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RESEARCH ARTICLE

Potential use of anti-thrombospondin-related apical merozoite protein (TRAMP) polyclonal antibodies in sandwich enzyme-linked immunosorbent assay (ELISA) for detection of *Plasmodium knowlesi*

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ABSTRACT

Plasmodium knowlesi, primarily a zoonotic malaria species is the most common malaria pathogen in the Southeast Asia especially in Malaysian Borneo, Malaysia. Due to morphological resemblance of *P. knowlesi* to other human *Plasmodium*, the sensitivity for microscopic detection of *P. knowlesi*, which is the gold standard, is compromised. Thus, efforts have been made in finding alternatives for the disease diagnosis. This study described the potential use of anti-PkTRAMP polyclonal antibodies in sandwich ELISA for *P. knowlesi* detection. Anti-PkTRAMP polyclonal antibodies raised from mice and rabbit were first evaluated for their binding capability towards native proteins in *P. knowlesi* lysates using Western blot. These mice and rabbit polyclonal antibodies were then used in the sandwich ELISA as capture and detection antibodies, respectively. *P. knowlesi* A1H1 culture was utilised to determine the limit of detection (LOD) of this assay. Its clinical performance was determined by testing with archived human malaria and uninfected samples. Western blot analysis affirmed the polyclonal antibodies reactivity to *P. knowlesi*. The LOD obtained from three replicated assays was at 0.015% parasitaemia. The assay has 76% sensitivity and 75% specificity for *P. knowlesi*. Its positive and negative predictive values were 76% and 75%, respectively. No cross reactivity with *P. falciparum* and healthy samples was observed, except for *P. vivax* where 10 out of 12 samples were detected. In conclusion, anti-PkTRAMP polyclonal antibodies can be useful in detecting *P. knowlesi*. Regardless, the full potential of anti-PkTRAMP antibodies for diagnostic purposes need to be explored further.

Keywords: Malaria; *Plasmodium knowlesi*; Thrombospondin-related apical merozoite protein (TRAMP); sandwich enzyme-linked immunosorbent assay (ELISA); polyclonal antibodies.

INTRODUCTION

The four common human malaria species are *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae* and *Plasmodium ovale*. However, it is now known that humans also contract malaria from *P. knowlesi,* a zoonotic malaria species (Singh *et al*., 2004). Diagnosing *P. knowlesi* infection accurately in humans is crucial as it can lead to hyperparasitaemia in short period and severe complications such as severe acute kidney damage, jaundice, and respiratory distress (William *et al.,* 2011; Kotepui *et al.,* 2020). Microscopic examination of blood smears reveals the presence of *P. knowlesi* parasites, which often resemble those of other malaria species such as *P. falciparum* and *P. malariae*, making it challenging to differentiate them solely based on morphology and may lead to inaccurate diagnosis. Additionally, in infections with very low parasitaemia levels or mixed infections, the minority parasite species may be overlooked (Giha *et al.,* 2005). This leads to delays in delivering effective therapy and can be fatal (Barber *et al.,* 2013). Early and accurate diagnosis is therefore essential.

Currently, the diagnosis of *P. knowlesi* infection typically involves a combination of clinical suspicion; microscopy, rapid diagnostic tests (RDTs) and molecular methods such as polymerase chain reaction (PCR). RDTs are the most convenient method and widely used for rapid malaria diagnosis. Several RDTs have been developed that can detect all four human malaria species and *P. knowlesi* such as OptiMAL-IT and BinaxNOW™ Malaria. These commercialised RDTs use pan-specific antibodies for the detection of *P. knowlesi*, but they demonstrated poor sensitivity and specificity in detecting *P. knowlesi* in both fresh and frozen samples. Besides, these kits cannot always accurately distinguish between the different *Plasmodium* species (van Hellemond *et al.,* 2009; Foster *et al.,* 2014). To date, there is no commercially available RDT can specifically detect *P. knowlesi*. Hitherto, PCR has been used as a complementary method to confirm species identification, especially in regions where *P. knowlesi* is prevalent. A nested PCR has been developed (Singh *et al.,* 2004) for *P. knowlesi* detection but the test was reported to cross react with *P. vivax* (Imwong *et al.,* 2009). This method also requires skilled personnels and expensive equipment, making it impractical in fields

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and resource-constrained situations. Given the limitations of the currently available approaches, there is a need to discover a better option to detect *P. knowlesi*.

In contrast to traditional diagnostic methods such as microscopy and PCR, there is growing interest in exploring alternative approaches such as the enzyme-linked immunosorbent assay (ELISA). ELISA is a highly sensitive and specific immunological assay commonly used in clinical laboratories for detecting various antigens or antibodies. This method is appealing due to its simplicity and high throughput. It is also cost-effective as compared to PCR. The principle of ELISA involves the binding of a specific antigen or antibody to a solid-phase surface, followed by the addition of an enzyme-linked detection reagent that produces a measurable signal. The ELISA can be adapted to detect a wide range of pathogens, including malaria parasites, by targeting specific antigens or antibodies associated with the infection. However, at present, there is no commercialised ELISA available specifically for *P. knowlesi.* For instance, Malaria Ag CELISA (Cellabs, Australia) and Genedia malaria antigen ELISA (Green Cross Co., Republic of Korea) are ELISA kits that are available in the market, but they are only suitable for the detection of *P. falciparum* by targeting the parasite's antigen, histidine rich protein 2.

In the case of *P. knowlesi*, several antigens have been identified as potential targets for ELISA-based detection. *Plasmodium knowlesi* thrombospondin-related apical merozoite protein (PkTRAMP) is a transmembrane protein displayed on the merozoite surface. It localises in the merozoite's rhoptry bulb and relocates to the surface of merozoite prior to erythrocyte invasion. It is then secreted after the attachment of merozoite to the host's erythrocyte (Thompson *et al.,* 2004). This 43 kDa protein functions to recognise the human erythrocyte's receptor for successful invasion (Knuepfer *et al.,* 2019). This protein was expressed not only in the merozoites, but also in the other asexual erythrocytic stages of the parasite such as schizonts and trophozoites, making it a good candidate for detection by the antibodies. There was evidence that the antibodies raised against the ortholog protein, *P. falciparum* TRAMP could inhibit merozoite invasion into erythrocyte, causing stunted parasite growth *in vitro* (Siddiqui *et al.,* 2013). This study describes the potential use of anti-PkTRAMP polyclonal antibodies in a sandwich ELISA for the detection of *P. knowlesi* in whole blood samples.

MATERIALS AND METHODS

Expression and purification of recombinant protein

Escherichia coli BL21 (DE3) pLysS strain was used to express the recombinant PkTRAMP from the previously constructed recombinant plasmid (Ng, 2023). A single colony of *E. coli* containing the recombinant plasmid PkTRAMP-pET30a(+) was inoculated into Luria Bertani (LB) broth with 34 µg/ml kanamycin and 34 µg/ml chloramphenicol, incubated with shaking overnight at 37°C. The overnight culture was then diluted to 600 nm = 0.1 and then allowed to multiply in 37°C shaking incubator until its optical density (OD) reached 0.4–0.6. Protein expression was induced using 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the culture was let to propagate for 6 hours at 37°C. The cells were then harvested via 10 minutes centrifugation at 6 500 rpm. Next, the recombinant protein purification was conducted using ProBond™ purification system (Invitrogen, USA) under hybrid condition to preserve the protein activity. Pelleted culture cells (50 ml) were resuspended in 8 ml of 6 M guanidium cell lysis (pH 7.8) to allow cell lysis for 10 minutes on ice. The pellet was then sonicated until the lysate was clear for thorough lysis. Then, the lysate was poured into the purification column prepared in denaturing condition to allow binding of the His-tagged protein to the nickel-NTA agarose resin. The column was washed a few times with denaturing binding buffer (pH 7.8), denaturing wash buffer (pH 6.0), and native wash buffer (pH 8.0) as the manufacturer's instruction. Finally, native elution buffer (pH 8.0) was used to elute the protein and the Bradford Assay kit (Bio-Rad, USA) was utilised to determine the concentration of the purified recombinant PkTRAMP.

Attainment and purification of polyclonal antibodies

PkTRAMP anti-sera of mice and rabbit used in this study were attained from previous study (Ng, 2023) at the Department of Parasitology, Universiti Malaya. Female BALB/c mice (6 8 weeks old) were immunised with 30 µg of purified recombinant PkTRAMP in complete Freund's adjuvant (1:1 ratio), whereas New Zealand white rabbit (16 weeks old) was immunised with 100 µg of the recombinant protein. Two booster shots were given to the mice in the 1 month of immunisation period, while the rabbit was given 3 booster shots in the 14 weeks of immunisation period. At the end of immunisation, the sera were acquired from the blood of the organisms. Proteus Protein A antibody purification column (Bio-Rad, UK) was used to purify mice and rabbit polyclonal antibodies from the collected sera according to the manufacturer's procedure. First, the Protein A resin (in basic condition, pH 9.0) will immobilise the antibodies. After washing unbound contaminants, two different elution buffers were used to elute the antibodies depending on the type of IgG desired; 0.1 M sodium citrate buffer (pH 5.5) for mouse IgG1 elution and 0.2 M Glycine/HCl buffer (pH 2.5) for the elution of mouse IgG2, mouse IgG3 and rabbit IgG. Finally, the elution was neutralised using 1 M Tris/HCl buffer (pH 9.0).

Determination of sample size

To determine the sample size needed for this study, values for sensitivity (Se) were priorly set whereby the null hypothesis, H_o; Se =70% and the alternative hypothesis, H_A; Se = 90%. At 95% confidence (α) and 80% power (β), the minimum sample size was determined using the following equation described by Hajian-Tilaki (2014).

$$n = \frac{\left[Z_{\frac{\alpha}{2}}\sqrt{P_0(1-P_0)} + Z_{\beta}\sqrt{P_1(1-P_1)}\right]^2}{(P_1 - P_0)^2}$$

where,

n = number of samples

$Z_{\alpha/2}$ = upper $\alpha/2$ percentile of the standard normal distribution

Z_{β} = upper β percentile of the standard normal distribution

P_o = value of sensitivity under null hypothesis

P_1 = value of sensitivity under alternative hypothesis

From the calculation, 33 *P. knowlesi* samples were needed for this study.

Attainment of blood samples

In this study, archived whole blood samples of mono-infection malaria samples collected between the year 2016 to 2022 were used. The samples are comprised of 41 *P. knowlesi,* 10 *P. falciparum* and 12 *P. vivax*. All samples chosen were diagnosed positive by both microscopy and nested polymerase chain reaction (PCR). Blood samples taken from 18 healthy individuals that have never been exposed to malaria were used as the control group.

Blood sample/culture preparation for sandwich ELISA

First, 1X PBS was used to wash each blood/culture sample (25 µl) three times and was spun at 800 × g at 4°C for 5 minutes to remove the PBS each time. The pelleted blood sample was then resuspended in 0.1% (v/v) saponin in PBS and placed on ice for 10 minutes. Next, to remove the saponin, the sample was centrifuged at 1 800 × g at 4°C for 10 minutes. After that, the sample was washed 4 times with 1X PBS by centrifugation at the same speed and time. Finally, 4 volumes of 1X PBS were used to resuspend the processed samples containing the crude protein lysate and these samples are ready to be used in the sandwich ELISA.

Western blot using PkTRAMP polyclonal antibodies

Both mice and rabbit polyclonal antibodies were tested through Western blot to ensure that the raised antibodies can bind to native PkTRAMP. Crude protein lysates of healthy donor blood, *P. knowlesi*-infected clinical blood sample and *P. knowlesi* culture (1% parasitaemia) were resolved in 12% SDS-PAGE with PkTRAMP purified protein (50 ng) as positive control under reducing condition. Electrophoresis was run to separate the proteins and then transferred to polyvinylidene difluoride (PVDF) membrane. Blocking of membranes were conducted overnight in 5% blocking buffer (5% skimmed milk in Tris buffered saline (TBS)) at 4°C. The membranes were placed in two separate containers and then probed with mice anti-PkTRAMP polyclonal antibodies (10 µg/ml) and rabbit antibodies (10 µg/ml) separately in 2.5% blocking buffer. They were incubated at room temperature for 1 hour. Next, 0.2% tween-20 in TBS (TBST) were used to wash the membranes 3 times with and incubated for 1 hour at room temperature with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2 500 dilution) for rabbit polyclonal antibody. When mice polyclonal antibody was tested, biotin labelled goat anti-mouse IgG (1:2 500 dilution) was used, followed by alkaline phosphatase conjugated streptavidin (1:2 500 dilution), both incubated for 1 hour at room temperature. Finally, 3,3',5,5'-tetramethylbenzidine (TMB) was used to develop the colour for rabbit polyclonal antibody test and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) for mice polyclonal antibody test.

Limit of detection of the sandwich ELISA using purified recombinant protein and *in vitro* culture

A serial concentration of the purified recombinant PkTRAMP from 40 000 pg/ml to 78 pg/ml were tested to generate the standard curve for limit of detection (LOD) determination. In this test, 1X PBS was used as the blank. Three technical replicate assays were run on different days for reproducibility. From the result obtained, the best concentration of PkTRAMP to be utilised as the positive control during clinical evaluation was selected. The same procedure was conducted using A1H1 *P. knowlesi* culture lysate to determine the LOD of the sandwich ELISA. Culture was diluted to parasitaemia ranged from 3% to 0.0005% using parasite-free whole blood in 50% haematocrit. The parasite lysate was prepared using the method described above. The LOD was derived from the plotted standard curve by calculating the $OD._{LOD}$ as shown below (Dixit *et al.,* 2011).

$$OD._{LOD} = AVG._{Blank} + (2\ STD._{Blank})$$

where,
$OD._{LOD}$ = Absorbance corresponding to the LOD
$AVG._{Blank}$ = Average absorbance of blank
$STD._{Blank}$ = Standard deviation of blank

Sandwich ELISA for PkTRAMP detection

In this sandwich ELISA, mice polyclonal antibodies were utilised as capture antibodies and rabbit polyclonal antibodies for detection. First, 50 µl of 1 µg/ml mice anti-PkTRAMP polyclonal antibodies in sodium bicarbonate buffer (pH 9.6) were loaded in the 96-well microplate and incubated overnight in 4°C. The plate was blocked with 1% (w/v) BSA in PBS for 2 hours at 37°C and then washed 3 times with 0.1% Tween-20 in PBS (PBST). The abovementioned prepared blood samples (50 µl) were added in duplicates into the wells and incubated in 37°C for 1 hour. After that, the wells were washed 3 times and incubated with 50 µl of 1 µg/ml rabbit anti-PkTRAMP polyclonal antibodies in 1% BSA in PBS for 1 hour at 37°C. Next, the plate was incubated with 50 µl of HRP-conjugated goat anti-rabbit IgG (1:2 500 dilution) for 1 hour at 37°C after it was washed 5 times with 0.1% PBST. After 5 times wash, TMB (50 µl) were added into the wells then incubated for 25 minutes at room temperature in the dark. Finally, the reaction was stopped using 2 N sulphuric acid (50 µl) and the absorbance was measured at 450 nm.

Data and statistical analysis

The average absorbance measurement of 10 healthy samples plus twice the standard deviation was used to calculate the assay's cut-off value. To evaluate the clinical sensitivity, specificity, positive and negative predictive values of the assay, all archived malaria samples along with a new set of healthy samples (n=18) were tested in reference to nested PCR and microscopy result. The kappa value was used to assess the degree of agreement (McHugh, 2012) of the results between the sandwich ELISA results and the reference techniques.

Ethical approval

Ethics approval for the use of patient samples in this study was granted by National Medical Research Register, Ministry of Health Malaysia (NMRR ID-21-02338-AGZ (IIR)). All patients signed a letter of consent.

RESULTS

Expression and purification of recombinant PkTRAMP

Expression of recombinant PkTRAMP in *E. coli* was successful. As shown in Figure 1A and 1B, the arrows show the recombinant PkTRAMP bands resolved in 12% SDS-PAGE gel stained with Coomassie brilliant blue at its expected size (~38 kDa) during expression and after purification process.

Western blot using anti-PkTRAMP polyclonal antibodies

The binding ability of anti-PkTRAMP mice and rabbit polyclonal antibodies towards native PkTRAMP present in *P. knowlesi* A1H1 culture and archived patient's blood sample were assessed. Results showed that both mice and rabbit polyclonal antibodies were reactive towards the crude protein lysates from *P. knowlesi* culture and clinical sample (Figure 2A and 2B) whereby there were several bands observed in their respective lanes. Both antibodies detected the recombinant PkTRAMP at its expected size ~38 kDa (Lanes 4 in Figure 2A and 2B). A band of ~40 kDa appeared in *P. knowlesi* culture (Lane 3, Figure 2A) when rabbit polyclonal antibodies were used. In Figure 2B, protein bands of the same size were also observed in *P. knowlesi* patient archived blood (Lane 2) and *P. knowlesi* culture (Lane 3) when probed using mice polyclonal antibodies. Nevertheless, both rabbit and mice polyclonal antibodies did not react to healthy blood protein extract (Lanes 1 in Figure 2A and 2B).

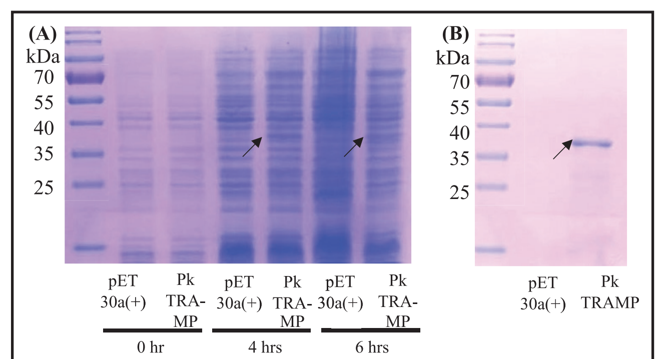

Figure 1. SDS-PAGE gel images. Migrated proteins from *E. coli* crude lysate collected at 0 hr, 4 hrs, and 6 hrs intervals during recombinant PkTRAMP expression (A). Recombinant PkTRAMP band after purification (B). Arrows show the recombinant PkTRAMP bands at expected size ~38 kDa. pET30a(+) is an empty vector served as the negative control.

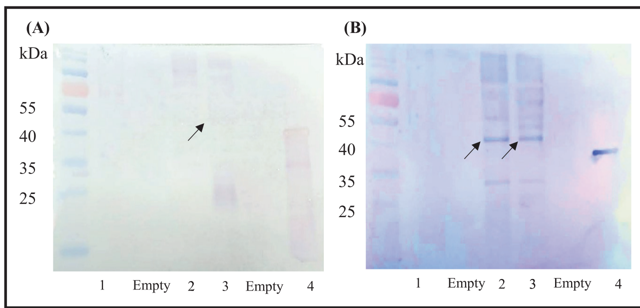

Figure 2. Western blot results demonstrating the binding capability of the polyclonal antibodies to recombinant and native PkTRAMP in *P. knowlesi* infected erythrocyte lysates. Lane 1. uninfected blood protein extract, Lane 2. *P. knowlesi*-infected patient sample, Lane 3. *P. knowlesi* A1H1 culture (1% parasitaemia) and Lane 4. recombinant PkTRAMP (50 ng). Rabbit polyclonal antibodies (A). Lane 1, no band observed. Lane 2, several bands were observed. Lane 3, ~40 kDa band size was observed (arrow). Lane 4, recombinant PkTRAMP at ~38 kDa was observed. Mice polyclonal antibodies (B). Lane 1, no band observed. Lane 2 & 3, several bands were observed including ~40 kDa bands (arrows). Lane 4, recombinant PkTRAMP at ~38 kDa was observed.

PkTRAMP sandwich ELISA

The LOD for the recombinant PkTRAMP determined the concentration of recombinant PkTRAMP to be utilised as positive control in the sandwich ELISA. The standard curve shown in Figure 3 was plotted from the data of 3 replicates and their detection limit were determined. It was calculated that the LOD of the assay was at 804.5 pg/ml of recombinant PkTRAMP. The absorbance at this point is very much lower than the cut-off of the sandwich ELISA (OD=0.2408) obtained from the readings of 10 healthy samples. Hence, the most suitable concentration to be used as the positive control is 2 500 pg/ml of the recombinant protein as it has slightly higher absorbance and distinguishable from the healthy samples' response. On the other hand, three replicates were conducted to determine the LOD towards native PkTRAMP from the erythrocyte lysate of *P. knowlesi* culture. The LOD of the assay obtained from the standard curve of parasitaemia against absorbance generated was at 0.015% parasitaemia (Figure 4).

Figure 5 illustrates the absorbance point of each clinical sample tested by PkTRAMP sandwich ELISA and Table 1 shows the result summary of this assay. It demonstrated 76% sensitivity detecting 31 out of 41 *P. knowlesi* samples. Moreover, its specificity was at 75% where 30 non-*P. knowlesi* samples (18 healthy, 10 *P. falciparum* and 2 *P. vivax* samples) were undetected by the assay. The remaining 10 *P. vivax* samples were tested positive in the assay, signifying cross reactivity. Its positive and negative predictive values were 76% (31/41) and 75%, (30/40), respectively. This PkTRAMP sandwich ELISA has a moderate agreement with the reference method reflected by a kappa value of 51%. Table 2 recorded the clinical performance values of this assay. Additionally, Spearman's correlation test revealed there was a fair correlation (rs=0.309, p=0.055) between absorbance and parasitaemia when *P. knowlesi* clinical samples were tested. Figure 6 shows the plot of absorbance against the parasitaemia of *P. knowlesi* clinical samples. On the other hand, there was a strong correlation (rs=0.965, p<0.001) observed between the two variables when *P. knowlesi* culture was used.

As the current sandwich ELISA cross reacted significantly with *P. vivax*, the clinical performances of the assay were evaluated for the detection of *P. knowlesi* and *P. vivax* concurrently. The sensitivity and specificity for the were 77% (41/53) and 100% (28/28), respectively. The positive and negative predictive values were at 100% (41/41) and 70% (28/40), respectively. The agreement with the reference method was increased with kappa value of 70% indicating a good level of agreement.

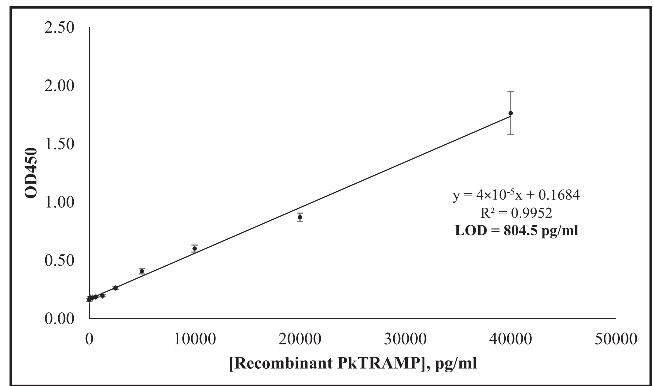

Figure 3. Plot of the mean absorbance versus recombinant PkTRAMP concentration of the 3 replicate assays.

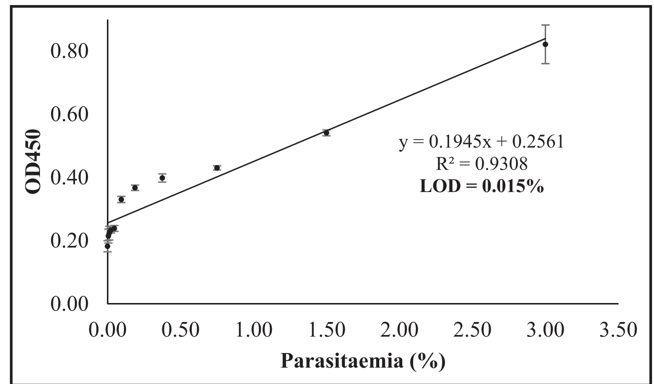

Figure 4. Plot of the mean absorbance versus parasitaemia of *P. knowlesi* culture from 3 replicate assays.

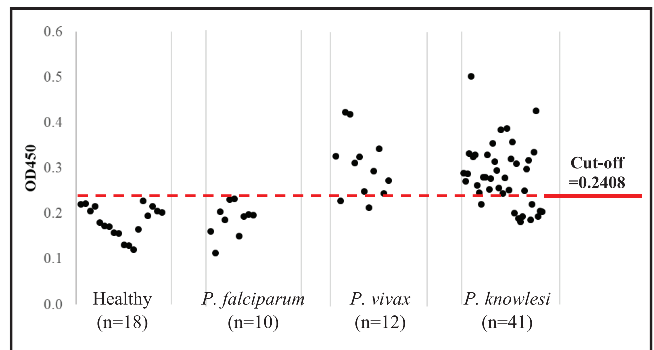

Figure 5. Data points of clinical samples assessed with PkTRAMP sandwich ELISA. Dashed line is the cut-off point at OD_{450}=0.2408.

Table 1. Detection of *P. knowlesi* using PkTRAMP sandwich ELISA method

Blood samples	PkTRAMP Sandwich ELISA result	
	Detected samples	Undetected samples
P. knowlesi (n=41)	31	10
Non-*P. knowlesi* samples		
Healthy (n=18)	0	18
P. falciparum (n=10)	0	10
P. vivax (n=12)	10	2

Table 2. Clinical performance of PkTRAMP sandwich ELISA for *P. knowlesi* detection in reference to microscopy and nested PCR

Test	Calculation	Value
Sensitivity	$\frac{31}{31 + 10} \times 100\%$	76%
Specificity	$\frac{30}{30 + 10} \times 100\%$	75%
Positive predictive value	$\frac{31}{31 + 10} \times 100\%$	76%
Negative predictive value	$\frac{30}{30 + 10} \times 100\%$	75%

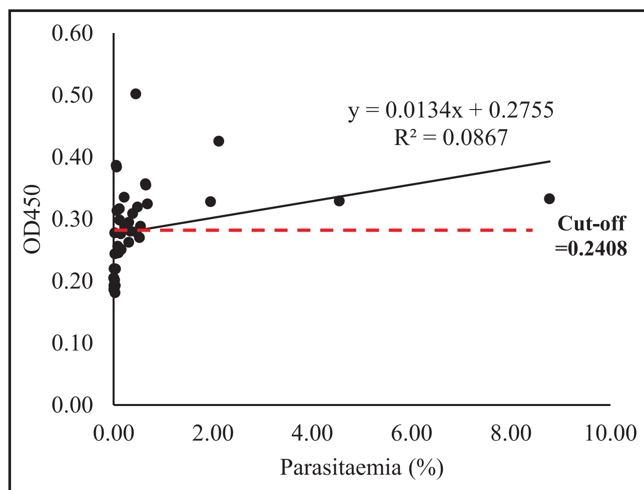

Figure 6. Plot of absorbance versus parasitaemia of *P. knowlesi* clinical samples tested with PkTRAMP sandwich ELISA. Dashed line is the cut-off point at OD_{450}=0.2408.

DISCUSSION

In this study, sandwich ELISA method was chosen in hope to increase the sensitivity and specificity. With the use of two antibodies raised from different animal models, they might recognise different epitopes on the antigen. This could reduce antibodies competition for the same binding sites and simultaneously increase the chance of antigen-antibody binding by recognising more epitopes. In addition, the polyclonal antibodies used exhibit multi-epitope binding properties which will increase the probability for successful antigen-antibody binding (Ascoli & Aggeler, 2018). Approximately ~40 kDa protein bands were observed in both *P. knowlesi* samples' lanes (clinical and culture sample) in the Western blot of mice and rabbit anti-PkTRAMP polyclonal antibodies. These protein bands might resemble of the native PkTRAMP which is a 43 kDa protein in its natural form. The other faint unknown bands appeared in *P. knowlesi* lanes may be the result from nonspecific binding to other proteins in the total protein lysates, or the binding of the antibodies to the denatured forms of the targeted protein due to external factors during sample preparations or storage.

It is preferred for an assay to have a low LOD to enable detection of parasite even at low parasitaemia. The detection limit of microscopy is 0.001% parasitaemia while the LOD of PCR is 0.00002% to 0.0001% parasitaemia (Tangpukdee *et al.,* 2009; Britton *et al.,* 2016). The Malaria Ag CELISA (Cellabs, Australia), a commercialised sandwich ELISA for *Plasmodium* sp. detection has an LOD of 0.001% parasitaemia. It is noted that the LOD of the current sandwich ELISA is higher when compared with the reference methods and the commercialised sandwich ELISA. However, because Malaria Ag CELISA uses monoclonal antibodies for both capture and detection in this instance, enable it to have a considerably lower LOD. Khusmith *et al.* (1992) utilised both monoclonal antibodies (MAb) and polyclonal antibodies (PAb) in sandwich ELISA for *P. vivax* detection in their study. Two pairs of capture-detection antibodies were examined; MAb-MAb and PAb-MAb. It was discovered that the MAb-MAb (0.007% parasitaemia) pairing in their sandwich ELISA had a LOD significantly lower than the pairing of PAb-MAb (0.27% parasitaemia). Evidently, the detection limit of an assay may be lowered with the use of monoclonal antibodies. Hence, monoclonal antibodies may be helpful to improve the LOD of this PkTRAMP sandwich ELISA. Besides, monoclonal antibodies may offer minimal false positive reactions, reduced cross reactivity, reproducibility and enable standardisation in testing (Yokoi *et al.,* 2002; Joshi *et al.,* 2005).

In this current study, there was no evidence of cross-reactivity with healthy and *P. falciparum* samples, but it was observed in *P. vivax* samples. This is probably due to high amino acid sequence homology of *P. knowlesi* TRAMP with *P. vivax* TRAMP (88% homology). On the other hand, *P. falciparum* TRAMP shared 60% homology with PkTRAMP. As such, higher protein homology increases the chance of cross reactivity as the proteins may have similar binding sites for antibody binding. Serological cross reactivity among antibodies against the other proteins of *P. knowlesi* and *P. vivax* such as merozoite surface protein-1_{42}, apical membrane antigen-1 and GPI-anchored micronemal antigen were also observed in other studies (Drew *et al.,* 2004; Muh *et al.,* 2018*,* 2020). Additionally, with the use of polyclonal antibodies that are capable to recognise multiple epitopes (Lipman *et al.,* 2005), the susceptibility for unspecific binding increases (Cao *et al.,* 2003). Regardless, this sandwich ELISA exhibited 100% specificity towards healthy samples which highlights its ability to distinguish uninfected samples from malaria samples. As this sandwich ELISA cross reacted significantly with *P. vivax*, the assay may be useful for simultaneous detection of *P. knowlesi* and *P. vivax* since the predominant human malaria species in the Asian region is *P. vivax,* while *P. knowlesi* is endemic to Southeast Asia, especially Malaysia. Its effectiveness was highlighted by the fact that the overall performance has improved when identifying *P. vivax* and *P. knowlesi* than *P. knowlesi* alone. Nonetheless, it is crucial to enhance the sensitivity of the assay to above 90% (Parikh *et al.*, 2008).

It was found that *P. knowlesi* clinical samples that exhibited parasitaemia below or slightly higher than 0.015% were tested negative in the sandwich ELISA (parasitaemia ranging 0.001% to 0.037%). Markedly, the levels of parasitaemia in the of these samples did not precisely match their absorbance values. So, the parasitaemia of these samples were not accurately corresponded to the concentration of the targeted protein in the samples. This was corroborated by Spearman's correlation test which revealed that there was only a fair correlation between the parasitaemia and absorbance when testing *P. knowlesi* clinical samples. These samples, including the samples with parasitaemia higher than the LOD but were tested negative, may have undergone the freeze-thaw effect that led to protein degradation. Additionally, fluctuations in temperature during the transportation and storage of the samples may also contribute to the cause of protein degradation. There was evidence in previous study that showed a significant reduction of absorbance in sandwich ELISA for *P. falciparum* HRP2 samples that had undergone 5 cycles of freeze-thawing (Kifude *et al.,* 2008). When fresh P. *knowlesi* culture was used, strong correlation was observed between the absorbance and parasitaemia, postulating that using fresh clinical samples might improve the clinical performance of the sandwich ELISA.

This test has demonstrated enticing sensitivity in detecting *P. knowlesi*, despite its performance appearing to be inferior to other currently available diagnosis techniques. Nevertheless, high sensitivity is a crucial factor in diagnosis of *P. knowlesi* malaria, which calls for early identification and timely treatment to prevent the illness from getting worse. By using target antigen-affinity purified polyclonal antibodies, the assay's clinical performance may be enhanced. Monoclonal antibodies can also be the alternative as they are sensitive to a specific epitope, sustainable and reproducible. With the use of these antibodies, the background interference of this sandwich ELISA may be reduced and therefore enhancing the signal from positive sample. The limitation of this study is that *P. ovale* and *P. malariae* samples were not tested using this sandwich ELISA due to limited samples since these samples are rare as there were zero indigenous human malaria occurrence since 2018 in Malaysia (WHO, 2023).

CONCLUSION

In conclusion, PkTRAMP has the attributes as a biomarker for *P. knowlesi* detection. Even so, the full potential of anti-PkTRAMP antibodies has yet to be explored. Further studies need to be conducted to enhance the sandwich ELISA performance. This may be achieved by utilising monoclonal and/or affinity purified polyclonal antibodies. The use of fresh clinical blood samples may also help to avoid the possibility of acquiring false negative results.

Conflict of interests

The authors declare that they have no conflict of interest.

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