

EPIDEMIOLOGICAL AND MOLECULAR ANALYSIS OF *TOXOPLASMA GONDII* IN FAECAL SAMPLES OF CATS OBTAINED FROM HOUSE OF MATERNAL IN BALI

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Background: toxoplasmosis is a zoonotic-parasite disease caused by *T.gondii* that spread in many countries. Recently, this disease is one of the major public health problems associated with severe enocomical and social impacts such as miscarriages, hydrocephalus, blindness and mental retardations. The human parastic infection are closely related with the presence of infected cats around their houses, in which the animals are the definitive host releasing the parasites into the environment via their feces. The aim of this research was to determine the correlation between morphological prevalence and molecular detection of *T.gondii* in cats feces. **Methods:** detection of molecular was carried out by applying microscopic and a specific polymerase chain reaction (PCR). A case-control study using 40 maternal with positive serology of *T. gondii* and 40 maternal with negative serology were selected by purposive sampling method. Similarly, 80 faecal sample of cats were also obtained from each house of the women. The major risk factors considered as the important rule for human toxoplasmosis such as age, education, occupation, contact with soil, cat's age, un-owned cats and oocyst-positive feces were determined by questionnaire. **Results:** The epidemiological analysis showed that the highest risk factor was oocyst-positive feces (OR= 8.143; $p= 0.003$), followed by education (OR= 3.414; $p= 0.045$), contact with soil (OR= 2.255; $p= 0.073$), un-owned cats (OR= 2.25; $p= 0.210$), age (OR=2.23; $p=0.074$), occupation (OR= 1.556; $p= 0.348$) and cat's age (OR= 1.138; $p= 0.799$). However, the pregnant women with serologically positive to *T. gondii* had a very high probability of miscarriages (OR= 18.857; $p= 0.0001$) compared to whom with serologically negative. Moreover, logistic regression model analysis revealed that only oocyst-positive feces and education contributed a significant factor in causing toxoplasmosis among pregnant women. Microscopic observation of 80 faecal samples indicated that only 14 (17.5%) contained typical oocyst of *T. gondii*. All of the 8 oocyst of *T. gondii* positive-faecal samples were further confirmed with PCR, and we found that 4 (50%) were positive. **Conclusion:** This study concluded that the presence of oocyst-positive feces in the environment in conjunction with education level were considered to play significant contribution to the accident of toxoplasmosis in pregnant women.

Keywords: Epidemiology; zoonosis; risk factor; *Toxoplasma gondii*; PCR.

INTRODUCTION

Toxoplasmosis is a serious zoonosis disease caused by *T. gondii* protozoa infection. This disease can spread from animals to humans through the infective stadium contained in the warm-blooded animals. Cats are the main host for the *T.gondii* which can spread the infective *T. gondii* oocyst directly, contaminate their surroundings and potentially spreading the disease to humans or other

animals as the intermediate host. According to Dubey (2009), cats are definitive host of *T.gondii* and have crucial role in the *T.gondii* epidemiology. They are also the main source of the environment's contamination of the infective *T. gondii* oocyst.¹

Some of epidemiological studies showed the association of human habits that increase the risk of *T.gondii* infection in human beings in different countries. For instance, most of *T.gondii* infections in Brasil and in some developing countries are caused by the intake of oocyst-contaminated drinking water or vegetables.²⁻⁴ The seroprevalence of toxoplasmosis in human beings at several areas in Indonesia has also been reported, which were between 3.1 % to 64 %.⁵ From those epidemiology studies, some researchers believe that the eating

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habits such as eating *lawar* (raw vegetables) in Bali, is the determinant factor for the parasite infection.⁵ It is also in accordance with study by Sukaryawati's (2011) that in Mengwi, Bali, Badung Bali, 41.8% of pregnant women was found to be positively or had been positively infected by *T. gondii*.⁶ Based on the above studies, it is important to conduct a research on the existence of cats around their houses and the contamination of *T.gondii* oocysts in the surroundings through the cat's feces.

In Bali, cats living around houses and public places defecate in the surroundings, and can therefore be the potentials risk factor for spreading of toxoplasmosis infection in human. A study is required to determine as to whether the areas contaminated by cat's feces contaminated with *T.gondii* oocysts is the risk factor for toxoplasmosis in human in Bali. According to Damriyasa *et al* (2000), the sporulated *T. gondii* oocysts in the contaminated areas are the potential source of infection in human or other mediator hosts such as livestock. Livestock later could also become the source of infection to humans.⁷ This data is essentially needed in the strategy to reduce the spreading of toxoplasmosis from animals to human beings. This study was focused on cats as the potential source of spreading, the environments contaminated by cat's feces with infective *T.gondii* oocysts, and the risk factors of toxoplasmosis spreading to pregnant women. Aims of this study were to know the prevalence of toxoplasmosis and to detect *T.gondii* oocysts in cats feces molecularly by using the B1 gen amplification.

MATERIALS AND METHODS

Study Design

A retrospectively case-control method was used in this study (from January 2013 to January 2014). The case group was 40 serologically positive maternal which were confirmed to be infected by *T. gondii*. The control group were 40 serologically negative maternal confirmed by laboratory examination in Denpasar, living at similar geographic areas. Both case and control groups were confirmed by ELFA (Enzyme Linked Fluorescent Assay) test. Additional data for the maternal women were collected by interviews and structured questionnaire. Prior to the study, all of maternal had signed the Informed consent to approve their participation in this research.

Detection of *T. gondii* oocysts

A number of 80 cats captured around both the case and the control groups were kept in separated cages. Approximately 10 grams of fecal sample from each cat were placed into pots containing 10% calcium bicromate. The cats' feces were then examined under a microscope using the floating concentrate method to see the existence of the infective *T. gondii* oocyst by cats feces with

positive *T. gondii* oocysts were placed in microcentrifuge tube and further molecularly analyzed.

Molecular Detection of *T. gondii* oocysts by PCR (polymerase chain reaction)

The cats' feces microscopically positive of *T.gondii* oocysts proceeded to DNA examination by using the PCR to sure that the oocysts were *T.gondii*, not the other parasite.

DNA Extraction

Genomic DNA was extracted and isolated using according to the manufacturer protocol.⁸

PCR reaction

PCR reaction mixture (25 µl) contained of 2 µl DNA template 5 µl PCR buffer, 1.5 µl MgCl₂, 0.2 µl dNTPs, 0.1 µl Taq polymerase, 1 µl F and R Primer, and 14.2 µl dH₂O. After vortex for 15 seconds, it was centrifuge at 10.000 rpm speed for 5 minutes, solvent were added to the R and F primer with 1:10 dilution. The detection and identification of *T. gondii* was carried out by using B1-B22 primer (F): 5'-AACGGGCGAGTAGCACCTGAGGAGA-3' and B1-B23 (R): 5'-TGGGTCTACGTCGATGGCATGACAAC-3'. Amplification was performed in the Thermalcycler Model TC25/H machine using an initial denaturation of 95°C for 5 min, followed by 35 cycles with the reaction condition as follows: denaturation at 93°C for 60 sec, annealing at 60°C for 60 sec and extension at 72°C for 30 secs with the final extension at 72°C for 10 min.⁹

Electrophoresis Gel

Following PCR reaction, quality and quantity of the PCR products was determined by the electrophoresis. 1.5% agarose (75 mg + 50 ml TAE buffer) was added with 2µl DNA Stainer. Then, 5 µl of PCR product with 2 µl loading dye (merk loading dye nya). The first column was filled with 100 bp DNA size markers to know the length of targetted band. Elektrophoresis was done at 100 volt for 30 mins. The visualized band was captured with the UV transilluminator ultraviolet (Fotodyne Inc., Foto/UV 21 type) and photographed using digital camera with UV filter (Canon, power shot A 630 type).

Statistical Analysis

The statistical analysis was done by calculating the OR (Odds Ratio) value of each examined risk factor, and by bivariat analysis using the *chi square* test to analyse the significance of the association between the toxoplasmosis incidents on maternal and the risk factor to get the X², 95 % CI and OR values. The risk factors that statistically significant were related with the toxoplasmosis incidents on maternal ($p < 0.25$), followed by the

multivariate analysis using the logistic regression test to get the OR value and $p < 0.05$ value, also to get the final model of logistic regression. The molecular based data found were used to strengthen the result of the microscopic test to ensure the oocyst found were really the *T. gondii* oocysts. All the statistical analysis were done using the STATA version 12.1 software.

RESULTS

Among 43 samples qualified for inclusion and exclusion criteria, only 40 samples as the case group agreed to participate in the research. While for the control group, among 60 qualified samples, only 42 samples agreed to participate. Then, to balance the number of samples for the case and control groups, 40 of the control group determined in random and adapted according to the geographic area from the case group.

General characteristic

Overall, there were 80 samples in this research, consist of 40 case samples and 40 control samples. The ages of samples were between 19 to 48 years old. The average age was 30.33 years old (30.325 ± 6.788) in the control group and 34.08 years old (34.075 ± 6.154) in the case group. Based on Table 1, 60% of toxoplasmosis positively infected samples were more than 31 years old but did not significantly different with the control group ($OR = 2.25$; $p = 0.074$). For the level of education, 90% of the control group had adequate level (high school and university graduated), while only 72.5% in the case group. Yet, the amount of samples who had low level of education (elementary and junior high school) case group were 15% larger and statistically significant ($OR = 3.414$; $p = 0.045$) (Figure 1). Considered of risky occupation (farmers and housewives), 40% of the case group and 30% of the control group had risky occupation, which were not statistically significant ($OR = 1.556$; $p = 0.348$). the effects of toxoplasmosis infection was miscarriages, 80% samples in the case group had miscarriages and only 17.5% in the control group had been miscarried, and it was statistically significant ($OR = 18.857$; $p = 0.000$) (Figure 2)

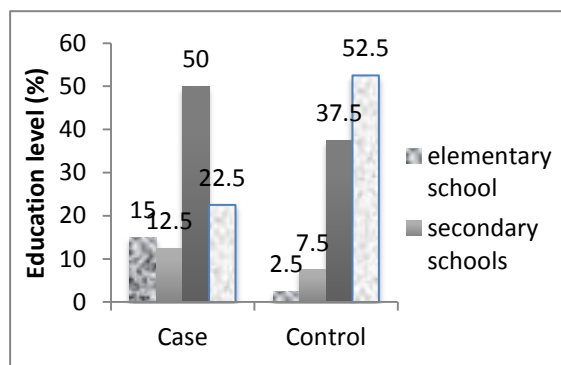


Figure 1

Sample Distribution based on the Education Level

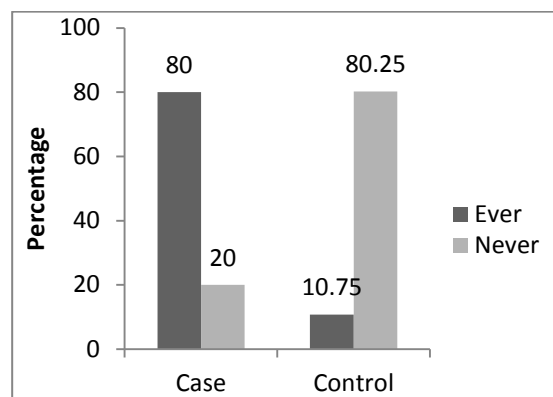


Figure 2

Sample Distribution based on the Miscarriage Occurrence

Risk factor related to contacts with soil

Based on table 1, 62.5% of the case group often have contacts with soil in their daily activities, while there were only 42.5% of the control group have contacts with soil. It was statistically insignificant ($OR = 2.255$; $p = 0.073$). It was revealed because 40% of the case group and 30% of the control group had risky occupation that often required them to have contact with soil (farmers and housewives).

Risk factor related with the existence of cats around the house

From the Table 1, 80% of the case group did not have cats as pets but there were many wild cats around their houses, while in the control group 90% did not have cats as pets but there were also many cats around their houses. Yet, it was statistically insignificant ($OR = 2.25$; $p = 0.21$). For the condition of cat's feces around their houses, whether it were cleaned or not, 72.5% of the case group did not clean the cat's feces, while in the control group 65.5% did not clean the cat's feces, and it was statistically insignificant ($OR = 0.885$; $p = 0.81$). The microscopic analysis to the cat's feces to examine the existence of *T.gondii* oocysts that contaminate the surroundings revealed that 30% of the cats' feces in case group positively carried the *T.gondii* oocysts, while in the control group only 5% positive, it was statistically significant ($OR = 8.143$; $p = 0.003$).

Multivariate analysis

The risk factors qualified to be included in the multivariate analysis were the samples' age, the samples' education level, contacts with soil, the status of cat existence, and the cats' feces that positively carried the *T.gondii* oocysts with $p < 0.25$ as showed in Table 2.

Table 1
The result of bivariate analysis to the risk factors of toxoplasmosis on maternal in Bali

Risk factor	Case	Control	OR (95% CI)	p
	Frequency (%)	Frequency (%)		
Age (years)				
< 31	16 (40)	24 (60)	2.25 (0.841-6.072)	0.074
> 31	24 (60)	16 (40)		
Education Level				
Inadequate (< high school)	11 (27.5)	4 (10)	3.414 (0.879-16.027)	0.045**
Adequate (≥ high school)	29 (72.5)	36 (90)		
Occupation status				
Risky	16 (40)	12 (30)	1.556 (0.559-4.369)	0.348
Safe	24 (60)	28 (70)		
Contact with soil				
Yes	25 (62.5)	17 (42.5)	2.255 (0.841-6.096)	0.073
No	15 (37.5)	23 (57.5)		
Cat's Status				
Pets	8 (20)	4 (10)	2.25 (0.535-11.094)	0.210
Wild	32 (80)	36 (90)		
Cats' feces				
Positive	12 (30)	2 (5)	8.143 (1.581-78.729)	0.003**
Negative	28 (70)	38 (95)		

**statistically significant ($p < 0.05$)

Table 2
Independent variables qualified for the logistic regression analysis

No	Variable	OR	95% CI	p
1	Age	2.250	0.841-6.072	0.074
2	Education level	3.414	0.879-16.027	0.045
3	Contact with soil	2.255	0.841-6.096	0.073
4	Status of cats' existence	2.250	0.535-11.094	0.21
5	Cat's feces with positive <i>T. gondii</i> oocyst	8.143	1.581-78.729	0.003

The logistic regression model

After the model analysis, the following risk factors have strong influence to the toxoplasmosis incidents experienced by maternal in Bali such as education level (OR= 0.430; $p = 0.005$) and the cat's feces positively carried the *T. gondii* oocysts (OR= 8.725; $p = 0.008$) with p value < 0.05 (Table 3).

Table 3
Independent variables with strong influence toward the toxoplasmosis incidents to the maternal in Bali.

No	Variable	OR	95%CI	P value
1	Education level	0.430	0.239 – 0.774	0.005**
2	The cat's feces that positively carried <i>T.gondii</i> oocyst	8.725	1.743 – 43.671	0.008**

**statistically significant ($p < 0.05$).

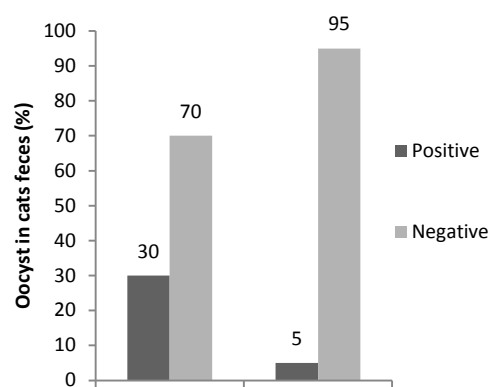


Figure 3
The distribution of the *T.gondii* oocyst in cats' feces found at the samples' houses

Microscopic test and PCR (polymerase chain reaction)

The prevalence of *T.gondii* oocysts in the cat's feces in this research was 30% in and 5% for the case group and the control group, respectively. The *T.gondii* oocysts that have not yet sporulated can be seen in picture 4 A while picture 4 B shows the oocysts that have already sporulated. Picture 5 shows the result of the *T.gondii* DNA examination with B1 gen amplification, the PCR product revealed the band at 370 bp position.

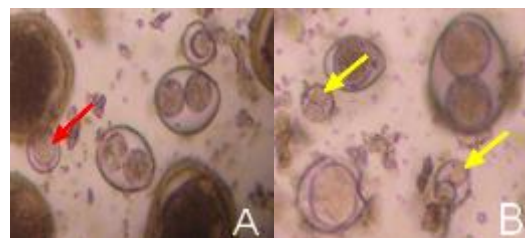


Figure 4
The microscopic analysis of (A) *T.gondii* oocysts that have not yet been sporulated, (B) *T.gondii* oocysts that have already been sporulated.

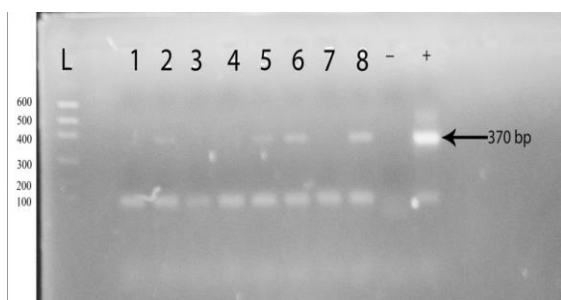


Figure 5

The result of the PCR product electrophoresis, L = marker (DNA ladder 100 bp) (+) = positive control, (-) = negative control, Column 1-8 isolat *T.gondii* oocysts of the cats in Bali, the arrow shows the specific PCR product at 370 bp position.

DISCUSSION

Toxoplasma gondii is an infective and zoonotic disease caused by parasite agen. In Indonesia, especially in Bali, the spread of this disease is very potential, since there are many cats, including wild cats that become the definitive host live in abundance. The existence of wild cats is a potential risk factor of the toxoplasmosis disease's spreading to their surroundings, other animals and human beings. The efforts to prevent and control the disease should be done in order to prepare outbreak of the disease. Therefore, the data about the risk factors of toxoplasmosis disease is necessary to be evaluated for early warning to prevent the outbreak. In general, the samples infected by toxoplasmosis were more than 31 years old (60%). This result was similar with the research conducted in Thailand that reported the highest prevalence of toxoplasmosis is within the 20 – 40 years old group. However, it was different with the result of the researches by Rai *et al* (1996)¹⁰ in Korea and China which stated that the prevalence of toxoplasmosis were mostly happened to women in the oldest age group. There are a lot of studies discussed about the relation between the human's toxoplasmosis seroprevalence with age. It describes that the risk of toxoplasmosis infection could happen anytime during human lifespan.¹¹ Mostly of the samples were highly educated The percentage of the elementary school graduated samples were quite low, but they were potentially infected by toxoplasmosis because lower education level means less knowledge, comprehension, and proper behavior. It is in accordance with the result of a research by Liu *et al* (2009)¹², that low level of education is a risk factor of toxoplasmosis infection. Similar research in Brazil revealed that pregnant women with low level of income and education have higher risk to be infected by toxoplasmosis.¹³ The same fact also reported by Avelino *et al* (2004)¹⁴ that pregnant women in west area of Brazil with low level of education and income, had higher risk of toxoplasmosis infection. Lower level of education correlates with the level of knowledge and less healthy behavior. The

unhealthy behavior in this discussion were doing the high risk work, such as farming, gardening, raising livestock and other works that require a lot of contacts with soil without using protecting equipment so that they might increase the possibility to be infected by toxoplasmosis. In this circumstances, health education, especially about zoonosis diseases such as toxoplasmosis, should be designed to prevent, control, and eliminate the disease. In this research, 40% samples in the case group and 30% in the control group had a risk to be infected by toxoplasmosis because of their daily work (farmers and housewives), but it was statistically insignificant ($p=0.348$).

The sample's occupation in this research, farmers and housewives, were closely related with activities that required contacts with soil. In this study, the contacts with soil showed insignificant result as risk factor (OR= 2.25; $p=0.073$). It is in accordance with a research in Sudan conducted by Khalil *et al* (2014)¹⁵, some pregnant women involved in Sudan who contacts with soil oftently and proved that it was not a risk factor of taxoplasmosis infection ($p=0.2 >0.05$). There was not significant relation between pregnant women who worked in the garden around their houses with toxoplasmosis infection incidents ($p=0.29 > 0.05$). Weigel *et al* (1999)¹⁶ found different result in Europe, almost 17% pregnant women infected by toxoplasmosis because of contacts with soil. Similar result also found by Spalding *et al* (2005)¹⁷ who stated that contacts with soil were high risk factor of toxoplasmosis infection for pregnant women. According to Dubey (2000)¹⁸, direct contact with cats not always caused *T.gondii* infection, cats' feces that contaminated surroundings environment are more dangerous. The contamination of the environment by cats' feces is a serious risk factor of the spreading of toxoplasmosis to human beings or animals. The very large population of cats in Bali, mostly wild cats, is a potential risk factor in the spreading of toxoplasmosis infection in this area.

In this research, the wild-cats found were mostly under a year old. Therefore, the cats did not have resistance to the *T.gondii* increasing the possibility of infected *T.gondii*. The existence of many wild cats also increases the possibility of the environmental contamination by the cat's feces. According to Amany *et al* (2012)⁹, the cats under one year old produced an abundance of *T.gondii* oocysts because they are starting to hunt once they were weaned off. The result of a research by Kapperud *et al* (1996)¹⁹ revealed that pregnant women who often have daily contacts with kittens under one year old have more possibility to get infected by toxoplasmosis (OR= 3.6; $p=0.04$) compared with pregnant women who do not have contacts with cats.

Result related to the status of cats' existence that were wild cats, 85% and 90% in the case group and

the control group, respectively (OR= 2.25; $p= 0.21$). Kapperud *et al* (1996)¹⁹ also found that there was no significant difference between domestic and wild cats related to toxoplasmosis incidents in pregnant women in Norway, because in fact, there were no difference in the way of treating both the domestic and the wild cats. Cats were released around houses without any health care/treatment. A study conducted in Egypt described that 97.4% wild cats *T.gondii* oocysts positive in the feces contaminated their surroundings.²⁰ This condition was almost similar to the condition of wild cats' population in Bali. One thing special about the existence of cats in Bali, is a belief that Balinese people are not allowed to kill cats, intently or not. It is clear that this condition would increase the population of cats in Bali. The large population of the wild cats would increase the contamination of the environment caused by cat's feces which is correlated with the widespread of toxoplasmosis infection to human beings or other animals.

The prevalence of *T.gondii* oocysts in the cats' feces was 30% and 5%, in the case group and control group, respectively. Overall, the whole prevalence was 17.5% (n= 80). This condition was higher than the condition found in a research done in Egypt by Amany *et al* (2012)⁹ where the prevalence of *T. gondii* oocysts in the cat's feces was 2%. Similar prevalence were found in some countries or areas, such as 12% in South Germany²; 15% in Australia;²² 4.3% in Chile²³; 10% in Qatar²⁴ and 50% in Egypt.²⁵ The 17.5% prevalence of *T.gondii* oocysts found in the cats' feces is a high potential of the toxoplasmosis infection spreading. It is also supported by the cats' habit to bury their feces underground or sand. It will sporulated and could survive several months or even several years. Besides, the oocysts has floating ability that gives more opportunity to spread everywhere when the rainy season comes and it may contaminate foods, drinking water, and others.

Several methods was used to detect the existence of *T. gondii* oocysts such as microscopically and bioassay, although the sensitivity and specificity of those two methods were still doubtful. To support the microscopic result, PCR reaction using primer to detect B1 gen was done. The purpose of the molecular examination being done was to ensure the presence of *T.gondii* no other parasites. PCR results showed that the specificity of *T.gondii* oocysts (Figure 5). Study by Amany *et al* (2012)⁹ also used molecular detection B1 gen from the naturally cat's feces. There were also studies done in Egypt using primer which detected B1 gen on pregnant women serum and lambs²⁶ and in contaminated vegetables and fruit.²⁷

Toxoplasmosis positive-Pregnant women have 18.8 times higher risk to miscarry than those who were not infected by toxoplasmosis (OR= 18.857;

95% CI= 5.450-68.235; $p= 0.000$). Study in Sudan by Khalil *et al* (2014)¹⁵ stated that toxoplasmosis infected-pregnant women have 3.3 times higher risk to miscarry than those who were not infected with toxoplasmosis (OR= 3.3; 95%CI= 1.5-7.3; $p= 0.002$). It can be suggested to pregnant women to avoid contact with cats' feces because 17.5% of cats' feces in Bali carried *T. gondii* oocysts, and also reduce the activities that required contacts with soil or by using gloves. However, our research showed insignificant result related to the toxoplasmosis incidents in the samples' contacts with soil. It could be explained that almost all samples washed their hands after contacts with soil. In this research, it can be confirmed that the toxoplasmosis infection on maternal in Bali was associated with the low level of education, and because there were many cats' feces that carried the *T.gondii* oocyst spread and contaminated the environment's surroundings that support the oocyst to be sporulated.

CONCLUSION

The effects of toxoplasmosis infection are very serious and become a great and widespread health problem in society. In correlation to the result of this research, we suggested to the community, especially the Balinese that some actions should be done. It is important to control the population growth of cats, especially wild cats, using gloves when having contacts with soil to avoid contacts with cats' feces and increase the education level in the hope to increase the Balinese knowledge and behavior, mostly in health problems. Those steps would be able to support the strategy to control, prevent or even eliminate the toxoplasmosis infection in Bali.

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