

# Evaluation of monoclonal antibody (IgG2bMAb) for detection of coproantigen from experimentally infected rats with *Strongyloides ratti*

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## ABSTRACT

### Background and Aim:

Highly sensitive and specific diagnostic assay for the detection of *Strongyloides* is needed due to the intermittent and low concentration of eggs, larvae and adult worms that can be found in a faecal specimen. In some cases, repeated sampling of the faecal specimen is required to obtain satisfactory and reliable results. The aim of the study is to develop and evaluate monoclonal antibody-based Sandwich ELISA for the detection of coproantigen associated with *Strongyloides* infection using *S. ratti* as a model.

**Place and Duration of Study:** Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, University Malaysia, Between September 2018 and March 2019

**Methodology:** The monoclonal antibody was raised against a soluble antigen of the infective filariform larvae (iL3) of *S. ratti*. The monoclonal antibody produced (IgG2bMAb) was evaluated for cross-reactivity against homologous and heterologous helminth antigens such as excretory-secretory (ES), infective larvae (iL3) and coproantigen of *S. ratti*, adult worms of *A. caninum*, *A. suum*, *T. canis* and *T. cati*.

**Results:** An IgG2bMAb was observed to react with 30 kDa proteins associated with all homologous antigen from iL3, ES and coproantigen of *S. ratti* and cross-reacted with one heterologous antigen from adult worm of *A. caninum* at the same molecular weight. There was no cross-reaction observed with other heterologous antigens from adult worms of *T. canis*, *T. cati* and *A. suum*. The sensitivity of IgG2bMAb for the detection of *S. ratti* was 85% in Sandwich ELISA. Cross-reaction was observed with hookworm antigen that caused by *A. caninum* in Western immunoblotting.

**Conclusion:** The results indicated that IgG2b have an immunodiagnostic property as IgG2bMAb and was able to detect antigens from coproantigen related to *S. ratti* with 85% sensitivity based on Sandwich ELISA) even though cross-reaction was observed with *A. caninum*. These findings will be very useful to tackle many cases of multiple worms' infections such as both strongyloidiasis and hookworm.

*Keywords:* Monoclonal antibody, *Strongyloides ratti*,

## 1. INTRODUCTION

Strongyloidiasis is a parasitic intestinal infection caused by *S. stercoralis* and *S. fuelleborni* in humans with *S. stercoralis* being the most common parasite associated with human infections (25). This parasite is responsible for some of the zoonotic infections worldwide and is predominantly found in tropical and subtropical countries such as in Africa and Southeast Asia, where the disease is endemic (12). Despite the severity of the infection among immunosuppressed and elderly patients, the disease is still considered as one of the most neglected tropical diseases (35). Fatal hyperinfection

associated with disseminated infection has been widely documented, especially in the immunosuppressed patients undergoing therapy with a corticosteroid (15,29,7). Strongyloidiasis is still one of the most difficult parasitic infections to diagnose due to intermittent and low parasite concentration apart from being misdiagnosed as hookworm infection (1). The commonly used diagnostic methods are based on clinical manifestations or demonstration of the parasite using standard parasitological techniques such as agar plate, Baermann and microscopy (11). Antigen-based serological and molecular-based methods have been used for detection of strongyloidiasis (10, 22, 16). In this study, the use of monoclonal antibody-based Sandwich ELISA for the detection of coproantigen of *S. ratti* was evaluated.

## **2. MATERIAL AND METHODS**

### **2.1 Preparation of soluble antigens from homologous and heterologous helminths**

ES and iL3 of *S. ratti* were harvested from the in vitro culture of iL3 in Dulbecco's Modified Eagle's Medium (DMEM)-serum free medium (Merck, USA) as described earlier (24). The adult worms of *A. suum*, *T. cati*, *T. canis* and *A. caninum* were collected from the intestines of an infected pig, kittens, puppies and dogs respectively. Soluble antigens were prepared from the adult worms by freezing and thawing in liquid nitrogen. Then, the as-prepared antigens were homogenised and sonicated in cold phosphate buffered saline solution with pH 7.2 (28). The soluble antigens were then separated by centrifugation at 5000 rpm at 4°C and protein concentrations were determined using Bradford Protein Assay Kit (Thermo Fisher Scientific, USA) based on the manufacturer instructions. The antigens were then supplemented with protease inhibitor (Merck, USA) and stored in aliquots at -80°C.

### **2.2 Immunization of mice**

The monoclonal antibody was developed against the soluble antigen of iL3 larvae of *S. ratti* (Kohler & Milstein, 1975). Blood samples were collected every two weeks from the tail vein of each mouse before and after immunisation and were used as controls for positive and negative sera (13,37). Five to eight weeks old female Balb/c mice were immunised intraperitoneal with 50 µg/mL of the soluble antigen emulsified in Complete Freund's adjuvant (Merck, USA). At day 14 and 21, boosters were given with the same antigen concentration (50 µg/mL) emulsified in an equal volume of Incomplete Freund's adjuvant (Merck, USA). Blood samples were collected at 21 days after booster immunisation and were analysed for antibody titers against similar antigen using Indirect ELISA (21,20,9). The mouse with the highest antibody titer was selected for the development of hybridoma cells. Parental myeloma cells of Balb/c mice origin, Sp2/0-Ag14 (ATCC® CRL-1581™) was used to stabilize hybridoma cells for the continuous secretion of monoclonal antibodies (13,37). The cells were cultured in 5 mL of DMEM (Merck, USA), containing 10% fetal bovine serum (Meck, USA) in a 25 cm<sup>3</sup> tissue culture flask (Thermoscientific, USA). Then, the cells were incubated in a 5% CO<sub>2</sub> at 37°C (Galaxy B+ Incubator, RS Biotek, Scotland). Similarly, the spleen of the selected mice was aseptically removed and washed in 35 x 10 mm sterile tissue culture dishes (Thermoscientific, USA), containing a DMEM-serum-free medium. The spleen was disaggregated into a single cell suspension by flushing with a three mL sterile syringe and a 25 G needle containing a DMEM-serum-free medium. In preparation for the cell fusion, the 2 x 10<sup>7</sup> of the parental myeloma cell and 6 x 10<sup>7</sup> viable splenocytes were mixed in a ratio of 1:3 (18,37).

### **2.3 Fusion of hybridoma cells**

Fusion was done in a 50 mL conical tube and was centrifuged for 10 minutes at 1500 rpm. The resulting supernatant was completely discarded (Wayne et al., 2006). Fusion was performed by disrupting the cell pellets by gently tapping the bottom of the tube. 1 mL of 50% polyethylene glycol (Merck, USA) was continuously added to the tube over the period of 1 minute under stirring. Then, 4 mL of DMEM-serum-free medium was added to the fusion mixture with continuous gentle stirring over a period of 4 minutes and the tube was incubated for 15 minutes in water bath at 37°C. About 30 mL of Complete Growth Medium (STEMCELL Technologies, USA) was slowly added to the tube and the cells were centrifuged at 1500 rpm for 10 minutes. The fused cells were re-suspended in 10 mL of Hybridoma Recovery Medium (STEMCELL Technologies, USA) and transferred to a 75cm<sup>2</sup> tissue culture flask containing new 20 mL of Hybridoma Recovery Medium (STEMCELL Technologies, USA) and incubated for 3 days in a 5% CO<sub>2</sub> at 37°C (Galaxy B+ Incubator, RS Biotek, Scotland). The hybrid clones were then selected in a culture medium supplemented with hypoxanthine-aminopterin-thymidine (HAT) and sub-cloned by limiting dilutions (37). Indirect ELISA was then used to screen for antibodies producing hybrid cells for continuous in vitro culture (9,20,21).

### **2.4 Isotyping of monoclonal antibody**

Indirect ELISA was used to confirm the antibody-secreting hybridoma cells (9,20,21) and Western immunoblot (23,33). Intraperitoneal injection of  $1 \times 10^6$  hybridoma cells was done for the production of ascites fluid. The ascites fluid was collected and centrifuged at 5000 rpm at 4°C for 15 minutes and the supernatant was collected and stored at -20°C. Isotyping of the supernatant from the in vitro culture of hybridoma cells and ascites fluid were done using Mouse Monoclonal Antibody Isotyping Kit (Merck, USA) based on the manufacturer instructions.

## 2.5 Mycoplasma test

Supernatant from the in vitro culture of hybridoma cells and ascites fluid was tested for contamination with mycoplasma using Universal Mycoplasma Detection Kit (ATCC, USA) based on the manufacturer instructions.

## 2.6 Indirect ELISA with homologous and heterologous antigens

The reaction of the monoclonal antibody against homologous antigens (iL3, ES and coproantigen of *S. ratti*) and heterologous antigens (from the adult worms of *A. suum*, *T. cati*, *T. canis* and *A. caninum*) were done using Indirect ELISA (9,20,21). 0.5µg/mL of each heterologous antigen was coated on the ELISA plates separately (Thermo Fisher, USA) and was incubated at 4°C overnight. 100µL of Blocking Reagent (Merck, USA) were added to the coated ELISA plates and were incubated for 30 minutes at 37°C. The coated ELISA plates were washed three times with 100µL PBS-Tween 20 (pH of 7.2) (9). Then, the plates were reacted with monoclonal antibody and incubated for 30 minutes at 37°C. The plates were then washed three times with 200µL PBS-Tween 20 (pH 7.2) and 100µL ALP Goat-anti mouse IgG (Merck, USA) was then added as secondary antibody and incubated at 37°C for 45 minutes. P-Nitrophenyl-phosphate (pNPP) (Thermo Fisher, USA) was used as the substrate at 405 nm (Dynex Technologies, USA) after 30 minutes incubation at room temperature. The reaction was stopped after adding 100µL of 0.75 M NaOH.

## 2.7 Western Immunoblotting with homologous and heterologous antigens

Each homologous and heterologous antigen was loaded in the separated wells of the SDS-PAGE system (Sambrook et al., 1989) and then the gel was run for 1–2 h at 100 V (Mini Trans-Blot, Bio-Rad USA). The antigens were then transferred to an Immobilon® PVDF Membranes (Merck, USA) using a Semi-dry blotter (Merck, USA) as instructed by the manufacturer. The membranes were then soaked in a blocking solution, incubated with agitation for 1 hour and washed three times with agitation in PBS for 10 minutes. After that, the resulting membranes were then reacted with the monoclonal antibody and incubated with agitation for one hour at room temperature and washed three times with agitation in PBS for 10 minutes. (33,23). Next, the membranes were further incubated in ALP Goat-anti mouse IgG (Merck, USA) as a secondary antibody with agitation for 3 hours at room temperature, washed three times with agitation in PBS for 10 minutes and incubated with agitation in SIGMAFAST™ BCIP®/NBT (SIGMA-ALDRICH, USA) for another 30 minutes at room temperature. The reaction was terminated by washing the membranes with distilled water.

## 2.9 Preparation of rat fecal specimens for Sandwich ELISA

Fifty (50) faecal samples collected from 20 experimental-based inoculated rat with *S. ratti* (24) as well as 30 clean and uninfected rats as normal controls were used for the sensitivity test and standardisation of Sandwich ELISA. Faecal specimens collected from each rat were mixed with 1mL PBS (pH 7.2) in an Eppendorf tube. The mixture was then homogenised and spin at 5000 rpm for 10 minutes. The resulting supernatant was collected into new Eppendorf tubes and the pellets were discarded (26). The supernatant was kept at -80°C before further analysis was conducted using Sandwich ELISA (19,31).

## 2.10 Sandwich ELISA for detection coproantigen of *S. ratti*

ELISA plates were coated with 5µg/mL of rabbit polyclonal antibodies that raised against iL3 larvae of *S. ratti* and incubated overnight at 4°C. 100µL of Blocking Reagent (Merck, USA) was added and then were incubated for 30 minutes at 37°C. The plates were then washed three times with 200µL PBS-Tween 20 (pH 7.2). Then, 100µL of supernatant from faecal specimen was added and incubated for 1 hour at 37°C. The plates were then washed three times with 200µL PBS-Tween 20 (pH 7.2), reacted with monoclonal antibody, incubated for 30 minutes at 37°C (Schmidt et al., 2012) and washed three times with 200µL PBS-Tween 20 (pH 7.2) before added 100µL ALP Goat-anti mouse IgG (Merck, USA) that acts as secondary antibody. The mixture was then incubated at 37°C for 45 minutes. P-Nitrophenyl-phosphate (pNPP) (Thermo Fisher, USA) was added after incubated for 30 minutes at room temperature. The reaction was terminated by adding 100µL of 0.75 M NaOH into the mixture (19).

### 3. RESULTS

In this study, three monoclonal antibodies denoted as S1, S2 and S3 were developed against the soluble antigen of iL3 larvae of *S. ratti* (Figure 1). Monoclonal antibody S3 was selected for this study and renamed as IgG2bMAb (monoclonal S1 and S2 were not used in this study and were cryopreserved). Characterization of the immunoglobulin sub-classes based on the Monoclonal Antibody Isotyping Kit (Merck, USA) showed that monoclonal antibody S1, S2 and S3 were from the immunoglobulin sub-classes of IgG1, IgG2a and IgG2b respectively (Figure 1).

Western Immunoblotting analysis of IgG2bMAb against the two homologous antigens of ES and iL3 of *S. ratti* confirmed that IgG2bMAb was able to react with both the homologous antigens. IgG2bMAb reacted strongly to the antigen from iL3 compared to ES from the same larvae of *S. ratti* (Figure 2). Western immunoblotting analysis for the cross-reactivity with other species of heterologous intestinal helminth was done. Heterologous antigens such as the adult worm soluble antigens from *T. canis* (Tcn), *T. cati* (Tct), *A. caninum* (Ac) and *A. suum* (As) were tested against IgG2bMAb. IgG2bMAb was observed to cross-reacted strongly at 30 kDa with *A. caninum* (Ac) antigen by producing similar band intensity with the positive control antigen from iL3 of *S. ratti* (Sr) used in this study (Figure 3).

There was no visible cross-reaction detected with *T. canis* (Tcn), *T. cati* (Tct) and *A. suum* (As) antigens by IgG2bMAb (Figure 3). IgG2bMAb was further tested in Sandwich ELISA for the immunodiagnostic detection of coproantigen of *S. ratti* in rat's faecal specimen. Thirty clean and uninfected rats were used as the negative control for the standardisation of the cut-off-point used in the Sandwich ELISA (Mean  $\pm$  3 SD = 0.266) (Figure 4). Twenty positively inoculated rat's faecal specimens (previously subcutaneously inoculated with  $1 \times 10^5$  iL3 larvae) (24) was used for the sensitivity study in Sandwich ELISA. In this study, IgG2bMAb detected 17 positive coproantigen from 20 inoculated faecal specimens of experimentally inoculated rats with iL3 of *S. ratti* with three false negatives detected below 0.266 (Figure 4). Thus, the sensitivity of IgG2bMAb based Sandwich ELISA for detection of coproantigen of *S. ratti* in rat's faecal specimens was 85% as calculated from the detection rate of  $17/20 \times 100$  (Figure 4).

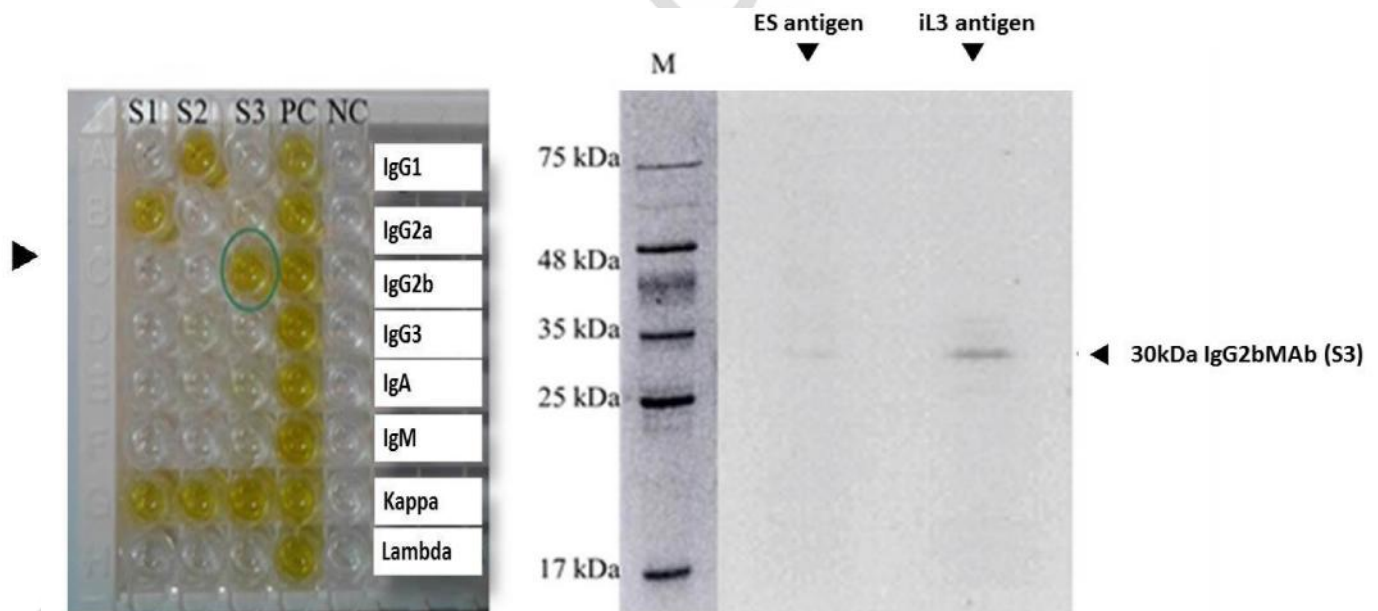
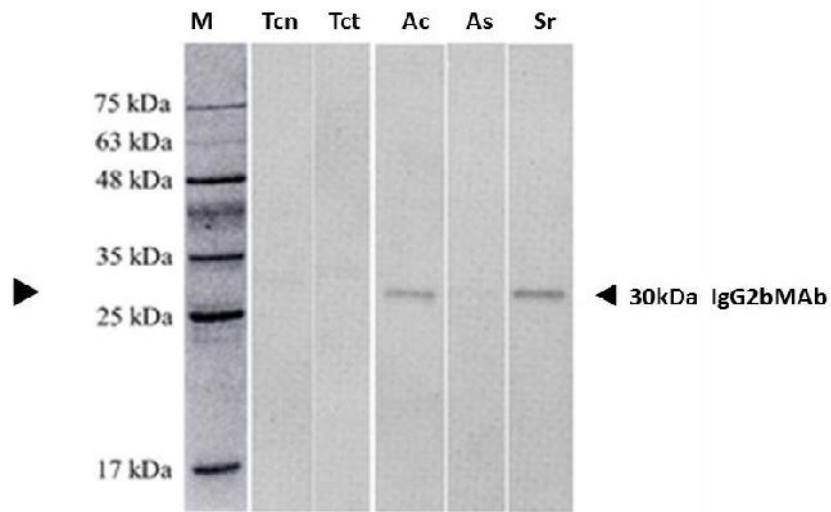
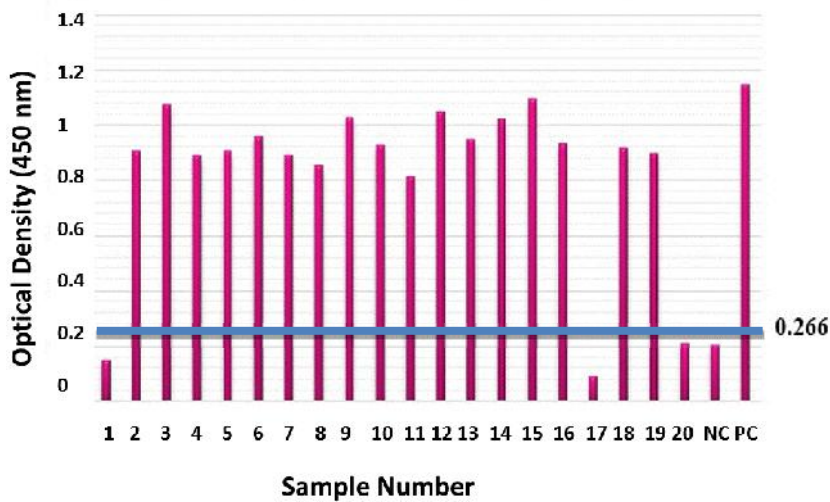


Figure 1: Characterization of the immunoglobulin sub-classes based on Isotyping Kit

Figure 2: Western immunoblotting analysis of IgG2bMAb against ES and iL3 of *S. ratti*



**Figure 3: Western immunoblotting analysis for the cross-reactivity with other species of heterologous intestinal helminth**



N=20 infected rat wit *S. ratti* sandwich ELISA, positive detected = 17 sandwich ELISA. False negative detected =3, Sensitivity of reaction

**Figure 4: Sandwich ELISA for the immunodiagnostic detection of coproantigen of *S. ratti* in rat's faecal specimen**

#### 4. DISCUSSION

Strongyloidiasis is frequently underdiagnosed because many of the cases are asymptomatic and current diagnostic methods are lacking sensitivity. The early detection of this disease is still one of the major obstacles in the infection prevention. Conventional diagnostic methods are insensitive due to the poor and inconsistent parasite load in stool samples. World Health Organization estimated that about 30–100 million people are infected worldwide, and the precise data is still remained unknown, especially in the endemic countries which are associated with poor sanitary conditions. Acute and chronic infections have been reported widely in children, elderly and the immunosuppressed patients. Fatal infections mostly occur in the immunosuppressed patient associated with corticosteroids chemotherapy. In this group of patients, the majority of the cases are underdiagnosed due to asymptomatic infection and lack of defined symptoms, hence resulting in fatal disseminated infection. In this study, *S. ratti* was used to represent *S. stercoralis* based on the

morphological and similarity in genome up to 80%. Three monoclonal antibodies (S1, S2 and S3) were produced and characterizations of the antibody isotypes were IgG1, IgG2a and IgG2b for S1, S2 and S3 respectively (Figure 1). S3 (later renamed as monoclonal IgG2bMAb) was chosen to be evaluated in the monoclonal IgG2bMAb-based Sandwich ELISA for detection of coproantigen from *S. ratti* in faecal specimens based on experimental inoculated Wistar rats (S1 and S2 were cryopreserved). To our knowledge, this is the first study utilises IgG2b in the immunological detection of helminth's coproantigen in the faecal specimen.

Western immunoblotting confirmation for the detection of monoclonal IgG2bMAb against the prepared ES and iL3 antigens of *S. ratti* was conducted. The result showed that monoclonal IgG2bMAb was able to bind with 30 kDa protein of iL3 soluble antigen but low binding with ES antigen of *S. ratti* (Figure 2). The weak binding with ES protein was probably associated with the fluctuation of secreted ES by iL3 and the establishment of patency for serological detection of ES protein that was previously estimated around 6 to 7 weeks (34). This resulted in a low concentration of ES protein and detection with monoclonal IgG2bMAb. Nevertheless, this experiment confirmed that IgG2bMAb was able to detect the presence of *S. ratti* immunogenic protein at 30 kDa (Figure 2). Another study also confirmed that serum IgG reactivity against iL3 of *S. stercoralis* immunogenic proteins was at 28 kDa, 31 kDa and 41 kDa which were associated with a high quantity of *S. stercoralis* worm (6). In our study, the 30 kDa detected by monoclonal IgG2bMAb was between 28 kDa and 31 kDa, which was in agreement with the study.

Multiple intestinal helminths infection has been reported in endemic areas. These resulted in cross-reaction due to similar binding epitopes and may produce false positive results in an immunodiagnostic test (36,14,27). Hookworm infection caused by *Ancylostoma* spp and *Necator* spp. was known to cross-reacted with Strongyloidiasis in an immunodiagnostic test (2,3,4). To observe if similar cross-reaction occurs with monoclonal IgG2bMAb, heterologous antigens from commonly known intestinal helminths such as *T. canis* (*Tcn*), *T. cati* (*Tct*), *A. caninum* (*Ac*) and *A. suum* (*As*) were prepared. This is to evaluate if monoclonal IgG2bMAb cross-reacted with these intestinal helminths apart from *S. ratti* that may produce false positive results. In Western immunoblotting, monoclonal IgG2bMAb only cross-reacted with the antigen from the adult worm of *A. caninum* (*Ac*) at 30 kDa (Figure 3). Multiple intestinal helminth is commonly affected children, especially in rural areas in the developing world due to poor hygiene (5,8,17,32,8). The cross-reactivity with *A. caninum* suggests that monoclonal IgG2bMAb-based Sandwich ELISA can be used to detect multiple helminth infections, especially from the patient faecal specimen with multiple intestinal helminth infections such as both *Strongyloides* spp. and *Ancylostoma* spp. (Figure 3).

The sensitivity of monoclonal IgG2bMAb-based Sandwich ELISA for detecting coproantigen of *S. ratti* was evaluated with twenty faecal specimens that were positively confirmed being infected rats with *S. ratti* (Figure 4). The cut-off-point was determined based on Optical Density (OD) reading at 0.266 of Mean  $\pm$  3 SD of thirty uninfected normal control rats with monoclonal IgG2bMAb-based Sandwich ELISA. OD reading above 0.266 was considered positive for the presence of coproantigen of *S. ratti*, whereas OD reading below 0.266 was taken as negative. Monoclonal IgG2bMAb-based Sandwich ELISA was able to detect seventeen positives out of the twenty known infected rats used for this sensitivity test (Figure 4). Thus, the sensitivity of IgG2bMAb-based Sandwich ELISA for detection of coproantigen of *S. ratti* in rat's faecal specimens was 85%. Three faecal specimens from this group with OD reading below 0.266 were taken as false negative as these specimens were from known positive infected rats. These low OD reading most probably due to low parasite load in a faecal specimen of these three rats. The application of the soluble antigens of iL3 larvae of *S. ratti* used in this study for the production of monoclonal IgG2bMAb was able to detect 30 kDa coproantigens from all the three developmental stages of *S. ratti* which are the eggs, larvae and adults' stages in a faecal specimen. This is a significant finding as not all the stages of *S. stercoralis* can be seen in a faecal specimen that was sent to the diagnostic laboratory (2,3).

## 5. CONCLUSION

In this study, IgG2b proved to have an immunodiagnostic property as IgG2bMAb was able to detect antigens from coproantigen related to *S. ratti* (sensitivity was 85% based on Sandwich ELISA) even though cross-reaction was observed with *A. caninum*. These findings will be very useful to tackle many cases of multiple worms' infections such as both strongyloidiasis and hookworm but need further evaluation and study in the endemic area where multiple infections can be common.

## ETHICAL APPROVAL

Experimental on an animal was conducted through the approved protocol that was submitted to the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (UPM/IACUC/AUP-R026/2015). The procedure involves establishment and propagation of *S. ratti* in rats for the preparation of soluble antigens. These were later used in the immunisation protocol of Balb/c mice for the activation of B-cells and production of hybridoma cells.

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