Comparative Study of Methods used for the Isolation of *Toxoplasma gondii* *

by ADEL MOHAMMED ALY ABBAS, *Lecturer in Bacteriology, High Institute of Public Health, Alexandria, Egypt, UAR*

Serological methods, however useful in epidemiological surveys, are often unsatisfactory in the diagnosis of the cause of illness in individual patients; and the commoner an infection, the less satisfactory they are. Infection with *Toxoplasma gondii* is very common, estimates of its frequency varying from 25% to 90% of the human population in different countries. Correspondingly, serological diagnosis of clinical toxoplasmosis is difficult and uncertain. Greater, though not absolute, certainty attends the demonstration of the parasite. Rarely can this be done in histological sections; more commonly it requires isolation, and for this mice and other rodents, chick embryos and tissue cultures have been used. It seemed desirable to find out which of these systems and which technique was best for the isolation of *T. gondii* from the tissues of acutely, subacutely and chronically infected animals.

Materials and methods

The following inocula were used.

Acutely infected tissue. Spleen emulsion from mice dying 4 days after inoculation with the RH strain. The diluent was:

Medium 199 [a]	473.8 ml
7.5% $NaHCO_3$	14.7 ml
Calf serum	10 ml
Benzyl penicillin (100 IU/ml)	0.5 ml
Streptomycin sulfate (10 μg/ml)	0.5 ml
Polymixin B (100 IU/ml)	0.5 ml

An attempt was made to count the number of parasites. The result was 50 million per millilitre of spleen emulsion, which must have been an underestimate, for on this basis a quarter of a parasite would be infectious for mice (see Table 1).

Subacutely infected tissue. Spleen emulsion from mice inoculated 14 days previously with cysts of the Rabbit A strain.[b] The same diluent was used. The parasites were too scanty to count.

Chronically infected tissue. Brain suspension from mice inoculated 5 months previously with cysts of the Rabbit A or Lamb 18 strain.[b, c] Dilutions were prepared in normal saline with antibiotics. The parasite count gave 200 *T. gondii* cysts per millilitre of brain, which would mean that a hundredth of a cyst was infectious (see Table 3). This need not necessarily indicate underestimation but may have been due to cyst rupture with the release of large numbers of contained trophozoites.

Preliminary experiments

Before comparative tests on the different systems were performed, it was necessary to find the best way of using each of them.

11-day-old chick embryos were inoculated on the chorioallantoic membrane and 8-day embryos into the yolk sac with emulsions of tissues prepared as described above. The best time to find evidence of infection in chick embryos, whether inoculated on the chorioallantoic membrane or in the yolk sac, was found to be 7-8 days after inoculation with acutely infected tissue and 10 days after inoculation with subacutely and chronically infected tissue. No matter which route was used, pocks could be seen on the chorioallantoic membrane at these times. There was little to choose between the routes, but the chorioallantoic was slightly the better.

Trypsin-dispersed monolayer cultures of HeLa and HEp-2 cells were prepared in the usual way. Various growth media were used: one based on Hanks' salt solution with added lactalbumin hydrolysate, sodium bicarbonate, antibiotics and human serum; another

* Work performed during the author's tenure of a WHO Fellowship and forming part of a thesis for the degree of Ph.D. of the University of Sheffield, England.

[a] Morgan, J. F., Morton, H. J. & Parker, R. C. (1950) *Proc. Soc. exp. Biol. (N.Y.)*, **73**, 1-8.

[b] Beverley, J. K. A. (1959) *Nature (Lond.)*, **183**, 1348-1349.

[c] Beverley, J. K. A. (1959) *Nature (Lond.)*, **184**, 2041.

similar, but with calf serum; and a third based on Eagle's medium with sodium bicarbonate, antibiotics and calf serum. The cells were cultured without rolling for 7-10 days, and then the growth medium was removed, the cultures were inoculated with emulsions of acutely, subacutely or chronically infected tissues, and a maintenance medium was added. This was based either on Hanks' basic salt solution or on medium 199. Neither HeLa nor HEp-2 cells proved of practical value for the isolation of *T. gondii* from subacutely and chronically infected tissue. The parasite grew, but subsequent mouse passage was needed to reveal its presence. From acutely infected tissue, on the other hand, it grew well on both tissue cultures, HEp-2 being the better. The best time to examine the cultures was found to be the 10th day and the best way to find the parasite was by microscopic examination of maintenance medium and of flying cover-slips stained overnight by Giemsa's method. Cytopathic changes could not be relied on.

Mice were inoculated with the three tissue emulsions by the intracerebral, intraperitoneal and subcutaneous routes, the sensitivity of the methods being found to be in that order, except for chronically infected tissue for which the intraperitoneal route was best. But, although the most sensitive, the intracerebral route had disadvantages; it allowed of only a small inoculum and was unsuitable for material treated with antibiotics against bacterial contamination. Although the subcutaneous route was the least sensitive for the isolation of *T. gondii* from tissues free of bacterial contamination, it proved the best for the isolation of the parasite from tissues contamined with bacteria. Material of this type was mixed with antibiotics and inoculated into 42 mice by the intraperitoneal route. Of these, 22 died prematurely and *T. gondii* was isolated from only 4 of the 42. When a similar inoculum was given to 42 mice by the subcutaneous route only 6 died, and *T. gondii* was isolated from 14 of the 42. For material free of gross bacterial contamination the intraperitoneal route proved the most generally useful and was employed in the subsequent comparative experiments. Animals inoculated with acutely infected tissue were killed when moribund between 3 and 12 days after inoculation. Smears of peritoneal exudate, liver, spleen, lung and brain were stained overnight by Giemsa's method and examined for *T. gondii* trophozoites. Those inoculated with subacute or chronically infected tissues were killed at the end of 8 weeks and emulsions of their brains examined for *T. gondii* cysts. Some were moribund before this time. If less than 3 weeks had elapsed, smears of peritoneal exudate, liver, spleen, lung and brain were examined. After 3 weeks, brain emulsion was examined for *T. gondii* cysts.

Experiments

The best ways of using the three systems—chick embryos, tissue cultures and mice—having been determined, it was possible to compare them.

Dilutions of acutely infected tissue ranging from 10^{-2} to 10^{-8} were prepared. From each dilution 0.2 ml was inoculated on the chorioallantoic membrane of each of 8 chick embryos, 0.1 ml into each of 6 HEp-2 tissue culture tubes and 0.5 ml intraperitoneally into each of 6 mice.

Results

The results of the comparative tests on acutely infected tissue are shown in Table 1. Since it had been shown that tissue culture was of no practical value for the isolation of strains from subacutely and chronically infected tissue, comparison of methods for isolating *T. gondii* from such tissues was limited to chick embryos and mouse inoculation. The results are shown in Tables 2 and 3.

Discussion

The diagnosis of *T. gondii* infection has sometimes been established by isolation of the parasites in tissue culture,[d] but Kaufman[e] considered this method unsuitable since it required large inocula. Isolation in the chick embryo has also been successful.[f] The most commonly used method, however, has been the inoculation of the laboratory mouse, this being a cheap, easily handled, highly susceptible animal which is almost never found to be naturally infected. Jones et al.[g] compared the various methods used in the inoculation of mice with tissues suspected of containing *T. gondii* and found the intraperitoneal route to be the best. As far as the writer knows there has been no previous quantitative comparison of the efficiency of different methods of isolating *T. gondii* from infected tissues.

[d] Jacobs, L., Fair, J. R. & Bickerton, J. H. (1954) *A.M.A. Arch. Ophthal.*, **51**, 287.

[e] Kaufman, H. E. (1961) *Surv. Ophthal.*, **6**, 877-881.

[f] Morris, D., Levin, B. & France, N. E. (1955) *Lancet*, **2**, 1172-1174.

[g] Jones, F. E., Eyles, D., Coleman, N. & Gibson, C. L. (1958) *Amer. J. trop. Med. Hyg.*, **7**, 531-535.

TABLE 1

RESULTS WITH ACUTELY INFECTED TISSUE[a]

Dilutions	Chick embryos infected	Tissue cultures infected	Mice infected
10^{-2}	8/8	6/6	6/6
10^{-3}	8/8	6/6	6/6
10^{-4}	8/8	6/6	6/6
10^{-5}	8/8	5/6	6/6
10^{-6}	6/8	3/6	6/6
10^{-7}	6/8	2/6	6/6
10^{-8}	2/8	0/6	6/6

Mice: ID_{50} titre/0.5 ml: $10^{-8.5}$ ($10^{-8.8}$/ml)	316	Order of efficiency
Chick embryos: ID_{50} titre/0.2 ml: $10^{-7.3}$ (10^{-8}/ml)	16	
Tissue cultures: ID_{50} titre/0.1 ml: 10^{-6} ($10^{-7.1}$/ml)	1	

[a] ID_{50} titres calculated by the method of Reed, L. J. & Muench, H. (1938) *Amer. J. Hyg.*, 27, 493-497.

TABLE 2

RESULTS WITH SUBACUTELY INFECTED TISSUE

Dilutions	Chick embryos infected	Mice infected
10^{-1}	8/8	6/6
10^{-2}	5/8	6/6
10^{-3}	1/8	5/6
10^{-4}	0/8	3/6
10^{-5}	0/8	1/6
10^{-6}	0/8	0/6

Mice: ID_{50} titre/0.5 ml: 10^{-4} ($10^{-4.8}$/ml)	50	Order of efficiency
Chick embryos: ID_{50} titre/0.2 ml: $10^{-2.8}$ ($10^{-3.1}$/ml)	1	

TABLE 3

RESULTS WITH CHRONICALLY INFECTED TISSUE

Dilutions	Chick embryos infected	Mice infected
10^{-1}	6/8	6/6
10^{-2}	6/8	6/6
10^{-3}	5/8	6/6
10^{-4}	1/8	5/6
10^{-5}	0/8	1/6
10^{-6}	0/8	0/6

Mice: ID_{50} titre/0.5 ml: $10^{-4.5}$ ($10^{-4.8}$/ml)	10	Order of efficiency
Chick embryos: ID_{50} titre/0.2 ml: $10^{-3.5}$ ($10^{-4.2}$/ml)	1	

Conclusions

For isolation of *T. gondii* from acutely infected tissue, mice intraperitoneally inoculated were at least 16 times as sensitive as chick embryos and at least 316 times as sensitive as tissue cultures.

For isolation from subacutely infected tissue mice were 50 times as sensitive as chick embryos. Tissue cultures were of no value.

For isolation from chronically infected tissue mice were 10 times as sensitive as chick embryos. Tissue cultures were of no value.

Mouse inoculation was by far the best method for eventual diagnosis, and the most generally useful route was the intraperitoneal. This could advantageously be combined with the intracerebral when bacteriologically sterile specimens were inoculated. For specimens of doubtful sterility the subcutaneous route was best. Tissue culture was of little use. Chick embryos had the advantage of revealing infection sooner (10 days as against 6 weeks for inoculation of mice with all except highly virulent strains).