Hog Cholera V. Demonstration of the Antigen in Swine Tissues by the Fluorescent Antibody Technique

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SUMMARY

The fluorescent antibody technique was employed to detect hog cholera virus in tissue sections of various organs from experimentally infected swine. The method proved to be highly sensitive and infection could be detected in these animals as early as three days after inoculation with the virus. Best results were obtained when tissues were collected from young animals in advanced disease rather than from sows or from pigs in the early febrile phase. Tonsil, spleen and lymph node were the tissues of choice and were most satisfactory when removed from freshly killed animals rather than from those that had died.

RESUME

La technique d'immunofluorescence fut employée pour démontrer la présence du virus de la peste porcine dans des sections de divers organes provenant de porcs infectés expérimentalement. La méthode s'est montrée très sensible et a permis de démontrer l'infection de ces porcs dès le troisième jour après l'inoculation. De meilleurs résultats furent obtenus avec les tissus prélevés de jeunes sujets montrant des signes avancés de la maladie, plutôt qu'avec des tissus provenant de truies de reproduction ou de porcs au début de la phase thermique de la maladie. Les amygdales, la rate et les ganglions lymphatiques furent les tissus de choix et s'avérèrent plus satisfaisants lorsque prélevés chez des animaux fraîchement tués de préférence à ceux trouvés morts.

Studies previously reported (1) indicated that the fluorescent antibody technique was a reliable method for detection of hog cholera virus in tissue cultures inoculated with suspensions of spleen from infected swine. Using this technique with appropriately selected specimen material a positive diagnosis of hog cholera, in most instances, can be made in 24 to 48 hours.

Work of Stair *et al* (2) suggested that staining of cryostat-cut sections of tissue from infected swine might provide a more rapid diagnostic method. Therefore, studies were undertaken to determine the reliability of the method and factors influencing selection of specimen material.

Materials and Methods

Experimental Animals

These studies were commenced shortly after those employing the tissue culture technique and utilized many of the same animals. All were white bacon-type swine of the Landrace or Yorkshire breeds, or their crosses. Excepting those in series 1, which were secondary specific pathogen free (SPF) swine produced at the laboratory, all were purchased locally. Normal control tissues were obtained from pigs picked at random in an abattoir serving eastern Ontario and western Quebec.

Series 1 — This preliminary trial was an attempt to determine the sensitivity of the technique using spleen, lymph node and kidney tissues from animals infected with a standard virulent strain and the American 1030 (neurotropic) strain of hog cholera virus. Thirty-three secondary SPF pigs weighing less than 100 pounds were exposed by intramuscular injection with 5 ml. of defibrinated infective swine blood. All were killed or died between the 4th and 8th day post inoculation. There were 13 control animals.

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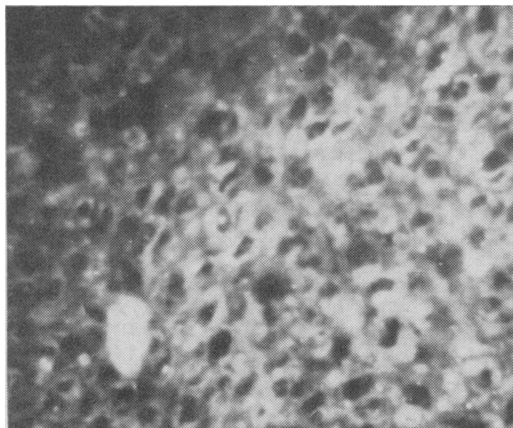

Fig. 1. Lymphoid tissue of the tonsil showing the fluorescent cytoplasm of the cells surrounding dark unstained nuclei. Most of the cells show some fluorescence but this appears most intense in cells in the center of the field where exciting illumination is strongest. The large white mass was debris and showed bright blue autofluorescence microscopically in contrast with the yellow-green of the specific fluorescence. X320. Infected spleen and lymph node sections have a similar appearance.

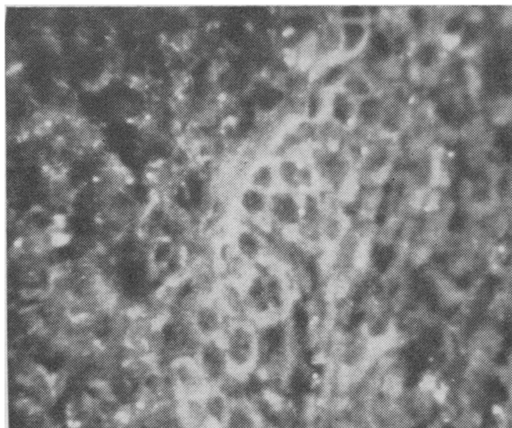

Fig. 2. Epithelium of a tonsillar crypt is shown crossing the field vertically at the center. The dark nuclei of epithelial cells are in contrast with the fluorescent cytoplasm. Fluorescence is also present in lymphoid cells below the epithelium but lacks intensity because of the poorer illumination at the periphery of the field. X320.

Series 2 — This group, used in the tissue-culture studies previously reported (1), was planned to determine the best time for collection of specimens during disease. Twenty-four young swine, purchased locally, were exposed to the standard strain of hog cholera virus by intramuscular inoculation with 5 ml. of defibrinated infective swine blood. Spleen, lymph node and kidney tissues were collected at intervals between the 3rd and 7th days post inoculation. Four additional pigs were used as controls.

Series 3 — This group of animals was also used in tissue-culture studies (1) to determine the influence of the size of the swine on effectiveness of the technique. Twenty-four older animals, ranging in weight from 100 to over 300 pounds, were exposed by the intramuscular route using 5 ml. of defibrinated infective pig blood. Spleen, lymph node, kidney and tonsil tissue were collected when disease was advanced or, in a few instances, after death. Tissues from 13 normal swine served as controls.

Preparation of Specimens

Preservation — Tissues were collected immediately after the animals were killed or as soon as possible after death. A portion of each tissue selected was frozen immediately on tissue holders for sectioning. In most instances tissues were also preserved to assess the possible effects of refrigeration or freezing on specimens during shipment. Two other portions of each tissue were placed in separate glass jars, one being stored in the refrigerator at 9°C for four or five days and the other in a mechanical freezer at -20°C until it was convenient to examine them.

Sectioning — Tissue was mounted on tissue holders using water or a drop of Lab-Tek O.C.T. compound[1] zone 1, and quickly frozen in a container of crushed dry ice. Frozen blocks were placed on the freezer bar of the cryostat to adjust to the cutting temperature of -20°C. A Lab-Tek cryostat* with the microtome set at two microns was used to cut the tissues. The sections were lifted on the small wire pickup device, placed on cold microscope slides and thawed in place by the warmth from a finger on the back of the slide. Fixation was for 10 minutes in a tank of acetone placed in a dry ice-alcohol bath.

Staining — Preparation of conjugates and staining techniques were the same as used in the tissue-culture studies (1). One slide from each tissue was stained with the immune conjugate and one with the normal. Number 1 coverslips were mounted on the sections using 10 per cent glycerol in 0.01M phosphate buffered saline (pH 7.4).

Microscopy

Examination of the preparations was by means of a Zeiss Standard Universal microscope equipped with an OSRAM high

[1] Lab-Tek Instruments Co., Westmont, Ill., U.S.A.

TABLE I — Results obtained by the fluorescent-antibody method in examination of tissues from swine infected with a standard strain of hog cholera (series 1)

Animal Number	Tissue Section Fluorescence[1]		
	Spleen	Lymph Node	Kidney
102	+++	++++	++++
252[2]	−	−	+
254	++++	+++	++++
266	++++	++++	++
267[2]	++++	++++	+
268	++++	+++	++
269[2]	++++	+++	+
270	++++	++++	++++
271	++++	++++	+++
272	++	++	unfit
273[2]	++	++	unfit
280	+++	+++	+
281	++	+++	−
284	+++	+++	−
285	+	+	−
288	++++	+++	++++
289	+++	++++	++++
292	++++	++++	++
298	+++	+++	+++
299	++++	++++	++++
controls[3] .			

[1]Tissue fluorescence judged as described in the test (Results).
[2]Died
[3]See controls Table II.

pressure mercury lamp HBO 200W and dark-field condenser. Exciter filters 38/2.5 (fixed) and BG12/4, and barrier filter 44 (440 millimicrons and above transmittance) were employed. Examinations were made using Neofluar 16X and 40X objectives and 8X oculars. The microscopist was not aware of the identity of animals from which tissues were being studied.

Results

The distribution of fluorescence within the cell was similar to that found earlier in pig kidney tissue-culture cells. Fluorescence was apparent in the cytoplasm, was not particulate, and was most intense adjacent to the nucleus (Figs. 1 and 2).

Location of Infected Cells and Assessment of Degree of Involvement of Tissues

In these experiments progressive involvement of various organs of infected animals could be followed by the FA technique. When young animals were killed soon after infection examination of spleen sections revealed a few large fluorescing cells scattered in the Malpighian corpuscles (evaluated as a one plus or $^{+}$ reaction). As the disease advanced, the number of these fluorescing cells increased until each corpuscle became a sharply defined focus ($^{++}$). Later, fluorescing cells became numerous beyond the corpuscles ($^{+++}$) and gradually involved the whole tissue section so that corpuscles could no longer be readily differentiated ($^{++++}$). Fluorescence was also observed in endothelial cells of the larger blood vessels of the spleen. A somewhat similar progressive involvement was observed in lymph nodes, starting with a few cells in the germinal centres and gradually spreading to include all round-cell elements of the organ. Development of infection in the lymph nodes was slightly delayed as compared with spleen.

In kidney tissue specific fluorescence tended to become apparent later than in the other tissues. First to be involved in this organ were widely scattered large irregular cells, thought to be reticulo-endothelial, lying between the tubules of the cortex and a few cells in the small round-cell foci below the capsule ($^{+}$). As the disease progressed increasing numbers of fluorescing intertubular cells were found, and small cells within the glomerular tuft also were stained ($^{++}$). In more advanced cases the capillaries between the tubules and in the tufts were outlined by their fluorescence, and early staining was found in the epithe-

TABLE II — Results obtained by the fluorescent-antibody method in examination of tissues from swine infected with the American 1030 strain of hog cholera (series 1)

Animal Number	Tissue Section Fluorescence[1]		
	Spleen	Lymph Node	Kidney
255	−	+	+
256	++	++	+
257	+	+	−
258	++++	++++	No Section
259	++	++	+
260	unfit	++	+++
264[2]	unfit	unfit	unfit
274	++	+++	+
275	++++	++++	++++
276	++	+++	+
277	unfit	unfit	unfit
278	unfit	unfit	unfit
279	unfit	unfit	unfit
Negative Controls 001-0013 incl.	−	−	−

[1]Fluorescence judged as described in the text (Results).
[2]Died

TABLE III — Effect of stage of disease on fluorescence in tissues (series 2)

Days PI[1] Killed	Number	Tissue Section Fluorescence[2]		
		Spleen	Lymph Node	Kidney
3	1	++	+	−
	2	++	+	−
	5	+	+	−
	8	++	+	−
	11	+	+	−
	15	++	+	−
4	24[3]	++	unfit	unfit
5	3	+++	+++	+
	7	+++	++++	+
	16	+++	+++	++
	18	++++	++++	+++
	22	+++	++	−
7	4	++++	++++	+++
	6	++++	++++	+++
	12	++++	++++	+++
	14	++++	++++	+++
	20	++++	++++	+++
	21	++++	++++	+++
8	23[3]	++++	++++	+++
9	9	++++	++++	+++
	17	++++	++++	++++
10	10	++++	++++	++++
	13	++++	++++	++++
	19	++++	++++	++++
Controls (4 animals)		−	−	−

[1]Post infection
[2]Tissue fluorescence judged as described in the text (Results).
[3]Died

lial cells of the tubules ($^{+++}$). Marked fluorescence in tubular epithelium was found only in late stages of infection ($^{++++}$). At this time endothelium of large blood vessels and epithelium of the renal pelvis were also involved.

Palatine tonsils were not studied as extensively as the other three tissues, but fluorescence there was particularly intense and was present when animals were killed three days post inoculation. Staining in the lymphoid component resembled that found in spleen and lymph node. In addition, fluorescence occurred in the epithelium lining the tonsillar crypts.

Parotid salivary glands were examined early in the studies but the practice was discontinued. These tissues did not appear to offer any advantage over those of the other organs, and were more difficult to section satisfactorily. It was also considered that they could be confused with fat, lymphoid tissue or other glands at the time of collection.

Other organs were studied in a few instances. In liver, fluorescence was observed in the Kuffer's cells. In lung, it was found in large cells of the alveolar exudate and in cells migrating through bronchial epithelium. Fluorescence was also observed in groups of round cells associated with blood vessels and bronchioles. Hippocampus was the only area of the brain examined and here fluorescence was found in cells of the perivascular cuffs. Antigen was detected in urinary bladder epithelium but not in that of the gall bladder although, in the latter tissue, scattered histiocytes in the tunica propria were stained. It did not appear that these organs would provide as satisfactory specimen material as tonsil, spleen, or lymph node. However, the findings in these various organs would probably vary depending upon the stage of infection.

Findings with Series 1 Swine

Animals in series 1 were killed (or died)

TABLE IV — Effect of size of the animal on amount of fluorescence in tissue sections (series 3)

Weight (pounds)	Pig Number	Tissue Section Fluorescence[1]			
		Tonsil	Spleen	Lymph Node	Kidney
100-150	33	++++	++++	++++	++++
	34	No tissue	++	+++	+++
	35	++++	+++	+++	++++
	36	++++	++++	++++	unfit
160-200	37	+++	++	+++	unfit
	38[2]	unfit	unfit	unfit	unfit
	39[2]	–	–	–	–
	40[2]	++	unfit	unfit	unfit
	45	+++	++	++	++
	46	++	++	+++	++
	47	++	+++	+++	+
	48[2]	++	+	–	++
210-250	41	++++	+++	+++	–
	42	++++	++++	++++	++++
	43	++++	++++	++++	+++
	44	++++	++++	+++	–
Sows (300)	29[2]	unfit	unfit	unfit	unfit
	30	++++	+++	+	–
	31	++++	++	++	unfit
	32[2]	unfit	unfit	unfit	unfit
	49	+	–	–	–
	50	+++	++	–	–
	51	–	+	–	–
	52	+++	++	–	–
Controls (13 animals)		–	–	–	–

[1]Tissue fluorescence judged as described in the text (Results).
[2]Died during the night

between the 4th and 8th days post inoculation. Results have been presented in two tables according to the virus strain employed to infect the pigs. Table I lists findings where the standard strain was used. The amount of fluorescence in spleen tissue was moderately good (++) to excellent (++++) in all but two instances. Lymph node was comparable with spleen. Kidney tissue was generally less satisfactory except when the animal had been infected for a long period. Table II shows results obtained with tissue sections from swine infected with the neurotropic strain. Fluorescing cells appeared to be more numerous in swine infected with the standard virus strain than in those of animals infected with the neurotropic strain. In this group all tissues examined from four animals were unsuitable for study, one (264) because of putrefaction and three (277, 278 and 279) for an undetermined reason. The tissues of these latter three animals did not appear to be decomposed when they were mounted on tissue holders for cutting yet in the tissue sections they appeared to be in an advanced state of autolysis. Excepting those declared unsuitable for examination, all cases of hog cholera produced by either strain of virus were detected. No false positive reactions were found among the 13 control animals. None of the tissues from infected animals reacted with the normal conjugate.

Findings with Series 2 Swine

Table III lists the reactions found in tissue sections when specimens (Series 2) were collected at different intervals after exposure (PI). On the third and fourth day PI fluorescence was observed in spleen tissue involving large cells of the Malpighian corpuscles. In the lymph nodes similar cells fluoresced but were less numerous. The kidneys were negative. From the fifth day PI onward distribution of fluorescence in spleen and lymph node sections increased and positive results were also obtained, with one exception, in all kidney specimens.

Findings with Series 3 Swine

The effect of size of the animal on find-

TABLE V — Comparison of results obtained when tissues of infected swine were processed immediately after collection and after storage at 9°C or −20°C

	Fresh tissues	Stored tissues	
		at 9°C	at −20°C
Infected pigs examined	74[1]	74	74
Pigs judged "positive"[2]	66(89.2%)[3]	65(87.8%)	63(85.1%)
Pigs judged "negative"	1(1.4%)	4(5.4%)	4(5.4%)
Pigs judged (unfit)	7(9.5%)	5(6.8%)	7(9.5%)
Positive spleens	65	62	61
Positive lymph nodes	62	58	46
Positive kidneys	45	44	33

[1]Figures are shown only for those instances in which all three methods of handling the tissues were employed.
[2]Judged "positive" on findings in sections of one or more of the following organs: spleen, lymph node, and kidney. Sections of tonsil were not prepared in all cases but in one instance was positive when the other three tissues were negative.
[3]Per cent of number of infected pigs examined is given in brackets.

ings in sections of various tissues (Series 3) are shown in Table IV. Tissues from the older sows, particularly lymph node and kidney, appeared to be slightly less reactive than those from younger animals. In two instances (Nos. 49 and 51) only one tissue, tonsil and spleen respectively, were weakly positive. This table also indicates that difficulty was experienced with decomposed specimens from six animals which had died during the night. Of these, in four instances tissues were judged unfit for examination. In one instance examination was attempted with negative findings, while in the other although results appeared positive tissue sections were difficult to interpret because of autolytic changes. Sections from the 160-200 pound group generally showed fewer fluorescing cells than those of the previous 100-150 pound group or the succeeding 210-250 pound group. This observation was not strictly in agreement with what would be expected if the intensity of infection decreases with age.

Effects of Tissue Preservation on Sensitivity of the Test

Table V allows comparison of findings when tissues were processed immediately after collection with those when the tissues were preserved by refrigeration and by freezing. An animal was judged as infected if sections from one or more of spleen, lymph node or kidney tissue showed specific fluorescence. The table indicates that only a few more cases of hog cholera were detected when fresh tissue specimens were examined, that is 89.2 per cent as opposed to 87.8 per cent when specimens were preserved by refrigeration and 85.1 per cent when they were preserved by freezing. More cases were judged "negative" when tissues were preserved. However, it was possible to detect at least 85 per cent of the cases using either one of the methods of preservation. Since tonsils were not examined in all experiments, they were not included in the table.

The lower part of Table V shows spleen and lymph node to have been more useful as specimen material than kidney tissue. While findings using tonsil tissue were not shown in Table V data in Table IV suggested that this organ would be at least as useful as spleen in the test.

No false positive results were obtained in any of the tissues from 30 normal control swine either when the specimens were fresh or preserved by refrigeration or freezing.

Non-specific Staining and Autofluorescence

Background fluorescence was rarely a problem but was encountered when autolysis was advanced and in some tissues preserved by freezing or refrigeration. The difficulty was reduced, and contrast of specific fluorescence increased, when sections were cut as thinly as possible. The single barrier filter used also increased contrast. When marked non-specific background staining occurred, it was recognized by its even distribution throughout the section. However, it tended to "mask" specific fluorescence and made interpretation more difficult. Occasionally cells were found in spleen, lymph node and kidney sections

which showed intense non-specific staining of the correct color, but these had a finely particulate appearance as if sprinkled with fluorescent dust. Similar staining occurred with the normal conjugate. This type of reaction was most often found in tissue which had undergone degeneration. Therefore, all granular or particulate fluorescence was disregarded even if the color was correct. Considerable non-specific uptake of the conjugate was sometimes found in necrotic debris in the tonsillar crypts but was easily identified as such.

Occasionally autofluorescence of kidney tubular epithelium was extremely intense and, while it would not be confused with specific staining because of the color (using No. 44 barrier filter), its intensity could mask specific fluorescence.

Autofluorescence of granulocytes was easily differentiated by the pale, almost white color (No. 44 barrier filter) and by the coarsely granular appearance of the cells. These cells were often numerous in lymph nodes.

Discussion

Findings with swine of series 1 were not strictly comparable with those in succeeding trials. Animals of this series were obtained from the secondary SPF swine colony and their temperature responses to the infective inoculum were atypical. Many animals developed high temperatures less than 24 hours after injection with infective material, some almost immediately. This possibly accounted for the somewhat erratic results obtained (Tables I and II) as compared with animals of series 2 (Table III). While tissues of only five secondary SPF pigs exposed to the American 1030 strain were fit for examination, there appeared to be less antigen in the tissues (Table II) as compared with those which received the standard strain (Table I).

This work employing direct sections and work previously reported using the tissue-culture technique (1) suggested that best results could be expected when specimens were collected from animals in advanced disease with falling temperatures rather than from earlier cases during the febrile phase of infection.

Work reported here and the earlier reports concerning the tissue culture-fluorescence technique (1), the agar gel-precipitation test (3,4) and the complement-fixation test (5) have all indicated that tissues of infected sows are less satisfactory specimens than those of infected younger pigs for demonstrating the presence of hog cholera virus. Whether this indicates less virus or the presence of an inhibitory substance in organs of the older animals remains to be clarified.

Few specimens may be received at diagnostic laboratories in fresh condition. From our studies it would appear that if tissues are refrigerated or frozen immediately after collection and kept so during transit they will provide satisfactory specimen material. Moveover, those unfit for direct section staining, should still be usable for tissue culture-fluorescence studies.

Using the direct tissue-section method positive results were available in less than three hours after receipt of the specimen while the tissue-culture inoculation method required 24 to 48 hours.

ACKNOWLEDGMENTS

The encouragement of Drs. P. J. G. Plummer, Director and J. F. Frank, Assistant Director, during the course of this study was appreciated.

The authors wish to acknowledge the technical assistance of Mr. A. Boyd in preparation of conjugates, cutting and staining of sections and photography and of Mr. M. Picard in provision of immune sera and infective tissues.

Appreciation is also expressed to Dr. G. Poirier, Inspector in Charge, and his staff, and to Canada Packers Ltd., Hull, P. Q. for provision of normal control tissues and to Dr. C. L. L'Ecuyer for SPF swine.

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