CULTIVATION OF THE HOG CHOLERA VIRUS

By CARL TENBROECK, M.D.

(*From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey*)

(Received for publication, July 7, 1941)

Although 9 years have passed since Hecke (1) reported the cultivation of the hog cholera virus, no confirmation of his work has yet appeared. This is probably in large part due to the fact that only swine can be used to demonstrate the virus and,—since the disease is so highly contagious,—to the necessity of keeping the inoculated animals in strict isolation.

Hecke used a number of swine tissues in his experiments. In hanging drop preparations containing choroid plexus or spleen he was able to demonstrate virus in the 15th passage. In flask cultures containing bone marrow in plasma plus Drew's solution virus was present in the 10th transfer, while it was demonstrated in the 20th transfer of lymph node in plasma plus Drew's solution and in the 14th transfer containing spleen. Media containing kidney did not favor the increase of virus. There was great irregularity in the tests for virus in the various transfers and in all cases it was eventually lost. The least amount of culture tested was a 1:1000 dilution. Long incubation periods in the inoculated swine were not uncommon, suggesting either an attenuation of the virus or cross-infection.

Hog cholera is an important disease and it seemed desirable to attempt to repeat and amplify the work already reported. Furthermore, since the virus is so highly specific for swine, it was believed that its culture would be difficult, and that, if it was successful, it might provide methods for the cultivation of some of the other specific viruses of man and animals. The results obtained are presented herewith.

Materials

A strain of virus used for vaccination in the central portion of the United States was obtained through the courtesy of Dr. Shope. It had probably been passed by inoculation through a series of swine and is highly virulent for them, causing a temperature and marked prostration in 2½ to 3 days after intramuscular injection. Swine that are killed after 3 or 4 days of fever show relatively few lesions: the lymph nodes are enlarged but seldom hemorrhagic, and a few hemorrhages are found under the capsule of the kidneys. If the animals are allowed to live for a longer period of time after injection, other characteristic lesions of hog cholera appear. The virus is neutralized by an anti-hog cholera serum produced in the eastern United States by one of the large commercial houses.

The swine used were raised on the grounds of the Department and were a cross between Chester Whites and Jersey Reds. The stock has not been vaccinated against hog cholera and has been free from disease. All inoculated animals were kept under strict isolation, one animal to a unit, and their temperatures were taken at least once daily. They were autopsied after they had had fever for 3 or more days.

Simms's (2) modification of Tyrode solution was used for tissue suspensions and dilutions were made in buffered salt solution. Fertile chicken eggs incubated 10 days and prepared by Burnet's technique (3) were used in some of the experiments.

Testicle tissue has been used because it is readily obtained without killing the animal and one pig will provide tissue for two experiments. Pigs weighing 60 to 90 lbs. were etherized and one testicle was removed. This was immediately brought to the laboratory, flamed, and uncooked tissue removed from the interior, minced with fine scissors, and transferred to culture medium.

Methods and Results

Culture on Embryonated Eggs.—When the work was started it seemed probable that some different technique would be necessary to propagate the virus, and it was decided to make use of the observation by Murphy (4) that mammalian tissue placed on the chorioallantoic membrane of embryonated eggs would live for a considerable period of time.

Virus, *i.e.* defibrinated blood from a pig bled 3 days after the development of temperature following inoculation with virus, plus Tyrode solution was mixed with minced swine testicle, and after standing about 10 minutes a small amount of tissue was deposited by means of a large calibre pipette on the chorioallantoic membrane of eggs containing 10-day embryos. The opening in the shell was covered with Scotch tape and the eggs were incubated at 37°C. for 3 days. The eggs were then opened, and the membranes removed, ground, and suspended in Tyrode solution to approximately a 10 per cent suspension. (If the activity of the suspension was to be determined 10 per cent suspensions of the membranes were made.) This suspension was mixed with fresh minced swine testicle and eggs were again inoculated. This process was repeated every 3 days. Occasionally the eggs became contaminated and it was necessary to go back to material from earlier transfers that had been preserved in the refrigerator or dried. The amount of dilution at each transfer cannot be calculated, but it is at least 1:10 and probably much greater. The membrane suspensions were tested for virus at intervals and the results are given in Table I.

It will be seen that virus was demonstrated up to the 13th transfer, when the experiment was discontinued, and that a large amount was present since 1×10^{-6} cc. of membrane suspension from the last transfer was sufficient to produce disease in a pig. The material from the 10th transfer, which is shown in the table to be highly infectious, was also injected subcutaneously into two pigs, each pig receiving at the same time, but in another area, 24 cc. of anti-hog cholera serum. One pig received 1×10^{-1} cc. and the other 1×10^{-4} cc. of the membrane suspension. Neither pig showed a temperature and later both were

found to be immune to the original virus. Since our pigs have always been susceptible, this experiment shows that the passage virus was neutralized by anti-hog cholera serum and that immunity followed the simultaneous injection of immune serum and virus.

There has been no evidence for the adaptation of the virus to the egg. On three different occasions during the course of the experiments membrane suspension that had been tested and found positive for virus was transferred to the chorioallantoic membrane of 10-day embryonated eggs without swine tissue. The eggs were incubated for 3 days, the membranes then suspended, and a fresh lot of eggs inoculated. After 3 days incubation the membranes from this lot of eggs were suspended and injected into swine. None of the swine developed a temperature and all were later shown to be susceptible to the

TABLE I

Tests for Hog Cholera Virus in Suspensions of Chorioallantoic Membranes on Which Were Deposited Minced Swine Testicle and Virus

Passage	Amount of 10 per cent membrane suspension that produced typical hog cholera	Remarks
	cc.	
6	1×10^{-4}	Higher dilutions not tested
9	1×10^{-5}	10^{-6} cc. not tested; 10^{-7}, 10^{-8}, and 10^{-9} cc. negative
10	1×10^{-6}*	10^{-7} and 10^{-8} cc. negative
12	1×10^{-6}	Higher dilutions not tested
13	1×10^{-6}	" " " "

* The protein nitrogen in the 10 per cent membrane suspension was 0.55 mg. per cc.

virus. From eggs in the 12th transfer membranes and embryos were removed and a 10 per cent suspension was made of each. 1×10^{-1} cc. of the embryo suspension produced acute hog cholera, whereas 1×10^{-4} cc. did not. The membrane suspension was titrated at the same time and 1×10^{-6} cc. caused acute hog cholera. The virus in the embryo suspension evidently was a contamination from the membranes, for if the virus had invaded the embryo we would expect that it would be present in a greater concentration than could be demonstrated.

Two tests were made to determine whether the passage virus would increase in chick embryos inoculated intravenously by the method described by Eichhorn (5). The starting material was a proved infectious suspension from the 12th passage on the chorioallantoic membrane in the presence of swine testicle. In one experiment this material was injected intravenously into a set of eggs and after 24 hours a 10 per cent suspension of the embryos was made and 0.1 cc. of a 10^{-2} dilution was injected into each egg in a second set. A 10 per cent suspension of the embryos in this second set was made after 24 hours incubation

and 1 cc. injected into a pig, with negative result. The experiment was repeated and the eggs were kept 3 days after inoculation instead of 24 hours. An embryo suspension from the 2nd passage failed to produce hog cholera when injected into a susceptible pig.

Search was made for a contaminating virus in material from the 11th transfer. Guinea pigs and rabbits given intracerebral and subcutaneous injections showed no rise in temperature and no evident illness. Mice injected intracerebrally lived for a month, and when they were finally disposed of were in good condition. Five mice, while under ether anesthesia, were inoculated intranasally with virus. Two were killed after 3 days and the remaining three after 20 days, and the lungs of all were found to be negative. The virus produced no lesions on the chorioallantoic membrane of eggs that contained 10-day embryos. These negative results make it extremely unlikely that a contaminating virus was present.

Flask Cultures.—Along with the transfers in eggs as described above, cultures in Maitland's medium were carried.

3 cc. of Tyrode solution and 1 cc. of sterile swine serum were combined in a 50 cc. Erlenmeyer flask. To this was added a small amount of minced swine testicle and 0.5 cc. of hog cholera virus. After 3 days incubation 0.5 cc. of the first culture was transferred to freshly prepared medium. After 4 transfers the serum was omitted, the medium then consisting of 4.5 cc. of Tyrode solution plus swine tissue and 0.5 cc. of the previous culture. The dilution was then 1:10 at each transfer.

Virus was demonstrated in the cultures made at the 3rd, 6th, 9th, and 14th transfers. In the 14th transfer, 1×10^{-5} cc. of culture produced acute and characteristic hog cholera.

Culture on Serum Agar.—At about the time the above work was completed, reports of the culture of a number of viruses on serum agar plus minced embryo tissue began to appear (6–8). All these experiments were based on the study made by Zinsser and Schoenbach (9) and the application of these studies to the culture of typhus rickettsiae. A trial was made and it was found that the hog cholera virus also could be cultured in this way. The medium was the same as that described by Zinsser *et al.* (10) except that Simms's (2) modification was substituted for the regular Tyrode solution and serum from normal swine was used.

The serum was filtered through Seitz pads and stored in sterile containers. Two parts of this were mixed with 3 parts of double strength Tyrode solution, passed through a sterile Berkefeld N filter, and then mixed with 3 parts of sterile 3 per cent Difco agar in distilled water. The medium was transferred to Kolle flasks with a neck shaped to hold a No. 8 rubber stopper or to test tubes measuring 1.8 × 18 cm. These tubes, as well as the Kolle flasks, were closed with rubber stoppers after inoculation.

Minced swine testicle to which the inoculum had been added was spread over the surface of the solid serum agar. After 2 or, usually, 3 days incubation at 37°C. the tissue was washed off into 10 cc. of buffered salt solution or Tyrode to each Kolle flask. This was usually diluted approximately 1:10 and passed through sterile Berkefeld filters before fresh inoculations were made.

The starting material was not the original hog cholera virus but was the material from the 13th passage on eggs. An additional 13 passages on the serum agar have been made and virus was demonstrated by swine inoculation as shown in Table II. The dilutions for swine inoculation were made in buffered salt solution without serum and the swine were immediately injected intramuscularly.

TABLE II

Tests for Hog Cholera Virus in Suspensions of Tissue from 3-Day Cultures on Swine Serum Agar

Passage	Material	Protein nitrogen per cc. of undiluted material	Least amount causing hog cholera
		mg.	*cc.*
1	Not centrifuged	0.71	1×10^{-7}
9	" "	—	1×10^{-5}*
10	Centrifuged and supernatant used	0.5	1×10^{-6}*
11	" " " "	0.83	1×10^{-6}**
12	" " " "	—	1×10^{-7}
13	" " " "	0.57	1×10^{-6}

* Higher dilutions not tested.

** A pig receiving 1×10^{-2} cc. of this material intramuscularly in the hind leg and 24 cc. of anti-hog cholera serum subcutaneously in the axillae showed no illness, *i.e.* the virus was neutralized.

SUMMARY

The work of Hecke on the cultivation of hog cholera virus was confirmed with ease. Virus was grown in the presence of fresh minced swine testicle in flasks containing Tyrode solution, on the chorioallantoic membrane of embryonated eggs, and on the surface of swine serum agar. In flasks it was grown for 14 transfers; while on eggs it was grown for 13 transfers, followed by an equal number of transfers on agar, making 26 transfers in all. Only one strain of virus was used and we do not know whether all strains can be cultivated so readily or whether we were particularly fortunate in the selection of the strain used. Neither do we know whether swine testicle is better than other tissues for growth.

The cultured virus produces characteristic hog cholera when injected into swine, and its effect can be neutralized with commercial anti-hog cholera serum. No evidence of attenuation of the virus was obtained, the last culture being

highly virulent when small amounts were injected. No evidence for the adaptation to the egg could be secured, since passages without swine testicle on the membrane or intravenously for 2 transfers resulted in a loss of the virus. No contaminating virus that might favor the cultivation could be detected by animal or egg inoculation.

Not only has the virus been cultivated but it has been demonstrated in large amounts in the culture. Four suspensions containing slightly over 0.5 mg. of protein nitrogen[1] produced typical hog cholera when 1×10^{-6} cc. was injected, and one suspension made in the same way was active in one-tenth this amount. Few titrations on what is commonly known as hog cholera virus, *i.e.* the serum from acutely ill pigs, are available. We made one such titration and produced a delayed disease with 1×10^{-5} cc. of infectious serum. It seems probable that the culture virus is more active than the commonly used virus and that its practical use in hog cholera vaccination and hyperimmunization would result in a considerable saving.

All of the methods used yielded active cultures, but the serum agar method is the one of choice since larger amounts of suspension can be obtained with less labor.

BIBLIOGRAPHY

1. Hecke, F., *Centr. Bakt., 1. Abt., Orig.*, 1932, **126,** 517.
2. Simms, H. S., cited by Sanders, M., *Arch. Path.*, 1939, **28,** 541.
3. Burnet, F. M., *J. Path. and Bact.*, 1933, **37,** 107.
4. Murphy, J. B., *J. Am. Med. Assn.*, 1912, **59,** 874.
5. Eichhorn, E. A., *Science*, 1940, **92,** 245.
6. Kurotchkin, T. J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41,** 407.
7. Cheever, F. S., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42,** 113.
8. Pang, K. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43,** 755.
9. Zinsser, H., and Schoenbach, E. B., *J. Exp. Med.*, 1937, **66,** 207.
10. Zinsser, H., FitzPatrick, F., and Wei, H., *J. Exp. Med.*, 1939, **69,** 179.

[1] These determinations were kindly made by Dr. Roger M. Herriott.