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Development and evaluation of a recombinant *Bartonella henselae* outer membrane protein (BHp26)-based enzyme-linked immunosorbent assay (ELISA) for serodiagnosis of cat scratch disease

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ABSTRACT

Aims: The diagnosis of cat scratch disease (CSD), a disease caused by *Bartonella henselae*, is challenging and often hampered by the lack of appropriate laboratory assays in developing countries due to limited resources. Currently, the indirect immunofluorescence assay (IFA) is the mainstay for CSD diagnosis. However, IFA kits are costly as limited samples can be tested on one slide and reading of the immunofluorescence results is subjective. In this study, the sensitivity and specificity of a recombinant *B. henselae* outer membrane protein (BHp26)-based enzyme-linked immunosorbent assay (ELISA) was assessed for serodiagnosis purposes.
Methodology and results: *Bartonella henselae* outer membrane protein (*BHp26*) gene was cloned into a pBAD-TOPO expression plasmid and transformed into a TOP10 *Escherichia coli* host. The recombinant protein BHp26 was purified using an affinity chromatography approach in an AKTA purifier 10 system. The immunogenicity of the purified recombinant protein was evaluated using Western blot (WB). A recombinant outer membrane protein-based enzyme-linked immunosorbent assay (ELISA) was developed for detection against *B. henselae* antibodies in human sera. The recombinant protein-based ELISA demonstrated 57.7% agreement and 25% sensitivity as compared to IFA. A high specificity (94%) was exhibited when the ELISA was tested against 50 patients' sera with positive findings to other infectious causes, including dengue, rickettsiae, leptospira, legionella and mycoplasma. Using the ELISA developed in this study, 14% (7/50) of urban blood donors and 9.1% (5/55) of healthy individuals from rural areas had IgG antibodies detected against *B. henselae*, suggesting previous exposure to the pathogen.
Conclusion, significance and impact of study: In view of the rising incidence of CSD, the recombinant outer membrane protein-based ELISA will be helpful for screening a large sample size of human sera for serosurveillance study.

Keywords: Bartonella henselae, cat scratch disease (CSD), outer membrane protein (p26), Western blot, ELISA

INTRODUCTION

Cat scratch disease (CSD), a disease caused by *Bartonella henselae*, is featured primary granulomatous skin lesions and acute regional lymphadenopathy after a cat scratch or bite (Breitschwerdt, 2017). The disease affects many organs, including the eyes, liver, central nervous system and bones, and may present as bacillary angiomatosis, peliosis hepatis and bacteremia among immunocompromised individuals (Bass *et al.*, 1997; Cheslock and Embers, 2019). Complications such as prolonged fever, hepatosplenic disease, encephalopathy and ocular disease have been reported (Florin *et al.*, 2008; Chomel *et al.*, 2009; Cheslock and Embers, 2019; Kalogeropoulos *et al.*, 2019). Fleas, lice and ticks are known to harbour bartonellae; hence, people who have direct contact with infected animals and animal ectoparasites such as fleas and ticks are at high risk of contracting the disease (Breitschwerdt, 2014; Cheslock and Embers, 2019). High *B. henselae* seropositivity has been reported in populations with frequent animal contact, including veterinarians, cattle breeders, farmers, forestry workers and aboriginal people (Sayin-Kutlu *et al.*, 2012; Blacksell *et al.*, 2015; Zając *et al.*, 2015; Cheslock and Embers, 2019).

The serological approach is the mainstay of CSD diagnosis as *B. henselae*, a risk group 2 pathogen, is a Gram-negative, intraerythrocytic and fastidious organism that is not routinely cultured in the clinical laboratory. The indirect immunofluorescence assay (IFA), which makes use of *B. henselae* whole-cell antigen, is available commercially as a reference diagnostic method for *Bartonella* infection. However, as limited samples can be tested on a single IFA slide and reading of IFA results are

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often subjective, alternative serological assay, such as enzyme-linked immunosorbent assay (ELISA) that enable multiple specimens to be tested at one time, should be explored.

ELISA is an automated semiquantitative assay that provides objective determination of antibody detection for many infectious diseases. As recombinant protein antigens can be expressed on a large scale in *Escherichia coli*, this offers an advantage bypassing the need to culture fastidious organisms for the preparation of native antigens. Hence, this has prompted us to explore the development of recombinant outer membrane protein-based enzyme-linked immunosorbent assays for serological studies of *B. henselae* infection. A previous study has shown the potential use of an ELISA (IgG) based *B. henselae* 26-kDa immunogenic protein (p26) for diagnosis of *B. henselae* infection in naturally-infected cats (Werner *et al.*, 2008). This study aims to investigate the immunoreactivity of the *B. henselae* recombinant protein (BHp26) in humans and the feasibility of developing recombinant outer membrane protein (BHp26)-based ELISA to facilitate serodiagnosis of *B. henselae* infection.

MATERIALS AND METHODS

Human serum samples

Ethical approval (MEC ID No.: 20159-1658) was obtained for the use of human serum samples prior to the commencement of this study. Positive and negative sera were randomly chosen from patients' samples stored at the Diagnostic Microbiology Laboratory, University Malaya Medical Center, after prescreening using a commercial IFA kit (Focus Diagnostics, USA). The tests were performed in accordance with the manufacturer's instruction, using *B. henselae* cultivated in Vero cells that were fixed on the IFA slides (supplied in the kit). The samples included (i) 32 *B. henselae* IFA-negative patients' serum samples (antibody titre <1:64) for cut-off determination of the cut-off value of ELISA assay and (ii) 26 serum samples obtained from suspected CSD patients (n=17) and healthy individuals (n=9) for comparison of IFA, WB and ELISA methods.

To establish the specificity of the ELISA, 50 serum samples from patients with positive serological findings for dengue (n=10), typhus fever (n=10), leptospirosis (n=10), legionellosis (n=10) and mycoplasma infection (n=10) were evaluated. Additionally, a total of 50 urban blood donors and 55 healthy individuals from rural areas (39 farm workers and 16 aboriginal villagers) collected from a previous study, Kho *et al.* (2017), were also screened using the ELISA.

Cloning and antigenic analysis of *BHp26* gene fragment

An immunodominant outer membrane protein gene (783 bp) of *B. henselae*, abbreviated as *BHp26*, was amplified from *B. henselae* strain used in the IFA kit (Focus Diagnostics, USA). The primers for amplification of BHp26 were designed according to the *B. henselae* sequence (GenBank accession number: DQ270028) published by Werner *et al.* (2006). Amplification was performed in a 25 µL reaction mixture containing forward primer (5' ATGAAAAAAGTAATTTTCAAACCG 3'), reverse primer (5' ATTGATAGCAAATACTATGGTG 3'), Platinum high fidelity *Taq* DNA polymerase (Invitogen, USA), *B. henselae* DNA extract, 10× high PCR buffer, 50 mM $MgSO_4$ and 10 mM dNTP mix. The PCR reaction was started with an initial denaturation at 94 °C for two min, followed by denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 sec, extension at 68 °C for one min for 35 cycles and a final extension step at 72 °C for 10 min in a Veriti thermal cycler (Applied Biosystems, USA). The PCR product was purified using GeneAll Expin DNA purification kit (GeneAll, South Korea) and inserted into the pBAD-TOPO TA expression vector with C-terminal polyhistidine (6xHis) tag (Invitogen, USA), following the manufacturer's instruction. The plasmid was then transformed into an *Escherichia coli* host (One Shot® TOP10 chemically competent *E. coli*). The nucleotide sequence of cloned plasmid was determined using ABI PRISM 377 Genetic Analyser (Applied Biosystems, USA) and analyzed using BioEdit Sequence Alignment Editor Software (version 7.2.5.0) and search for similarity using the BLAST function of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) (Hall, 1999).

The amino acid sequence of the gene insert was submitted to SVMTriP website (http://sysbio.unl.edu/SVMTriP/prediction.php), an online server for linear antigenic epitope prediction, for analysis of the putative antigenic region of BHp26 (Yao *et al.*, 2012). The result of a positive score indicates a putative antigenic epitope.

Expression and purification of BHp26 protein

The optimal arabinose concentration to induce recombinant protein expression was determined by adding 10-fold serially diluted L-arabinose (0.2-0.00002%) into five 10 mL recombinant *E. coli* cultures. After induction for four h at 37 °C, 250 rpm, the cultures were centrifuged and the pellets were suspended in binding buffer and sonicated with 30 sec burst followed by 30 sec cooling on ice for 10 cycles in Branson Ultrasonic instrument (USA). The crude sample was centrifuged at 15 000× *g* for 15 min at 4 °C. The supernatant was then analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The expressed protein was purified using HisTrap FF column (GE Healthcare, USA), which had been pre-packed with Ni Sepharose 6 Fast Flow, in an AKTA purifier 10 system (GE Healthcare, USA) monitored by the UNICORN 5.0 software (GE Healthcare, USA). The column was first equilibrated with binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 40 mM imidazole, pH 7.4) with a flow rate of 5 mL/min prior to the loading of the protein sample. The recombinant protein was collected in

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25 1 mL fractions in elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4) for Western blot analysis.

A purified protein sample was digested using trypsin and the resulting peptides were subjected to MALDI TOF/TOF 5800 mass spectrometer analysis (Sciex, Framingham, USA) (Shevchenko *et al.*, 1996; Shevchenko *et al.*, 2006). The spectra were then analysed to identify the protein from SwissProt sequence databases using Mascot search engine (Perkins *et al.*, 1999).

Western blotting (WB) analysis

Purified proteins separated in the SDS-PAGE gel were transferred to a polyvinylidene fluoride (PVDF) membrane. After immersion in a blocking buffer (PBS, 0.05% Tween 20, 5% skim milk) for an hour followed by washing with PBST (PBS, 0.05% Tween 20) for five min twice, the PVDF membrane was cut into strips and incubated with sera diluted 1:100 with blocking buffer at 4 °C overnight. The membrane strips were washed with PBST for 15 min thrice and immersed in a blocking buffer containing KPL peroxidase-labelled anti-human IgG (H+L) antibody (Sera Care, USA) diluted 1:3000 for two h. HRP-conjugated anti-V5 antibody and anti-His antibody (Invitrogen, USA) were added as positive controls. The strips were washed with PBST for 15 min thrice and stained in DAB (3,3′-diaminobenzidine tetrahydrochloride) solution (Thermo Fisher Scientific, USA) until the desired colour development was achieved.

Development and evaluation of IgG ELISA

A 96-well microplate (Greiner Bio-One, Austria) was coated overnight with 100 µL per well of 20 µg/mL purified protein at 4 °C. After washing thrice with 200 µL PBST (PBS, 0.05% Tween 20) followed by incubation in 200 µL blocking buffer (PBS, 0.05% Tween 20, 5% skim milk) for an hour and washing twice with PBST. Human serum pre-adsorbed with 100 µg/mL of *E. coli* lysate (100 µL per well) was then added to each well and the plate was incubated at room temperature for an hour. After washing four times in PBST, the wells were added with 100 µL KPL peroxidase-conjugated anti-human IgG (H+L) antibody (Sera Care, USA) diluted 1:8000 in blocking buffer. Following washing step four times with PBST, the wells were added with 100 µL ABTS peroxidase substrate system (Sera Care, USA) for color development. The reaction was stopped after incubation in the dark for one h. The absorbance of each well was read at wavelength 405 nm using a microplate reader (BioTek, USA). The above ELISA protocol was optimized using two pooled *B. henselae*-positive and negative patient sera.

Using 32 *B. henselae* IFA IgG-negative patients' sera, the mean OD ± 3SD was determined as the cut-off value for the ELISA. The agreement, sensitivity and specificity of the ELISA were determined by comparing the ELISA results to IFA by using two-by-two contingency tables using 26 IFA-positive and negative sera (Stites and Terr, 1991). Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences) software program, version 28 (SPSS Inc., Chicago, IL) to determine if significant differences exist among various patient groups in terms of the seropositivity against *B. henselae*. Chi-square test was used and p value ≤ 0.05 was considered as statistically significant.

RESULTS

Sequence determination and putative antigenic analysis of *BHp26* gene

Analysis of the cloned *BHp26* gene sequence (GenBank accession number: OP589188) showed the closest similarity (99.5%, 731/735) with that of *B. henselae* strain BM1374165 87 (GenBank accession number: HG969191) of human origin and *B. henselae* strain U4-11 (GenBank accession number: DQ270026) of feline origin. The *BHp26* gene sequence differed by three nucleotides in comparison with *B. henselae* F1 p26 (GenBank accession number: DQ270027), which was expressed as an antigen in a capture ELISA for diagnosis of feline *B. henselae* infection (Werner *et al.*, 2008). Prediction using SVMTriP online server showed the presence of two antigenic epitopes in BHp26 protein, i.e., AKTIAEAADLKLGKVIKINE at the position 183-202 (score=1.0) and VKMAMIALTLLAASPITHAE at the position 14-33 (score=0.556).

Expression of BHp26 protein and mass spectrometry analysis

Two protein fragments with molecular weights of ~25 and ~22.6 kDa were noted in all arabinose-induced *E. coli* cultures except for the culture with the lowest concentration of arabinose (0.00002%). The highest level of the recombinant protein expression was induced with 0.2% arabinose. The ~25 kDa protein was excised for mass spectrometry analysis and the spectra of the expressed protein were searched using MASCOT against *B. henselae* database (Swiss-Prot). The protein was identified as *B. henselae* outer membrane protein (UniProt accession numbers: A0A0R4J8D2) and immunogenic protein (UniProt accession numbers Q0H392 and Q0H391), thus confirming that the protein was correctly expressed.

Comparison of three serological approaches (IFA, Western blot and ELISA) for the detection of *B. henselae* antibodies

The expressed protein (~25 kDa) was obtained for WB and ELISA testing after purification using immobilized metal affinity chromatography. To reduce the background readings generated due to *E. coli* contaminant proteins in BHp26 antigen preparation, serum samples were subjected to pre-treatment with *E. coli* lysates prior to use

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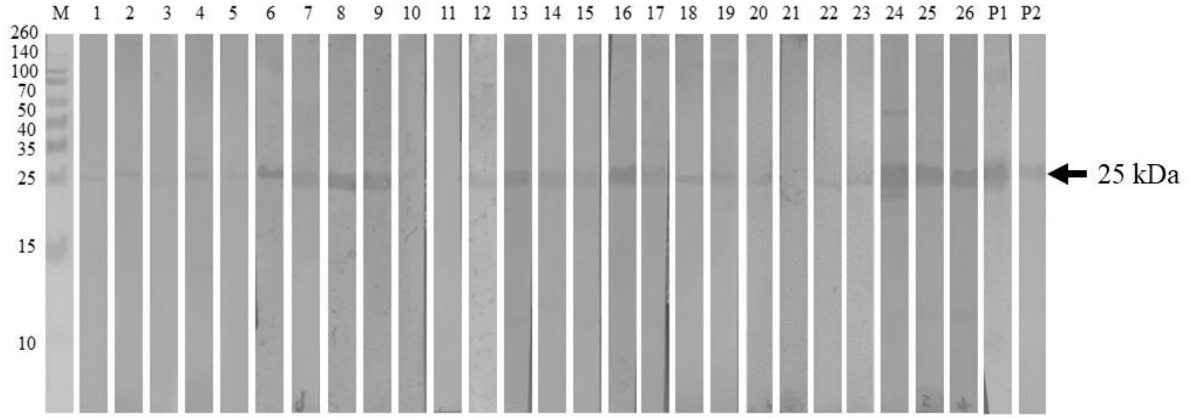

Figure 1: IgG Western blot analysis of BHp26 using sera from 17 patients, six blood donors and three farm workers' sera (refer to Table 1 for the information on the sera tested). Arrows show the position of the purified BHp26. P1, HRP-conjugated anti-V5 antibody; P2, HRP-conjugated anti-Histidine antibody.

Table 1: Comparison of IFA, Western blot and ELISA results of 17 patients, six blood donors and three farm workers.

Lane	Serum reference number	Source	IFA (titres)	Western blot	ELISA	IFA and Western blot	IFA and ELISA	Western blot and ELISA	IFA, Western blot and ELISA
1	Mal	Patient	- (<1:64)	-	-	A	A	A	A
2	8798	Patient	- (<1:64)	+	-	D	A	D	D
3	Jon	Patient	- (<1:64)	-	-	A	A	A	A
4	0878	Patient	- (<1:64)	+	-	D	A	D	D
5	1743	Patient	- (<1:64)	+	-	D	A	D	D
6	4639	Patient	+ (1:64)	+	-	A	D	D	D
7	5016	Patient	+ (1:64)	+	-	A	D	D	D
8	4185	Patient	+ (1:64)	+	-	A	D	D	D
9	2460	Patient	+ (1:64)	+	+	A	A	A	A
10	1490	Patient	- (<1:64)	-	-	A	A	A	A
11	5277	Patient	- (<1:64)	-	-	A	A	A	A
12	2426	Patient	+ (1:64)	+	-	A	D	D	D
13	4946	Patient	+ (1:64)	+	-	A	D	D	D
14	1995	Patient	+ (1:64)	+	-	A	D	D	D
15	6314	Patient	+ (1:64)	+	-	A	D	D	D
16	7972	Patient	+ (1:64)	+	-	A	D	D	D
17	9324	Patient	+ (1:64)	+	-	A	D	D	D
18	9773	Blood donor	- (<1:64)	+	+	D	D	A	D
19	9819	Blood donor	- (<1:64)	+	-	D	A	D	D
20	9754	Blood donor	- (<1:64)	+	-	D	A	D	D
21	9756	Blood donor	- (<1:64)	-	-	A	A	A	A
22	9761	Blood donor	- (<1:64)	+	-	D	A	D	D
23	9765	Blood donor	- (<1:64)	+	-	D	A	D	D
24	SM016	Farm worker	+ (1:64)	+	+	A	A	A	A
25	PS045	Farm worker	+ (1:64)	+	+	A	A	A	A
26	G011	Farm worker	- (<1:64)	+	+	D	D	A	D

-, Negative; +, Positive; A, Agreement; D, Discordant.

in the ELISA in this study. Eight (30.8%) of the 26 serum samples shared the same results with IFA upon testing using WB and ELISA (Table 1). Of 12 *B. henselae* IFA-positive serum samples, all (100%) tested positive by Western blot, but only three (25%) were positive by ELISA.

Figure 1 shows the IgG WB analysis of BHp26 of the serum samples. Of 14 *B. henselae* IFA-negative serum samples, 12 (85.7%) were tested negative by ELISA while only five (35.7%) tested negative by Western blot. The agreement between *B. henselae* IFA and BHp26 WB tests was 65.4% (n=17), while the sensitivity and

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Table 2: Comparison of the agreement, sensitivity and specificity of BHp26 IgG Western blot and ELISA, as compared to *B. henselae* IgG IFA.

Serological test	Agreement	Sensitivity	Specificity
	No. (%)	No. (%)	No. (%)
Western blot	17 (65.4)	12 (100)	5 (35.7)
ELISA	15 (57.7)	3 (25)	12 (85.7)

specificity of BHp26 WB are 100% and 35.7%, respectively. The agreement between *B. henselae* IFA and BHp26 ELISA was 57.7% (n=15), while the sensitivity and specificity of BHp26 ELISA were 25% and 85.7%, respectively (Table 2).

Evaluation of BHp26 IgG ELISA

The ELISA cut-off value (1.506) was determined based on the average OD ± 3SD of 32 *B. henselae* IFA IgG-negative patients' sera. Of 50 serum samples with positive serological findings for other infectious causes, *B. henselae* antibody was only detected from one each of the dengue, typhus fever and leptospiral seropositive patients, using the ELISA. None of the patients seropositive for legionellosis or mycoplasma infection were positive by the ELISA. Hence, a high specificity (94%) of the ELISA was observed in this study. Further evaluation using ELISA showed that 14% (7/50) of urban blood donors and 9.1% (5/55) of healthy individuals from rural areas had IgG antibodies detected against *B. henselae*. However, no significant difference was found in the *B. henselae* seropositivity between these two groups of people (p=0.430).

DISCUSSION

The CSD incidence rises with increasing animal-human contact, especially in areas where there is a high infestation of fleas and ticks (Breitschwerdt, 2014). As the clinical manifestation of *B. henselae* infection mimics many infectious diseases and chronic diseases, CSD may be left underdiagnosed or under-reported in the tropical region (Florin *et al.*, 2008; Noden *et al.*, 2014). The IFA was first introduced for the serodiagnosis of *B. henselae* infection in the 1990s (Regnery *et al.*, 1992). Unfortunately, the assay is not routinely used in clinical laboratories from developing countries due to limited resources, thus adding more challenges in the diagnosis of *B. henselae* infection. A previous study reported that IgG antibodies against *B. henselae* outer membrane protein BHp26 could be a useful serodiagnostic marker for feline infection (Werner *et al.*, 2008). Hence, the potential application of the *B. henselae* BHp26 protein for the serodiagnosis of human infection was assessed for the first time in this study.

The antigenicity of the expressed BHp26 recombinant protein in this study was first determined using the linear antigenic epitope prediction method. Two putative antigenic epitopes in the recombinant protein have been identified in this study. Comparatively, *B. henselae*-infected Vero cells are fixed on IFA slides, allowing the antibodies to bind to the surface antigen in its native form. Thus, many more antigenic sites are exposed for antibody binding with different level of specificity. The limited antigenic site of the recombinant protein may explain the lower sensitivity (25.0%) and agreement (57.7%) of the ELISA as compared to IFA in this study (Table 2). In contrast, the recombinant protein-based ELISA exhibited high specificity (85%) upon evaluation using sera with known IFA status. The specificity of the ELISA was as high as 94% when the assay was subjected to testing using sera from 50 patients who were seropositive to other infectious causes, including dengue, rickettsiae, leptospira, legionella and mycoplasma.

Since a majority of serum samples investigated in this study had low IFA titres (1:64), against B*. henselae*, this might be another reason affecting the ELISA sensitivity. A previous study reported that low-affinity IgM and IgG antibodies generated during early host immune response are not readily detectable in ELISA due to rapid dissociation rates and extensive washing in the procedures (Liang *et al.*, 2007). In this study, WB analysis showed a 100% sensitivity as compared to IFA (Table 2). Hence, it will be ideal to have another serological approach, such as WB, to confirm the diagnosis for CSD, which has been compounded due to the non-specificity of the signs and symptoms which mimic many diseases of infectious and noninfectious etiologies (Klotz *et al.*, 2011). A CSD patient may develop generalized symptoms such as fatigue, myalgias, headaches, generalized pains and insomnia (Rising *et al.*, 2016). After exposure to the host, the organism is known to disseminate hematogenously throughout the body, involving liver, spleen, respiratory tract, gastrointestinal tract, heart, renal and central nervous system (Adal *et al.*, 1994; Florin *et al.*, 2008). In this study, *B. henselae* antibody was detected from one each of the patient groups that were seropositive to dengue, typhus fever and leptospirosis. While the serological cross-reactivity between *Bartonella* and *Rickettsia* species (causative agent for typhus fever) has been documented, cross-reactivity between *B. henselae*, dengue virus or leptospiral organisms has not been reported, thus suggesting a possibility of mixed infection in these patients (Hollingdale *et al.*, 1978; da Costa *et al.*, 2005).

One of the benefits of having ELISA for testing is on its ability to facilitate the screening of a large number of human sera in epidemiology studies. In this study, higher seropositivity of *B. henselae* was noted amongst blood donors from urban areas (14%) than healthy individuals from rural areas (9.1%), albeit no significant difference was found in the *B. henselae* seropositivity betwen these two groups of people. High *B. henselae* seropositivity in

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blood donors has also been reported in Korea (15%) (Kwon *et al.*, 2017), Poland (23%) (Łysakowska *et al.*, 2019) and China (19.6%) (Sun *et al.*, 2010), with significant association with exposure to cat and occupations such as farm dweller and veterinary personnel. As *B. henselae* may cause asymptomatic bacteremia, the detection of antibodies in urban blood donors and healthy individuals from rural areas suggests that exposure to *B. henselae* is not uncommon in the Malaysian population.

CONCLUSION

This is the first study to describe the potential application of a recombinant outer membrane protein (BHp26)-based serological assay for human infection. Due to the variation in the format of serological approaches, this study reports low sensitivity but high specificity of the ELISA in comparison with IFA. Future investigation should include the use of a codon-optimized synthetic gene to improve the level of recombinant protein expression. There is clearly a need to improve the sensitivity of the ELISA by including more target antigen epitopes, or to include western blot to enhance the diagnostic accuracy of CSD.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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