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# Profiles of IgM, IgG and IgE immune responses of mice against p14, p31 and p71 proteins following immunization with crude cystic fluid of *Cysticercus bovis*



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## ABSTRACT

**Background:** The presence of IgM, IgG and IgE antibodies has been widely used as important markers for the differentiation of acute and chronic infection in many infectious diseases, but limited studies reported on *Cysticercus bovis* infection are available. This study aimed to determine the IgM, IgG and IgE responses of mice against crude cystic fluid, p14, p31 and p71 antigens as the possible diagnosis of acute and chronic cysticercosis in hosts.

**Methods:** Six mice were immunized four times at 10 days interval with *Cysticercus bovis*. Individual p14, p31 and p71 proteins were separated by using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and elution of proteins from gel with elution buffer. The crude cystic fluid, p14, p31 and p71 were used as antigens for enzyme-linked immunosorbent assay (ELISA) to measure the IgM, IgG and IgE antibody titers in mice sera before and after the first, the second, the third and the fourth immunizations.

**Results:** No IgM, IgG and IgE against crude cystic fluid, p14, p31,

and p71 was detected in sera samples collected before the first immunization. Moderate to high titers of IgM antibodies against crude cystic fluid, p14, p31 and p71 were identified following the first, the second, the third and the fourth immunizations. IgG titers against crude cystic fluid, p14, p31 and p71 was still at a low level following the first immunization and started to increase following the second, the third and the fourth immunization. Generally, low titers of IgE antibodies against crude cystic fluid, p14, p31 and p71 were detected after each immunization. IgE responses only increased slightly against crude cystic fluid and p71 antigens after the third and the fourth immunizations. The IgM, IgG and IgE responses of mice against crude cystic fluid and p71 antigens were higher than those against p31 and p14 antigens.

**Conclusions:** The presence of IgM without IgG and IgE can indicate acute infection, whereas the presence of IgM and IgG with slight increase IgE might indicate a chronic infection of *Cysticercus bovis*

**Keywords:** IgM/IgG/IgE responses, p71, p31, p14, *Cysticercus bovis*

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## INTRODUCTION

*Cysticercus bovis* is the larva of *Taenia saginata*, and it is normally found in cattle following infection with the proglottids of adult tapeworm through contaminated grass or other goods. In the infected cattle, the *Cysticercus bovis* cysts can persist for months or years in various organs or tissues such as liver, muscles (cardiac and skeletal), diaphragms, lungs, kidneys and lymph nodes.<sup>1</sup> Humoral and cellular immune responses, both specific and non-specific, play an essential role in the destruction and removal of parasites from the infected cattle. The presence of antibodies, especially IgG, against *Cysticercus bovis* has been detected and used in the development serological diagnosis for the parasite infection in cattle.<sup>2</sup> However, no study has reported on the use of IgM, IgG and IgE classes of antibodies

for serological diagnosis of *Cysticercus bovis* infection in cattle, especially as markers for acute and chronic infections.

The presence of IgM and IgG antibodies has been widely used as important markers for the differentiation of acute and chronic infection in many infectious diseases.<sup>3,4,5</sup> Meanwhile, IgE is the indicator of allergic reactions, including those caused by parasitic infections.<sup>6</sup> In parasitic infection, the allergen released by the parasites can activate B cells to produce IgE antibodies. The binding of IgE with its receptors on the surface of Mast cells can trigger degranulation and the release of histamines from Mast cells.<sup>7,8</sup> Such histamines play important roles in the immunological destruction or removal of parasites from the bodies.<sup>9</sup> The increase in IgE levels have been reported in some

parasitic infections such as *Malaria falcifarum*<sup>10</sup> and *Cysticercus cellulose* infections in human.<sup>11</sup>

The cysts of tapeworm larva composed of immunogenic proteins which are capable of inducing an immune response in the infected hosts. The immunogenic proteins can be found in the wall and fluid of cysts as well as in the body of the parasites and are capable of inducing both cellular and antibody responses in the infected animals.<sup>12</sup> Antibodies against 260 kDa, 150 kDa, 130 kDa, 67 kDa, 60 kDa, 55 kDa, 50 kDa, 23 kDa, 18 kDa and 14 kDa have been detected *Cysticercus bovis* infected cattle and have been for the development of diagnostics for the detection of the infection in cattle.<sup>2</sup> Immunizations of mice with crude cystic fluid of *Cysticercus bovis* also induced antibody responses against 16.81 kDa; 19.22 kDa; 20.98 kDa; 27.41 kDa; 34.02 kDa; 38.31 kDa and 54.94 kDa proteins.<sup>13</sup> Recently, three of those immunogenic proteins (14 kDa, 31 kDa and 71 kDa) have been isolated and purified by using SDS-PAGE. The potential uses of the proteins for detection of IgM, IgG and IgE antibodies were examined to develop a serological test for acute and chronic infection of *Cysticercus bovis* in cattle. Therefore, the aim of this study is to determine the IgM, IgG and IgE responses of mice against crude cystic fluid, p14, p31 and p71 antigens as the possible diagnosis of acute and chronic cysticercosis in hosts.

## MATERIALS AND METHODS

### Immunization of Mice with Crude Cystic fluid of *Cysticercus bovis*

Crude cystic fluid was extracted by cutting into pieces of *Cysticercus bovis* cyst collected from three Bali Cattle experimentally infected with the proglottids of adult tape-worm.<sup>14</sup> The cuts of the cysts were suspended with phosphate-buffered saline (PBS) (final concentration of 20% w/v) and were centrifuged at 5000 g for 10 minutes. The supernatant fluid was used for four times, 10 day-interval immunizations of mice. The cystic fluid antigen was mixed into emulsion using complete Freund adjuvant (first immunization), in incomplete Freund adjuvant (the second and the third immunizations) and without adjuvant (the fourth immunization). Six female, 7 weeks old mice were used, and sera samples were collected before the first immunization and seven days after each immunization.

### Isolation and Purification of Individual Proteins from *Cysticercus* Cystic fluid

Cystic fluid of *Cysticercus bovis* was diluted (3 samples: 1 sample buffer) in sample reducing buffer (1.3% SDS, 5% mercaptoethanol, 0.0625 Tris-

HCl pH. 6.8, 10% glycerol, 0.001% bromophenol blue). The individual cystic fluid proteins were separated by loading 1 ml diluted cystic fluid into sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel using large electrophoresis cell (TV400Y Standard Twin-Plate Maxi-Gel Electrophoresis Units Scie-Plus, UK). The SDS-PAGE was conducted according to Laemli procedure<sup>15</sup> using 150-volt power supply for approximately 4 hours. The gel was then cut horizontally into 15 pieces, and each gel cut was homogenized by grinding it in a mortar using piston. The individual proteins were eluted from the gel homogenate by adding 3 ml elution buffer (5 mM DTT, 50 mM Tris-HCl pH7.9, 0.1% SDS, 0.15 M NaCl, 0.1mM EDTA, 1mM PMSF) and incubation for overnight at 4°C. The elute containing individual proteins were collected by centrifugation at 1000 x g for 5 minutes and precipitation with acetone (1 elute: 4 acetone). The precipitate was collected by centrifugation as above, air-dried and diluted in 0.5 ml phosphate-buffered saline (PBS) pH 7.4.

### Antibodies

Polyclonal antibody used in this study was pooled sera collected from six mice at seven days after the last of four times immunizations with *Cysticercus bovis* as described above. The antibody was used for detection of cystic fluid antigen and also as a positive control for ELISA and Western blotting assay. Pooled sera collected before immunization of mice were used as a negative control. Both positive and negative control sera have been tested and titrated by ELISA, and their specificity against *Cysticercus bovis* antigens has been confirmed by Western blotting assay (Data not shown).

### Detection of Cystic Fluid Individual Protein by ELISA

The presence of individual proteins in elutes of each gel cut was tested by ELISA using polyclonal antibody. Each elutes from 15 gel cuts was coated into wells of ELISA microplate for overnight using carbonate-bicarbonate coating buffer (10 mM NaHCO<sub>3</sub>, 3mM Na<sub>2</sub>CO<sub>3</sub>, pH. 9.6). After incubation for overnight at 4°C and twice washes with PBS-T (PBS containing 0.1% Tween 20), polyclonal antibody (diluted 1:500 in PBS-T) was added into each well, and the microplate was incubated for one hour at 37°C. Following three times washes, 100 µl antimouse IgG-horse radish peroxidase (HRP) was added into each well, and the microplate was incubated for 1 hour at 37°C. The presence of antibodies-antigen binding in wells was visualized by adding 100 µl TMB substrate (KPL-USA). OD of the substrate was read by ELISA reader after adding 50 µl stop solution (1 N H<sub>2</sub>SO<sub>4</sub>).

## Western blotting

assay

The western blotting assay was performed to determine the purity of individual proteins isolated from gel cuts. Western blotting assay was conducted according to the procedures as described by Dunn 1984 using carbonate-bicarbonate transfer buffer system in minitransblot cell (Bio-Rad, USA). Following SDS-PAGE analysis in mini cell system (Bio-Rad, USA) according to Laemli (1970) procedures<sup>15</sup>, the separated proteins in the gel were transferred onto nitrocellulose membrane using carbonate-bicarbonate transfer buffer (10 mM NaHCO<sub>3</sub>, 3mM Na<sub>2</sub>CO<sub>3</sub>, pH 9.9, 20% methanol). The nitrocellulose membrane was blocked with 3% skim milk in Tris-buffered saline (TBS/150 mM NaCl, 100 mM Tris-HCl pH.7.4) for 1 hour at 37°C. The nitrocellulose membrane was then soaked mouse anti-*Cysticercus bovis* polyclonal antibody diluted in 3% skim milk in TBS for 18 hours at room temperature. Following 3 times washes with TBS, anti-mouse IgG-Alkaline phosphatase diluted 1:500 (KPL USA) was added, and the presence of proteins in nitrocellulose membrane was visualized by adding BCL/NBT (5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium) substrate.

## Titration IgM, IgG and IgE Antibody in Sera Samples of Mice

The titers of IgM, IgG and IgE antibodies in mouse serum against crude cystic fluid, p14, p31 and p14 antigens were examined by indirect biotin-streptavidin ELISA. One hundred ul of crude cystic fluid (diluted 1/50) and purified individual proteins

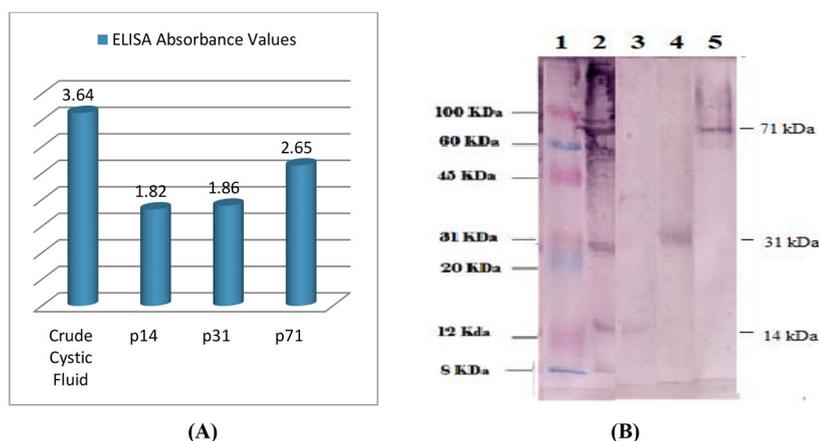
(p14, p31 and p71) of *Cysticercus bovis* (diluted 1/20) were coated into wells of ELISA microplates as above. The wells were washed three times with PBS-T and blocked with 3% skim milk in PBS as described above. Five-fold dilution (1/100, 1500, and 1/1525) of sera samples in PBS-T containing 2% skim milk were prepared. One hundred diluted sera samples from each dilution were added into wells and incubated for one hour at 37°C. Biotinylated anti-mouse IgM (eBioscience), IgG (KPL-USA), and IgE (e-Bioscience) were added and incubated for 1 hour at 37°C. After three times washes with PBS-T as above, 100 µl streptavidin-HRP (KPL) was added and incubated as above. The presence of IgM, IgG and IgE was visualized by adding 100 µl TMB (tetramethylbenzidine) into each well. The colour development of TMB substrate was stopped by adding 50 µl of 1 N H<sub>2</sub>SO<sub>4</sub>. The optical density (OD) of substrate was the read at 450 nm in an ELISA reader. The ELISA titers of each Ig classes were expressed sample per positive ratio calculated according to the following formula.<sup>16</sup>

$$ELISA \text{ ratio (ER)} = \frac{(OD \text{ of samples} - OD \text{ of negative control})}{(OD \text{ of positive control} - OD \text{ of negative control})}$$

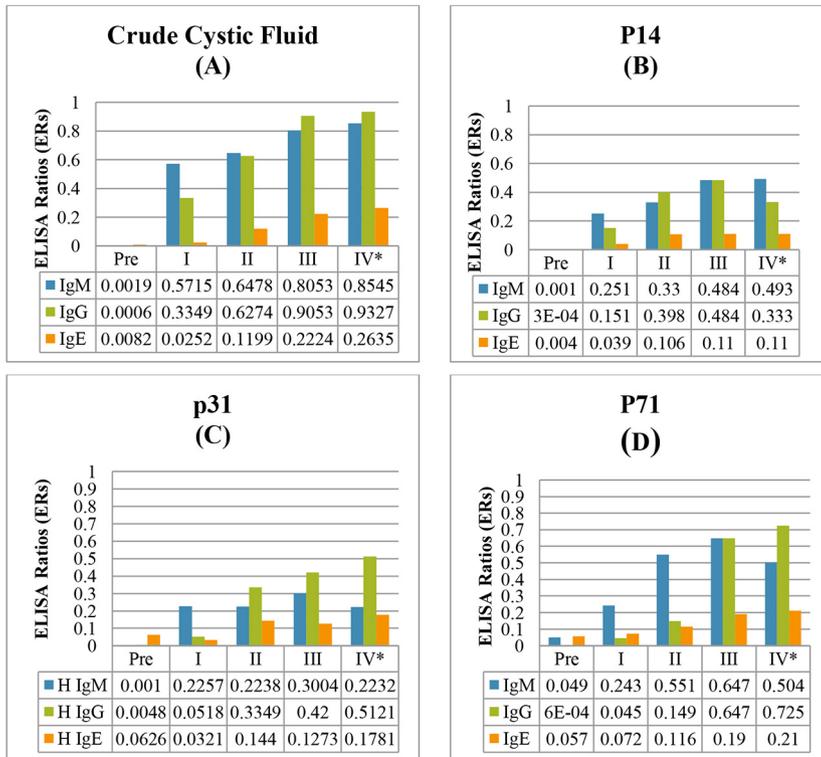
## RESULTS

One crude cystic fluid and three individual proteins (p14, p31 and p71) used as antigens on this study were tested by ELISA and Western blotting assay using polyclonal antibody. The OD of the four antigens were 1.82, 1.86, 2.54 and 3.65, respectively (Figure 1A). In Western blotting assay, multiple bands of proteins with the molecular of 91 kDa, 71 kDa, 68 kDa, 51 kDa, 31 kDa, 18 kDa and 14 kDa in crude cystic fluid sample, and single individual bands of 14 kDa, 31, kDa and 71 kDa proteins were observed (Figure 1B).

ELISA results showed that four times immunizations of mice with crude cystic fluid antigen-induced moderate to high levels of IgM and IgG antibody responses, but low levels of IgE antibody responses. No IgM, IgG and IgE antibody response against crude cystic fluid, p14, p31 and p71 antigens were detected in sera samples of mice collected before immunization. The moderate to high levels of IgM responses occurred after the first immunization and increased following the second and the third immunization and slightly declined following the fourth immunization. The patterns of IgM responses for crude cystic fluid, p14, p31 and p71 antigens were similar. However, in general crude cystic fluid and p71 antigens induced higher levels of IgM antibody responses than those against p31 and p14 antigens (Figure 2).



**Figure 1.** Profiles of crude cystic fluid, p14, p31 and p71 in ELISA and Western Blotting analysis. In Figure 1A, OD of crude cystic fluid, p14, p31 and p71 in ELISA were shown. In Figure 1B lane 1: prestained standard marker proteins, lanes 2-5: crude cystic fluid, p14, p31, and p71, respectively.



**Figure 2.** Profiles of IgM, IgG and IgE responses of mice against crude cystic fluid, p14, p31, and p71 antigens of *Cysticercus bovis* after immunizations with crude cystic fluid as immunogens. IgM, IgG and IgE titers against crude cystic fluid (A), p14 (B), p31 (C) and p71 (D).  
\*) Pre, First, Second, Third and Fourth immunizations.

The patterns of IgG responses against crude cystic fluid, p14, p31 and p71 antigens were slightly different from those of IgM responses. Following the first immunization, the levels of IgG antibody response of mice against crude cystic fluid, p14, p31 and p71 antigens were generally still deficient and slightly increased after the second immunization. The levels of IgG antibody responses increased sharply following the third and the fourth immunizations. The levels of IgG antibody responses against crude cystic fluid and p71 antigens were generally higher than those against p31 and p14 antigens (Figure 2). The levels of IgE antibody responses against crude cystic fluid, p14, p31 and p71 antigens were generally low and only slightly increased after the third and the fourth immunizations especially against crude cystic fluid and p71 antigens (Figure 2).

The IgM, IgG and IgE antibody titers of mice sera against crude cystic fluid antigen before immunization, following the first, the second, the third and the fourth immunizations were as follows. Before immunization, the ELISA ratios (ERs) of IgM, IgG and IgE antibodies against crude

cystic fluid antigen were 0.0019, 0.0006, and 0.0082 respectively and after the first immunization, they were 0.5715, 0.3349 and 0.0252 respectively. Following the second immunization, they increased to 0.6478, 0.6274 and 0.0252 respectively, and to 0.809, 0.953 and 0.2214 respectively after the third immunization. The titers of IgM, IgG and IgE against crude cystic fluid antigens after the fourth immunization were 0.8543, 0.9237 and 0.2635, respectively (Figure 2A).

Before immunization, the ERs of IgM, IgG and IgE in mouse sera against p14 antigen were 0.001, 0.000318, and 0.003685 respectively and after the first immunization they were 0.25086, 0.150826 and 0.03875 respectively. Following the second immunization, they increased to 0.32986, 0.3981 and 0.1062 respectively, and to 0.4844, 0.49200 and 0.10967 respectively after the third immunization. The ERs of IgM, IgG and IgE antibody after the fourth immunization were 0.4932, 0.3326 and 0.1098, respectively (Figure 2B). Similar to those against p14, the ERs of IgM, IgG and IgE antibody in mouse sera before the first immunization were 0.001, 0.0048, and 0.0626 respectively and after the first immunization, they were 0.2257, 0.0518 and 0.0321 respectively. Following the second immunization, the ERs of IgM, IgG and IgE in mouse sera were 0.2238, 0.3349 and 0.144 respectively and after the third immunization, they were 0.3004, 0.4200 and 0.1273 respectively. The ERs of IgM, IgG and IgE after the fourth immunization were 0.02232, 0.5121 and 0.1781, respectively (Figure 2C).

The IgM, IgG and IgE antibody titer of mice sera against p71 antigen were generally higher than those against p14 and p31 antigens, but lower than those against crude cystic fluid antigen. Before immunization, the ERs of IgM, IgG and IgE in mouse sera against p71 antigen were 0.0049, 0.00064, and 0.0572 respectively and after the first immunization they were 0.24788, 0.04543 and 0.07279 respectively. Following the second immunization, they were 0.55051, 0.14924 and 0.011601 respectively and after the third immunization they were 0.4708, 0.64708 and 0.1905 respectively. The ERs of IgM, IgG and IgE against p71 antigen after the fourth immunization were 0.50369, 0.72522 and 0.21045 respectively (Figure 2D).

## DISCUSSION

The larva of *Taenia saginata* is typically found as cysts (known as *Cysticercus bovis*) in tissues or organs of infected cattle and can persist for a prolonged period of times, causing a disease known as cysticercosis. The presence of *Cysticercus bovis* cysts in tissues such as lung, muscles, liver, diaphragms and heart muscles<sup>1</sup> can induce immune

response which can be useful for the development of a diagnostic test for the disease. The presence of antibodies against *Cysticercus bovis* has been detected in the infected cattle and has been used for the development of serological diagnostic for cysticercosis in cattle.<sup>2</sup> In this study, the IgM, IgG and IgE responses of mice against crude cystic fluid, p14, p31 and p71 antigens were examined by ELISA to explore the potential use of those proteins as antigens for serological diagnosis of acute and chronic infection of *Cysticercus bovis* in hosts.

The antigens used for ELISA test were crude cystic fluid and three purified individual proteins (p14, p31 and p71) isolated from SDS-PAGE gel as described above. ELISA test showed that all three individual proteins were reactive with polyclonal antibody. The reactivity of the proteins isolated from SDS-PAGE gel with the homologues antibodies is important for the development of the diagnostic test. In the SDS-PAGE technique, SDS is used to unfold and to give negative charge of the proteins. This process converts native proteins into a primary structure which can disrupt the ability of the antigen to bind with antibody, and restoring the proteins into their native structure is required to regain their ability to bind with the homologous antibody. It appears that acetone precipitation step to remove the SDS from protein molecules has restored the antigenicity of proteins isolated from SDS-PAGE gel.<sup>17</sup> The use of proteins isolated from SDS-PAGE gel as ELISA antigen for the development of a serological test for *Fasciola gigantica* has been reported.<sup>18</sup>

Immunization of mice with crude cystic fluid-induced high level of both IgM and IgG antibody responses, but low level of IgE responses. Moderate to high levels of IgM responses level were detected following the first immunization, increased following the second immunization, and stable following the third and the fourth immunization. The titers of IgM against crude cystic fluid and p71 antigen were higher than those against p31 and p14 antigens. The result indicates crude cystic fluid and p71 were more capable inducing high level of IgM and therefore more potential to be used as antigens for detection of IgM in the infected host than those of p14 and p31 antigens. As IgM responses usually occur very early, i.e. after the first immunization, the presence of IgM can likely be used as an indicator of acute infection.<sup>19</sup> In primary response, naïve B cells bearing IgM and IgD as B-cell receptors (BCRs)<sup>20</sup> releases IgM as a quick response against invading pathogen including parasite antigen. However, high IgM was observed after repeated boosters indicating that IgM level in serum persisted after three times boosters. The result suggests that, although it occurred early, the presence of IgM alone cannot be used as an indicator for acute infection.

Slightly different patterns of IgG level as compared to the patterns of IgM level were observed in this study. Following the first immunization, IgM titers were moderate to high, whereas IgG titers were still relatively very low. Relatively high IgG titers were observed only after the second immunization, increased after the third immunization and were steady after the fourth immunization. The results indicate that IgG antibody occur later than IgM in mice immunized with crude cystic fluid of *Cysticercus bovis*. The result demonstrates that the release of IgG requires antigen processing by antigen presenting cells (APCs) and establishment of B-cell memory bearing IgG molecules as BCR on the surface of the cells.<sup>21</sup> Repeated immunizations (boosters) have enabled the processing of antigens by APCs and establishment of B cell memory bearing IgG molecule as BCRs on the surface of the cells.<sup>22</sup> Upon repeated exposures with the same antigens, B cell memory released IgG molecules as a response to invading pathogen such as *Cysticercus bovis*

Antigen processing by APCs also enabled many immune cells to produce cytokines involved in driving B-cells to secrete different classes of antibodies. Cytokines produced by many different immune cells such as IL-4, IL-6, IL-2, and IFN- $\gamma$  influenced the Ig isotype released by B cells. IL-4 produced by Th2 and other immune cells drives Ig class-switching to IgE and IgG1, whereas IFN- $\gamma$  drives Ig class-switching to IgG2a or IgG3.<sup>23</sup> Production of IL-4 by Th-2 which drives Ig class-switching to IgG1 or IgE also occurs via antigen processing, whereas IgM release from B cells can occur without antigen processing.<sup>24</sup> The presence of many different cytokines following antigen processing is influenced by many factors such as types of antigen, the use of an adjuvant, and frequencies and duration of antigen exposures to the immune cells.<sup>2</sup>

The IgE responses of mice against crude cystic fluid, p14, p31 and p71 antigens were relatively low throughout this study. IgE level only increased slightly after the third and the fourth immunizations, especially against crude cystic fluid and p71 antigens. The increase of IgE levels in serum against a particular antigen is usually associated with allergic reactions, including those caused by parasitic infection.<sup>6,7</sup> A slight increase in IgE level, especially against crude cystic fluid and p71 antigen combined with IgM and IgG titers may be useful in the development of a diagnostic test for acute and chronic *Cysticercus bovis* in a host.

By observing the titers of IgM, IgG and IgE titers in the sera samples of mice after four times immunizations, it is clear that the presence of high titers IgM alone with low level or without IgG

appeared to indicate the acute infection. The high level of IgG and IgM can indicate chronic infection. In other parasitic infection such as *Toxoplasma gondii* and viral infections such as dengue virus, Epstein-Barr, parvovirus and hepatitis B, IgM level is associated with acute infection, and IgG level is related to chronic infection.<sup>25-28</sup> Meanwhile, IgE as indicator of parasitic infection may be helpful in differing parasitic infection such as *Cysticercus bovis* from other pathogens such as viruses and bacteria. The increase of IgE titers has been observed in *Malaria falciparum* infection and other parasitic infections.<sup>10,29</sup>

## CONCLUSIONS

The presence of IgM without IgG and IgE can indicate acute infection, whereas the presence of IgM and IgG with slight increase IgE might indicate a chronic infection of *Cysticercus bovis*.

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## ETHICAL APPROVAL

This study has been approved by the Ethical Commission for the Use of Animals in Research and Education of the Faculty of Veterinary Medicine, Universitas Udayana, Bali, Indonesia, with Ethical Clearance No.275b/KE-PH-Lit -3/2016.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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## AUTHOR CONTRIBUTION

All of the authors are equally contributed to the study from the study framework, data gathering, until reporting the result of the study.

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