PRODUCTION AND EVALUATION OF *RICKETTSIA TYPHI* RECOMBINANT 17-KDA PROTEIN FOR SERODIAGNOSIS OF MURINE TYPHUS

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ABSTRACT

Murine typhus or endemic typhus is a rickettsiosis caused by *Rickettsia typhi* and transmitted to human via fleas from rodents, cats and dogs. Common clinical manifestations are fever, malaise, myalgia, nausea, and vomiting, with presence or absence of rash and eschar, and lymphadenopathy, which also present in other febrile illnesses such as scrub typhus, leptospirosis and melioidosis. Murine typhus can be found worldwide and its re-emergence has been reported in Southeast Asia. Serodiagnosis of murine typhus currently relies on an immunofluorescence assay (IFA). Despite precision and accuracy, IFA uses antigens prepared from intracellular culture of *R. typhi* which requires biosafety level 3 facility. The use of whole cell lysate antigens also leads to the difficulty in protocol standardization. Thus, a development of sensitive, specific, standardizable, rapid and affordable murine typhus serodiagnostic method is needed. In this study, laboratory scale-production of recombinant 17-kDa antigen of *R. typhi* (rRty17-kDa) was successfully done using bacterial cloning system, confirming by Western blot and mass spectrometry. Purified rRty17-kDa was evaluated for its reactivity with patient sera positive for antibodies to murine typhus, scrub typhus, leptospirosis and melioidosis by indirect ELISA. Results showed cross-reactivity of rRty17-kDa with sera from healthy donors, leptospirosis and melioidosis patients. Sensitivity of 15.0% and 65.0%, and specificity of 87.5% and 42.5% for IgG and IgM ELISA were observed, respectively, which overall do not meet the requirement for an ideal diagnostic test. The results suggested an inadequacy of rRty17-kDa for a potent murine typhus serodiagnosis in its present form.

Keywords: Murine typhus, *Rickettsia typhi*, recombinant protein, 17-kDa outer membrane protein, ELISA

INTRODUCTION

Murine typhus or endemic typhus has historically been a long-known rickettsial disease.

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Its outbreaks have been reported worldwide, especially in Southeast Asian, Mediterranean, and southern United States regions (Chaliotis *et al*, 2012). The disease is caused by *Rickettsia typhi*, a small obligated intracellular Gram-negative bacterium of the family Rickettsiaceae. Transmission of *R. typhi* generally involves rats and the rat flea, *Xenopsylla cheopis*, although the cat flea, *Ctenocephalides felis*, was also reported as a competent vector (Azad *et al*, 1997). In some areas, cats, dogs, rodents and opossums were found to act as reservoirs of the disease vectors (Blanton

et al, 2016; Civen and Ngo, 2008; Liddell and Sparks, 2012). Murine typhus usually produces mild or self-limiting symptoms lasting for 3-7 days. Fever, rash and headache are typical. Chill, myalgia, malaise and anorexia can be presented. Complications such as anemia, hematuria or proteinuria may also be seen in lower frequency (Tsioutis *et al*, 2017). Reported mortality rate was low as 1% with use of appropriate antibiotics, and 4% without use of antibiotics (Civen and Ngo, 2008). Unusual and fatal manifestations have been reported (Carr *et al*, 2014; Hudson *et al*, 1997; Perez *et al*, 2018; Sakamoto *et al*, 2013; Malheiro *et al*, 2017). Due to the non-specific clinical representations shared with other febrile illnesses such as dengue, leptospirosis, typhoid and melioidosis, murine typhus remains under-appreciated and under-diagnosed (Paris and Dumler, 2016). A quick and accurate diagnosis is importantly required for facilitation and administration of the disease treatment.

Diagnosis of murine typhus and other rickettsioses can be done by bacterial culture, molecular detection of the bacterial DNA and serological diagnosis. Bacterial culture is useful for a definitive diagnosis but the technique takes times, requires an invasive sample collection and a high biosafety level facility. Nucleic detection methods such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) assays are developed for rickettsial detection in whole blood and buffy coat. Primers were designed for pan-rickettsial and species-specific detection (Luce-Fedrow *et al*, 2015; Parola *et al*, 2005; Robinson *et al*, 2019). Real-time assay was also developed (Renvoisé *et al*, 2012). Frequently used gene targets include 16S rRNA, citrate synthase (*gltA*), 17-kDa lipoprotein, and outer membrane protein (*omp*A and *omp*B). None of the genes have been proved to be more effective than others (Luce-Fedrow *et al*, 2015; Paris and Dumler, 2016; Renvoisé *et al*, 2012).

A classical immunoassay to detect *Rickettsia* spp. is Weil-Felix test which based on a detection of antibodies to *Proteus* bacterial antigens that cross-react with rickettsiae (Cruickshank, 1927). The test lacks sensitivity and specificity and generally no longer recommended. The adaptation of serological methods for rickettsial diagnosis includes rapid diagnostic tests (RDTs) based on immunochromatographic assay and dot blot, enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA). IFA is considered as the gold standard method for rickettsial serodiagnosis. It is used to detect both IgM and IgG in patient sera against rickettsial antigens. Four-folded seroconversion in paired-sera is used to justify the infection (Dhawan *et al*, 2020; Paris and Dumler, 2016). However, in resource-limited settings, IFA can be considered expensive, needs a fluorescence microscope and training of personnel to conduct the test. A biosafety level 3 facility is required for the intracellular bacterial culture for antigen preparation; and the antigen purification and preparation processes are technically challenging. These limitations lead to a need for development of a more affordable diagnostic tool. ELISA is a widely used serologic assay that can also applied for both IgG and IgM detection. Ability of screening large number of sera samples, relatively low cost, technically easy to perform, and reproducibility of results are some interesting features of ELISA to contemplate for successful serodiagnosis (Luce-Fedrow A *et al*, 2015; Robinson *et al*, 2019). ELISAs for rickettsial serodiagnosis, especially scrub typhus, have been developed using whole bacterial cell and recombinant protein antigens (Chao *et al*, 2011; Dasch *et al*, 1979; Robinson *et al*, 2019). For murine typhus, an outer membrane protein B (OmpB) of *R. typhi* was identified as an immunodominant antigen and its recombinant protein was expressed in *E. coli* expression system and tested for murine typhus serodiagnosis (Chao *et al*, 2008). ELISA for murine typhus diagnosis based on recombinant OmpB is also available with an excellent sensitivity and specificity for combined IgM and IgG tests on paired specimens (95.0% and 98.3%, respectively), however, sensitivity of the IgM ELISA from acute specimens only was poor (45.0%) (Lokida *et al*, 2020).

Based on the lack of an ideal diagnostic test for murine typhus and intention to improve a test for the disease diagnosis, this study aimed to produce recombinant 17-kDa protein from *R. typhi*. 17-kDa protein has been first described in *Rickettsia rickettsii*, the causative agent of Rocky Mountain Spotted Fever (Anderson *et al*, 1987). The protein has been identified as a surface exposed lipoprotein presented on outer membrane of *Rickettsia* spp. including *R. typhi*, and absent in members of other genera such as *Bacillus*, *Proteus*, *Neisseria* and *Escherichia* (Anderson, 1990; Anderson and Tzianabos, 1989). Antigenicity of the rickettsial 17-kDa protein was suggested by its reactivity with immune sera from experimentally immunized animals or infected human (Anderson, 1990). Our preliminary Western blot data also showed that *R. typhi* whole cell lysate antigens reacted with pooled murine typhus-positive sera with a strong reactive band of size smaller than 20 kDa, suggesting for the protein as a good target in murine typhus serodiagnosis. To our knowledge, the *R. typhi* 17-kDa protein had not been evaluated before for using in serodiagnosis, therefore, we chose *R. typhi* 17-kDa protein as a target for protein expression in this study. The protein was produced through the procedure of gene cloning and subsequent expression of the protein using *Escherichia coli* as expression vector. A potential sensitivity, specificity and cross reactivity of the recombinant protein for detection of murine typhus infection by ELISA was evaluated using rickettsioses and other febrile disease sera.

MATERIALS AND METHODS

PCR amplification of *R. typhi* 17-kDa surface antigen gene

R. typhi was cultured and inactivated in a biosafety level 3 facility at Mahidol-Oxford Research Unit, Faculty of Tropical Medicine, Mahidol University and provided as bacterial pellet. Genomic DNA of *R. typhi* was extracted from the bacterial pellet using TIANamp genomic DNA kit (TIANGEN Biotech, Beijing, China) and used as a template for PCR amplification of 17-kDa surface antigen gene.

PCR primers were designed to amplify a 418 bp fragment of *R. typhi* 17-kDa (Rty17-kDa) gene (GenBank accession number AE017197.1). A forward primer was 17kDa: 5´-TAATGG*CTCGAG*TGGTATGAACAAACA-3´ containing *Xho*I restriction site. A reverse primer was rev17kDa: 5´-ATTCGC*GAATTC*CCATTGCCCGTCAGG-3´ containing *Eco*RI restriction site. PCR amplification was accomplished in 25 μL of PCR reaction containing 12.5 μL of 2´ Go-Taq Green Master Mix (Promega Corporation, Madison, WI, USA), 1 μL of 10 μM 17kDa primer, 1 μL of 10 μM rev17kDa primer, 1 μL of *R. typhi* genomic DNA and 9.5 μL of sterile DW. DNA amplification condition was set up as: initial denaturation at 95°C for 2 min, follows by 30 cycles of subsequent denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, and final extension at 72°C for 5 min. PCR product was mixed with Novel Juice DNA Gel staining reagent (GeneDireX, Inc., Taoyuan, Taiwan), resolved on 1.5% agarose gel, and visualized in a gel documentation system (Molecular Imager Gel Doc XR+ imaging system, Bio-Rad, Hercules, CA, USA).

Construction of recombinant plasmid containing *R. typhi* 17-kDa surface antigen gene

Rty17-kDa PCR fragment was purified using TIANquick MIDI Purification Kit (TIANGEN Biotech). pRSET B vector (Thermo Fisher Scientific, Waltham, MA, USA) was purified from *E. coli* host using PureLink Quick Plasmid Miniprep Kit (Thermo Fisher Scientific). The purified PCR product and vector were double digested with *Xho*I and *Eco*RI enzymes (New England Biolabs, Ipswich, MA, USA), purified, and ligated in Mighty Mix (Takara Bio Inc., Shiga, Japan) to produce recombinant plasmid containing Rty17-kDa gene incorporated with a 6× Histidine-tag at the 5' end. The ligation mix was transformed into *E. coli* DH5a host strain by electroporation using EC100 Electroporator (E-C Apparatus Corporation, Philadelphia, PA, USA). The electroporation condition was 1 pulse at 1.8 kV in 0.1 cm cuvette.

Positive *E. coli* clones were selected by ampicillin resistance on LB agar containing 100 μg/mL ampicillin (LB-Amp agar). The presence of Rty17-kDa gene fragment was confirmed by PCR and nucleotide sequencing. The recombinant plasmid was named pRSET-rRty17-kDa.

Recombinant *R. typhi* 17-kDa protein expression and purification

Sequencing confirmed pRSET-rRty17-kDa was transformed into BL21 (DE3) pLysS *E. coli* expression host by heat-shock method. Positive clones were selected by ampicillin and chloramphenicol resistance on LB agar containing 100 μg/mL ampicillin and 40 μg/mL chloramphenicol (LB-Amp-Chloram), and confirmed by PCR. The bacteria were named BL21-pRSET-rRty17-kDa.

Recombinant *R. typhi* 17-kDa protein (rRty17-kDa) expression was induced in BL21-pRSET-rRty17-kDa *E. coli* cultured in 200 mL LB-Amp-Chloram broth by addition of isopropyl b-D-1-thiogalactopyranoside (IPTG) to 1 mM final concentration, and incubated at 37°C for 3 h at 180 rpm in a shaking incubator. After induction, the bacterial cell pellet was collected by centrifugation at 8,000 ×*g* for 15 min at 4°C and resuspended in 3 mL lysis-equilibration-wash (LEW) buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). Three milliliters of B-Per™ Complete, Bacterial Protein Extraction Reagent (Thermo Fisher Scientific) was added, and the mixture was mixed by pipetting and incubated for 2 h at room temperature with gentle rocking. The mixture was centrifuged at 8,000 ×*g* for 15 min at 4°C, and the supernatant were collected as "supernate". Five milliliters of a freshly prepared 8 M urea were added to the remaining cell pellet and mixed by pipetting. The cell lysate was centrifuged and the supernatant was collected as "urea 1". Three milliliters of 8 M urea were added to the remaining cell pellet and mixed by pipetting. The cell lysate was centrifuged and the supernatant was collected as "urea 2". The small amount of residue cell pellet was mixed with 500 μL of 8 M urea by pipetting and collected as "pellet". Supernate, urea 1, urea 2 and pellet were

resolved by SDS-PAGE on 10% separating gel and stained with Coomassie brilliant blue. A presence of 6× Histidine-tagged protein was determined by Western blotting with anti-6´ Histidine-tag antibody (Abcam, Cambridge, MA, USA). All samples were stored at -20°C until purification.

The recombinant protein in urea fraction was purified with HisPur™ Ni-NTA Superflow Agarose (Thermo Fisher Scientific) in a 10-ml purification column (Bio-Rad) under denaturing conditions (8 M urea) at room temperature, and eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl and 8 M urea, pH 8.0) containing 10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 100 mM and 250 mM imidazole. The presence of 6× Histidine-tagged protein was confirmed by Western blotting, and the purified recombinant protein sample was sent for mass spectrometry analysis at the Department of Molecular Tropical Medicine and Genetics, Faculty of Tropical Medicine, Mahidol University. The purified rRty-17kDa was dialyzed with 0.1% PBS at 4°C and lyophilized using a Freeze Dryer (Labconco Corporation, Kansas City, MO, USA). The lyophilized protein was stored at -70°C in vials. Before use, the powdered protein was resuspended in sterile distilled water by gentle tapping and pipetting. Resuspended protein was aliquoted in vials and stored at -20°C.

Serum samples

The use of stored leftover sera in this study was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (approval no. MUTM 2019-072-01). Antibody status and number of samples are shown in Table 1. A murine typhus antibody status was determined by immunofluorescence (IFA) assay which is considered as a reference method.

Indirect ELISA

Each well of a Nunc 96-well Immuno plate (Thermo Fisher Scientific) was coated with 50 μL of rRty-17kDa diluted in carbonate buffer (pH 9.6), at 0.76 mg/well. Wells coated with only coating buffer (no antigen) were included for all samples. The coated plates were left uncovered at 37°C in

Table 1 Antibody status and numbers of serum samples used in this study.

Serum samples	Murine typhus antibody		Number
	IgG	IgM	
Murine typhus-antibody positive (IgG+, IgM-)	+	-	10
Murine typhus-antibody positive (IgG-, IgM+)	-	+	10
Murine typhus-antibody positive (IgG+, IgM+)	+	+	10
Scrub typhus-antibody positive	-	-	30
Leptospirosis-antibody positive	ND	ND	33
Melioidosis-antibody positive	ND	ND	26
Apparently healthy donor	ND	ND	10

ND, not done

an incubator overnight. Plates were washed with 150 mL of 1´ PBS-0.05%Tween 20 (1´ PBS-T) 3 times and all wells was blocked with 100 μL of 1% BSA in 1´ PBS-T at 37°C for 1.5 h. The plates were then washed with 1´ PBS-T 3 times and 50 μL of serum samples diluted at 1:100 with diluent (0.2% gelatin, 0.2% BSA in 1´ PBS-T) was added. Blank wells containing diluent instead of serum were included. The antigen-antibody reaction was allowed to take place at 37°C for 1.5 h. Serum incubation was followed by washings with 1´ PBS-T 3 times. Subsequently, HRP-conjugated anti-human IgG diluted at 1:2000 or anti-human IgM diluted at 1:4000 with diluent was added (50 μL/well) and the reaction was incubated at 37°C for 1.5 h. Plates were washed thoroughly 4 times with 1´ PBS-T. Color was developed by adding ABTS® Peroxidase substrate (KPL International Limited, Gaithersburg, MD, USA), 50 μL/well. Reaction was stopped with 1% SDS, 25 μL/well. An optical density (OD) was measured at a wavelength of 405 nm by microplate reader, immediately after incubation for 30 min in dark.

For analysis, OD of test wells was subtracted by OD of blank and OD of the correspondent no antigen well. Pooled murine typhus-negative serum was included and used for cut-off setting. Positive was read if the OD was ≥ 2× average OD of pooled murine typhus-negative serum wells.

Data analysis

For cross reactivity determination, indirect rRty17-kDa ELISA results were expressed as average OD for each group of disease sera and compared by analysis of variance (ANOVA). P-value < 0.05 was considered significantly different. Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) were calculated as follow: sensitivity = [TP / (TP + FN)] ´ 100, specificity = [TN / (TN + FP)] ´ 100, PPV = [TP / (TP + FP)] ´ 100, and NPV = [TN / (TN + FN)] ´ 100, where TP is true positive, FP is false positive, TN is true negative, FN is false negative.

RESULTS

PCR amplification and cloning of *R. typhi* 17-kDa surface antigen gene

Rty17-kDa gene without signal sequence (size 418 bp) was PCR amplified from *R. typhi* genomic DNA using cloning primers (17kDa and rev17kDa) containing *Xho*I/*Eco*RI sites. Purified Rty17-kDa PCR product and pRSET B vector (2.9 kb) were double digested with restriction enzymes *Xho*I and *Eco*RI, and gel purified (Figure 1A). The purified Rty17-kDa fragment was ligated with pRSET B vector and transformed into DH5α *E. coli* host for recombinant plasmid DNA propagation. Positive

bacterial colony was checked by colony PCR. Four positive clones were retrieved. Recombinant plasmids were named pRSET-Rty17-kDa1-4. Their sequences were confirmed by DNA sequencing. pRSET-Rty17-kDa1was used for further protein expression.

Prediction of recombinant gene insertion site in pRSET B vector and product size

Based on the sequencing result, the site of Rty17-kDa gene insertion in pRSET B vector and protein expression was depicted (Figure 1B), and the expression of a recombinant protein (rRty17-kDa) of 171 amino acids, corresponding to 513 nucleotides, was predicted (Figure 1C). A predicted molecular weight of the rRty17-kDa is 18.2 kDa.

Expression and purification of rRty17-kDa

pRSET-Rty17-kDa-1 was extracted from DH5a *E. coli* host and transformed into *E. coli* expression host, BL21 (DE3) pLysS. The protein expression in bacterial culture was induced by 1 mM IPTG. The bacterial cells were lysed and rRty17-kDa protein was extracted with 8 M urea. Four fractions were obtained, supernatant, urea 1, urea 2 and pellet (Figure 2A). A presence of 6× Histidine-tagged rRty17-kDa was screened by Western blotting with anti-Histidine (Figure 2B). Strong positive His-tag bands were observed in urea 1 and urea 2 fractions at ~20-kDa, indicating the presence of recombinant protein in inclusion bodies. The pellet fraction also showed a weaker His-tag positive. Noticeably, multiple bands were observed in a marker lane at positions over 50 kDa
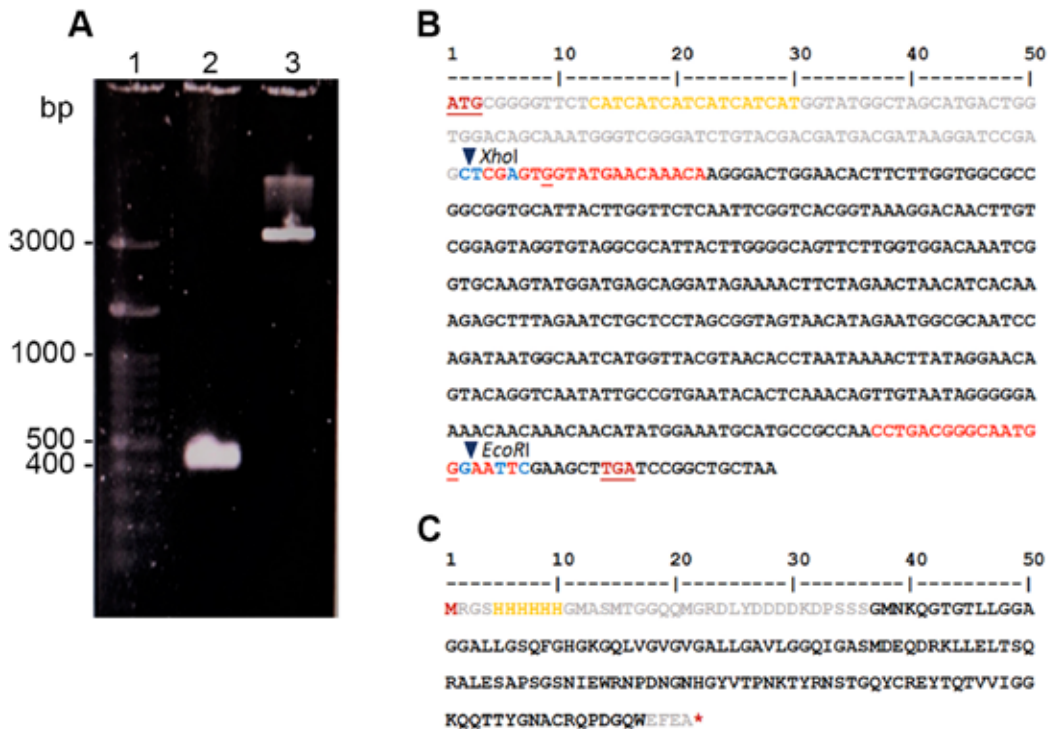

Fig 1 (A) Agarose gel electrophoresis of purified Rty17-kDa PCR product and purified pRSET B vector after *Xho*I/*Eco*RI double digestion. Lane 1, 100 bp DNA marker; lane 2, purified Rty17-kDa PCR product (418 bp), lane 3, purified pRSET B vector (2.9 kb). (B) Diagram illustrated Rty17-kDa gene insertion site in pRSET B vector. ATG indicates a protein expression start site. Grey letters indicate nucleotide sequences of pRSET B vector. Yellow letters indicate histidine tag in pRSET B. Cloning primers with restriction sites for *Xho*I and *Eco*RI are indicated in red and blue letters. Black letters indicate the inserted gene sequence. TGA indicates a stop codon. (C) Sequences of the predicted rRty17-kDa protein.

in Figure 2A, which could be a spillover occurred during a sample loading step.

Urea 1, urea 2 and pellet fractions were pooled and subjected for protein purification by Ni-NTA column chromatography. The protein was purified and eluted with increased concentrations (10 mM, 20 mM, 30 mM, 40 mM, 50 mM, 100 mM and 250 mM) of imidazole (Figure 3A). Before elution with imidazole, wash fractions (with 8 M urea) were collected as flow-through and wash in separate tubes. By Western blot analysis, positive His-tag bands were observed in 50 mM, 100 mM and 250 mM imidazole lane. These fractions were pooled and lyophilized for further use in ELISA assay. After purification, the purified protein was subjected to mass spectrometry analysis. The mass spectrometry analysis showed the matching of rRty17-kDa with protein accession no. AFQ36777.1, *Rickettsia typhi* partial 17 kDa outer membrane protein from NCBI database.
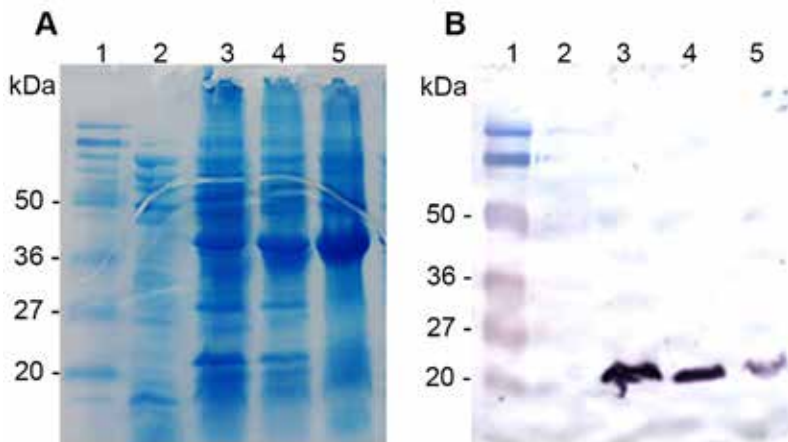

Fig 2 **(A)** SDS-PAGE and **(B)** Western blot analysis with anti-Histidine-Tag antibody of the expressed rRty17-kDa from urea extracts. Lane 1, prestained protein marker; lane 2, supernatant of bacteria lysate collected prior to urea extraction; lane 3, urea extract fraction 1; lane 4, urea extract fraction 2; lane 5, bacteria pellet collected after urea extraction.
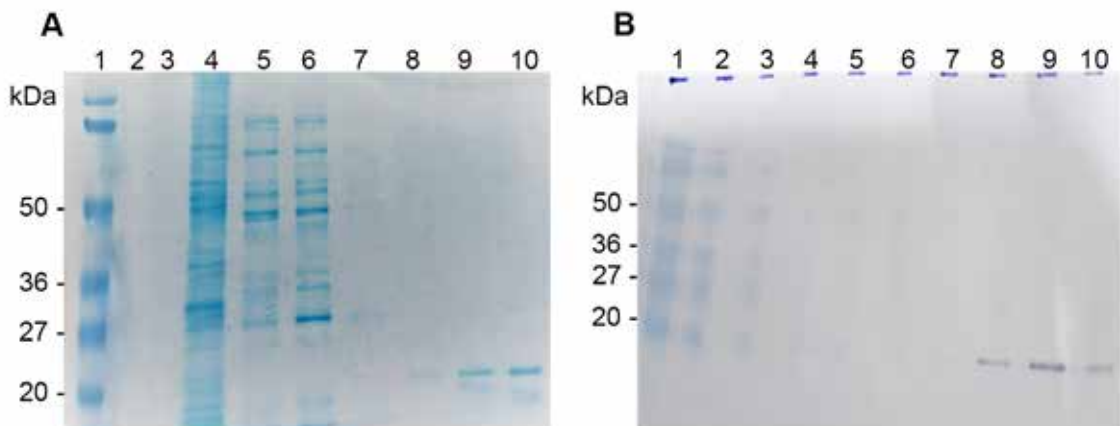

Fig 3 **(A)** SDS-PAGE and **(B)** Western blot analysis with anti-Histidine-Tag antibody of the purified rRty17-kDa. Lane 1, prestained protein marker; lane 2, flow-through; lane 3, wash; lane 4, 10 mM; lane 5, 20 mM; lane 6, 30 mM; lane 7, 40 mM; lane 8, 50 mM; lane 9, 100 mM; lane 10, 250 mM imidazole.

Determination of rRty17-kDa reactivity with disease sera from murine typhus, scrub typhus, leptospirosis and melioidosis patients by indirect ELISA

Indirect IgG and IgM ELISAs were performed with optimized rRty17-kDa protein coating concentration (0.76 mg/well). Sera from patients with different disease status (Table 1) were tested. A significant higher reactivity of rRty17-kDa with leptospirosis-positive, melioidosis-positive and normal control sera was observed compared with murine typhus-positive sera when tested for IgG (Figure 4A). The highest cross-reactivity was observed with normal sera. A significant higher reactivity of rRty17-kDa with normal control sera was also observed compared with murine typhus-positive sera when tested for IgM (Figure 4B). The results suggested that the rRty17-kDa is not able to differentiate between murine typhus and other febrile diseases (scrub typhus, leptospirosis and melioidosis) using ELISA.

Sensitivity and specificity of indirect IgG and IgM ELISAs

For sensitivity and specificity determination, only sera with known murine typhus antibody status tested by the standard method immunofluorescence assay (IFA) were included in the analysis (Table 1 and 2). Results of IFA were used to define true positive and true negative. Sensitivity and specificity calculated for IgG assay were 15.0% and 87.5%; PPV was 37.5% and NPV was 67.3%. Sensitivity and specificity calculated for IgM assay were 65.0% and 42.5%; PPV was 36.1% and NPV was 70.8%. It was apparent from the values of sensitivity and specificity from both IgG and IgM ELISA that the rRty17-kDa in its current form is not suitable for diagnosing murine typhus. Although a high specificity was observed with IgG ELISA, it was accompanied by a very low sensitivity. For IgM ELISA, generally low sensitivity and specificity ($\leq 65\%$) were observed. To sum up, as both high sensitivity and specificity are needed for an ideal diagnostic test, the results suggested an inadequacy of the rRty-17kDa-based ELISA for an effectual serodiagnosis of murine typhus.

DISCUSSION

The current study was aimed to produce recombinant antigen of *R. typhi* for using in a serological diagnosis of murine typhus. Though a plethora of diagnostic approaches are available for
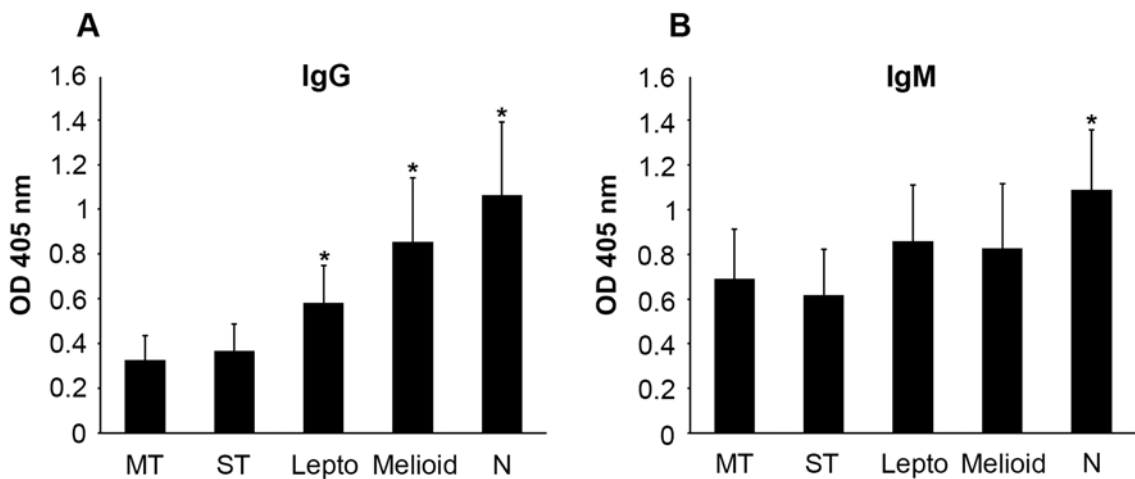
Fig 4 Reactivity of rRty17-kDa with disease sera determined by indirect **(A)** IgG and **(B)** IgM ELISA. OD 405 nm is an optical density measured at wavelength 405 nm. Stars (*) represent groups of sera with statistical different OD compared to MT at $p < 0.05$. MT, murine typhus; ST, scrub typhus; Lepto, leptospirosis; Melioid, melioidosis; N, normal control.

Table 2 Numbers of murine typhus-antibody positive and negative serum samples tested by IgG and IgM ELISAs compared with murine typhus IFA.

IgG ELISA	Immunofluorescence assay (IFA)		Total
	No. of positive	**No. of negative**	
No. of positive	3	5	8
No. of negative	17	35	52
Total	20	40	
IgM ELISA	**Immunofluorescence assay (IFA)**		**Total**
	No. of positive	**No. of negative**	
No. of positive	13	23	36
No. of negative	7	17	24
Total	20	40	

detection of rickettsioses, there is always a need to develop a novel, ready-to-use and inexpensive test, preferably targeting diagnoses to the species level. Thus, a 17-kDa protein gene from *R. typhi* was selected to be cloned, expressed, and produced in prokaryotic (*E. coli*) expression system and purified by affinity column chromatography. ELISA was done to evaluate the sensitivity, specificity and cross-reactivity of the recombinant protein with patient sera.

One limitation of the study lies in the necessity of performing screening and subsequent confirmation to recognize a potential target for recombinant protein production. Prior to this study, an initial screening was performed by Western blotting of *R. typhi* whole cell lysate antigens reacted with pooled murine typhus-positive sera (data not shown). A strong reactive band was detected, and suggested a presence of immunodominant protein of size smaller than 20 kDa. As it was reported in previous study that 17-kDa surface antigen gene was successfully used for rickettsial diagnosis by PCR-based molecular assays (Paris and Dumler, 2016), and to our knowledge, the *R. typhi* 17-kDa protein had not been evaluated before for using in serodiagnosis, we chose *R. typhi* 17-kDa protein

as a target for protein expression in this study. It would be more confident if the immunoreactive band from Western blot analysis had been confirmed of the protein identity, by technique such as mass spectrometry, before subsequent protein cloning and expression.

After purification, the rRty17-kDa identity was confirmed by mass spectrometry. However, a low reactivity with murine typhus sera was observed in both IgG and IgM ELISAs. This could due to an incomplete protein refolding (Martinez-Alonso *et al*, 2008), such as the desired protein may acquire a stable soluble conformation after 8 M urea treatment condition used in this study, but the active site structure remained unsuitable for activity with antibody in the positive serum. It is often noted that high level expression of recombinant protein in *E. coli* results in formation of inclusion bodies (Singh *et al*, 2015), as was also occurred in this study. For renaturation of inclusion body proteins, protein refolding methods such as pulse renaturation processes, size exclusion chromatography and adsorption chromatography, can be applied. The principle of these methods is physical separation of partially folded protein molecules during buffer exchange, as a result

protein-protein interaction is reduced, which in turn lower aggregation and improve recovery of protein as potential soluble and bioactive forms (Singh and Panda, 2005). It has also been reported that peptide tags, such as histidine tag, can negatively interfere tertiary structure or biological activity of the fused chimeric proteins (Chant *et al*, 2005; Khan *et al*, 2012). Cross reactivity of the rRty17-kDa was observed with sera from scrub typhus, leptospirosis, melioidosis patients, and even with health sera by indirect ELISA assay. It is possible that the cross reactivity may originate from background noise reaction caused by the hydrophobic binding of immunoglobulin complexes in sample specimens to the plastic surfaces, which may produce false positive results (Terato *et al*, 2016). Use of frozen and leftover sera from previous studies could be stated as a limitation for this study, as degradation of antibody in sera may have occurred due to repeated freeze-thaw cycles during the experiment. Although sera positive for murine typhus and scrub typhus used in the study were referenced by IFA method, status of some serum samples were not known, such as sera positive for leptospirosis, melioidosis and healthy people were not characterized for possibility of presence of any other infection including murine typhus. To enhance performance with any immunogenic protein, uses of recently collected serum samples which are characterized for disease status and stage of disease are recommended.

Finally, in an effort to express a recombinant protein for using in a development of a potential serodiagnostic approach for murine typhus detection, though the cloning, expression, production and purification of recombinant 17-kDa surface antigen from *R. typhi* was successful, evaluation of the recombinant protein reacted with patient sera by ELISA had not shown any discernible diagnostic capability so far. Therefore, serodiagnosis of murine typhus with the recombinant 17-kDa surface antigen in its current conformation is not recommended. Further improvements of the expression and protein refolding system may be required for the recombinant Rty17-kDa protein and

identifications of novel immunogenic target proteins by methods such as immunoproteomics or *in silico* immunogenic site prediction will help in the future development of murine typhus immunodiagnosis.

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