Detection of Toxoplasma gondii in cat’s internal organs by immunohistochemistry methods labeled with-[strept] avidin-biotin

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Abstract

Aim: The aims of the study are to detect the presence of Toxoplasma gondii antigen and to determine its distribution location in several organs of domestic cat using immunohistochemistry (IHC) method with Labeled-[Strept] Avidin-Biotin (LAB-SA).

Material and Methods: Four domestic cats aged 1-2 years were used as sample in this research. The sample divided into two groups with two cats each. Cats in Group I were positive Toxoplasma based on serologically screening test, while cats in Group II were orally infected with $1 \times 10^6$ Toxoplasma oocyst. All samples then necropsied, and the organs including brain, liver, kidney, duodenum, jejunum, ileum, lungs, and spleen were collected for IHC method with LAB-SA.

Result: The result showed that Toxoplasma antigens were detected in ileum of both serologically positive domestic cat and the experimentally infected cats. Toxoplasma was also observed in kidney of serologically positive domestic cat. In the serologically positive domestic cat, necrotic lesions were found on ileum, kidney, and liver, whereas in experimentally infected cat, the lesion was only found on ileum.

Conclusion: The presence of Toxoplasma antigen is successfully detected in several organs of domestic cat using IHC method with the LAB-SA.

Keywords: cat, immunohistochemistry, labeled-[strept] avidin-biotin, Toxoplasma gondii.

Introduction

The obligate intracellular parasite Toxoplasma gondii infects a broad range of mammalian and avian hosts including approximately one-third of the human population [1-4].

Toxoplasmosis is a globally spread zoonosis with a clinical impact on the unborn fetuses and the immunosuppressed individuals, furthermore, it is regarded as one of the leading causes of death in food-borne illness [5]. Toxoplasmosis is a worldwide reported zoonotic infection caused by the protozoon Toxoplasma gondii [6]. T. gondii cause a lifelong chronic infection in host by impede and suppress the immune system [7,8].

The domestic and other felids have been proven as the major reservoir host of T. gondii infection. It is generally assumed that cats play a major role in transmitting T. gondii through fecal contamination of soil, food, and water because they can excrete millions of oocysts in short period of time (1-2 weeks) [9]. The infection can be obtained by eating raw or undercooked meat containing cysts or consuming food or water contained with sporulated oocysts [10,11] or by ingestion of raw milk and milk products [12].

Several laboratory methods have been developed to detect antibody in serum of infected cat such as polymerase chain reaction, enzyme-linked immunosorbent assay, latex agglutination test, indirect hemagglutination test, indirect fluorescent antibody test, and immunochromatography (IC). Although these tests are expensive and require a specialized laboratory, they are more sensitive and specific [13,14].

IC is a rapid, simple, sensitive, and specific diagnostic tool to detect specific antibodies from T. gondii infection in cats in field conditions [15]. Fecal flotation technique is used to detect oocyst in fecal samples as well. Although flotations are a reference method for the detection of T. gondii oocysts, it has been suggested that an alternative test is also needed due to
the microscopic examination is time-consuming and needs an experience microscopist [16].

The study of detecting parasites within host organs could not be conducted easily. To overcome the limitation of conventional methods, new diagnostic methods are devised, such as histopathology and immunohistochemistry (IHC) method [17,18]. The most widely used and highly sensitive immunohistochemistry (IHC) method is avidin-biotin method or commonly called avidin-biotin complex (ABC) [19].

The most commonly used avidin-biotin method is labeled avidin-biotin (LAB) or labeled streptavidin avidin-biotin (LSAB) with biotinylated secondary antibody and three reagents from peroxide or alkaline phosphate. This method has higher sensitivity compared to other ABC methods [20].

The purpose of this study is to detect the appearance of T. gondii antigen and to determine its distribution location in several organs of domestic cat using IHC method with labeled-(Strept) avidin-biotin (LAB-SA).

Materials and Methods

Ethical approval

The study was approved by the Ethics Committee of Ethical Clearance for Pre-clinical Research, Integrated Research and Testing Laboratory, Gadjah Mada University, Yogyakarta, Indonesia (approval no. 00111/09).

Sample preparation

Four domestic cats aged 1-2 years were used as sample in this research. The sample divided into two groups with two cats each. Cats in Group I were positive Toxoplasma based on serologically screening test (tested by Feline Anigen Toxoplasma Ab Rapid Test Kit), while cats in Group II were orally infected with 1×10⁶ Toxoplasma oocyst. The cats were euthanized using an intravenous injection of 20% pentobarbitone solution as a euthanized material which highly recommended by World Society for the Protection of Animals, London. This method is considered best practice because it consistently produces a death when used as the sole means of euthanasia. Then, a necropsy was performed on both individuals, followed by the collections of the organs including brain, liver, kidney, duodenum, jejunum, ileum, lungs, and spleen for IHC method with LAB-SA (Invitrogen® LAB-SA Detection System).

Results

IHC

Results of the examination of several organs by IHC staining are shown in Figure-1a and b:

Macrophages are observed in some organs such as liver, lungs, and kidney of cats which serologically positive Toxoplasmosis. Thus, indicating chronically infected. The result than supported by enzyme-linked fluorescent assay (ELFA) examination which positively reacted to immunoglobulin (Ig) G (Immunoserology IgM anti-Toxoplasma: Reactive: 8.8 IU/ml). Meanwhile, in cats infected with Toxoplasma, macrophages are only found in ileum. This result indicates that the infection is in an active state.

Figure-1: Cat intestine with Toxoplasma positive immunohistochemistry staining. (a) Macrophages in ileum that serologically positive of Toxoplasma (scale bar=20 µm) (arrow); (b) macrophages in the ileum in cats with an experimentally infected cat of Toxoplasma. (Scale bar=20 µm) (arrow).
acute stage. ELFA examination to strengthened the result was then carried out which showed the positive reactions of all serum to IgM (Immunoserology IgM anti-Toxoplasma: Reactive: 0.71 index).

Some clinical symptoms data have been collected from the examination of body temperature, breath, and pulse frequencies. The clinical symptoms in both serologically positive and experimentally infected cat with toxoplasmosis are included increased breathing (ranging between 20-80/min and 41-120/min, respectively) and increased heart beat frequency (81-110 and 110-170 bpm, respectively). The results showed that all samples still in normal standard of healthy cat. However, the clinical data were not specific enough to diagnose cat with toxoplasmosis.

**Discussion**

The IHC examination results on several organs of experimentally infected cat show that the only part of the small intestine which showed positive result was the ileum (Figure-1a and b), whereas on the other organs were not found. Immunohistochemistry positive result in serologically *Toxoplasma* positive cat was found intracytoplasmic of epithelial cell of kidney tubules (Figure-2b) and ileum, while on the negative control were not revealed (Figure-2a). Positive reaction was shown by dark brown color, which indicated antigen-antibody binding. The positive reaction in the ileum of both groups existed inside the macrophages.

IHC positive results in this study show a dark brown color. Broad spectrum with diaminobenzidine (DAB) chromogen produces dark brown color on the binding of antigen-antibody *Toxoplasma* on stained tissue. According to Bionisch [19], DAB would block all the residual primary antibodies from the first immune reactions.

Streptavidin biotin (SB) method is faster, sensitive, and clean with strong color intensity, besides there are no color disruptions by the poor quality of the background. The SB method is 4-8 times more sensitive compare to avidin-biotin method, and 8-16 times more sensitive, and accurate than Papanicolaou (PAP) method. In addition, LAB-SA provides superior sensitivity to that of the avidin-biotin complex (ABC) [3,4,7,9]. The LAB-SA method is also sensitive than peroxidase (PAP) methods [3,7] and has the advantage of a single universal labeling reagent (enzyme conjugated streptavidin), instead of specific PAP complexes for each animal system.

The results obtained are almost the same identical to what had been done previously by Ramosvara [20] in a study where all infected tissue of 14 specific-pathogen-free (SPF) cats had been examined histologically for all stages of *Toxoplasma* life cycle. Ileum is the most common sites of infection, although in the case of eight SPF cats the entire small intestine was infected. Infection appeared to be concentrated on the epithelial cells of villi tip. In infected cells, the parasite was found between the core and border.

In this research, the positive IHC reaction was seen by the appearance of macrophage cells in both ileum and kidney. This is because *T. gondii* was phagocytized by macrophage cells, creating the possibility of *T. gondii* antigens to exist in the organ when IHC staining is performed using primary antibody and secondary antibody. According to Hutchison et al. [21], Cazabone et al. [22] antigen will circulate the parasite products produced at the time the parasites invade macrophages. The presence of this antigen indicating the infection is acute and still active. Antigen circulation, in principle, has the same composition and structure with soluble antigen.

*T. gondii* infection can generate non-specific and specific immunity. Non-specific immunity is conducted by macrophages and natural killer (NK) cells. Specific immunity is proven by the ability of *T. gondii* to induce humoral and cellular response. Cellular response against *T. gondii* infection is more dominant than humoral response. At the initial infection, macrophages infected by *T. gondii* tachyzoites produced interleukin (IL-2), tumor necrotic factor-α, IL-β, and IL-15. Cytokine IL-12 activates NK cells to produce interferon-γ (IFN) [23].

On further infection, when adaptive immunity is activated, tachyzoites that infect macrophages or other cells that act as antigen-presenting cells (APC) was digested by lysosomes. *T. gondii* tachyzoites antigens are then presented by major histocompatibility complex II molecules on the macrophages or APC membrane. The appearance of antigen is recognized and captured by receptors (Th-cell receptor [TCR]) owned by T helper lymphocytes (Th cells). The bond between antigen and TCR triggered Th cells to produce IL-2. *T. gondii* tachyzoites also triggers APC to produce IL-12. IL-12 produced by macrophages and APC working in synergy with IL-2 to cause Th cell differentiation into Th1. Th1 cells produced IFN-γ. Antigen and receptor bond activates CD8 + T cells to produce IFN-γ. The existence of IL-2 produced by Th1 cells also reinforced the activation of CD8 + T cells, resulting in more production of IFN-γ. IFN-γ produced by NK cells, Th1 or CD8 + T encourages macrophages to function as microbicidies [23].

Macrophages are the predominant leukocytes beside neutrophil, eosinophil, basophil, monocyte, and 110-170 bpm, respectively). The results showed that all samples still in normal standard of healthy cat.
and lymphocyte [24]. Monocyte in blood circulation will differentiate into macrophage in the tissues. Monocyte or macrophage is the most dominant to be infected by *Toxoplasma* then followed by neutrophil and lymphocyte. The ability of tachyzoites is to reproduce decrease when it infected the neutrophil, but it will increase again when the tachyzoites infected other cells or tissues. All types of cells in various organs, except erythrocytes, can be infected by *T. gondii*. Thus, it is assumed that the cells in the ileum were also infected, which was the reason of macrophage activity increased.

The presence occurrence of blood (Figure-3) in feces of infected cat is caused by damages on intestinal wall Bañales et al. [25] stated that tachyzoites multiply in the lamina propria of the intestine and eventually spread throughout the entire body. Tachyzoites cause tissue damage and eventually develop into bradyzoites stage after which they form tissue cysts. Numerous positive lesions were detected in all organs characterized by necrotic lesions (Table-1; Figure-4a and b). All findings are shown in Table-1. Domestic cat serologically *Toxoplasma* positive, necrotics lesions were mainly observed in the ileum, kidney, and liver. While in domestic cat infected by oocyst *Toxoplasma*, the necrotics lesions were mainly observed in the ileum, this caused by the stage of the infection is still preliminary or it is an acute infection, thus the parasite is not spreading to all organs yet. Figure-4 shows the necrosis of the cells of ileum and kidney of cat. The necrosis was in karyolysis category, described by the loss of nucleus in some cells.

**Conclusion**

The appearance of *Toxoplasma* antigen is successfully detected in several organs of domestic cat using IHC method with LAB-SA.

**Author’s Contributions**

MH and RWN supervised the overall research work. JP, SH, RYS, BS, and DA participated in sampling, made available relevant literatures and executed the experiment and analyzed the tissue and data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the content.

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**Competing Interests**

The authors declare that they have no competing interest.

| Organ   | Domestic cat serologically *Toxoplasma* positive Necrotic lesions | Detection of *T. gondii* by IHC | Domestic cat infected by oocyst *Toxoplasma* Necrotic lesions | Detection of *T. gondii* by IHC |
|---------|---------------------------------------------------------------|---------------------------------|-------------------------------------------------------------|---------------------------------|
| Ileum   | +                                                            | +                               | +                                                           | +                               |
| Jejunum | -                                                            | -                               | -                                                           | -                               |
| Duodenum| -                                                            | -                               | -                                                           | -                               |
| Kidney  | +                                                            | -                               | -                                                           | -                               |
| Lung    | -                                                            | -                               | -                                                           | -                               |
| Liver   | +                                                            | -                               | -                                                           | -                               |
| Spleen  | -                                                            | -                               | -                                                           | -                               |

IHC=Immunohistochemistry, *T. gondii*=*Toxoplasma gondii*
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