Immunodiagnosis of paramphistomosis using monoclonal antibody-based sandwich ELISA for detection of *Paramphistomum gracile* circulating 16 kDa antigen

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SUMMARY

In this study, we have produced a monoclonal antibody (MoAb) against 16 kDa antigen of *Paramphistomum gracile* (16 kDaAgPg), and developed an accurate sandwich enzyme-linked immunosorbent assay (sandwich ELISA) for the detection of circulating 16 kDaAg in the serum and fecal samples from cattle naturally infected with *P. gracile*. MoAb 1D10 was immobilized on a microtitre plate, and the antigen in the samples was captured and detected with biotinylated rabbit anti-16 kDaAgPg antibody. The lower detection limit of sandwich ELISA was 3·5 pg mL^{-1}, and no cross-reaction with other parasite antigens was evaluated. The reliability of the assay was examined using the serum and fecal samples from cattle naturally infected with *P. gracile, Fasciola gigantica, Moniezia benedeni, Trichuris* sp., *Strongyloides* sp., strongylids and non-infected animals. The sandwich ELISA showed the sensitivity, specificity and accuracy at 98·33, 100 and 99·55% (serum samples), and 96·67, 100 and 99·09% (fecal samples). Therefore, this detection method is a rapid and excellent potential assay for the accurate diagnosis of paramphistomosis.

Key words: *Paramphisomum gracile*, paramphistomosis, 16 kDa antigen, monoclonal antibody, sandwich ELISA, immunodiagnosis.

INTRODUCTION

Paramphistomosis is well-known helminth parasites of ruminants, i.e. cattle, buffaloes, goats and sheep, which is caused by digenetic flukes belong to the superfamily of Paramphistomoidea (Gupta *et al*. 1978). This disease is found in many regions, particularly in Africa, Asia, Australia, eastern Europe and Russia, which results in major economic losses in agriculture (Gupta *et al*. 1978; Nikitin, 1979; Hanna *et al*. 1988; Anuracpreeda *et al*. 2008, 2012, 2015). The occurrence of this disease, mainly with *Paramphistomum gracile*, has also been reported (Chethanon *et al*. 1985; Prasitirat *et al*. 1997; Panyarachun *et al*. 2013; Anuracpreeda *et al*. 2017). Presently, a transitory diagnosis of paramphistomosis has been performed by conventional method through the detection of the fluke's eggs in the feces of infected animals, in conjunction with the history and clinical signs of the disease. However, this method often results in misdiagnosis and lack sensitivity, particularly in low levels of adult fluke burden or during the prepatent period (Horak, 1971; Bida and Schillhorn van Veen, 1977). As well, the antigen-detection method was found to be more sensitive and specific (Zheng *et al*. 1990; Anuracpreeda *et al*. 2009*a*, *b*). In the present study, we have developed the monoclonal antibody (MoAb)-based sandwich enzyme-linked immunosorbent assay (sandwich ELISA) for diagnose active *P. gracile* infection by detecting the 16 kDa antigen in both serum and fecal samples of naturally infected animals. The use of this assay method provides the immunodiagnosis of paramphistomosis by *P. gracile* in ruminants with high sensitivities, specificities and accuracies.

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MATERIALS AND METHODS

Ethics statement

Experiments on animals were approved by the Animal Care and Use Committee (SCMUACUC), Faculty of Science, Mahidol University, Thailand, and were specifically used for this study. Serum and fecal samples from naturally infected cattle were collected from fields in many areas of Thailand with monoinfections of *P. gracile*, other trematode, cestode and nematode parasites.

Parasite samples and antigen preparations

Live *P. gracile* adult worms were collected from the rumen of naturally infected cattle or water buffaloes killed at the local slaughter houses, Pathum Thani

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Table 1. Cross-reactivity testing of a sandwich ELISA to various crude antigens from trematode, cestode and nematode parasites.

Antigens[a]	OD at 450 nm[b]
Trematode parasites	
Paramphistomum gracile, 16 kDaAg	3·441
P. gracile	2·923
P. cervi	0·098
Orthocoelium parvipapillatum	0·089
Fischoederius cobboldi	0·079
Gastrothylax crumenifer	0·074
Carmyerius spatiosus	0·077
Cotylophoron cotylophorum	0·085
Gigantocotyle explanatum	0·082
Eurytrema pancreaticum	0·086
Fasciola gigantica	0·068
F. hepatica	0·078
Schistosoma spindale	0·076
S. mansoni	0·079
S. japonicum	0·083
S. mekongi	0·079
Opisthorchis viverrini	0·073
Cestode parasites	
Avitellina centripunctata	0·069
Moniezia benedeni	0·065
Nematode parasites	
Haemonchus placei	0·068
Trichuris sp.	0·087
Setaria labiato-papillosa	0·065
Reagent blank	0·148

[a] The protein content of each parasite antigen preparation was adjusted to 20 μg mL^{-1}, and a 50-μL volume was used for analysis.
[b] Mean OD was determined in triplicates performed on three separate occasions.

Province, Thailand. Other trematodes, cestodes and nematode parasites were obtained for the cross-reactivity study as shown in Table 1. The whole body (WB) antigen of all parasites and excretory–secretory (ES) antigen of *P. gracile* were prepared as per the method described by Anuracpreeda *et al.* (2006, 2013*a*, *b*, 2016*b*, *c*, *d*, *e*; Panyarachun *et al.* 2010). The 16 kDa antigen of *P. gracile* (16 kDaAgPg) was obtained as per the method described earlier by Anuracpreeda *et al.* (2016*a*, 2017). Protein concentrations of antigen preparations were determined by Lowry's method (Lowry *et al.* 1951).

Generation and purification of MoAbs and polyclonal antibodies (PoAbs) against 16 kDaAgPg

We previously described the production and characterization of hybridoma clones of MoAbs against 16 kDaAgPg according to the method of Anuracpreeda *et al.* (2011, 2014, 2017). Briefly, the hybridoma clones expressing MoAb against 16 kDaAgPg were produced by fusion of mouse myeloma cells (P3 × 63-Ag8·653) with splenocytes from inbred BALB/c mouse immunized with 16 kDaAgPg, using polyethylene glycol. The immunization of BALB/c mice comprised 3 subcutaneous injections with 25 μg of 16 kDaAgPg. The first immunization was emulsified in complete Freund's adjuvant, and subsequent injections used incomplete Freund's adjuvant. The MoAbs were initially screened for their reactivity by indirect ELISA, and the highly reactive hybridomas were cloned by limiting dilution techniques. Hybridoma clone (1D10) producing a high antibody titre against 16 kDaAgPg was selected and the specific MoAb isotypes were determined by indirect ELISA. In the cross-reactivity study, proteins (10 μg) in WB from *P. gracile* as well as WB from other trematode, cestode and nematode parasites were separated using 12·5% SDS–PAGE, and transferred onto nitrocellulose (NC) membranes for immunoblotting by the selected MoAb 1D10 according to the method of Anuracpreeda *et al.* (2016*a*). Polyclonal anti-16 kDaAgPg for the detection of antigen captured by the immobilized MoAb was produced by immunizing New Zealand White rabbits with 16 kDa antigen of *P. gracile* as per the method of Anuracpreeda *et al.* (2016*c*). The IgM fraction of MoAb and IgG fraction of PoAb were purified by affinity chromatography, and the purified IgG of PoAb was subsequently biotinylated as previously described (Anuracpreeda *et al.* 2016*c*).

Evaluation of the lower detection limit and the specificity of sandwich ELISA

The method described previously by Anuracpreeda *et al.* (2016*b*) was used to evaluate the lower detection limit of sandwich ELISA. The 16 kDa antigen and WB as well as ES antigens of *P. gracile* were titrated with a serial dilution method. The endpoint of detection limit was considered to be the lowest amount of parasite antigen still exhibiting the positive optical density (OD) values. To examine cross-reactivity testing, the specificity of ELISA was analysed by using WB antigens from other trematode, cestode and nematode parasites. Each of these antigens was prepared at different concentrations and detected possible presence of 16 kDa antigen.

Detection of circulating 16 kDaAgPg by sandwich ELISA

The sandwich ELISA was performed as per the method described previously by Anuracpreeda *et al.* (2016*b*, *c*). For each step, 50 μL well^{-1} was added unless otherwise mentioned. microtitre plate was coated with 10 μg mL^{-1} rabbit anti-mouse IgG diluted in carbonate buffer. After washing, 10 μg mL^{-1} purified MoAb 1D10 was added. Then, the plate was blocked with 150 μL well^{-1} of 5% skimmed milk in phosphate buffered saline (PBS) for 1 h at 37 °C. After washing as described earlier, the reference antigens or samples were added (triplicate wells), and incubated for 3 h at 37 °C. Thereafter, 2 μg mL^{-1} biotinylated rabbit IgG antibody against 16 kDaAgPg

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was added, and TMB substrate solution was used as a chromogen to detect streptavidin-conjugated peroxidase reaction. The enzyme reaction was stopped by adding of 1 N HCl, and determined from the OD value measured at 450 nm using an automatic microplate reader.

Evaluation of results and statistics

The cut-off value was calculated according to the method of Anuracpreeda *et al*. (2016*b*), as the mean OD of negative controls plus three times the standard deviations (S.D.). The data were considered no significant if *P*-value greater than 0·05, and considered to be highly and very highly significant if *P*-value <0·05 and 0·01, respectively. According to Galen (1980), standard diagnostic indices including sensitivity, specificity, predictive values, false positive and negative rate, and accuracy were calculated.

RESULTS

The specificity of MoAb 1D10 against 16 kDa antigen with other trematode, cestode and nematode's antigens

In both indirect ELISA and immunoblotting assay, MoAb 1D10 showed strong reaction with the 16 kDa antigen in WB of adult *P. gracile* at molecular weight 16 kDa, while showing no cross-reaction with WB antigens from other parasites.

The lower detection limit and the specificity of sandwich ELISA

This assay could detect the 16 kDa antigen and native 16 kDa antigen in WB and ES fractions of *P. gracile* as low as 3·5, 60 and 120 pg mL^{-1}, respectively (Fig. 1A). Moreover, this test was highly specific for the 16 kDa antigen, and the cross-reactions with other parasite antigens were not observed (Table 1).

Immunodiagnosis of naturally infected and control serum or fecal samples by sandwich ELISA

Serum or fecal samples from the 220 cattle were collected and examined by sandwich ELISA, including 60 samples from paramphistomosis, 60 from fasciolosis, 10 from monieziasis, 10 from trichuriasis, 10 from strongyloidiasis, 10 from strongylid infections and 60 from non-infected animals. It was evident that 98·33% (59/60) (Fig. 1B) and 96·67% (58/60) (Fig. 1C) of paramphistomosis serum and fecal samples were tested positive, respectively, while all serum or fecal samples from those infected with other infections and non-infected controls were found to be 100% (160/160) negative (Fig. 1B and C). Hence, the diagnostic sensitivity, specificity, positive predictive value, negative predictive value, false positive rate, false negative rate and accuracy

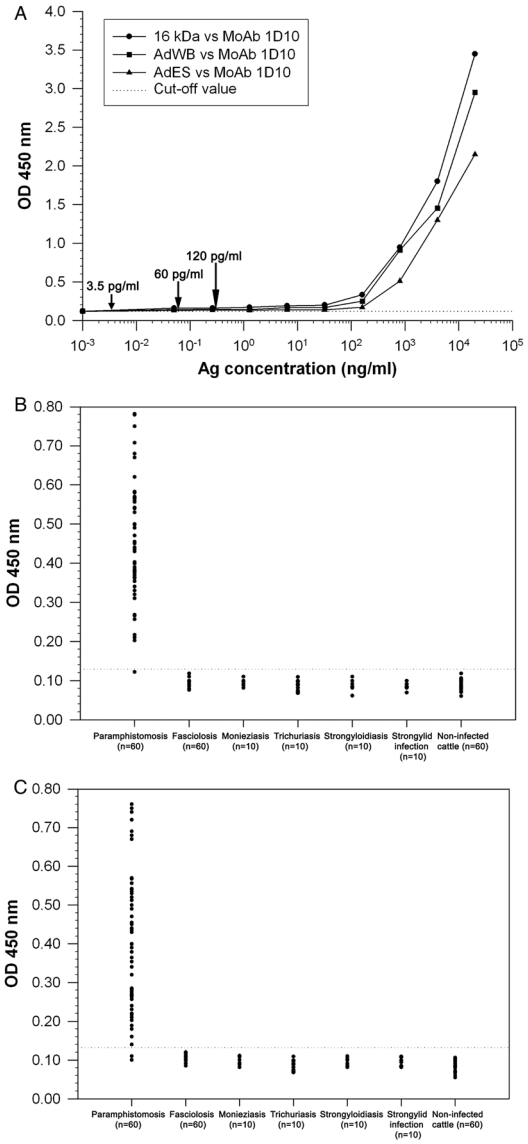

Fig. 1. (A) The sandwich ELISA denotes the analysis of the lowest concentrations of 16 kDa antigen (line with black circle), native 16 kDa antigen in WB (line with black square) and ES (line with black triangle) fractions of adult *Paramphistomum gracile*. The arrows indicate the lowest concentrations of 16 kDa antigen that could still be detected. (B) Analysis of the relative levels of circulating 16 kDa antigens in the sera from cattle with monoinfections as measured by a reliable MoAb-based sandwich ELISA (OD values at 450 nm). Serum samples obtained from cattle with paramphistomosis, fasciolosis, monieziasis, trichuriasis, strongyloidiasis and strongylid infection were compared with control sera obtained from non-infected cattle. The horizontal dotted line represents the cut-off value for a positive detection. (C) Analysis of the relative levels of circulating 16 kDa antigens in the fecal samples (fecal supernatant) from cattle with monoinfections as measured by a reliable MoAb-based sandwich ELISA (OD values at 450 nm). Fecal samples obtained from cattle with paramphistomosis, fasciolosis, monieziasis, trichuriasis, strongyloidiasis and strongylid infection were compared with control sera obtained from non-infected cattle. The horizontal dotted line represents the cut-off value for a positive detection.

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were found to be 98·33, 100, 100, 99·38, 0, 1·67 and 99·55% for serum samples, and be 96·67, 100, 100, 98·77, 0, 3·33 and 99·09% for fecal samples. In addition, a positive correlation was found between egg counts in feces of *P. gracile*-infected animals and the OD values of sandwich ELISA in both serum ($r^2 = 0{\cdot}628$, $P < 0{\cdot}01$) and fecal samples ($r^2 = 0{\cdot}548$, $P < 0{\cdot}01$).

DISCUSSION

This study was the first to develop a highly sensitive and specific MoAb-based sandwich ELISA which is utilized for detection of circulating 16 kDa antigen in the serum and fecal samples from cattle naturally infected with *P. gracile*. The effectiveness of the ELISA implies the presence of 16 kDa antigen in the *P. gracile*-infected cattle's serum and fecal samples, as has been reported previously (Anuracpreeda *et al*. 2014). In our study, this assay could detect 16 kDa antigen and native 16 kDa antigen in WB and ES fractions of adult *P. gracile* at the concentrations as low as 3·5, 60 and 120 pg mL^{-1}, respectively. The detection limits of our assay are lower than those reported earlier in other closely related trematode parasites. For instance, a capture ELISA was used to detect the ES antigens in the samples of animals infected with *Fasciola hepatica* at the detection limits at 0·25 (for mice serum), 0·3 (for sheep feces) and 0·6 ng mL^{-1} (for cattle feces) (Langley and Hillyer, 1989; Mezo *et al*. 2004). In addition, the detection limits of this assay are lower than those of our previous reports when the circulating antigens were detected in the sera of mice and cattle infected with *Fasciola gigantica* (Anuracpreeda *et al*. 2009*a*, 2013*a*). Moreover, it was reported that a sandwich ELISA was utilized to detect the ES antigens in both serum and fecal samples of patients infected with *F. gigantica* at the concentration levels 3 and 15 ng mL^{-1}, respectively (Espino and Finlay, 1994; Demerdash *et al*. 2011), which were still higher than in this study. Moreover, no cross-reactivity was observed when the ELISA was employed to detect various concentrations of other parasite antigens (Table 1).

It was quite interesting to note that the diagnostic sensitivity, specificity and accuracy of this MoAb-based sandwich ELISA were very high as in naturally infected cattle sera at 98·33, 100 and 99·55%, whereas in fecal samples they were 96·67, 100 and 99·09%, respectively. From our results, it is obvious that this sandwich ELISA would be successfully able to detect the presence of the 16 kDa antigen in both serum and fecal samples of animals infected with *F. gigantica*. Moreover, a positive correlation was found between egg counts in feces of *P. gracile*-infected animals and the OD values of sandwich ELISA in both serum and fecal samples. This result was similar to the positive correlation reported by Espino and Finlay (1994) and Estuningsih *et al*. (2009).

In conclusions, this sandwich ELISA exhibited high efficiencies and precisions, and could be a very useful tool not only for the rapid diagnosis of paramphistomosis in livestock, but also for the seroepidemiological screening and monitoring of chemotherapeutic efficacy of animals from different areas. More importantly, this assay method may be modifiable into a more convenient diagnostic kit, and could be useful in laboratories lacking well-trained microscopists.

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