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Toxoplasmosis in Animal and Laboratory Diagnosis

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The protozoa known as *Toxoplasma gondii* was discovered by Nicolle and Manceaux in 1908 in the North African rodent *Ctenodactylus gondi*. Since then morphologically identical parasite have been discovered in many mammals, birds and reptiles in all parts of the world. Sabin (1939), however, demonstrated that the morphological and immunol-

ogical identity of the toxoplasma strains originated from a large number of different animals, including man.

While the early interest in this organism was purely zoological, indisputable evidence of the occurrence of toxoplasmosis in man was presented by Wolf et al. in 1939. Among the wild animals, toxoplasma has been isolated from hares, rats and pigeons. In domestic animals or in animals living in close contact with human as pets, the infection has been demonstrated in rabbits, chicken, swine, cows, sheep, mink, dogs and cats. Especially, swine toxoplasmosis has become as an important problem, both from an economic and a public-health point of view in Japan.

Toxoplasmosis was first reported in swine by Farrell et al. in Ohio (1952). Sanger and Cole (1955) isolated toxoplasma from some offspring of a naturally infected sow. Outbreaks of the porcine infection have also been reported by Momberg-Jorgensen in Scandinavia (1956). Swine toxoplasmosis, with obvious clinical signs, has been reported by Nobuto et al. (1960) and Sato (1958) in Japan. Folkers (1962) published studies on toxoplasmosis in pigs with special reference to pathogenicity and immunity.

Nobuto et al. and Folkers described the clinical symptoms that were noted in susceptible pigs and were characterized by fever, dyspnoe with rapid respiration of the abdominal type, reduced appetite, lassitude and cyanosis of the ears and legs.

Haematological examination revealed marked variations in the total white blood cell count during the course of disease, but generally an increase of the number of leucocytes was observed. There was also in most cases an increase of the percentage of neutrophile leucocytes with a shift to the left of the nuclei of neutrophilie leucocytes which resemble to the changes observed in hog cholera. In toxoplasmosis, however no significant changes appeared on the reticulocytes, and this fact is very important for the differential diagnosis to hog cholera. Sanger et al. (1953) found clinical disease in experimentally infected calves and heifers an dintrauterine transmission of the infection and also reported clinical disease due to toxoplasma in four herds of cattle in Ohio.

Cole et al. (1954) identified toxoplasma as the cause of an outbreak of a ovine fatal disease, involving the respiratory and central nervous systems. Beverley and Watson (1961, 1962) and Beverley and Mackay (1962) have described high rate of toxoplasma prevalence in sheep, and isolation of the parasite from aborted fetuses, placentas, and still born lambs in England.

Among domestic birds, chickens and ducks sometimes suffered from the severe disease associated with toxoplasma infection. Toxoplasmosis is wide spread in dogs and cats (Feldman and Miller 1956).

Morphology of Toxoplasma gondii

The parasite, when extracellular, presents a crescentic or arc-shaped form, with one end

Fig. 1 Proliferative form (trophozoite) Giemsa staining.

pointed and the other more rounded (trophozoite) (Fig. 1). It varies in size, being an average 4-7 μ in length, and 2-4 μ in width. The nucleus is located near the rounded end. Localization in the tissue is always cytoplasmic, and multiplication can only take place within living cells.

Within cells, the organisms divide by binary fission. Reproduction of toxoplasma by internal budding (endodyogeny) was described by Goldman et al. (1958) on the basis of silver-impregnated staining. Multiplying parasites fill up the cytoplasm of the host cell
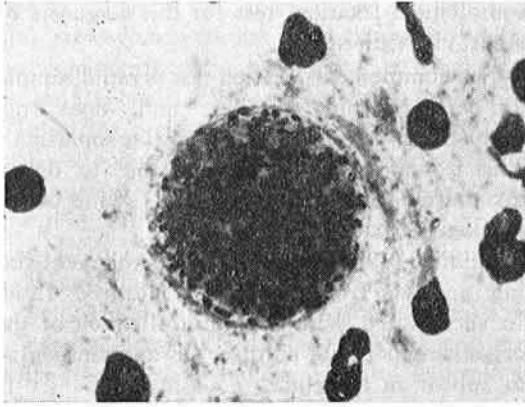
Fig. 2 Toxoplasma Cyst Giemsa staining

and form terminal colony which are surrounded by the residual wall of the host cell. The cell may rupture and the liberated organisms invade new cells. This occurs in acute infection in susceptible animals or in animals with very low resistance.

The true cysts in the chronic infection contain numerous parasites within a wall that has been formed of interaction of parasite and host rather than of parasite alone(Fig.2). In this form, the parasite can maintain itself within the host for years.

Giemsa staining is generally employed for the detection of the organism in a smear preparation. It is however often insufficient when applied to a sample of infected tissue harboring only a few organisms.

The fluorescent antibody technique was first applied to the detection of *Toxoplasma gondii* by Goldman in 1957. Recently, Tsunematsu, et al. and Ito et al. (1964)worked to determine whether the technique is more suitable for direct examination of the organism in infected tissue than any other routine staining method. When a sample was stained under optimal conditions, the toxoplasma was recognized in a typical form of crescent greenish yellow in color on the back ground without brightness. Toxoplasma organisms were specifically recognized in the liver, spleen, lungs, kidneys, mesentric lymph nodes, and pancreas in large numbers. Moreover, they were demonstrated in films of infected mouse blood with success. Cysts of toxoplasma were also recognized in the brain of

the mouse inoculated with the Beverley strain. With this method, Fukazawa et al. (1964) reported that toxoplasma organisms could be demonstrated constantly in the smear preparation of the lungs and lymphnodes of swine at slaughter inspection. Ito et al. (1966) reported an easy and constant method for the demonstration of parasitemia in the infected animals. One to 2 ml of heparinized blood is added to 10 time its volumes of 0.1% saponin PBS and 20 minutes later the mixture is washed with PBS by centrifuge and then the supernatant is discarded. The resultant sediment is resuspended in a very small amount of PBS i.e., 0.2 ml and a thickened preparation is made on a thin slid with it. After drying and fixing with acetone, it is stained with the fluorescent antibody.

Koyama et al. (19) stained living toxoplasma organisms by means of the acridine orange. The nucleus of the proliferative form is fluorescent greenish yellow, and the cytoplasma is red. At high magnification, it became apparent that the red fluorescence of cytoplasma was due to many red spherical granules in it and these granules gradually faded in the course of time, and finally disappeared. The phenomenon might practically be applied to distinguish living organism from the dead ones according to the existence of these granules.

Isolation of the parasite

Direct microscopy alone is very uncertain because of the possibility of confusing toxoplasma with other microorganisms such as Sarcocystis, Ieishmania or Encephalitozoon.

The isolation of the parasite is the most reliable evidence of infection. The most suitable method is to inaculate mice which are very susceptible and in which spontaneous infections are very rare. The material to be examined may be lungs, liver, lymphnodes and brain. In the isolation of organism from chronic cases, 30 grams of muscle or brain are used after digestion with 0.25% trypsin-saline in 60 minutes at room temperature 4 or 5 weeks after injection, a small piece of brain of the mouse is examined under micro-

scope to detect the presence of the toxoplasmic cyst (Hanaki et al. 1963).

Immunological tests
Sabin-Feldman dye test (1948) :
This test is based on the principle that extracellular toxoplasma lose their normal ability to stain with alkaline methylen blue after having been incubated with serum containing toxoplasmic antibodies. A heat-labile accessory factor, which is present in fresh human serum, is necessary for the action of antibody on the organism.

The sera to be tested are inactivated at 56 °C for half an hour. Serum dilutions are prepared in saline and, after addition of a mixture of accessory factor serum and toxoplasma, the tubes are incubated for one hour at 37°C. Alkaline methylen blue (pH 11) is then added and, after shaking the tubes, a drop of each dilution is examined microscopically under a cover slip. The stained and unstained organisms are counted (a total of 100) and 50% end-point is determined. The test should be carried out with proper positive and negative controls, in which the negative may stain of the parasites more than 90% and the positive stain less than 90%.

In practice, the dye test is rather complicated to perform because it requires as an antigen a constant fresh supply of living toxoplasma, usually in the form of peritoneal exudate from mice infected with RH strain of toxoplasma. Furthermore, it is necessary to have human donors to provide accessory factor serum devoid of toxoplasmic antibodies. This serum is heat-labile and should be stored in −80°C deep-freezer. Finally, a certain skill and adequate safety measures are necessary when handling the living parasites in order to avoid laboratory infections.

Complement-fixation test (CF)
Warren & Russ (1948) prepared the antigen by repeated freezing and thawing of cholioallantoic membrane from chick embryo infected with toxoplasma. The test is carried out in the same way as the commonly used complement-fixation test for the diagnosis of other virus diseases.

The complement-fixation test is rapid, simple to perform and specific, and does not involve the handling of living toxoplasma. This test, however, is not reliable to detect toxoplasmosis in domestic animal (swine, cattle, chicken, etc).

Shimizu (1961) reported the complement-fixing antigen from the tissue cultured fluid. In accordance with the multiplication of the organism in tissue culture, CF antigen begins to appear in the fluid.

He concluded that the tissue-culture-fluid antigen should be widely employed in the future as one of the best CF antigens of toxoplasma.

Complement-fixation inhibition test (CIF)
Harboe et al. (1957) found that sera from adult chickens experimentally infected with toxoplasma inhibited complement fixation in a mixture of serum of rabbit infected with toxoplasma and toxoplasmic antigen made from infected egg menbranes.

Nobuto et al. (1960) and Robertson et al. (1963) used the complement fixation inhibition test or the indirect complement fixation test for swine and calf sera. Prior to the CFI test, appropriate amounts of immune guinea pig serum and antigen to be used in the final test were determined by the direct CF method. In the CFI test, two fold serial dilution of swine serum (to be tested) heated at 60°C for 20 minutes before the test, were made first by using Ca, Mg and Na compound. To each tube containing 0.1 ml of serum dilution was added equal amount of 2 units of diluted antigen which had been determined from the preliminary titration. These mixtures were incubated at 37 °C for one hour. Then, 0.1 ml of indicator serum and 0.2 ml of exactly 2 unit of complement was added to each tube, and incubated overnight at 4 °C. The tube was taken out from the refrigerator and 0.2 ml of sensitized red cells was added to each tube, which was re-incubated in a water bath at 37 °C for 30 minutes.

Suzuki (1960), using the CFI test, studied almost 2,000 serum samples collected from

nearly all parts of Japan. The results from this survey showed that toxoplasma antibodies are common in swine throughout the country: in all, about 8.6 percent reacted positively. While this method facilitates diagnosis, it is still a complicated technique and requires specialized laboratory resources, as does the dye test.

Hamagglutination test (HA)

Jacobs and Lunde (1957) reported details of a hamagglutination test, in which sheep red cells treated with tannic acid and exposed to an extract containing the water-soluble components of toxoplasma from the peritoneal exudate of a mouse become agglutinated by the homologous antibody. This test gives promise of a safe, quite simple and practical method for the routine diagnosis of toxoplasmosis. HA test, however has the disadvantage of requiring freshly sensitized cells which cannot be stored satisfactorily for more than a few days. In addition, the sera from swine and small experimental animal could not be tested by Jacobs' original method because it sometimes causes non-specific hemagglutination in this system.

Nobuto & Hanaki (1963) succeeded in facilitating a new reproducible method for preparation of stable sensitized erythrocytes for

toxoplasma HA test which could be authorized by standard and distributed by a central laboratory. In this method sheep blood cells were fixed in alcohol-formalin and coated with antigen by using bis-diazo-benzidine. The resultant sensitized blood cells were freeze dried. Two percent normal swine serum with PBS heated for 10 minutes at 110°C was used as diluent instead of normal rabbit serum in the Jacobs' original method. In this diluent the sensitized blood cells were suspended beforehand. Then this suspension was employed to make a 1:4 dilution of the blood sample to be tested. Reading of results is made as follows, lower than 1:64 dilution =negative, 1:64 dilution=suspected positive, 1:256 dilution and higher=positive. (Fig. 3)

To collect a blood sample from a pig, prick a blood vessel of the flap of the ear, and saturate such a filter-paper strip as shown in figure (Fig. 4) with 0.1 ml of blood gushing out. This filter-paper strip consists of two
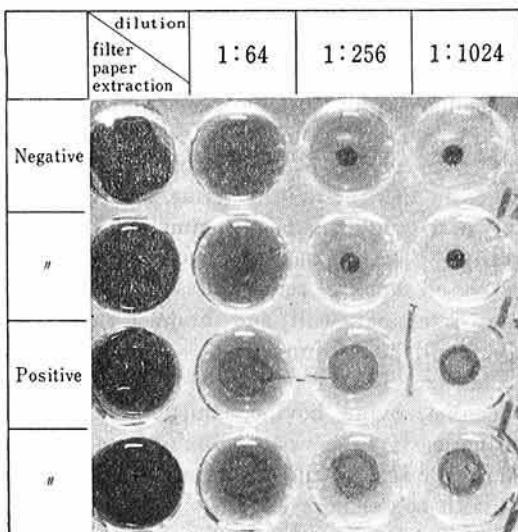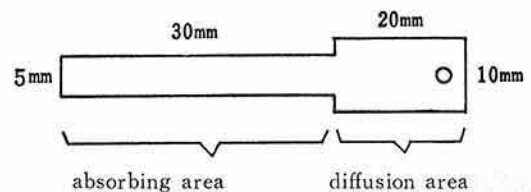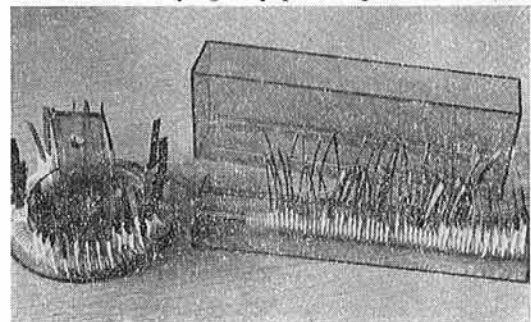
Fig. 4 Filterpaper strip (Nofuto)

Drying of paper strip

Fig. 3 HA-test pattern (Nobuto-Hanaki)

areas, a blood-absorbing and a blood-diffusion area. If the absorbing area is saturated with blood, the excessive blood will spread to the diffusion area. Therefore, a constant amount of blood always is absorbed in the absorbing area of the strip. The strip is dried, with
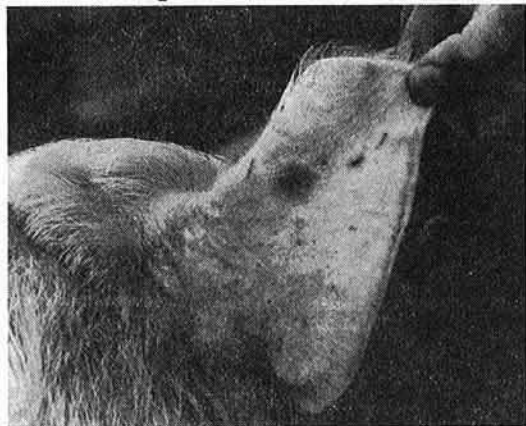
the absorbing area directed upward. The dried strip can be stored at room temperature for more than two months. It is very convenient to transport and preserve blood samples by this method. The filter paper can be used for collection of blood of any other mammals and chickens. The extraction of antibodies from dried filter paper is inhibited when the absorbed blood in the filter paper is exposed to a formaldehyde gas in the drying process.

Skin test

In persons who posses antibodies against toxoplasma, an intracutaneous injection of toxoplasmic antigen (toxoplasmin) is followed by an allergic skin reaction. This was first described by Frenkel in 1948 and its easiness to pertorm makes the toxoplasmin test a useful aid in the diagnosis of latent toxoplasmosis. However, distinct skin reactions could not be observed on the porcine toxoplasmosis with human toxoplasmin.

Nobuto et al. (1964) succeeded in eliciting a distinct skin reaction on infected swine by the use of concentrated antigen (Fig. 5). The antigen was concentrated and purified by means of acetone-ether treatment, the method

Fig. 5 Positive Skin test

basically described by Lunde and Jacobs for the preparation of powdered HA antigen. A potency test of prepared antigen was performed on the skin of the side of experimentally infected swine and the optimal dilution was made to contain two units of

reaction doses in final 0. 2 ml antigen. Then 2. 2 ml of this diluted antigen was lyophilized in 3 ml ampoules.

In practice, 0. 2 ml of reconstituted antigen was injected intracutaneously into the ear, and 24 hours after injection the test was read as positive when erythema or swelling was present in a diameter of more than 15 mm. There was a high correlation between TSC skin test and dye test except in the early stage of the infection.

Pathological findings

Nobuto et al. (1960) described in their studies on porcine toxoplasmosis that characteristic lesions were observed macroscopically in the lungs and lymphnodes and consisted of imcomplete retraction, edematous or serous swelling of the lungs, hyperemia, scattered foci of necrosis in lymphnodes. Hemorrhage was seen in the spleen and lymphnodes (gastric, hepatic and mescenteric lymphnodes) and minute hemorrhage in the kidneys under its capsule. Furthermore, follicular swelling and a grayish ulcerative or necrotic foci were scattered on the mucous membrane of the colon.

Microscopically, the lesions were characterized by miliary foci of necrosis, edema, and granulomatous inflammation involving the lungs, lymphnodes, liver and spleen. In the brain, microglial granuloma associated with focal necrosis was observed, and perivascular proliferation of mononuclear cells and edematous subependymitis also developed.

Terminal colony (pseudo cysts) and a trophozoite (proliferative form) of the parasite were seen either in the alveoli or in macrophages in the interstitial tissue where edematous and granuloatous inflammation occured usually. Terminal colony were also easily discovered among reticulartissue of lymphnodes, intestinal wall and brain. Cysts were only found in the brain.

Koestner (1960) reported the neuropathology of canine, ovine bovine, and porcine toxoplasmosis. It is a very valuable description and author agrees with his opinion in comparison with hog cholera lesion on toxoplasmosis.

a. The perivascular cuffing in the brain is

more extensive in hog cholera than in toxoplasmosis. The predominant cells in the former are the lymphocytes, in the latter the microglia. The cuffing in hog cholera rarely extend beyond the piaglial membrane : in toxoplasmosis they are often confluent with a necrotic focus or a glial nodule. Necrosis is indicated by disintegration of cells and repair is rarely seen in hog cholera but is commonly seen in toxoplasmosis.

b. The glial nodules in toxoplasmosis are a mixture of the glial elements, monocytes, and perithelial cells. The microglia being the predominant cell type in toxoplasmosis is almost exclusively in the lesions of brain of hog cholera.

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Rice Transplanting Machine

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Rice seedliags transplanted by hand now in Japan range from those younger ones with 2.5 leaves to those with 8 leaves. And some of paddy fields where they are transplanted are so wet and muddy as a farmers body sinks as deep as to his waist while others are so dry and so hard soil as it cannot be made finer with hoe. It would be most desirable that a rice transplanting machine could work efficieiently under any of such conditions of both seedlings and soil. But it is actually impossible to produce such machine. The machine on sale therefore is limited in efficiency according to different conditions, to which it is applied. Namely, the machine does not work at all or works rather in efficiently unless it is employed under a certain condition suited to its function.

The rice transplanting machine now under study or on sale can be roughly classified into the one for seedlings with roots washed and the other for those with soil clods or their roots.

Transplanting machine for seedlings with

roots washed

Rice seedlings with 4–6 leaves are picked out from a nursery at first. If they are picked out from a upland nursery, mud attached to their roots is struck off while those from an irrigated nursery are cleaned of mud from their roots by washing.

At any rate the seedlings are kept clean of mud from their roots in the same way as those used for traditional hand transplanting. When the machine loaded with seedlings runs, most of its working process is similar to the hand transplanting. Fig. 1 shows a upright type power machine for transplanting seedlings with their roots washed in two rows at a time. This type appeared in the market in 1965. The working process consists of the following stages: seedlings are carefully put upright in the seedling box; the clutch is on; the planting claw is opened and put into the seedling box; the claw is closed to grasp a hill of seedlings; the claw pulls out the hill and moves toward the field; it puts the hill into the surface of the field with soil levelled by a levelling board; then the claw opens again and returns to its original position,