

Toxoplasmosis Prevalence in Sheep in *Daerah Istimewa Yogyakarta*

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Abstract. A research on toxoplasmosis prevalence in sheep was conducted in Daerah Istimewa Yogyakarta. The objective of the research was to understand the prevalence level of toxoplasmosis in sheep using skin test method by taking the membrane protein of tachyzoit produced in vivo. The research was initiated by producing the tachyzoit membrane protein at the testing animals, later the obtained protein was prepared and used in the skin test method. At the end of the research agglutination test was conducted to confirm the diagnosis using card agglutination test. An optimal dosage of tachyzoit membrane protein used in sheep as the basic material of the skin test was 1.5 mg/ml/head. Result showed the reaction of skin was thickening and the duration after being injected intradermally varied from 12 to 30 minutes in various sizes from 8 to 19 millimetres. The skin test method showed that the prevalence level of toxoplasmosis in Yogyakarta was more than 70%.

Key Words: toxoplasmosis, prevalence, skin test

Introduction

Toxoplasmosis is one of many zoonosis diseases i.e. the disease that naturally can infect either animals or human. The clinical symptoms of the disease are not apparent but causing many losses to either the infected human or animals. In the medical domain for example, the care of toxoplasmosis infection always haunts women mainly the pregnant ones. In animals, toxoplasmosis has caused many economical losses significantly for it causes abortion, premature death and congenital disorder. These losses have not yet included the high cost maintenance of the animal farming both small and industrial scale. In this case, animals play a very important role as one of the infection forms. Human can get infected by consuming food containing toxoplasma oocyst or well-cooked meat which directly contains bradyzoite or one of toxoplasma phases, contacting with an open wound containing oocyst or pets like cats, dogs and birds. Besides, there are many other methods of infection potential to toxoplasmosis in human and animals (Nurcahyo, 2001). Cats

and some Felidae groups play an important role as the key of toxoplasmosis development and spreading since they act as the definitive hospes in toxoplasmosis. Various researches in cats worldwide detect antibody towards toxoplasmosis 20-90%. In general, the toxoplasmosis diagnoses are classified into three, namely clinical, biological and laboratorial diagnosis. The diagnosis will be difficult if the clinical symptoms of toxoplasmosis resemble other infectious and non infectious diseases. Accordingly, having another convincing diagnosis by isolating the parasite and inoculating the suspected tissue in mice or other sensitive testing animals is essential (Soulsby, 1982). Each diagnosis has its own strengths and weaknesses. Serological diagnosis is for detecting the presence of antibody IgM and IgG in serum, as well as the presence of antigen in the hospes body. A diagnosis is sometimes not sensitive particularly to patients with less immune response. Furthermore, histological examination sometimes does not find any parasite as the change in toxoplasmosis is not specific (Spencer, 1972). The diagnosis by convention is

generally not sensitive or it often causes fake positive as the use of antigen from foreign countries is different from the isolat from Indonesia. Also, the serological test provided in markets is still costly due to the limited antigen provision and the costly cattle antigen. Most farmers in Indonesia still disregard the animal health problem; consequently they are unwilling to spend for diagnosis and medication. The diagnosis has gone through many developments such as Sabin-Fieldman Test (SFT), Indirect Fluorescence Antibody Test (IFAT), Enzyme Linked Immuno Sorbent Assay (ELISA), Complement Fixation Test (CFT), Latex Agglutination Test (LA), Indirect Agglutination Test (IHA), Direct Agglutination Test (DA), Western Blot and Polymerase Chain Reaction. Each diagnosis technique above has its own advantage and disadvantage.

Toxoplasmosis in sheep and goats have significant meaning considering the Indonesian high interest of satay. It is concerned that a not well-cooked satay contains active bradyzoite cyst which can infect the feeder. The clinical symptoms of toxoplasmosis in sheep and goats are not clearly visible, it may be seen fever up to 41.5°C, anemia, cough, diarrhea, lost appetite, paralysis, trembling thigh muscle and nerve symptom. If the infection continues, it will lead to abortus, defective born or death.

The toxoplasmosis prevalence in Indonesia is predicted to increase as the lifestyle change of the society takes place (Nurcahyo, 2001). Meanwhile the prevalences in animals are seropositive 75.6% in dogs and 72.7% in cats in Jakarta, 61% in goats in Borneo, 51% in pigs in East Java, 50% in Irian Jaya (Ma'aruf, 1990). The occurrences in sheep and pigs in Yogyakarta are 50 and 40% respectively (Hartati et al., 1993). The serological test of toxoplasmosis using Toxoscreen DA test in Girimulyo and Kulon Progo result in 40% Etawah goats positive toxoplasmosis (Hartati, 1994). According to data from Animal farming department year 2003 in Sleman regency, there is the prevalence

70% in sheep and goats infected toxoplasmosis (Nurcahyo, 2004). At human the occurrence of toxoplasmosis worldwide is relatively high. It is estimated almost 500 millions human worldwide serologically suffer from toxoplasmosis. In USA it is reported 30-60% adults are infected toxoplasmosis (Frenkel, 1990). Whereas, a prevalence study in Japan states one third of adults are by latent infected toxoplasmosis (Matsuura et al., 1992). Toxoplasmosis in the form of congenital is the most dangerous one and causing serious problem. Estimated in USA is 3000 babies born per year suffering from congenital toxoplasmosis and charging USD 31-40 millions of medication care, not included the spiritual loss of abortus or baby death.

Toxoplasmosis in goats and sheep have significant role as many abortus cases occur mainly to sheep. About 20-100% sheep in the world is seropositive to toxoplasmosis. Meanwhile the fawn in England are 2.2% seropositive. The source of toxoplasmosis infection in sheep farming mostly comes from the food contaminated with toxoplasma oocyst and the infection could last for 2 years. The objective of the research is to see toxoplasmosis prevalence in sheep in Daerah Istimewa Yogyakarta using tachyzoit membrane protein which can be produced in vivo as the basic material for the skin test.

Materials and Methods

The production of tachyzoit membrane protein

To produce membrane protein, 30 adult mice aged 5-6 weeks were used and injected with local isolat tachyzoit *T. gondii* by intraperitoneal with a dosage 1×10^7 so after 72-96 hours the mice showed ill symptoms marked by standing fur, being weak, having no appetite, declining breathe frequency and rapid heartbeat. The mice were later cut open and had their abdominal cavity washed with physiological NaCl solution to get the tachyzoit. From the washing experiment carried out three

times using 5 ml NaCl for each time, a total of tachyzoit in a volume of 15 ml could be obtained. The tachyzoit was then infected to 60 adult mice with a dosage 1×10^7 to get more tachyzoit using the same previous method. The result of the abdominal washing was then centrifuged 3000 rpm at temperature 4°C for 10 minutes. Pellet obtained was washed three times using PBS containing Tris-amonium-chlorid pH 7.4 to lyse various erythrocyte contaminants. Afterwards the exudate was incubated by shaking at temperature 37°C for 5 minutes to lyse erythrocyte, added 40 ml PBS into the suspension, stirred carefully and centrifuged 3000 rpm for 10 minutes. The washing process was repeated at least 4 times to eliminate foreign protein. Following the centrifugation, were resuspending the cells in 100 ml PBS. Using needle size 27, the suspension was carefully absorbed so the pellet in the form of peritoneal cells would not be taken. The suspension containing free trophozoit was later filtered using Milipore sized $3\ \mu\text{m}$ to eliminate the remaining cell. The cell was calculated using haemocytometer (Boeckel & Co., Germany) and the product obtained is at the least $1 \times 10^9/\text{ml}$ out of 100 mice. Parasite trophozoit was then centrifuged at 3000 rpm for 10 minutes and washed 5 times using PBS pH 7.4.

Protein preparation

After cleanly washed, the remained tachyzoit in pellet was resuspended in a minimum of 3 ml PBS containing protease inhibitor tosyllysine chloromethyl keton (TLCK, Sigma, USA) in $10\ \mu\text{g}/\text{ml}$ Phenylmethylsulfonyl fluorides (PMSF, Sigma, Co) with final concentration 1 mM to avoid protein degradation. The organism suspension is dissonicated in ice at amplitude 14 micron with 10×1 minute pulsation and centrifuged in $10.000 \times g$ (18.000 rpm) for 3 minutes towards the pellet and the cell remained as well as non-soluble materials. The pellet from the

centrifuged result was ready to extract the membrane protein. The pellet was washed with PBS pH 7.4 and centrifuged at 11.000 rpm for 4 minutes to get rid of other soluble protein layers. The pellet was resuspended in 1 ml 0.5% Nonidet-P 40 (Sigma Co.) in PBS pH 7.4, placed in temperature 4°C for 1 hour and shaken at times. The mixed suspension was centrifuged at $10000 \times g$ (18.000 rpm) for 20 minutes in cold centrifuge. In addition, the supernatant was moved and dialysis was carried out for a night towards PBS pH 7.4. After dialysis, membrane antigen was divided in aliquot and stored in temperature -70°C for the upcoming test. The concentrations of soluble and non-soluble protein were counted using BioRad Micro Assay system.

The skin test (intra dermal) of membrane protein

The membrane protein from the tachyzoit from the above method was applied to sheep. The sheep were marked with signs/numbers to adjust to intradermal test result using the serological examination. The fur around the end tail in a diameter of five cm was clean-shaved. Intradermally 1.5 ml antigen toxoplasmosis was injected in the central area using Rautmann automatic injection pump. After 15-30 minutes, the injected part was examined. The diameter of thickening skin occurred was measured with cuttimeter. The injected area was free from any skin touch, alcohol or antiseptic until the measurement was done. The diagnosis was regarded positive if the diameter of skin thickening was the same or more than 5 mm, and it was negative if the diameter was less than 15 mm.

Agglutination test

Blood was taken from about 30 cattle suspected Toxoplasmosis using skin test. Serological examination took place in Parasitological laboratory or at the field using Card Agglutination Test as further validation of

Toxoplasmosis in cattle. Therefore, the result procured was well proven either by skin test or serological test.

Results and Discussion

The parasite replication

Tachyzoit is a form of acute toxoplasmosis which can be isolated from the peritoneal cavity of the mice either by in vivo or in vitro. Parasite replication (tachyzoit) is needed particularly to get the membrane protein of toxoplasma which will be used as the diagnosis material. In this research after the mice were injected tachyzoit through intraperitoneal, the mice died within 3-4 days depending on the dosage given and the strain used, followed by necropsy.

It was known that the strain stored in glycerine solution was less infective and it could cause ascites to mice in a longer time than the active strain from peritoneal solution or the strain from the storage in RPMI solution. The tachyzoit coming from the storage in temperature -20 °C was usually less infective and needed to be repassed to other mice which would die less than a week. The tachyzoit then replicated and accumulated from 8 to 16 organisms in the hospes cell (Soulsby, 1982).

The result showed the production of the membrane protein of a mouse with a dosage 1×10^7 Takizot was 13.58 mg. Yet the total still could vary depending on isolat toxoplasma, type and age of the testing animals used. At high dosage the mice generally died and only survived for a few days after the infection. The death was due to the very young age of mice (3 months) so the immune system had not fully grown in coping with toxoplasmosis infection. Other factors were furrow and isolat toxoplasma used. Krahenbuhl and Remington (1982) stated that the hospes resistance towards toxoplasmosis infection depended on the species, the hospes age and the level of toxoplasma strain virulence. Gandahusada (1990) also mentioned that the death and the damage of the animal body tissue infected with toxoplasmosis depended on the total parasite and the age of the animal tested. The death and the tissue damage of younger animals were faster than those of the older ones.

The optimal dosage of tachyzoit membrane protein used in sheep to diagnose skin by causing hypersensitivity reaction based on observation was 1.5 mg/ml/head. The hypersensitivity did not appear at other dosages.

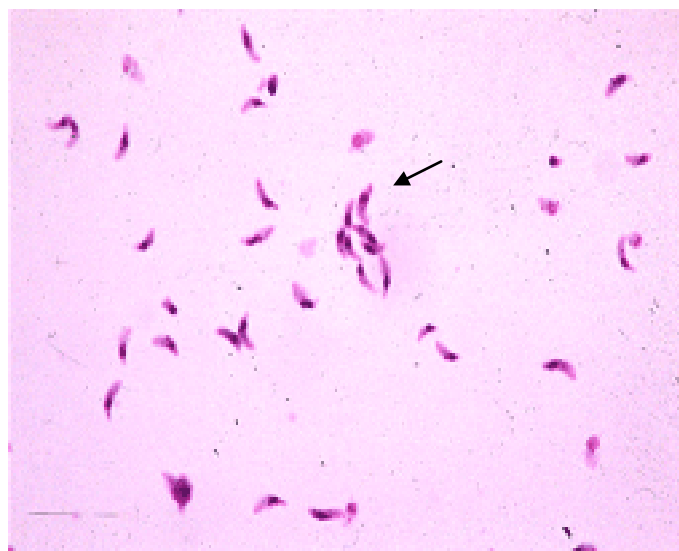


Figure 1. Tachyzoit collected from liquid of mice ascites (Giemsa painting)

The application of skin test

The sampling was done in 5 regencies in Daerah Istimewa Yogyakarta with different geographical types: Kabupaten Gunung Kidul, Kulon Progo, Bantul, Sleman and Kotamadya Yogyakarta. Samples of 30 sheep and goats at random were taken from each location. The previous survey had been done to more of goat sampling, considering the positive reactions found in goats. The samples were mainly taken from the densely cattle-populated area which has the potential of toxoplasmosis hospes. The optimal dosage observed of 1.5 ml antigen toxoplasma was given. After 15-30 minutes, skin thickening and ossification in the injected area showed positive reaction.

The diagnosis was positive if the diameter of skin thickening was 15 mm or more, and negative for less than 15 mm (Figure 2 and 3). The observation showed that the reaction of skin thickening and the duration after intradermally injected toxoplasmosis protein varied from 12 to 30 minutes in various sizes from 8 to 19 millimetres. The various duration and diameter was caused by the immunological

response in each hospes caused by the toxoplasma infection. Reaction occurred to the hospes that could develop the immune response toward the injection. The hypersensitivity reaction in the sheepskin happened as the effect of the antibody formation against the infection. The diagnosis was based on the allergic reaction formed after the intradermal injection.

The serological test

After the skin test, the cattle blood was taken for the serological examination with Card Agglutination Test (CATT) in Parasitological Laboratory Veterinary Faculty Gadjah Mada University or at the field. The method resulted within 10-15 minutes until the card dropped with serum and the reaction kit became dry. The examination functioned as a reference and comparator of skin test diagnosis whether the goats suffered from toxoplasmosis or not. The result of the skin test applying antigen of toxoplasmosis membrane was then compared to that of serological examination to get the sensitivity and the specificity of toxoplasma membrane antigen (Armitage, 1973).



Figure 2. The positive reaction is marked by the presence of skin thickening as the effect of the intradermal injection of tachyzoite membrane protein



Figure 3. The negative reaction is marked by the absence of skin thickening as the effect of the intradermal injection of tachyzoite membrane protein

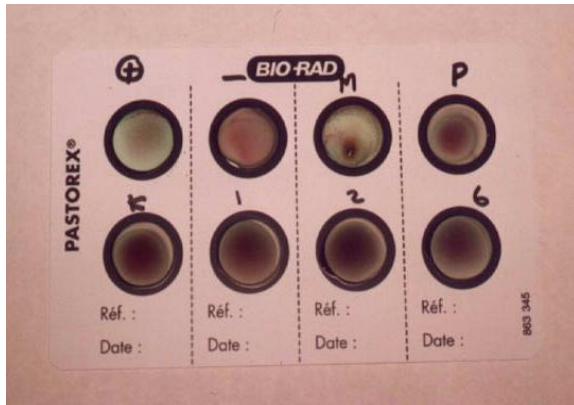


Figure 4. Example of serological examination result using CATT method to detection toxoplasmosis in sheep

In Figure 4 the serological examination using CATT method presented that the animal with positive toxoplasmosis was marked with the colour close to positive control colour i.e. green (code M), while others close to the positive control were brownish green (P). The assessment of only uncertain colours or skin thickening less than the determined diameter can be classified positive or fake negative. The animal not suffering from toxoplasmosis was indicated with the brownish colour on the surface of the diagnosis card. CATT method was basically quite effective to detect toxoplasmosis in sheep and goats. Further explanation could be seen in attachment 1 to 5. However, devices used in this method were relatively costly to apply for cattle.

The toxoplasmosis prevalence

The research on toxoplasmosis prevalence was essential to monitor the development of toxoplasmosis since the welfare increased and the social lifestyle changed. The increase of cat population in some areas as the definitive hospes of toxoplasma caused a larger spreading of the disease. Some cats seen around the location of sheep/goat farming led to a more rapid and perfect spread through their faeces that might contain toxoplasma oocyst.

The result of the toxoplasmosis prevalence in some regencies in Daerah Istimewa Yogyakarta showed a worrying result considering the samples taken in average showed seroprevalence, and the skin test was high or above 70%. The highest result in Figure 5 was in Kulon Progo, a hot hill area known for its goat and sheep farming, followed by Gunung Kidul and other regencies. Cats found in the goat/sheep farming were predicted as a trigger to the high prevalence number.

The prevalence determination from skin test method showed nearly the same result for the five areas of examination. However, it was apparent that the examination using the serological method showed a big difference between Kulon Progo (95.6%), Bantul (74.1 %) as well as Yogyakarta (74.1 %).

It was possible to happen although Yogyakarta was densely populated. However, due to the limited goat/sheep farming business and better healthy lifestyle in Yogyakarta and Bantul than in Kulon Progo, the rate of the toxoplasmosis prevalence in cattle was relatively lower. Gunung Kidul (81.5%) as the dry area during the dry season also led to the high rate of the toxoplasmosis in goats/sheep. The 76.7% prevalence in Sleman needed more concern as the regency had many farms of goats, sheep and cows.

The skin test sensitivity about 95.2% for Gunung Kidul was the highest rate, followed by Sleman (90.5%), Yogya (90%), Bantul (85.7) and Kulon Progo (75%). Meanwhile the specificity rate in Kulon Progo was the highest i.e. 90%, followed by Gunung Kidul, Bantul and Sleman 77.8%. Kotamadya Yogyakarta with 70% specificity rate turned out to be the lowest among the four regencies.

As a diagnosis tool, the skin test method must meet the criteria to select diagnostic assay to solve the problem faced particularly in toxoplasmosis prevention in animals. The

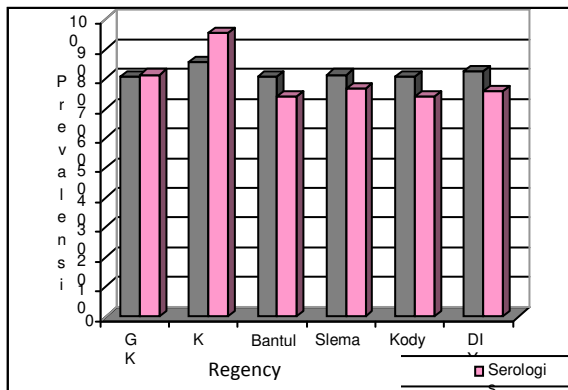


Figure 5. Histogram on toxoplasmosis prevalence in some regency in DIY using skin test method and serological CATT method.

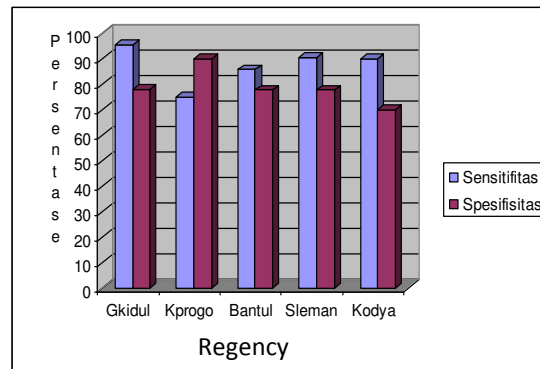


Figure 6. Histogram on sensitivity and specificity of skin test diagnosis and serological diagnosis on sheep toxoplasmosis in DIY

sensitivity of skin test used in the research was a diagnostic test focusing on the use of toxoplasmosis antigen to detect the immune response of the animals suffering from toxoplasmosis. As stated by Burgess (1988), the sensitivity related to the ability to detect antigen in a small number or to detect small immune response. However, the skin test made in this research could not distinguish acute or chronic toxoplasmosis for the result was only based on the hypersensitivity reaction that comes up as the effect of intradermal injection of toxoplasma antigen. This links to the basic material taken from the stadium tachyzoite whose emergence in toxoplasma phase is in acute phase. Whereas in chronic phase the stadium often involved in immunological process is bradyzoite. In the other hand, the bradyzoite is so hard to obtain from the animal tissue which is infected with toxoplasmosis, for its location is in tissue or organ so it is difficult to isolate the protein.

The specificity in toxoplasmosis diagnosis is closely related to the ability in distinguishing antigen from other parasites. The difficulty faced in diagnosing toxoplasma parasite is because toxoplasma is facultative parasite especially in the intermediary hospes which is in almost all mammals and fowl. The current study showed that in some places the

sensitivity was so high but the specificity was lower. The knowledge in the skin test application as the diagnostic material of toxoplasmosis would result better when conducted more samplings, therefore the statistic test could be evaluated further in term of the performance. Besides, estimating the sensitivity and the specificity involved in the identification of ill and healthy animals would be better when using standardized standard method such as ELISA or PCR which is believed as the golden method to diagnose a disease. The weakness of the research was the use of comparison method i.e. serological method, which turned out to be the same with the skin test method to detect the presence of active antibody in the immune response to toxoplasmosis. The uses of ELISA and PCR in the veterinary world are still considered a costly thing especially if it is related to the economical condition of the farmers.

Conclusions

Based on the research it could be concluded that the skin test method of the hopes for toxoplasmosis diagnosis on sheep and goats with accurate, fast and efficient result. Generally, for a toxoplasmosis diagnosis skin test and agglutination methods showed the same result. The toxoplasmosis prevalence

using skin test and agglutination method come up with Gunung Kidul 80.8% (81.5%), Kulon Progo 85.7% (95.6%), Bantul 80.8% (74.1 %), Sleman 81.5% (76.7%), Kota Yogya 80.8 % (74.1 %) and Daerah Istimewa Yogyakarta 82.6% (75.9%). The sensitivity and specificity of toxoplasma antigen for toxoplasmosis diagnosis in sheep/goats in Gunung Kidul 95.2 and the specificity is 77.8%; Kulon Progo 75 and 90%; Bantul 85.7 and 77.7%. Sleman 90.5 and 77.7%; Kota Yogya 90 and 70%. The suggestion to give is the maximal result could be gained by having comparison test using molecular methods such as ELISA and PCR technique toward skin test method so the accuracy level as the alternative diagnosis was found instead of the present toxoplasmosis tests.

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