when 2 pigs were allowed direct contact for a few hrs (pigs 7361 and 7527).

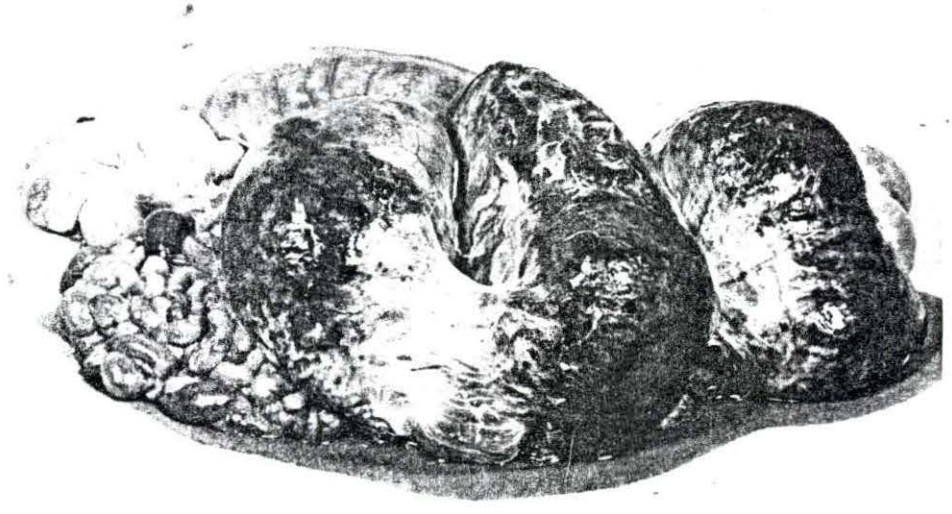
A marked difference existed between the results obtained with each of these isolates. In a single passage, isolate 7 appeared to increase in virulence and cause an acute reaction in the contact pig. Isolate 300 immunized the first contact and apparently did not spread to the second contact. Isolate 331 spread to all 3 contacts and in the process of serial passage increased somewhat in virulence. Nevertheless, it continued to cause a relatively long and low grade reaction.

The reason for the lack of spread of isolate 300 from pig 8499 to pig 8538 may be explained by correlating information in Table 17 with that in Table 18. On the first day of contact between these pigs, pig 8499 no longer had a viremia as evidenced by the FATCT. It seems likely that pig 8499 had recovered from its initial infection with HCV and was no longer disseminating virus.

It is evident from Table 18 that in the later stages of illness prior to death, leukocyte counts were not always a good indication of HCV infection. It is also evident that with these particular viruses a pig's

Fig. 7. Stomach and intestines from pig 8376

Fig. 8. Stomach and intestines from pig 8385



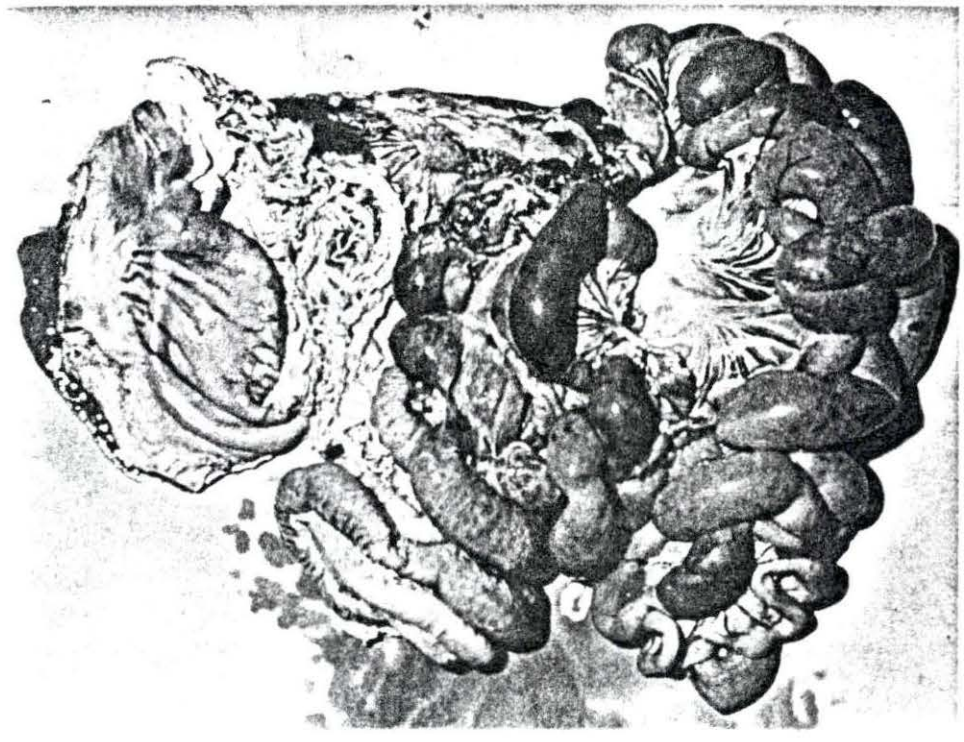


Fig. 9. Lung from pig 8376

Fig. 10. Lung from pig 8385

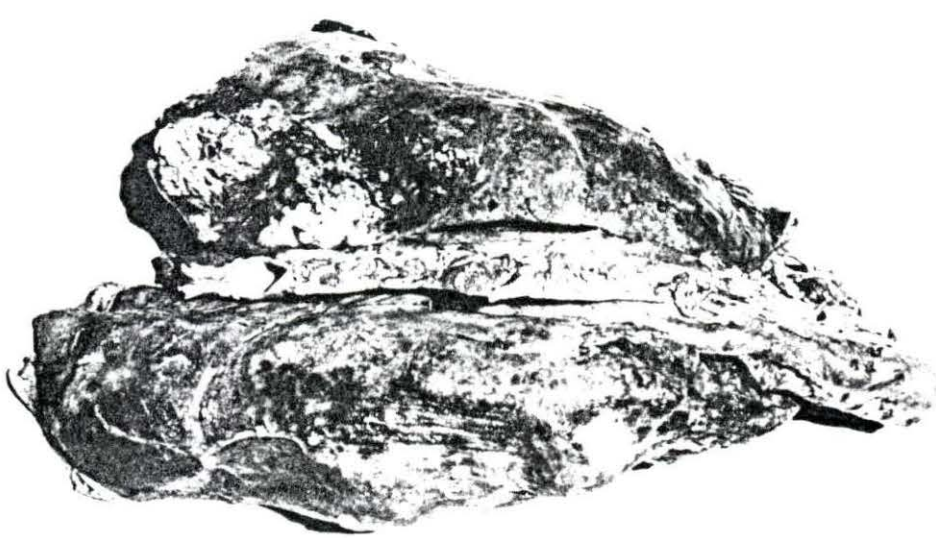
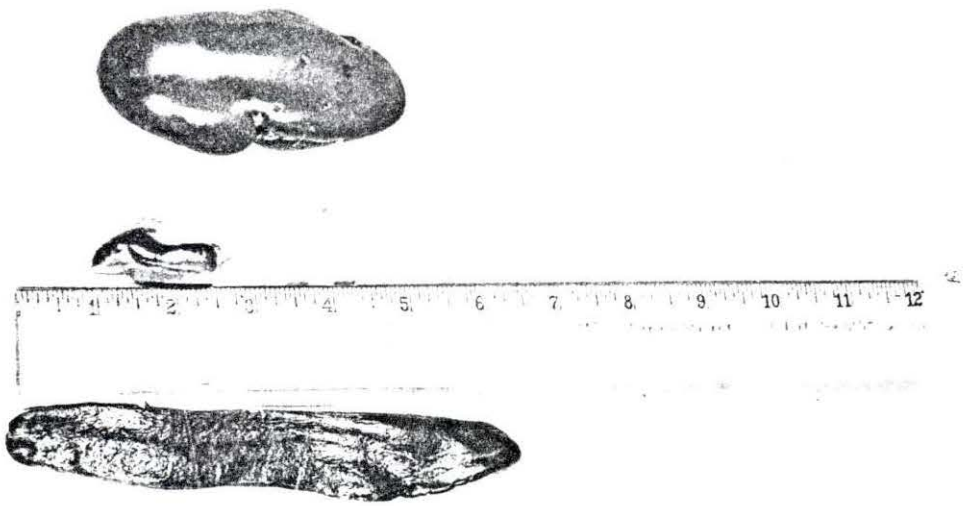


Fig. 11. Kidney, adrenal and spleen from pig 8376

Fig. 12. Kidney, adrenal and spleen from pig 8385



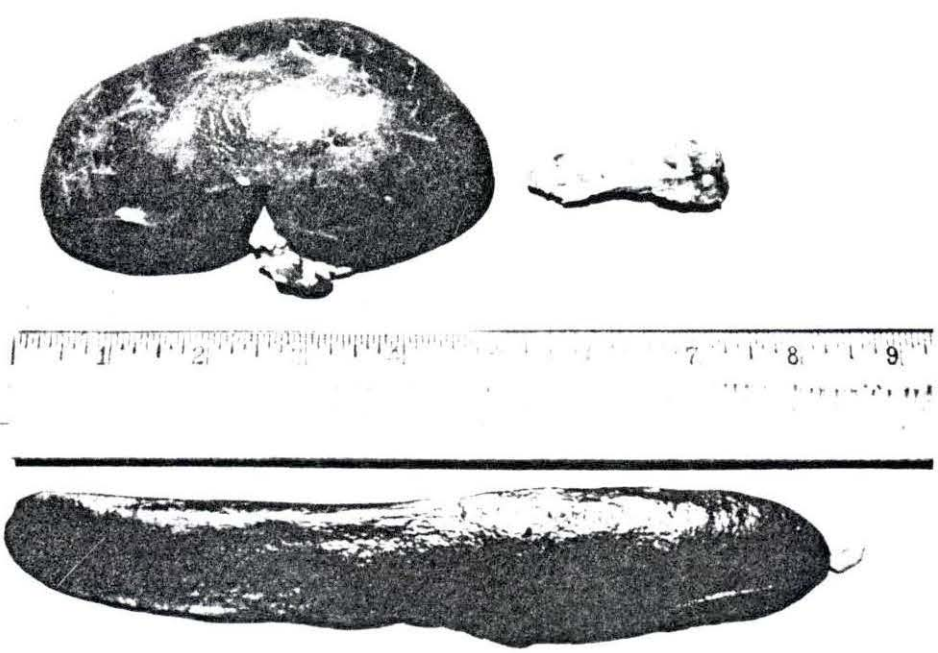


Table 17. Contact experiments with 3 field isolates of HCV classified as L^4

Isolate number	SIT (pig) number	Test pig description: Type	Test pig description: Weight (lbs)	Method of infection	Clinical reaction of infected pigs: Incubation time (days)	Clinical reaction of infected pigs: Days of illness preceding death	Clinical reaction of infected pigs: Total days (exposure to death)
7	7361	SPF-2	40	Injection of specimen 7	2	54	60
	7527	York	40	Exposure to pig 7361 (51 days after 7361 had been injected with specimen 7)	2	10	12
300	8376	SPF-2	44	Injection of specimen 300	1	91	104
	8499	SPF-1	45	Exposure to pig 8376 (99 days after 8376 had been injected with specimen 300)	No clinical reaction except 2 days of slightly elevated temperature (104 and 104.2 F)[a]		
	8538	SPF-1	48	Exposure to pig 8499 (20 days after 8499 had been exposed to pig 8376)	No clinical reaction[b]		

[a]Challenged 41 days after exposure. Immune.

[b]Challenged 21 days after exposure. Died following challenge.

Table 17 (Continued)

Isolate number	SIT (pig) number	Test pig description Type	Weight (lbs)	Method of infection	Clinical reaction of infected pigs: Incubation time (days)	Days of illness preceding death	Total days (exposure) to death)
331	8385	SPF-2	33	Injection of specimen 331	1	46	100
	8498	SPF-1	40	Exposure to pig 8385 (99 days after 8385 had been injected with specimen 331)	8	36	44
	8537	SPF-1	49	Exposure to pig 8498 (20 days after 8498 had been exposed to pig 8385)	3	24	27
	8547	SPF-1	50	Exposure to pig 8537 (21 days after 8537 had been exposed to pig 8498)	6	24	30

Table 18. Laboratory tests with samples obtained from pigs infected with field isolates of HCV 300 and 331

Isolate number	SIT (pig) number	Source of infection	Day samples obtained[a]	Clinical reaction[b]	Temperature	Leukocyte count	FATCT results[c] Blood	Feces	Saliva
300	8376	Specimen 300	73	Slight	103.8	....	+	....	....
			85	Slight	102.6	124,800	+	-	+
	8499	Exposure to pig 8376	9	None	102.8	14,000	+	....	....
			20	None	102.6	21,500	-	....	....
			30	None	102.4	21,650	-	....	....
			41	None	....	18,600	-	....	....
	8538[d]	Exposure to pig 8499	10	None	102.2	25,800	-	....	....
			21	None	....	18,400	-	....	....

[a]The number of days after injection of a specimen or exposure to an infected pig.

[b]On the particular day samples were obtained.

[c]+ = positive; - = negative.

[d]Apparently not infected. Died following subsequent challenge with Ames HCV.

Table 18 (Continued)

Isolate number	SIT (pig) number	Source of infection	Day samples obtained[a]	Clinical reaction[b]	Tempera-ture	Leukocyte count	FATCT results[c] Blood	Feces	Saliva
331	8385	Specimen 331	73	None	104.2	....	+	....	....
			85	Slight	105.2	31,400	+	+	+
	8498	Exposure to pig 8385	9	Slight	105.0	6,450	+	....	....
			20	Slight	103.4	4,550	+	....	....
			30	Slight	104.4	12,400	+	....	....
			41	Slight	105.8	24,750	+	....	....
	8537	Exposure to pig 8498	10	Slight	106.6	2,100	+	....	....
			21	Slight	104.6	3,750	+	....	....

observed clinical reaction did not always correspond to that which might have been expected from its temperature and leukocyte count.

Field Case Histories

The large number of HCV's classified as immunizing was somewhat unexpected. Particularly since the experimentally infected pigs were highly susceptible to HC. Were these isolates "naturally occurring" or was there some other possible source of infection, e.g., recent vaccination with modified-live HCV (MLV)? The histories which accompanied specimens from which I^1 viruses were isolated are presented in Table 19. Those from which I^2 viruses were isolated are presented in Table 20. In many instances pigs had been recently vaccinated or treated with MLV prior to obtaining samples. However, in other instances, there was no indication of the introduction of MLV into the herd.

Interpretation of Swine Inoculation Tests

It was possible to interpret most of the SIT's in a straight-forward manner. However, a few of the tests presented some difficulties.

A sewage line stoppage resulted in the partial flooding of isolation cages containing pigs injected with FATCT-negative specimens 112 and 114. The sewage contained excreta from HCV infected pigs. Both of the test pigs involved were sick when blood samples were obtained several days following the accident.

The blood sample from the first pig injected with specimen 112 was FATCT negative. Since any final SIT result would have been clouded by the

Table 19. History and remarks pertaining to field cases from which I[1] HCV's were isolated

Case number	Treatment prior to obtaining samples	Additional history and remarks
6	Serum[a]	Vaccinated 16 days prior to outbreak with MLV without serum.
10	Serum and MLV 24 hrs before samples were obtained	No additional information.
39-51	Serum and MLV	First clinical signs on 9-2-63. Treated 9-3-63. Several large hogs died about 9-15-63.
49	Serum, penicillin and streptomycin	One hundred and forty-seven suckling pigs sick 3 weeks ago. Recovered. Weaned and then got sick again.
58	No information	Twenty-six, 2-week-old pigs sick. Farrowed by 4 sows given serum alone about 3 months ago.
71	Serum and MLV 2 days before samples were obtained	Thirty pigs purchased from sales barn 5 weeks ago. Mixed with 121 pigs which had been recently vaccinated.

[a]Anti-HC serum unless otherwise specified.

Table 19 (Continued)

Case number	Treatment prior to obtaining samples	Additional history and remarks
90	Sulfa, liquemycin, penicillin, streptomycin erysipelas serum	No vaccination on this farm since the last HC outbreak, 25 years ago. No pigs added to the herd for some time. Pigs were sick for 2 weeks before lesions suggestive of HC were observed.
145	No information	Vaccinated with MLV about 2 weeks ago.
146	No information	Both sows and their pigs sick. Typical lesions of HC.
162	No information	Feeder pigs supposed to have been vaccinated 1-20-64. Purchased 1-21-64, sick 1-27-64. Leucopenia and lesions of HC.
232	No information	First sick 3-21-64. Samples received 3-26-64. Pneumonia, petechiae on heart, kidney and bladder, gastroenteritis. Temperatures 98-104 F.
261	No information	Typical HC lesions and symptoms.
275	None	Not vaccinated. Typical HC lesions.
333	Some pigs treated with serum and MLV	Spleens from 3 pigs. One had been treated with MLV and serum, 2 had not been treated. (Two of the 3 spleens were FATCT positive.)
342	Serum	Continuous farrowing system practiced on this farm. Pigs are vaccinated shortly after weaning. Last group vaccinated 3-1-64. All sickness in suckling pigs under 2 weeks of age. First observed sick on 3-22-64.

Table 20. History and remarks pertaining to field cases from which I^2 HCV's were isolated

Case number	Treatment prior to obtaining samples	Additional history and remarks
8	None	Recently vaccinated with MLV without serum.[a]
18-19-20	No information	No information.
22-44	Serum	Not vaccinated. Sows became ill in late gestation and were given only serum. Specimen 22 from a sow. Specimen 22 received 8-1-63. Pigs farrowed by these sows became ill at about 5 weeks of age (9-9-63). Specimen 44 was a spleen from 1 of the pigs (I^2 HCV from both specimens).
54-56	Serum and MLV	No information.
87	Serum and MLV	Typical HC, symptoms and lesions.
91	None	Not vaccinated. Typical lesions of HC.
104	Serum and antibiotics	Some of the pigs vaccinated 2 weeks ago with MLV without serum. Typical HC lesions.
109	Neomycin and terramycin	Samples from 6-week-old pig not vaccinated. Lesions suggestive of HC.
117	Sulfathiazole, furacin and antibiotics	Vaccinated 12-12-63. Samples received 12-20-63. A statement was made in the history that this appears to be a recurring condition associated with a continuous farrowing system.

[a]Anti-HC serum.

Table 20 (Continued)

Case number	Treatment prior to obtaining samples	Additional history and remarks
158	No information	Sample from 5-week-old pig. Some of the sows were vaccinated with MLV about 3 weeks before farrowing. Pigs born hairless. Live pigs anemic and icteric. No typical HC lesions.
171	No information	Vaccinated. No statement as to when pigs were vaccinated.
173	No information	Not vaccinated. Clinical signs not typical of HC but lesions suggest HC.
181	No information	Petechiae on kidney. Anemic.
183	Serum	Not vaccinated.
186	No information	Some pigs vaccinated twice with MLV and serum. Last vaccination 2 weeks ago. Leucopenia in 7 of 8 pigs.
207	None	Not vaccinated. Petechiae on kidney, bladder, and lungs.
214	None	Part of the herd was vaccinated on 1-24-64 with MLV and serum. Samples from 3-week-old pigs received on 3-18-64. Typical lesions of HC. Leucopenia 400-5,000 WBC's/mm^3.
222	Serum	Not vaccinated. Petechiae on kidneys, necrotic tonsils.
240	No information	Not vaccinated. Pigs die suddenly. Temperatures about 103 F. Petechiae on kidneys. Lesions of pneumonia. Clinical diagnosis of enterotoxemia.

Table 20 (Continued)

Case number	Treatment prior to obtaining samples	Additional history and remarks
262	No information	Leukocyte counts on 2 pigs, 5,720 and 1,672 WBC's/mm^3.
289	Antibiotics	Illness in group of baby pigs 1-2 weeks old. One sow slightly ill. No lesions suggestive of HC in pigs autopsied on the farm, but petechiae observed on the kidneys of 1 pig sent to the Iowa State University Diagnostic Laboratory. Sows vaccinated 2 years ago. No pigs vaccinated on the farm during the last 4 months. Two months ago, 6 sows farrowed 40 pigs. The pigs went through the same type of illness. Twenty-nine of the pigs died.
311	Penicillin and streptomycin	Not vaccinated. Illness in pigs about 8 weeks old. Leukocyte counts on 3 pigs, 14,550, 10,200 and 4,550/mm^3. Clinical signs: high temperature, constipation, anorexia and ataxia. Lesions: hemorrage of kidneys, bladder, lungs, and gastric serosa, marginal hyperemia of lymph nodes.
314	None	Illness in group of 50 pigs, 8 weeks old, not vaccinated. However, the first animal that died was a sow recently purchased at a sale barn. Temperatures from 104-105.5 F. Only lesions suggestive of HC were petechiae on the kidneys. All pigs have pneumonia.
316	Serum and MLV 24 hrs before obtaining samples	Illness in group of 8-week-old pigs. Not vaccinated. Temperatures 104-106 F. Clinical signs: Loss of appetite and diarrhea. Lesions: serous and hemorrhagic lymphadenitis, petechiae in medullary area of kidneys, widened costochondral junctions of ribs and catarral to necrotic enteritis.

Table 20 (Continued)

Case number	Treatment prior to obtaining samples	Additional history and remarks
322	No information	Samples from pig 4 months old. Vaccinated. Recently purchased at a sale barn. Average temperatures of pigs, 106 F. Clinical signs and lesions of HC.
323	None	Ten of eighty, 125-225 lb pigs are sick. Eleven have died. None have been vaccinated. Temperatures up to 107.5 F. Clinical signs: depression, anorexia and respiratory symptoms. Lesions: petechiae on kidney, bladder and larynx.
328	No information	Vaccinated 10 days ago with MLV without serum. Lesions: wide costochondral junctions, petechia in kidneys and serous lymphadenitis.
335	Tylosin in drinking water	Sample from 3-month-old pig. Pigs recently purchased from various sources. Clinical signs: diarrhea, staggered gait. Lesions: severe inflammation of stomach, massive pneumonic areas of entire lung, enlarged spleen, urinary bladder normal, mesenteric lymph nodes peripheral hemorrhage, and marked hemorrhage of serosal surface of intestines.
337	No information	Illness in group of 80 pigs, 2 months old. Vaccinated 2 weeks ago. Clinical signs: diarrhea and staggering. Temperatures 105-107 F.

prior history of contamination, the pig was killed 2 weeks after receiving the field specimen. At autopsy the pig did not have lesions indicative of HC. Eleven days after injection of the first pig, inoculum was again prepared from specimen 112 and injected into a second pig. This pig died 4 days later. There were no typical lesions of HC and the spleen was FATCT negative. Eight months later a third pig was injected with specimen 112. This SIT was negative. The conclusion was that HCV was not present in the specimen.

The field case history which accompanied specimen 112 lent some support to the above conclusion. The pigs had diarrhea, elevated temperatures and were sneezing. They had swine pox. Lesions were those of pneumonia and catarral enteritis. One week before the specimen was obtained, MLV and anti-HC serum had been administered to the herd.

The blood sample from the first pig injected with specimen 114 was FATCT positive. The pig died shortly after the blood sample had been obtained. Approximately 10 days after specimen 114 had been received, another specimen (specimen 120) was submitted from the same herd. Three ml of inoculum prepared from specimen 114 and 3 ml of inoculum prepared from specimen 120 were pooled and injected into a second pig. This SIT was negative. Eight months later a third pig was injected with specimen 114. This test was also negative. The conclusion was that the first pig injected with specimen 114 was infected with HCV by accidental exposure to contaminated sewage and that the specimen did not contain HCV.

A rather complete history accompanied specimens 114 and 120. The course of illness observed was that 1 or more pigs in a pen developed

anorexia and lethargy which persisted for 1-7 days. Temperatures were usually normal with an occasional high of 104 F. Within 3-7 days after the first clinical illness, pigs developed a severe hemorrhagic diarrhea and died within 8 hrs. Leukocyte counts were normal to slightly elevated and lesions were confined to the gastrointestinal tract.

The SIT results with specimens from cases 112 and 114-120 have been presented in considerable detail for 2 reasons. First, because they caused some difficulty in interpretation, but in addition, these were the only instances when pigs died after receiving a field specimen and the overall SIT result was recorded as negative.

Specimen 57 was too decomposed to examine by the FATCT; however, it was injected into a pig. The pig was clinically normal for 9 days after receiving the field specimen, but on the tenth and eleventh days it had a slight reaction and it died on the twelfth day. Samples were not obtained for FATCT examination. Gross lesions were not suggestive of HC. Since this specimen could not be used to satisfy the primary objective of this study, i.e., to compare FATCT and SIT results with field specimens, no additional SIT's were done. No conclusion was reached as to whether the specimen was SIT positive or negative; thus, for all practical purposes, it was treated as if it had not been examined by either the FATCT or the SIT.

Interesting results were obtained with the specimens from case 39-51. Specimen 39 was a blood sample and 51 was a spleen. Both were FATCT negative.

One of 2 blood samples taken from the pig injected with specimen 39 was FATCT positive. Following an obvious clinical reaction, the pig recovered and was challenged on 10-6-63. Temperatures taken on the first 3 days following challenge were all 102.8 F. No additional temperatures were taken but the pig continued to appear clinically normal. Sixteen days after challenge the pig was "released" from the experiment and the SIT was recorded as positive. The field isolate was classified as I^1. After its release the pig was placed in an isolation room with other pigs which had also resisted challenge. Without showing any prior clinical signs, the pig died on either the evening of 11-5-63 or early morning of 11-6-63. Some of the lesions observed at autopsy were suggestive of HC.

Two blood samples were taken from the pig injected with specimen 51. One was FATCT positive. The pig was sick for 27 days prior to its recovery and subsequent challenge. It died 3 days after challenge. One year later another pig was injected with specimen 51. It had a slight clinical reaction on the sixth, eighth, ninth and tenth days after receiving the specimen. No blood samples were taken from this pig. Twenty-four days after receiving the specimen, the pig was challenged. It died 10 days later.

Case 39-51 was recorded as FATCT negative and SIT positive.

CORRELATION OF FLUORESCENT ANTIBODY-TISSUE CULTURE AND SWINE INOCULATION TEST RESULTS

All 225 FATCT-positive diagnoses of HC were confirmed by SIT's. On the other hand, there were 8 cases of HC from which HCV was detected by SIT's but not by FATCT's done with the same field specimens. Eighty-three suspected cases were negative by both tests (Table 21). Results could not be compared for the remaining 22 cases because 1 or both tests were not done or the SIT was considered questionable (Table 22).

The majority of the 238 cases for which a laboratory diagnosis of HC was made, were confirmed in 3 ways. First, HCV was identified from the field specimen by the FATCT. Secondly, the disease was reproduced in a clinical or subclinical form and thirdly, HCV was reisolated and identified by the FATCT from the blood or spleen of the experimentally infected pig (Table 23).

A comparison was made between the relative virulence of field isolates of HCV and the frequency of their isolation and subsequent identification by the FATCT (Table 24). A comparison was also made between the relative virulence of field isolates and their titer in the field samples from which they were isolated (Table 25). The samples represented in Table 25 are the same as those in the previous Tables 3, 4, 5, 6 and 7. Not all the FATCT-positive samples are represented since the titer estimate was not initiated until sometime after this study had begun.

Table 21. Correlation of SIT and FATCT results on 316 suspected cases of HC

Number of cases	Test results SIT	FATCT	% correlation
225	Positive	Positive	96.6
8	Positive	Negative	
83	Negative	Negative	100

Table 22. Summary of SIT and FATCT results on 22 suspected cases of HC for which no correlation could be made

Number of cases	Test results SIT	FATCT
5	Positive	....[a]
3	Negative	....[a]
6	Questionable	Negative
8	....[b]	....[a]

[a]Samples decomposed or adulterated to the extent that no FATCT could be performed.

[b]Samples from these 8 cases were severely decomposed and no SIT was performed.

Table 23. Correlation of positive SIT results with FATCT results on field specimens and samples taken from experimentally infected pigs

Number of cases	SIT results Field specimens	FATCT results Field specimens	FATCT results Lab samples
203	Positive	Positive	Positive
17	Positive	Positive	Negative
5	Positive	Positive	....[a]
8	Positive	Negative	Positive[b]
5	Positive	....[c]	Positive
Total 238			

[a]No samples taken.

[b]In 2 instances a blood sample from the experimentally infected pig was FATCT negative but HCV was isolated and identified by FATCT from the spleen.

[c]Decomposed or adulterated specimens, no FATCT done.

Table 24. Comparison of the relative virulence of HCV's and the frequency of their isolation and identification by FATCT

HCV classification	Number of cases	Field samples: Spleen Number	Field samples: Spleen % positive	Field samples: Blood Number	Field samples: Blood % positive	Blood samples from experimentally infected pigs: Number	Blood samples from experimentally infected pigs: % positive
Lethal	193[a]	267/291[b]	91.8	142/219	64.8	198/203	97.5
Immunizing	45	57/67	85.1	36/73	49.3	27/52	51.9
Total	238	324/358	90.5	178/292	61.0	225[c]/255	88.2

[a]Field isolates from 193 cases killed experimentally infected pigs.

[b]Numerator = number FATCT positive; denominator = number examined by FATCT.

[c]Does not include 13 FATCT positive blood samples from pigs exposed by contact (Tables 17 and 18), injected with specimens from cases considered questionable (Table 14), and the first pig injected with specimen 114.

Table 25. Comparison of the relative virulence of HCV's and their titer in the field samples from which they were isolated

Virus classification	Type of sample	Number of samples	Average HCV titer/sample[a]	Average numerical value/sample[a]
Lethal	Spleen	230	3.5×10^4	3.8
	Blood	115	2.5×10^4	2.9
Immunizing	Spleen	46	1.7×10^4	3.1
	Blood	31	1.7×10^4	2.7
Total	Spleen	276	3.1×10^4	3.7
	Blood	146	2.4×10^4	2.9

[a]The methods for estimating the HCV titer and for calculating the average titer and numerical value are presented in PART I. FLUORESCENT ANTIBODY-TISSUE CULTURE TEST.

DISCUSSION

A close correlation was found between the results of SIT's and FATCT's, however, several advantages were noted for each type of test. Foremost is the fact that swine inoculation is a more sensitive method to detect HCV. Excluding the questionable cases, virus was detected from 8 cases only by SIT's. Another advantage of swine inoculation is that samples which are too decomposed or adulterated to be run by the FATCT, can be tested in swine.

On the other hand, there are several disadvantages of SIT's which more than offset the advantages. First is the expenditure that is necessary, not only for swine but also for the elaborate isolation facilities that are required. Secondly, the time factor must be considered. A significant number of SIT's required several weeks or even months to complete; in contrast, the FATCT was usually completed in 16-24 hrs. Of course, the disadvantages of swine inoculation discussed thus far were not uniquely revealed by this study. They are well known and have been stated elsewhere (62). However, 1 disadvantage of SIT's was emphasized during this study and that is the lack of clear-cut results which can occur in some instances. For example, if a pig is infected with a HCV of low virulence, the ensuing reaction is, to say the least, confusing. In addition, false negative results can occur. To exemplify this point, let us take a specific example. A blood sample was submitted from suspected case 158. The history gave little reason to believe that this was a case of HC, or so it was believed at the time.

Pigs 4-5 weeks of age were anemic and icteric. Some had been born hairless. The sample was examined by the FATCT and was found to contain a high titer of HCV. In the meantime the remainder of the sample, 3 ml, had been injected into a HC-susceptible pig. Following the injection, the pig had no definite clinical reaction. On the seventh day it was bled and HCV was again identified by the FATCT. Its temperature on the day of bleeding was 102.6 F. Since the pig had had no clinical reaction, it was challenged 14 days after receiving the field sample. It died 6 days later. The above information was then reported to the person who had submitted the sample. At that time an additional bit of information was received. Since HC had been diagnosed on the neighboring farm, the sows of the herd in question were vaccinated in the latter third of gestation. The original field sample had been used up for the FATCT and the first SIT; therefore, 2 additional pigs were injected with a blood sample from the pig previously injected with the field sample. Both of these pigs were found immune when subsequently challenged (Tables 13 and 15).

The few negative SIT results with specimens previously shown to contain HCV, cannot be particularily emphasized because the SIT was not employed to its fullest advantage. Ideally, both HC-susceptible and HC-immune pigs would be injected with the same suspect tissues (62). Also, at least 1 HC-susceptible pig should be injected with the suspect tissue plus anti-HC serum. Sufficient isolation facilities were not available

for performing all tests in such an elaborate manner. As it was, over 400 pigs were used in this study.

Concisely, what are the general attributes of the FATCT? There is no doubt that the FATCT is highly reliable, especially if certain procedures are followed, including the selection of samples. These procedures will be discussed subsequently. In addition, the FATCT is relatively economical, rapid, and easy to read.

Before reaching a conclusion about the percentage of field cases of HC from which virus was identified by the FATCT (such as the 96.6% in Table 21), 2 other factors must be considered. Both tests may have failed to detect HCV in suspect tissues. Furthermore, tissue may have been obtained from swine which were not infected with HCV even though other swine in the same herd were infected.

The correlation of results presented in Table 21 was made on a case basis. If it had been made on a specimen basis, the overall correlation would have been altered slightly. In 2 instances when 2 specimens were submitted from the same field case, both were positive by SIT's, whereas, only 1 of 2 was positive by FATCT's.

The success of the FATCT for the detection of HCV is contingent upon 3 factors: (1) a standardized and specific fluorescent antibody preparation, (2) HCV-susceptible, uniform cell cultures, and (3) satisfactory tissue samples from suspected cases of HC.

No difficulties should be encountered in preparing satisfactory fluorescent antibody, providing the elements responsible for non-

specific staining are effectively removed. Rabbit liver powder "sorption" has been satisfactory for this purpose. Once the fluorescent antibody is prepared it may be stored for a prolonged period at -60 C. One such preparation has been maintained at the NADL for over 2 years without a significant decrease in potency.

The best results are obtained with PK-15 cells if they are used for the FATCT within 3-4 days after they have formed a confluent monolayer. As these cells age, the specific staining decreases and the nonspecific staining increases. In addition, the cells continue to multiply, forming a multilayer of cell growth on the coverslip. This multilayer tends to "trap" the fluorescent antibody. As a result, there is an undesirable background of fluorescence which reduces the contrast between the specific staining of infected cells and the uninfected cells in the immediate vicinity.

Sample quality is particularly critical. If cell cultures are exposed to inoculum prepared from tissues that have undergone marked decomposition, the cells are rapidly destroyed. Sufficient dilution of the inoculum to minimize this toxic effect also results in the undesirable reduction in virus content per unit volume of the inoculum.

Multiple sampling is essential. Based on the information gained during this study, one can expect to isolate and identify HCV from approximately 90% of spleens obtained from swine herds where HC exists. Considering combined probabilities, there would be a 1 in 10 chance of obtaining a false negative result if only 1 spleen were submitted, 1 in

100 for 2 spleens, and 1 in 1,000 for 3 spleens. However, this reflects the average and probably does not accurately represent the reliability of the test for all cases of HC. More realistically, variabilities associated with each individual case should be considered. For instance, the treatment of swine with anti-HC serum could cause a significant reduction of the titer of HCV in the tissues and consequently decrease the chance for its detection by the FATCT.

Spleen is markedly superior to blood as a diagnostic sample and therefore blood would be indicated primarily when spleen either is not available or when blood can be obtained from swine which appear to be in the acute stage of HC, whereas, these same swine are not available for autopsy. A good rule of thumb is to submit the most samples from herds where the clinical findings are the most confusing. At least 3 spleens should be submitted from any suspected case.

Ideally, spleens should be obtained from swine with elevated temperatures and which are apparently in the acute stage of the disease. Less desirable but yet satisfactory samples could be taken from moribund swine or swine dead for only a short period of time prior to autopsy. In any case, the tissue should be shipped to the laboratory refrigerated or frozen to minimize decomposition in transit.

Anticoagulants should not be added to blood samples unless it has been determined that the anticoagulant or preservative contained therein is not toxic to cell cultures. The best procedure is to defibrinate the sample or allow it to clot and submit the serum.

A significantly greater percentage of blood samples from experimentally infected pigs were FATCT positive than those from confirmed field cases of HC (Table 24). There are several probable explanations for this difference. The laboratory samples were from pigs known to be infected with HCV, whereas, some of the field samples may have been taken from pigs not affected with HC. The majority of the experimentally infected pigs were bled during a period of marked clinical illness. This may not have been true for as many of the field samples. Hog cholera virus may have been destroyed by the decomposition of some of the field samples. Finally, many of the swine from which field samples were obtained had previously been treated with anti-HC serum.

It is also obvious from Table 24 that lethal HCV's were isolated much more frequently from the blood stream of experimentally infected pigs than were immunizing viruses. A lesser difference occurred between the blood samples from pigs naturally infected with these types of viruses. This discrepancy can logically be explained by assuming that the viruses classified as immunizing were causing a more severe reaction in the field. This assumption is confirmed by field case histories (Tables 19 and 20).

The FATCT could not be used to differentiate between lethal and immunizing field isolates of HCV. Even though less virus was present in tissues of swine naturally infected with HCV's classified as immunizing, this difference was not particularly striking (Table 25). Considering an individual case from which only a few samples might be submitted, it is

doubtful if any reliable determination of virulence could be based on the virus content of tissues.

The relative virulence of a particular HCV usually is of little significance in the field. The important factor is, of course, the severity of the disease it is able to cause under the particular field conditions in which it occurs. However, the inability to determine relative virulence by the FATCT brings out an important practical consideration. If samples are obtained from swine which have been recently vaccinated or treated with MLV, care must be exercised in drawing a conclusion from a positive test. The virus isolated and identified may not be responsible for clinical HC. In fact, irrespective of the circumstances, it should be emphasized that the FATCT detects the presence of HCV; it does not actually diagnose HC. The FATCT results plus additional laboratory and field information should be used in arriving at a diagnosis of the disease.

From the laboratory standpoint, the FATCT can most advantageously be used in conjunction with other types of tests which are based on evidence of the disease itself. For example, leukocyte counts and histopathologic examinations would yield valuable information which could be correlated with FATCT results.

The virulence classification of field isolates of HCV provided a general view of the relative virulence of naturally occurring HCV's. The application of the same method of classification to the reactions of pigs challenged with Ames HCV provided a base line of virulence to

which field isolates could be compared. The 2 major divisions of the classification scheme, i.e. lethal and immunizing, were of course fundamental, but the subclassifications were made somewhat arbitrarily except for L^1. When it was found that most of the pigs challenged with Ames HCV died after a clinical illness of 10 days or less, this subclassification was made in retrospect.

Lethal viruses were subclassified by the days of clinical illness preceding death rather than by the total days from exposure to death for 2 reasons. Many of the samples had undergone various degrees of decomposition and as a consequence the virus contained therein may have been attenuated slightly. In addition, the amount of virus each pig received varied with the HCV titer of the sample(s) from which the inoculum was prepared. It was assumed that if these factors did affect SIT results, they would be reflected more by the incubation period than by the duration of clinical illness.

Since the reaction of a pig to HCV infection depends on its innate resistance as well as the pathogenicity of the virus, it is probable that other types of pigs would have reacted differently to some of the field isolates. However, for the determination of relative virulence, it is believed that the classification data is generally reliable.

A few statements can, therefore, be made about the virulence of the 238 naturally-occurring HCV's which were isolated during this study. Approximately 30% of them were as virulent as Ames HCV, that is, they were classified as L^1. Approximately another 20% were classified

as immunizing and the remainder somewhere between these 2 extremes. There is 1 adjustment that could appropriately be made in the above figures. Upon examination of Table 11, it will be noted that a significantly lesser percentage of immunizing viruses were submitted by the Animal Health Division. To explain this difference it is necessary to divide the term of suspected cases into "3 degrees of suspected". Samples were received from cases which were probably HC, from cases which were possibly HC and from a few cases which were probably not HC. Samples submitted by the Animal Health Division were more frequently from cases in the first category above or in other words "selected cases" wherein the signs and lesions observed were typical of HC.

This difference is also reflected by the percentage of laboratory confirmed cases from the various sources (Table 10). It would be expected that highly virulent HCV's would cause the more typical clinical manifestation. Then to obtain a more representative overall picture of the virulence of naturally-occurring HCV's, one may consider only those isolates submitted by veterinary practitioners and the Iowa State University Veterinary Diagnostic Laboratory. Thus, it is found that over 30% of these field isolates were classified as immunizing.

Certainly few, if any, of the immunizing viruses would have been so classified on the basis of the reactions of naturally-infected pigs. Therefore, probably none of the viruses isolated during this study can be considered as avirulent and possibly all are potentially lethal under the appropriate circumstances.

More than 1 SIT was done with many of the field specimens. The various reasons for repeating tests have already been pointed out. Occasionally the time between the first and subsequent tests was over a year. This interval was not by design but occurred because the isolation facilities were usually fully utilized for a single SIT for each specimen at the time it was received. Consequently, the majority of the repeated tests were done when additional specimens were no longer being submitted from the field. During the time between the first and subsequent tests, specimens were stored at -60 C. However, most of the specimens were thawed or partially thawed once during their storage in the process of transferring them from one freezer to another. Therefore, most of the repeated tests were done with specimens that had been frozen and thawed twice (or 3 times if the specimen had been frozen for shipment to the NADL).

A SIT diagnosis of HC was considered questionable on 6 suspected cases because of the contradictory results of SIT's done for these cases. The specimens were all FATCT negative. Except for specimen 53, the first SIT done with each of them was positive, whereas repeated SIT's were negative. The first SIT with specimen 53 was considered incomplete, a second test was positive, and a third test was negative. Results of the individual SIT's and additional information pertaining to them have been presented in Table 14. There are several possible explanations for the contradictory results.

If it is assumed that these specimens did contain HCV, then it may also be assumed that they contained a minimal amount of virus. Since the FATCT was negative it seems reasonable that if virus was present, it was present in a low concentration. Therefore, repeated tests may have been negative because the small amount of viable virus was destroyed during storage of the specimen. It is also possible that the first inoculum contained a few infectious units of HCV, whereas, by chance, the inoculum that was subsequently prepared from the same specimen, did not contain infectious HCV.

If it is assumed that the specimens did not contain HCV, then what was the source of the virus which caused the positive SIT's? The test pigs may have been inadvertently exposed to HCV, possibly before they were placed in the isolation cages or in 1 instance, specimen 47, at the time of bleeding (Table 14). Another possibility is that the inoculum may have been contaminated with HCV during its preparation in the laboratory. It is probable that a minimal contamination would more likely be detected by the SIT than by the FATCT.

Any of the possibilities mentioned above could apply to 1 or more of the 6 cases. However, since no definite conclusion could be reached, these cases remain questionable.

Field isolates 109, 158, 219, 300, 331 and 342 failed to either kill or immunize the first pig injected with each of them. The corresponding specimens and consequently the suspected field cases would have been recorded as negative if HCV had not been identified by the FATCT from the

field specimens and the blood samples from 5 of the 6 test pigs. The knowledge that the specimens contained HCV prompted repeated SIT's with them. The repeated tests were all positive (Table 15). Since the repeated tests were positive, it appears that the first pigs may have failed to resist challenge with Ames HCV because they were incapable of a "normal" immunologic response. However, in at least 1 instance the pig failed to resist challenge for another reason.

Pig 8105 was challenged 27 days after it had been injected with isolate 300. The pig was slightly ill for 2 periods following injection of the isolate, once for 6 days and once for 7 days, but had been clinically normal for 8 days prior to challenge. Just prior to challenge a blood sample was taken. On the same day the pig's temperature was 102.2 F. When the pig died following challenge, the blood sample was examined by the FATCT and was found to contain HCV. It was, therefore, obvious that the pig had only apparently recovered and was challenged at a time when it still had a viremia from infection with isolate 300. The second pig injected with isolate 300, pig 8376, was not challenged. It died 104 days after it had received the field isolate (Table 16).

Blood samples were not obtained from the other pigs just prior to challenge. However, a similar situation may have occurred in at least 1 other instance. Pig 8148, which had been injected with isolate 331, had a reaction but apparently recovered and was challenged. It died following challenge. The second pig injected with isolate 331 was not

challenged. It died 100 days after it had been injected with the field isolate (Table 16).

Occasionally more than 1 specimen was submitted from the same field case but from different sources and/or on different days. If the accompanying information did not clearly indicate their common origin, the specimens were initially assumed to have come from different cases. Only after obtaining some additional information or after some additional "detective work" was it possible to correctly associate these specimens with the appropriate cases.

A similar problem occurred when more than 1 blood sample or several pieces of spleen were submitted from the same herd and there was no information to indicate whether the samples were from the same or different pigs.

The above problems occurred infrequently. Nevertheless they are stated as a point of information and to explain the reason for a slight difference between the results presented in this thesis and those presented in a brief preliminary report of these studies (66). Additional information and study of the data necessitated these minor changes.

In a few instances a choice had to be made for the purpose of recording the source of specimens from a suspected case of HC. For example, specimens 18, 19 and 20 were from the same case but specimens 18 and 19 were referred by the Iowa State University Veterinary Diagnostic Laboratory whereas specimen 20 was submitted directly to the NADL by a veterinary practitioner. In Tables 10 and 11, this case is listed for

the Iowa State University Veterinary Diagnostic Laboratory. In addition, a few specimens were apparently collected by veterinary practitioners but reached the NADL by an indirect route. These specimens were recorded as if they had been sent directly to the NADL by the practitioner. The previous statement, of course, does not apply to the specimens referred by the Iowa State University Veterinary Diagnostic Laboratory.

SUMMARY

An extensive evaluation of the fluorescent antibody-tissue culture test (FATCT) was conducted to determine its efficacy as a routine diagnostic test for hog cholera (HC). Tissues submitted from suspected or possible cases of HC were examined by the FATCT. Results thus obtained were compared to those obtained by the inoculation of HC-susceptible swine with a portion of the same tissues.

Spleen and blood samples were received from 338 suspected cases of HC occurring in 22 states. It was possible to compare the results of the FATCT and the swine inoculation test (SIT) for 316 of these cases. Hog cholera virus (HCV) was identified by the FATCT from 1 or more tissues from 225 cases. These positive FATCT results were confirmed by SIT's. Hog cholera virus was identified from 8 cases by SIT's but not by the corresponding FATCT's. Both types of tests were negative for 83 suspected cases.

A comparison of results could not be made for the remaining 22 cases from which samples were received. Tissues from these cases were either too decomposed or adulterated to examine by the FATCT or the SIT results were considered questionable.

A total of 918 spleen and blood samples were received from the above 338 suspected cases of HC. Sixty-eight of the samples were too decomposed or adulterated to examine by the FATCT. Hog cholera virus was identified by the FATCT from 502 (59%) of the remaining 850 samples.

Spleen was superior to blood as a diagnostic sample. Three hundred and fifty-eight spleens were submitted from swine herds wherein HC was proven to exist by the SIT. Hog cholera virus was identified by the FATCT from 324 (91%) of these spleen samples. In contrast, only 178 (61%) of 292 blood samples from these same herds were FATCT positive.

The majority of the HCV's which were isolated during this study killed experimentally infected pigs and were consequently classified as lethal. However, approximately 20% of the field isolates were of lesser virulence. That is, they immunized experimentally infected pigs against subsequent challenge with the lethal Ames strain of HCV.

The close correlation that was found between the results of FATCT's and SIT's should stimulate the acceptance of the FATCT as an in vitro method to identify HCV from naturally-occurring cases of HC.

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This study was conducted under Public Law 85-507.

APPENDIX A

Preparation of Carbonate-Bicarbonate Buffer (64)

Solution A - Na_2CO_3 - 5.3 gm

H_2O to make 100.0 ml

Solution B - $NaHCO_3$ - 4.2 gm

H_2O to make 100.0 ml

A pH of 9.0 should result from mixing 4.4 ml of solution A with 100 ml of solution B. However, it is sometimes necessary to add more of solution A to the mixture. The pH should be checked on a meter.

Preparation of Rabbit Liver Powder

1. Remove the omentum and gall bladders from fresh rabbit livers.
2. Homogenize the livers in a mechanical blender by short repeated activations of the blender to prevent overheating.
3. Extract the homogenized liver by washing it 3 x in 4 x its volume of acetone. After each washing, allow the liver to settle and then decant the supernatant fluid.
4. Collect the washed liver on a filter paper in a Buchner funnel.
5. Dry the liver on the filter paper with suction until the acetone has evaporated.
6. Break up the cake of liver that has formed on the filter paper and allow it to continue drying overnight at 37 C.
7. Finely divide the dried liver to a powder by homogenization in a blender.

APPENDIX B

Table 26. Individual FATCT and SIT results and virus classification

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
1	1/1[c]	2/2	1/1	+	L^2
2	1/1	0/1	1/2	+	L^3
3	Not examined[d]		....	Not examined[e]	
4	0/1	0/2	....	-	....
5	1/1	0/2	1/1	+	L^3
6	1/1	0/2	1/1	+	I^1
7	....	1/1	3/3	+	L^4
			....	+[f]	L^1

[a] In most instances these blood samples were obtained from experimental pigs injected with field specimens. Exceptions will be pointed out in appropriate footnotes. For example, see the following footnote "u".

[b] + = positive; - = negative.

[c] Numerator = number positive; denominator = number examined. Applies for both spleen and blood samples.

[d] All the samples of the specimen were either decomposed and/or adulterated to the extent that no FATCT could be performed.

[e] All the samples of the specimen were markedly decomposed and were not injected into a pig.

[f] Contact exposure. Not injected with specimen 7. Details presented in previous Table 17. Note here that when more than 1 SIT is associated with a particular specimen, the FATCT results with samples of that specimen are not repeated in the table.

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Field samples Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
8	1/1	0/1	....	+	I^2
9	0/1[c]	0/1	0/1	-	....
10	....	1/4	1/1	+	I^1
11	1/1	....	1/1	+	I^1
12	1/1	1/3	2/2	+	L^4
13	0/1	....	....	-	....
14	0/1	0/2	....	-	....
15	....	0/1	0/1	-	....
16	1/1	2/4	1/2	+	L^2
17	0/1	0/1	....	-	....
18[g]	....	....	....	-	....
19[h]	0/1	....	....	+	I^2
			0/1	+	I^2
			0/1[i]	....	....
			....	+[j]	I^2

[g]Specimen 18 included only lymph nodes which were FATCT negative.

[h]From same suspected field case of HC as specimen 18.

[i]Pig killed 6 days after injection with specimen 19. The blood from this pig was FATCT negative but the spleen was FATCT positive.

[j]Injected with blood from a pig injected with specimen 19.

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
20[h]	....	0/1	....	-	....
21	Not examined[d]		1/1	+	L^3
			....	+	L^4
22	....	1/1	....	+	I^2
23	0/1	....	....	-	....
24	0/2[c]	0/1	1/1	+	L^2
25	0/1	0/1	0/1	-	....
26	0/1	....	0/1	-	....
27	0/1	....	0/1	-	....
28	0/1	....	0/1	-	....
29	0/1	....	0/1	-	....
30	Not examined[d]		1/1	+	L^1
31	0/1	0/1	0/1	-	....
32	0/1	0/4	0/1	-	....
33	....	0/2	0/1	-	....
34	2/3	0/3	1/1	+	L^1
35	1/1	2/2	1/1	+	L^2
36	....	1/3[c]	1/1	+	L^1
37	0/2	0/1	0/1	-	....

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
38	1/1	....	1/1	+	KM[k]
39	0/1	....	1/2	+	I[1]
40	0/3	0/2	....	-	....
41	0/2	....	....	-	....
42	2/2	....	1/1	+	L[1]
43	1/1	....	1/1	+	L[2]
44[1]	0/1	....	0/1	+	I[2]
			....	+	I[2]
			0/1[m]	....	....
45	2/2	2/2	1/1	DB[n]	....
			....	+	L[2]
46	1/1	0/1	1/1	+	L[1]
47	0/1	0/1	1/2	+	L[1]
			....	-	....

[k]Killed in a moribund condition. Virus classified as lethal but not subclassified.

[l]From same suspected field case of HC as specimen 22.

[m]Pig killed 6 days after injection with specimen 44. Blood FATCT negative, spleen FATCT positive.

[n]Pig died within 24 hrs after bleeding. No virus classification made on this SIT. The SIT was repeated for the purpose of virus classification.

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
48[o]	....	1/1	....	....	....
49	2/2[c]	0/1	1/1	+	I[1]
50	1/1	0/1	1/1	+	L[2]
51[p]	0/1	....	1/2	-	....
			....	-	....
52	0/1	0/1	....	-	....
53	0/2	....	....	....[q]	....
			1/1	+	L[1]
			....	-	....
54	....	0/4	....	+	I[2]
55	....	0/3	1/1	+	KM[k]
			....	-	....
56[r]	1/1	....	1/1	+	KM[k]
57	Not examined[d]		....	....[s]	....

[o]From same suspected field case of HC as specimen 43.

[p]From same suspected field case of HC as specimen 39.

[q]SIT considered incomplete. Details presented in previous Table 14.

[r]From same suspected field case of HC as specimen 54.

[s]SIT considered incomplete. Details presented in previous text.

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	Field samples Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
58	2/2	2/2	1/1	DB^n	....
			....	+	I^1
59	0/2	0/3	....	-	....
60	0/1	0/1	....	-	....
61	1/1	0/3	1/1	+	KM^k
62	2/2	2/2	1/1	+	L^1
63	0/2	0/2	....	-	....
64	3/3[c]	2/2	1/1	+	KM^k
65	2/2	....	1/1	+	KM^k
66	1/1	....	....	+	L^1
67	1/1	1/1	1/1	+	L^1
68	0/1	0/1	0/1	-	....
69	0/1	....	....	-	....
70	2/2	2/2	1/1	+	L^1
71	2/2	0/1	1/1	+	I^1
72	....	0/1	....	-	....
73	1/1	1/1	1/1	DB^n	....
			....	+	L^1
74	0/2	....	....	-	....
75	0/1	0/1	....	-	....
76	0/1	....	0/1	-	....

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
77	2/2	....	1/1	+	L[1]
78	Not examined[d]		....	-	....
79	0/1	0/1	....	-	....
80	Not examined[d]		....	-	....
81	1/1	0/1	1/1	+	KM[k]
82	....	0/1	0/1	-	....
83	2/2[c]	....	1/1	....[t]	....
			....	+	L[3]
84	1/1	4/4	1/1	+	L[1]
85	1/2	2/4	....	+	L[1]
			1/1	+[u]	L[1]
86	0/2	0/4	....	-	....
87	1/1	1/3	0/2	+	I[2]
88	1/1	1/1	1/1	+	L[1]
89	0/2	....	0/1	-	....
90	1/1	0/2	1/1	+	I[1]
91	2/3	1/3	1/1	+	I[2]
92	....	2/2	2/2	+	L[4]

[t]SIT considered incomplete. Pig exsanguinated 27 days after injection with specimen 83.

[u]Injected with blood from pig injected with specimen 85.

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	Field samples Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
93	1/1	5/5	1/1	+	L[1]
94	Not examined[d]		....	Not examined[e]	
95	0/2	....	....	-	....
96	2/2	0/1	1/1	+	KM[k]
97	0/1	....	....	-	....
98	1/1	1/1	1/1	+	L[2]
99	0/2	0/2	....	-	....
100	2/4	1/2	1/1	+	KM[k]
101	....	0/3[c]	....	-	....
102	....	0/3	....	-	....
103	0/1	....	....	-	....
104	1/1	0/1	1/1	+	I[2]
105	2/2	....	1/1	+	KM[k]
106	2/2	2/2	1/1	+	L[2]
107[v]	0/1	0/1	....	-	....
108	1/1	1/1	1/1	DB[n]	....
			....	+	L[1]
109	1/1	1/1	1/1	-	....
			....	+	I[2]

[v]From same suspected case of HC as specimen 102.

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
110	1/1	....	1/1	+	L^2
111	2/2	2/2	1/1	+	L^2
112	0/2	0/2	0/1	....[w]	....
			....[x]	....[y]	....
			....	-	....
113	1/1	....	1/1	+	L^1
114	0/1	0/1	1/1	....[w]	....
			....	-	....
115	1/1	....	1/1	+	L^1
116	0/1[c]	....	....	-	....
117	1/1	0/1	....	+	I^2
118	1/1	....	1/1	+	KM^k
119	1/2	1/2	1/1	+	KM^k
120[z]	0/1	....	....	-	....

[w]Sewage line stoppage resulted in contamination of isolation cage. SIT repeated.

[x]No blood sample. Spleen FATCT negative.

[y]Pig died 4 days after injection with specimen 112. No lesions of HC. SIT repeated.

[z]From same suspected field case of HC as specimen 114. Pig injected with composite of inoculum prepared from specimens 114 and 120.

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
121	1/1	....	1/1	+	L^2
122	1/1	....	1/1	+	L^1
123	0/2	....	....	-	....
124	0/1	....	0/1	-	....
125	0/1	0/1	....	-	....
126	2/2	2/2	1/1	+	L^2
127	1/1	2/2	1/1	+	KM^k
128	1/1	....	1/1	+	L^2
129	0/1	0/1	....	-	....
130	0/1	....	....	-	....
131	0/1	0/1	....	-	....
132	0/1	....	....	-	....
133	0/1	....	0/1	-	....
134	1/1	....	1/1	+	L^2
135	1/1	....	1/1	+	KM^k
136	0/1[c]	....	....	-	....
137	1/1	....	1/1	+	KM^k
138	1/1	....	2/2	+	L^4
139	1/1	1/1	1/1	+	L^1
140	2/2	....	1/1	+	KM^k
141	1/1	1/1	1/1	+	KM^k

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Field samples Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
142	1/1	0/1	1/1	+	L^2
143	2/2	1/2	1/1	DB^n	....
			....	+	L^1
144	0/2	0/2	....	-	....
145	2/4	4/4	0/2	+	I^1
146	1/1	1/1	1/1	+	I^1
147	1/1	....	1/1	+	L^2
148	1/1	1/1	1/1	+	L^2
149	2/2	2/2	1/1	+	L^2
150	1/1	1/1	1/1	+	L^2
151	0/1	0/1	....	-	....
152	1/1	....	1/1	+	L^2
153	1/1	1/1	1/1	+	L^1
154	Not examined[d]		1/1	+	L^2
155	3/3[c]	3/3	1/1	+	L^1
156	0/1	0/1	....	-	....
157	1/2	1/1	1/1	+	L^1
158	....	1/1	1/1	-	....
			0/1	$+^{aa}$	I^2
			....	$+^{aa}$	I^2

[aa]Injected with blood from pig injected with specimen 158.

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
159	1/1	0/1	1/1	+	L^1
160	1/1	....	1/1	+	L^2
161	Not examined[d]		....	Not examined[e]	
162	2/2	1/2	1/1	+	I^1
163	1/1	....	1/1	+	L^2
164	1/1	1/1	1/1	+	L^1
165	0/4	....	....	-	....
166	2/2	0/2	1/1	+	L^2
167	0/1	0/1	....	+	I^2
			....	-	....
			0/1[bb]	....	....
168	2/2	....	1/1	+	L^2
169	1/1	1/1	1/1	+	L^1
170	1/1	0/5	1/1	+	L^2
171	1/1[c]	1/1	0/1	+	I^2
172	1/1	1/1	1/2	DB[n]	....
			....	+	L^2
173	1/1	0/1	1/2	+	I^2
174	1/2	3/4	1/1	+	L^1

[bb]Pig killed 6 days after injection with specimen 167. Both blood and spleen FATCT negative.

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Blood	Lab samples Blood[a]	SIT results[b]	Virus classi-
175	1/1	1/1	1/1	+	L^1
176	1/1	1/1	1/1	+	L^1
177	0/1	0/1	....	-	....
178	0/1	....	....	-	....
179	2/2	....	1/1	+	L^2
180	2/3	0/1	1/1	+	L^2
181	2/2	0/1	0/1	+	I^2
182	1/1	....	1/1	+	KM^k
183	1/1	5/5	0/1	+	I^2
184	1/1	0/1	1/1	+	L^2
185	0/1	0/1	....	-	....
186	1/3	1/3	0/1	+	I^2
187	0/1	0/1	....	-	....
188	Not examined[d]		....	Not examined[e]	
189	0/2	0/4	....	-	....
190	2/2[c]	2/2	1/1	+	L^2
191	1/1	2/2	1/1	+	L^2
192	2/2	3/5	1/1	+	L^2
193	Not examined[d]		....	Not examined[e]	
194	1/1	2/2	1/1	+	L^1
195	1/2	0/2	1/1	+	L^2

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
196	2/2	0/2	1/1	+	L^1
197	0/1	0/2	....	-	....
198	1/1	2/2	1/1	+	L^1
199	2/2	2/2	1/1	+	L^1
200	1/1	....	1/1	+	L^1
201	0/2	0/1	....	-	....
202	Not examined[d]		1/1	+	L^1
203	2/2	1/2	1/1	+	L^1
204	2/2	0/1	1/1	+	L^1
205	2/2	2/2	1/1	+	L^2
206	1/1	....	1/1	DB^n	L^2
			....	+	L^2
207	1/1	0/2	1/1	+	I^2
208	5/5[c]	3/5	1/1	+	L^1
			....	+	L^1
			....	+	L^1
209	1/1	....	1/1	+	L^2
210	2/2	2/2	1/1	+	L^1
211	1/1	1/1	1/1	DB^n	....
			....	+	L^2
212	3/3	1/1	1/1	+	L^2

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
213	0/1	0/2	1/1	+	L^1
			....	+	L^2
214	3/3	3/3	1/1	+	I^2
215	0/1	0/2	....	-	....
216	2/2	0/1	1/1	+	L^2
217	1/1	1/1	1/1	DB^n	....
			....	+	L^2
218	0/1	0/1	....	-	....
219	1/1	1/1	1/1	-	....
			....	+	L^2
220	1/1	0/1	1/1	DB^n	....
			....	+	L^2
221	2/2[c]	2/2	1/1	DB^n	....
			....	+	L^2
222	2/2	3/3	0/1	DB^n	....
			....	+	I^2
223	1/1	....	1/1	+	L^1
224	1/2	1/2	1/1	+	L^2
225	1/1	1/1	1/1	+	L^1
226	3/3	1/1	1/1	DB^n	....
			....	+	L^2

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
227	1/1	1/1	1/1	+	L^1
228	1/1	....	1/1	+	L^3
229	2/2	1/2	1/1	+	L^1
230	2/2	....	1/1	DB^n	....
			....	+	L^1
231	....	2/2	1/1	+	L^1
232	1/1	0/4	0/1	+	I^1
233	0/1	....	1/1	+	L^2
			....	+	L^2
234	1/3	1/3	1/1	+	L^2
235	1/1	....	1/1	DB^n	....
			....	+	L^1
236	....	1/2[c]	1/1	+	L^2
237	2/2	....	1/1	+	L^1
			....	+	L^2
			....	+	L^1
238[cc]	1/1	3/3	....	....	....
239	1/1	0/1	1/1	+	L^1
240	1/2	....	1/1	+	I^2

[cc]From same suspected field case of HC as specimen 231. No SIT done with this specimen.

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
241	1/1	....	1/1	+	L^2
242	2/2	....	1/1	+	L^2
243	2/2	2/2	1/1	+	L^1
			....	+	L^1
			....	+	L^1
244	5/5	1/1	1/1	+	L^2
245	0/2	0/2	....	-	....
246	1/1	1/1	1/1	+	L^2
247	1/1	....	1/1	+	L^2
248	1/1	0/1	1/1	+	L^1
249	2/2	2/2	1/1	+	L^1
250	1/1	1/1	....	+	L^1
251	1/1	....	1/1	+	L^1
252	1/1[c]	....	1/1	+	L^1
253	2/2	....	1/1	+	L^1
254	0/2	....	....	-	....
255	1/1	....	1/1	+	L^2
256	1/1	1/1	1/1	+	L^1
257	1/1	1/1	1/1	+	L^2
258	1/1	2/4	1/1	+	L^2
259	1/1	1/1	1/1	+	L^2

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	Field samples Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
260	1/1	1/1	1/1	+	L^3
261	1/1	0/1	1/1	+	I^1
262	2/2	2/2	1/1	+	I^2
263	Not examined[d]		1/1	+	L^1
264	0/1	0/1	....	-	....
265	1/1	0/1	1/1	+	L^1
266	0/1	0/1	....	-	....
267	2/2	1/1	1/1	+	L^2
268	1/1	1/1	1/1	+	L^2
269	1/1	1/1	1/1	+	L^1
270	1/1	1/1	1/1	+	L^1
271[dd]	0/4	0/3	....	-	....
272	Not examined		....	-	....
273	0/1[c]	....	....	-	....
274	1/1	....	1/1	DB[n]	....
			....	+	L^1
275	1/1	....	1/1	+	I^1
276	0/2	0/2	....	-	....
277	0/2	0/2	....	-	....
278	0/1	....	....	-	....

[dd] From same suspected case of HC as specimen 266.

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
279	0/1	....	1/1	+	L^1
			0/1	DB^n	....
			....	-	....
280	2/3	....	1/1	+	L^2
281	1/1	0/1	1/1	+	L^2
282	2/2	3/3	1/1	+	L^1
			....	+	L^1
			....	+	L^2
283	1/1	1/1	1/1	+	L^1
284	2/2	2/2	1/1	+	L^1
285	1/1	....	1/1	DB^n	....
			....	+	L^2
286	1/1[c]	....	1/1	+	L^3
287	2/2	2/2	1/1	+	L^2
288	0/1	....	....	-	....
289	1/1	....	0/1	+	I^2
290	2/2	....	1/1	+	L^2
291	1/1	....	1/1	+	L^2
292	0/2	0/1	1/1	+	L^2
			....	+	L^2
293	1/1	0/1	1/1	+	L^2

Table 26 (Continued)

Specimen number	FATCT results: Field samples Spleen	FATCT results: Field samples Blood	FATCT results: Lab samples Blood[a]	SIT results[b]	Virus classification
294	0/1	....	....	-	....
295	0/1	....	....	-	....
296	0/1	....	1/1	+	L^2
			....	-	....
297	2/2	....	....	+	L^1
298	0/2	....	....	-	....
299	2/2	0/2	1/1	+	L^3
300	1/1	0/1	2/2	-	....
			2/2	+	L^4
			1/4	+[ee]	I^2
			0/2	-[ee]	....
301	1/2[c]	1/2	1/1	+	L^2
302	Not examined[d]		....	Not examined[e]	
303	2/2	....	1/1	+	L^1
304	1/1	0/1	1/1	+	L^3
305	2/2	1/2	1/1	+	L^1
306	1/1	....	1/1	+	L^2
307	1/1	1/1	1/1	+	L^2
308	2/2	0/1	1/1	+	L^2
309	0/1	0/1	....	-	....

[ee]Contact exposure. Not injected with specimen 300. Details presented in previous Tables 17 and 18.

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Field samples Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
310	3/4	....	0/1	+	L^4
311	4/4	3/4	0/1	+	I^2
312	2/2	1/1	1/1	+	L^2
313	1/1	....	1/1	+	L^2
314	1/1	1/1	0/1	+	I^2
315	1/1	0/1	1/1	+	L^1
316	2/2	1/1	0/1	+	I^2
317	1/1	1/1	1/1	+	L^2
318	1/1	1/1	1/1	+	L^2
319	2/2	....	1/1	+	L^2
320	1/1	....	1/1	+	L^2
321	0/1[c]	0/1	....	-	....
322	1/1	....	0/1	+	I^2
323	2/2	....	1/1	+	I^2
324	1/2	....	1/1	+	L^2
325	0/1	....	....	-	....
326	0/1	0/1	....	-	....
327	2/2	....	0/1	+	L^1
328	1/1	1/1	0/1	+	I^2
329	0/1	0/1	....	-	....
330	3/3	2/3	1/1	+	L^2

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
331	13/13	....	1/1	-	....
			2/2	+	L^4
			4/4	+[ff]	L^4
			2/2	+[ff]	L^3
			....	+[ff]	L^3
332	1/1	....	1/1	+	L^2
333	2/3	0/1	1/1	+	I^1
334	1/1	1/1	1/1	+	L^2
335	1/1	....	1/1	+	I^2
336	0/1[c]	....	1/1	+	L^3
			....	+	L^1
			....	+	L^2
337	1/1	0/1	1/1	+	I^2
338	0/1	0/1	....	-	....
339	0/1	....	....	-	....
340	Not examined[d]		....	Not examined[e]	
341	1/1	0/1	1/1	DB[n]	....
			....	+	L^2

[ff]Contact exposure. Not injected with specimen 331. Details presented in previous Tables 17 and 18.

Table 26 (Continued)

Specimen number	FATCT results Field samples Spleen	Field samples Blood	Lab samples Blood[a]	SIT results[b]	Virus classification
342	1/1	1/1	0/1	-	....
			....	+	I^1
343	0/1	....	1/1	+	L^2
344	0/1	....	....	-	....
345	2/2	1/1	1/1	+	L^3
			....	+	L^2
346	1/1	1/1	1/1	DB^n	....
			....	+	L^1
			....	+	L^2
347	2/2	....	1/1	+	L^2
348	4/4	1/4	1/1	+	L^4