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Studies on the Detection of *Toxoplasma gondii* with Mouse Inoculation Method and Fluorescent Antibody Technic in Slaughtered Pigs

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Abstract

Ninety five pigs suspicious of *Toxoplasma*-infection were selected from 18, 867 ones killed at Isahaya City Slaughterhouse and used for the isolation of *T. gondii* with mouse inoculations of their hilar or hepatic lymph nodes and also for the microscopic detection of the parasite in the lymph nodes with direct fluorescent antibody technic. In the mouse inoculation method, *Toxoplasma* hemagglutination test was carried out with sera of mice killed 6 weeks after the inoculation of the lymph nodes into the mice. Further, *T. gondii* strains newly isolated were subinoculated into mice and hamsters to investigate their virulence.

The isolation rate of *T. gondii* was 8/95 or 8.4 %, while 33 of 95 (34.7 %) were positive in hemagglutination test. Fluorescent antibody technic indicated a positive response in 19 of 95 pig lymph nodes (20.0 %). Eight *T. gondii* strains were isolated and demonstrated a high virulence for mice and hamsters. In this paper, the methods used and the above-mentioned results are stated in detail and discussed.

Introduction

The recent knowledge of toxoplasmosis, a common communicable disease of medical importance between man and animals, has revealed that pigs are the most frequent source of the infection in man. Numbers of reports have been published concerning the incidence of this disease in pigs and the isolation of *Toxoplasma* parasite from them, as described later.

The most practical way to investigate the prevalence rate of toxoplasmosis in animals might be the isolation of the parasite by the

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mouse inoculation method but this method would have objections, since a number of mice and a considerable period of days are usually required until the final results are obtained. In recent years, the microscopic detection of the parasite with fluorescent antibody technic has been frequently attempted, as it is simple to perform and discloses an immediate result of examination. This technic, however, is found at the present time to sometimes provide a confusing result and still considered to involve some problems to be further examined on the technical procedure and the determination of result.

There have been many publications on the examination of pig sera with serological reactions of Sabin-Feldman's dye test, hemagglutination, *etc*. These data would be meaningful in advancing our understanding of the epidemiological aspect of toxoplasmosis but would not inform us the exact prevalence rate of this disease.

In this report, suspicious *Toxoplasma*-infected pigs were selected from slaughtered ones and their lymph nodes were used for the detection of the parasite with a mouse inoculation method and fluorescent antibody technic. Furthermore in the mouse inoculation method, hemagglutination test with sera from the inoculated mice were carried out in combination with the isolation of the parasite. In addition, *Toxoplasma* strains isolated in this experiment were subinoculated into mice and hamsters to examine their virulence in animals.

Materials and Methods

Materials

Ninety five pigs suspicious of toxoplasmosis were selected from 18,867 ones which were killed at Isahaya City Slaughterhouse in Nagasaki Prefecture during the period from December 1966 through March 1967.

These pigs displayed in their autopsy findings one or more combinations of edema, hypermia, swelling, hemorrhage, necrosis and other inflammatory signs in the lung, liver, intestine, lymph nodes or other organs. The hilar and hepatic lymph nodes excised from the pigs were examined for the parasite with a mouse inoculation method and fluorescent antibody technic.

Mice weighing 20 to 25 gm. and hamsters of approximately 100 gm. in body weight were used for inoculation. Fluorescein isothiocyanate-conjugated antitoxoplasma pig γ-globulin solution employed for staining the parasite was a product of Fuji Zoki Pharmac. Co. Sensitized red blood cells used in *Toxoplasma* hemagglutination test were supplied from Chemo-Sero Therapeutic Research Institute.

Methods

1. Mouse inoculation method for parasite isolation

Approximately 1 gm. of the hilar or hepatic lymph node of a pig was emulsified with 2 ml sterile in a mortar and filtrated through a sheet of sterile gauze. Then, 0.3 to 0.4 ml of the emulsion was inoculated intraperitoneally into each of 3 mice. One (M_1 mouse in Fig. 1) was sacrificed 6 weeks after the inoculation, and the brain excised from the mouse was crushed between 2 slide glasses and examined for cysts microscopically. Heart blood was collected with a blood absorbing filter paper (Toyo's) from the mouse for *Toxoplasma* hemagglutination test (HA).

To examine their peritoneal exudate for

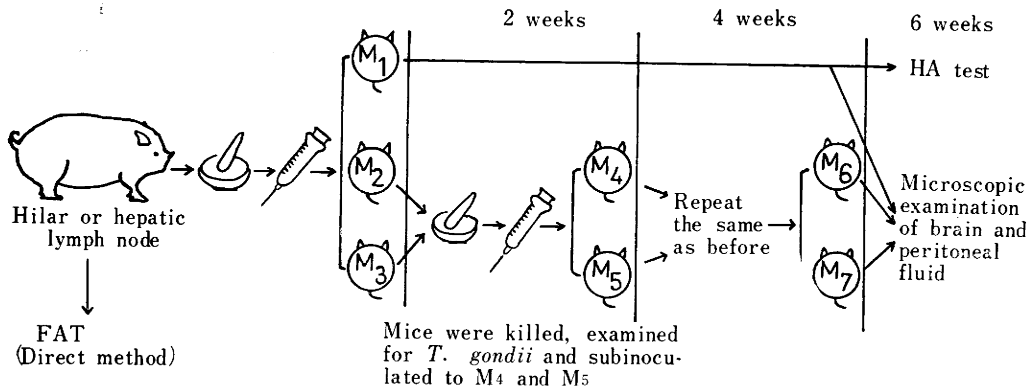

Fig. 1. Procedure of mouse inoculation method for the isolation of *Toxoplasma gondii* from pig lymph nodes

the proliferative form of *T. gondii*, 2 mice (M_2 and M_3 in Fig. 1) were killed 2 weeks after the inoculation. After a laparatomy was made along the median line of mouse, the peritoneal cavity was washed by 1 ml sterile saline with a small syringe. The peritoneal washings were collected in a tube and examined for the parasite microscopically. When no *Toxoplasma* parasite was detected in microscopic fields, a blind subinoculation with the materials taken from the mice was made into additional mice with a technic described below. The brains and livers of the mice (M_2 and M_3) were ground in a mortar, while the rest of the peritoneal washings and 2 ml sterile saline were added to them. After the emulsion was filtrated through a sheet of sterile gauze, 0.4 to 0.5 ml was inoculated intraperitoneally into each of 2 mice (M_4 and M_5). Two weeks later, M_4 and M_5 mice were examined and if necessary, subinoculated into M_6 and M_7 with the same method. For fear of any bacterial infection of the inoculated mice, the emulsions to be inoculated contained Dihydrostreptomycin sulfate J. P. in the concentration of 1 mg per ml and Penicillin-G potassium J. P. to 1,000 units per ml, although the entire procedure was made aseptically as much as possible. Once the inoculated mice demonstrated certain morbid symptom such as diarrhea, hair ruffling, ascites or emaciation, the ascitic fluid was examined for the parasite within 2 weeks after inoculation. When *Toxoplasma* parasites were detected in any of the inoculated mice, they were immediately subinoculated into mice for their virulence.

2. *Direct fluorescennt antibody technic (FAT) for parasite detection*

A Nikon ultraviolet microscope with a 200-watt mercury lamp was used in this experiment. Impression smear preparations were made by slightly impressing the cut surfaces of pig lymph nodes on slide glasses and stained with fluorescent antibody solution for 30 min. in an incubator of 37°C after dried and fixed with absolute methyl alcohol. They were then washed by dipping them in 1/100 M phosphate buffer saline solution, pH 7.5, to remove any excess dye material. The preparations were enclosed with drops of 90 % glycerin 1/100 M phosphate buffer saline, pH 7.5, under cover slips and examined microscopically for the parasite at 400 times magnification.

3. *Toxoplasma hemagglutination test (HA) with sera from inoculated mice*

Antigen solution for the test was prepared in accordance with the original method de-

veloped by Nobuto and Hanaki in 1964. In this test, use was made of sheep red blood cells which were sensitized by *Toxoplasma* antigen after treatment with Formalin-alcohol solution and further with Bis-Diazo Benzidine.

Sera were collected from M_1 mice 6 weeks after the inoculation of pig lymph nodes into them and diluted 64, 256, 1,024 and 4,096 times with saline. A standard for determining the result was selected on the appearance of a positive reaction for sera of 256 dilution.

4. *Appraisal of the virulence of isolated strains*

New *Toxoplasma* strains were used to investigate their virulence in animals after they had been subinoculated in mice more than 10 generations. Mice and hamsters inoculated with the strains were sacrificed shortly before death and the ascitic fluid quantities and the parasite numbers in the ascites were measured for the appraisal of their virulence. After pipetting the ascites of a mouse into a small graduated cylinder, the peritoneal cavity was carefully washed with 1 ml saline and the washings were again collected into the cylinder with a pipette. Accordingly, the total quantity of ascites was measured as it included the recovered amount of 1 ml saline washings. The total parasite number in ascites was given by multiplying the ascites quantity (mm^3) by the parasite number per mm^3 counted with a hemocytometer.

Results

1. *Parasite isolation with the mouse inoculation method and results of the HA test with sera from inoculated mice*

Isolation of *Toxoplasma* parasite with the mouse inoculation method was successful in 8 out of 95 cases examined and the isolation rate was 8.4%. Proliferative forms were detected in all 8 of the isolation-posive cases, whereas cysts were found only in 5 of them. The cysts were all detected in the brains of M_1 mice sacrificed 6 weeks after the inoculation. On the other hand, the detection of proliferative forms in the peritoneal exudates of inoculated mice was made in a case in the first generation of subinoculation, in 5 cases in the second and in 2 cases in the third. These results are summarized in Table 1.

It was understood from the results that blind subinoculations into mice would be necessary for detecting the parasites, proliferative forms in particular, when no parasite was detected in mice during the first generation of subinoculation.

The positive rate of M_1 mouse sera in HA was 34.7% (33 positive in 95 examined) and it was found to be much higher than the isolation rate of the parasite obtained with the mouse inoculation method. Eight positive cases in parasite isolation were all included in the HA-positive cases and no *Toxoplasma* parasite was detected in 62 HA-negative cases. Table 2 shows the results mentioned above.

2. *Parasite detection with direct fluorescent antibody technic FAT*

The parasite detection rate with FAT was 20.0% (19 positive in 95 examined). Of these 19 FAT-positive cases, 4 (21.1%) were positive for parasite isolation with the mouse inoculation method, while 4 (5.3%) of 76 FAT-negative likewise demonstrated a positive result in parasite isolation. Since the differnece between both the above-mentioned percentages

Table 1. Summarized results of the isolation of *T. gondii* from inoculated mice with the mouse inoculation method

Isolated strain	Examination period (weeks)			Results in subinoculated mice
	2	4	6	
No. 32	M_1 ———	———	→ C(−), HA++ ↘	{1/3*C(+) → D. Pf(+) 2/3 13D. Pf(+)
	M_2 − } →	{M_4 13D. **Pf(+) }	——— →	5−10D. Pf(+)
	M_3 − } →	{N_5 13D. Pf (+) }		
No. 72	M_1 ———	———	→ C(+), HA++	
	M_2 − } →	{M_4 14D. Pf (+) }	——— →	5—10D. Pf(+)
	M_3 − } →	{M_5 − }		
No. 105	M_1 ———	———	→ C(+), HA+	
	M_2 − ——→	{M_4 − } {M_5 − }	——→ C(−), HA− →	
	M_3 8D. Pf(+) ——→	M_3 7D. Pf(+)	——— →	5−10D. Pf(+)
No. 106	M_1 ———	———	→ C(−), HA++	
	M_2 − } →	{M_4 8D. Pf(+) }	——— →	5—10D. Pf(+)
	M_3 − } →	{M_5 8D. Pf(+) }		
No. 108	M_1 ———	———	→ C(+), HA++ ↘	{1/4C(+) → D. Pf(+) 3/4 D. Pf(+)
	M_2 − } →	{M_4 9D. Pf(+) }	——— →	5−10D. Pf(+)
	M_3 − } →	{M_5 9D. Pf(+) }		
No. 115	M_1 ———	———	→ C(+), HA++ ↘	{1/3C(+) → D. Pf(+) 2/3D. Pf(+)
	M_2 − } →	{M_4 − } →	{M_6 11D. Pf(+) } →	5−10D. Pf(+)
	M_3 − } →	{M_5 Pf(+) } →	{M_7 11D. Pf(+) } →	
No. 117	M_1 ———	———	→ C(+), HA++	
	M_2 − } →	{M_4 4D. Pf(−)***	{M_6 12D. Pf(+) } →	5−10D. Pf(+)
	M_3 − } →	{M_5 − ——→	{M_7 9D. Pf(+) } →	
No. 130	M_1 ———	———	→ C(+), HA+ ↘	{1/4C(+) → D. Pf(+) 3/4 D. Pf(+)
	M_2 − } →	{M_4 − } →	{M_6 13D. Pf(+) } →	5−10D. Pf(+)
	M_3 − } →	{M_5 − } →	{M_7 13D. Pf(+) } →	

* One of 3 inoculated mice
** The mouse died 13 days after inoculation
*** The mouse died from bacterial infection
Pf : Proliferative forms of *T. gondii* detected in the peritoneal exudate of inoculated mouse
C : Cysts detected in the brain of inoculated mouse

Table 2. Comparative results of the isolation of *T. gondii* from inoculated mice with the mouse inoculation method the positive rate of M_1 mouse* sera in hemagglutination test

Hemagglutination test of M mouse serum				Parasite isolation with mouse inoculation method			
	No.	%	(Fiducial rate %)**		No.	%	(Fiducial rate %)
Positive	33	34.7	(27.4−43.4)	Positive	8	24.2	(14.6−37.9)
				Negative	25	75.8	(62.1−85.4)
					33	100.0	
Negative	62	65.3	(56.6−72.6)	Positive	0	0.0	(0.0−5.9)
				Negative	62	100.0	(94.1−100.0)
					62	100.0	
Total	95	100.0					

* These mice were killed for the parasite examination 6 weeks after the inoculation of pig lymph nodes (see Fig. 1).
** 10% level

Table 3. Comparative result of the isolation of *T. gondii* from inoculated mice with the mouse inoculation method and the parasite detection in pig lymph nodes with fluorescent antibody technic

Fluorescent antibody technic on pig lymph nodes				Parasite isolation with mouse iuoculation method			
	No.	%	(Fiducial rate * %)		No.	%	(Fiducial rate %)
Positive	19	20.0	(14.2−27.6)	Positive	4	21.1	(10.4-40.1)
				Negative	15	78.9	(59.9−89.6)
					19	100.0	
Negative	76	80.0	(72.4−85.8)	Positive	4	5.3	(2.6−11.5)
				Negative	72	94.7	(88.5−97.4)
					76	100.0	

Note: Total parasite isolation rate was 8/95, 8.4%(5.0−14.6)
* 10 % level

was not considered as significant at a 0.05 probability level, it could not be concluded that the parasite isolation rate might be higher in FAT-positive cases than in FAT-negative ones.

3. *Virulence of the isolated strains*

Eight strains of *T. gondii* isolated from pigs were each successively subinoculated into mice and later hamsters to examine their virulence in animals. In summing up the results, it was recognized that all the strains were equally of high virulence enough to produce in mice and hamsters a fatal infection, since inoculated animals always died within 6 to 15 days after the inoculation and showed the accumulation of ascites which contained numbers of proliferative forms multiplying, regardless of the parasite number inoculated within

Table 4. Result of a comparative examination for the virulence of *T. gondii* strains newly isolated and the RH in mice

Strain	No. of parasites inoculated	No. of mice inoculated	Average survival days of inoculated mice	Ascites quantity in average ml	Average parasite numbers per mm^3	Total parasite numbers in average
No. 32	1×10^2	5/5	9.1	1.4	4.1×10^4	5.8×10^7
	1×10^3	5/5	8.7	1.7	1.9×10^4	3.2×10^7
	1×10^4	5/5	7.4	2.0	1.8×10^4	3.5×10^7
No. 72	1×10^2	4/5	8.8	1.3	2.3×10^4	2.9×10^7
	1×10^3	5/5	8.1	1.2	1.9×10^4	2.2×10^7
	1×10^4	5/5	6.8	1.5	2.7×10^4	4.1×10^7
No. 108	1×10^2	5/5	11.1	1.1	3.3×10^4	3.5×10^7
	1×10^3	5/5	8.4	1.6	2.5×10^4	4.0×10^7
	1×10^4	5/5	8.2	1.9	4.2×10^4	7.7×10^7
No. 117	1×10^2	5/5	9.1	1.4	2.3×10^4	3.2×10^7
	1×10^3	5/5	8.6	1.4	1.6×10^4	2.2×10^7
	1×10^4	5/5	7.6	1.2	1.7×10^4	2.1×10^7
RH	1×10^2	4/4	8.5	1.6	1.4×10^5	2.2×10^8
	1×10^3	5/5	8.3	1.3	1.2×10^5	1.5×10^8
	1×10^4	4/4	6.8	1.5	5.5×10^4	8.1×10^7

Table 5. Result of a comparative examination for the virulence of *T. gondii* strains newly isolated and the RH in hamsters

Strain	No. of parasites inoculated	No. of hamsters inoculated	Average survival days of inoculated hamsters	Ascites quantity in average ml	Average parasite numbers per mm^3	Total parasite numbers in average
No. 32	1×10^2	2/2	14.0	6.3	7.5×10^2	4.7×10^6
	1×10^4	3/3	8.0	2.0	1.8×10^4	3.6×10^7
No. 108	1×10^2	2/2	10.8	1.0	2.5×10^2	2.5×10^5
	1×10^4	3/3	11.3	4.0	1.4×10^3	5.6×10^6
No. 117	1×10^2	2/2	15.0	2.0	1.3×10^2	2.6×10^5
	1×10^4	3/3	9.5	2.6	1.4×10^4	3.6×10^7
No. 130	1×10^2	2/2	185.0 K*	—	—	—
	1×10^4	3/3	9.0	2.7	3.3×10^2	8.9×10^5
RH	1×10^2	2/2	10.8	1.0	2.5×10^2	2.5×10^5
	1×10^4	3/3	6.8	2.8	2.9×10^4	8.1×10^7

* Hamsters were killed on the 185th day and numbers of cysts were detected in both hamsters.

the range of 10^1 to 10^4.

It was especially noteworthy that even in the subinoculation into mice with the cysts isolated from the brains of M_1 mice, an acute infection was brought to the inoculated mice and proliferative forms were detected in their ascites in all mice with exception of 4 cases in the series of Nos. 32, 108, 115 and 130 pigs, in each of which cysts were detected in one of inoculated mice during the second generation of subinoculation (see Table 1).

Comparing with the RH strain, typical of high virulence, it was observed that the proliferative forms of these new strains growing in the ascites of inoculated mice were usually fewer in number than those of the RH, notwithstanding the former accumulated a larger quantity of ascites in mice than the latter. Some of the results are shown in Tables 4 and 5. In conclusion, any of the 8 isolated strains appeared to be almost equivalent or slightly inferior in virulence to the RH.

Discussion

It has been emphasized that the pig is an important source of *Toxoplasma* infection in man. A number of reports on the isolation of the parasite from pigs (Matsubayashi *et al.* 1957, Jacobs *et al.* 1957, Jacobs *et al.* 1960, Ishii *et al.* 1962, Maitani *et al.* 1966, Ruiz 1966, Zaman 1967, and Work 1967) or the serological survey on prevalence of this infection in slaughterhouse workers (Kobayashi *et al.* 1962, Murakami 1964 and Kawashima 1964) have been previously published.

Fluorescent antibody technic (FAT) has been widely used for the direct proof of *T. gondii* in animals, as it could give a distinct image of this parasite microscopically. But the fact that this technic sometimes gives false positive reactions or confusing results suggests that the mouse inoculation method may be still at present more reliable for the detection of the parasite than FAT. Abbas (1967) reported that the tissue culture method and the chick embryo cultivation for isolating the parasite were far inferior in parasite detection rate to the mouse inoculation method.

In the present experiments, attempts were made to detect *T. gondii* in the lymph nodes of suspicious *Toxoplasma*-infected pigs by the mouse inoculation method and FAT. Results obtained disclosed that the parasite detection rate was 8.4% with the mouse inoculation method and 20.0% with FAT (direct method), and that only 4 (21.1%) of 19 FAT-positive cases were positive in parasite isolation, whlie in 76 FAT-negative cases 4 isolation-positive (5.3%) were likewise observed. It was assumed in general that *T. gondii* could be isolated from mice inoculated with FAT-positive materials, if they were sufficient in number and virulent enough to infect mice, and that if the parasites in materials to be examined shoud be very few in number but of high virulence, there would be some cases in which FAT was negative but the parasite isolation was positive, and if the parasite situation should be reverse, the result would become contrary as well. Judging from this assumption, the results obtained were considered to be acceptable but it was not possible in this experiment to find a direct relationship between the results with both the parasite detection methods. A more detailed comparative study should be made on the

detection measure of *T. gondii*

Hemagglutination test (HA) which was carried out in the sera from mice inoculated with the pig lymph nodes indicated 34.7% positive rate and 8 cases positive in parasite isolation were all included in the HA-positive cases. The fact that there were as many as 25 cases in which HA was positive but the parasite isolation negative, would suggest the possibility that the parasites inoculated into mice might be too few to be isolated or totally destroyed in the mice. At the same time the results emphasize the necessity of study on the elaborate procedure of *Toxoplasma* parasite isolation.

Eight strains isolated were considered to be of high virulence for mice, as it was demonstrated in subinoculations of the strains in mice that they usually produced in mice an acute infection by the proliferative form and killed them in 6 to 15 days, even in the tests done with the strains originally isoloted as the cyst form.

Reports published on the isolation of the parasite with the diaphragm digestion technic stated that the parasites isolated were mostly the cyst form strains and of low virulence. Since the cyst form was principally harbored in the muscle and brain of the infected animal and was more resistant than the proliferative form, it might be reasonable to employ the digestion technic with the intention of isolating the cyst form. The inoculation method of lymph node emulsion used in this experiment was considered excellent for the isolation of both forms.

Consequently, no cyst form strain was isolated in this experiment. Case No. 59, however, which showed a positive HA but a negative parasite isolation in inoculated mice in the first and second subinoculations, might suggest that a cystic infection would arise in the inoculated mice. Further examinations on increased number of cases may give answers to the problems still remaining unsolved in this report.

Conclusions

Ninety five pigs suspicious of toxoplasmosis were examined for the isolation of *T. gondii* with mouse inoculations of their hilar or hepatic lymph nodes and the parasite detection in the lymph nodes with direct fluorescent antibody technic. *Toxoplasma* hemagglutination test also was carried out with sera from mice killed 6 weeks after the inoculation of the lymph nodes into them. In addition, *T. gondii* strains newly isolated were subinoculated into mice and hamsters for the investigation of their virulence. Results achieved are itemized as follows;

1. The isolation rate of *T. gondii* with the mouse inoculation method was 8.4% (8 positive in 95 examined) and proliferative forms were found in all 8 and cysts in 5 of the positive cases.
2. In hemagglutination test, 33 of 95 cases (34.7%) indicated a positive reaction and all 8 parasite isolotion-positive cases were included in the hemagglutination-positive cases.
3. In fluorescent antibody technic, 19 of 95 pig lymph nodes examined were positive (detection rate; 20.0%) and *T. gondii* could be isolated from only 4 of the 19 with the mouse inoculation method.
4. *T. gondii* strains isolated were considered to be equivalent or slightly lower in virulence for animals to the RH, since they were possible to produce on acute fatal infection in mice and hamsters.

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マウス接種法および螢光抗体法による屠殺豚からの *Toxoplasma gondii* の検出について

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摘　　　　要

長崎県諫早市立屠場に1966年12月より1967年3月までに搬入された豚18,867頭より，屠場獣医師の協力により選抜されたトキソプラズマ症の疑いある病変豚（肺水腫，肝壊死斑，腸充血など）95頭の肝または肺門リンパ腺の螢光抗体法（直接法）により原虫検出，マウス接種法による原虫分離，および接種マウスのＨＡ抗体価を測定し，それらの成績を比較検討した．また分離された株の毒性についてもＲＨ株と比較し検討した．

1. マウス接種法によるトキソプラズマ原虫分離は95例中8例（原虫分離率8.4%）で，いずれも栄養型が検出された．分離8株中5株は継代初期においては，シスト型も検出された．
2. マウス接種法によるＨＡ抗体価陽性（256倍以上陽性）は95例中33（陽性率34.7%）であった．原虫分離8株はいずれもＨＡ陽性であった．
3. 豚の肝または肺リンパ腺の割面スタンプ標本の，螢光抗体法（直接法）によるトキソプラズマ原虫の検出率は95例中19例（検出率20.0%）であった．この19例中4例からマウス接種法により原虫が分離できた．
4. 分離株のマウスおよびハムスターに対する毒性はＲＨ株と同じ程度かやや弱毒であった．