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Evaluation of the modified indirect hemagglutination assay using chicken red blood cells as a routine melioidosis detection in Maharaj Nakorn Chiang Mai Hospital

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ABSTRACT

Background: Melioidosis is a life-threatening illness caused by *Burkholderia pseudomallei*, which is endemic throughout Thailand. The indirect hemagglutination assay (IHA), a serological test, is widely used to diagnose melioidosis. However, the conventional IHA available in Thailand still requires more than 4 hours for measurement time, limiting its efficiency in clinical settings. This study addresses the need for a quicker and more efficient diagnostic method for melioidosis.

Objective: The study aimed to evaluate the agreement between a modified indirect hemagglutination (modified IHA) assay and the commercially available IHA kit for routine melioidosis diagnosis at Maharaj Nakorn Chiang Mai Hospital. Chicken red blood cells were used instead of sheep red blood cells to reduce diagnostic time and costs while maintaining high accuracy and reliability.

Materials and methods: A total of 368 serum samples were tested using the modified IHA assay, which utilized chicken red blood cells instead of sheep red blood cells used in the commercial IHA kit. The results were compared with those of the commercial IHA kit for melioidosis detection.

Results: The modified IHA assay showed 99.46% agreement, with an excellent kappa value of 0.98, compared to the commercial kit. In the validation step, the modified IHA assay correctly identified 100% (54 out of 54) of positive samples and 100% (54 out of 54) of negative samples. However, 0.63% (2 out of 320) false positives were observed in the diagnostic samples with the modified IHA assay.

Conclusion: The modified IHA assay may serve as a valuable alternative for the routine diagnosis of melioidosis because it is less time-consuming and more cost-effective than the commercial IHA kit. However, further studies with more clinical samples are warranted to confirm its utility.

Introduction

Melioidosis is an infectious disease of humans and animals caused by the environmental bacterium *Burkholderia pseudomallei (B. pseudomallei)*, a Gram-negative rod-shaped bacterium. It is a severe disease that is endemic with a high mortality rate in Northern Australia and Southeast Asia.[1] In Thailand, this disease is endemic in several regions, including the Northeast, Central, East, North, and South.[2,3] Patients are infected via skin contact, ingestion, and inhalation of *B. pseudomallei* in

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environmental sources, particularly soil, groundwater, and stagnant water.[1]

The clinical manifestations of melioidosis vary widely, ranging from asymptomatic infection to abscesses, pneumonia, disseminated disease, and death.[4] Notably, a high mortality rate of melioidosis, accounting for 42.3% (11 out of 26 patients), has been reported at Maharaj Nakorn Chiang Mai Hospital, Chiang Mai, Thailand.[5] The disease incubation period generally ranges from 1 to 21 days (mean 9 days).[6] However, this organism can remain latent in the human body for long periods.[7]

Standard laboratory diagnostic tests for investigating melioidosis include Gram's staining, bacterial culture, and the indirect hemagglutination assay (IHA).

Gram's staining can detect *B. pseudomallei* as a Gram-negative rod-shaped bacterium, but this test lacks sensitivity and specificity.[8] Bacterial culture remains the mainstay method for diagnosing melioidosis.[9] However, *B. pseudomallei* can sometimes be misidentified as other bacteria or dismissed as a contaminant. Although microbiological culture is the gold standard for diagnosis, it is a complex and time-consuming method. The IHA, a serological test, is widely used for the diagnosis of melioidosis by detecting antibodies raised against *B. pseudomallei*.[9]

Although it lacks standardization, it can be valuable for diagnosing febrile illness in people who have not lived in an endemic region. In Thailand, the diagnostic sensitivity of the IHA for melioidosis at admission has been reported as 73% and specificity as 64%.[10] The IHA has been used for the diagnosis of melioidosis for over 50 years, which remained a primary unchanged protocol. Nowadays, conventional and commercial IHA protocols use sheep red blood cells for detection, but the detection time remains more than 4 hours.

Chicken red blood cells are commonly used in hemagglutination assay (HA) and hemagglutination inhibition (HI) tests to measure hemagglutinating antibodies against viruses with lower cross-reactivity with human antibodies than mammalian red blood cells (e.g. sheep, horse, or cow).[11,12] Some publications on HA and HI protocols for quantifying influenza-specific antibody titers have reported that using chicken red blood cells can reduce the measurement time to less than 2 hours.[13] This study used chicken red blood cells to detect melioidosis in a modified indirect hemagglutination (modified IHA) assay.

This study evaluated the correlation between the modified IHA assay and the commercial IHA kit used for routine melioidosis diagnosis at Maharaj Nakorn Chiang Mai Hospital.

Materials and methods

Study population

This study used 368 serum samples from suspected melioidosis patients at Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University, collected between January 2021 and December 2022. The residual specimens from routine IHA for melioidosis detection were utilized to test the modified IHA assay. They compared it to the commercial IHA kit utilizing sheep red blood cells provided by the National Institute of Health, Department of Medical Science, Thailand, for melioidosis detection.

Chicken red blood cells preparation for modified indirect hemagglutination assay

Collection and storage of chicken red blood cells

Chickens, species Gallus gallus domesticus, were kindly supported by the Research Unit, Faculty of Medicine, Chiang Mai University. Five milliliters of chicken blood were collected from the brachial wing vein of a chicken aged 8-9 months by a veterinarian (AK.) under aseptic conditions (Figure 1). The chicken blood was then placed in a sterile tube containing an equal volume of Alsever's solution. The mixed blood was transported to the Immunology laboratory at a controlled temperature of 4 °C for further processing. Next, the mixed blood was washed in phosphate buffer saline (PBS) three times. The packed chicken red cells were suspended in PBS containing 0.05% glutaraldehyde to prepare the working solution as 1% washed cells (i.e., 10 µL of blood cells in 990 µl of PBS). The prepared 1% glutaraldehyde-treated chicken red blood cell suspension can be stored for up to three months at 4 °C, maintaining cell integrity for extended use in assays.

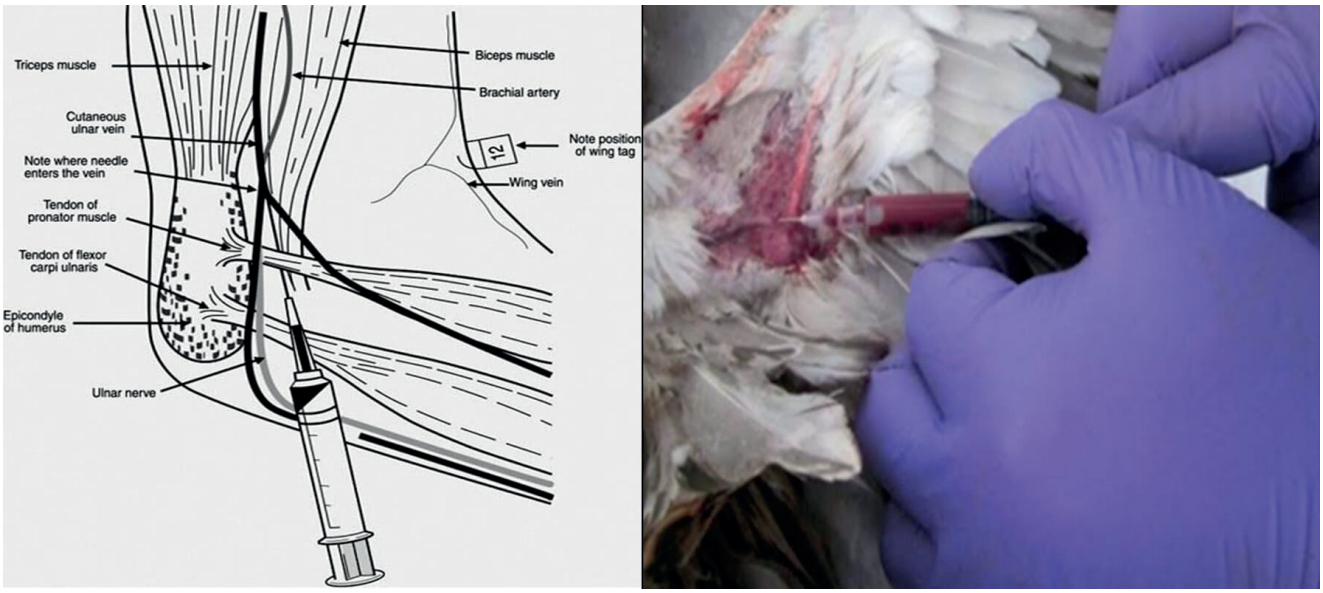

Figure 1. *Blood collection site at the brachial wing vein chicken.*

Chicken red blood cell sensitization

B. pseudomallei was isolated from a positive blood culture. The positive blood culture was Gram-stained, and the remaining blood culture was routinely processed by subculture onto MacConkey, blood, and chocolate agar plates. The bacteria grown on the plates were assessed for colony morphology, purity, and Gram-stain reaction. The isolated colony was then subjected to biochemical characterization (e.g., oxidase, indole, catalase, motility, triple sugar iron, urease, and carbohydrate fermentation tests).

The confirmed *B. pseudomallei* was subcultured on trypticase soy agar and then incubated at 37 °C for 24 to 48 hrs. For crude *B. pseudomallei* antigen preparations, the confirmed *B. pseudomallei* cells were optimized for the bacterial antigen dilution using McFarland number 3 ($9x10^8$ cells/mL) in 5 mL of PBS. The adjusted bacteria were then inactivated by autoclaving at 121 °C under a pressure of 15 psi for 15 minutes. Next, the optimal bacterial cell suspension was diluted to make the final dilutions of the antigens of 1:20, 1:40, 1:60, 1:80, 1:100, 1:120, 1:140, and 0 (no antigen) in PBS for titration of the sensitized red cells. The 1% washed chicken red blood cells were added to crude *B. pseudomallei* antigen suspension at a ratio 1:1, and the mixture was incubated at 37 °C for 1 hr. After incubation, the mixture was washed and stored at 4 °C until the modified IHA assay was used. The optimal bacterial antigen dilution was established for each new batch of bacterial antigen, representing a process of standardization of antigen concentration based on previous batches. The concentration of each antigen preparation was standardized with reference pooled sera before use in the assay to prevent batch-to-batch variation.

Titration assay

The positive control serum, pooled from three patients with culture-proven melioidosis who had established IHA titers around 1:1,280, was diluted two-fold, ranging from 1:20 to 1:10,240. Then, 25 µL of 1% washed chicken red blood cells from each antigen dilution were incubated with the positive control serum. Non-sensitized red cells were added as negative control. Then, the mixtures were incubated at room temperature for 2 hrs, following the commercial kit protocol, with observations taken every 15 minutes. The optimal antigen concentration was identified as the one that gives the correct (previously known) IHA titer for the pooled serum. The highest antibody dilution demonstrating complete or partial agglutination was recorded as the IHA titer. This study identified the optimal antigen concentration for the modified IHA assay as 1:100, ensuring accurate titer determination in subsequent analyses.

Validation of modified indirect hemagglutination test

To validate the modified IHA assay, 54 known positive and 54 known negative samples, including positive and negative controls from the commercial kit and patient samples, were evaluated for serum titers against *B. pseudomallei* antigen. Serum samples were prepared as detailed in Table 1, followed by incubation with either 1% sensitized chicken red blood cells for the modified-IHA assay or 1% sensitized sheep red blood cells for the commercial IHA kit. Non-sensitized red blood cells served as negative controls for both methods. The mixtures were incubated at room temperature for 2 hrs, with observations taken every 15 mins under the commercial kit protocol. An optimized incubation time of 45 minutes for the modified IHA assay was established based on results from known positive samples. The measurement steps for the commercial IHA kit and the modified IHA assay across four samples are shown in Table 1.

Table 1. Comparison of measurement steps between the commercial IHA kit and the modified IHA assay.

Steps	Commercial IHA kit	Modified indirect hemagglutination test
1. **Serum preparation** - Serum inactivation - Incubation of 5% uncoated cells with serum - Centrifugation for collecting the absorbed serum	**Yes** (At least 1 hour, depended on the number of samples)	**No**
2. **Sample dilution** - Two-fold dilution of absorbed serum - Addition of sensitized red blood cells, diluted serum, and negative and positive controls into a microplastic 96-well plate	**Yes** (At least 1 hour, depended on the number of samples)	**Yes** (At least 1 hour, depended on the number of samples)
3. **Indirect hemagglutination test** (incubation time)	**Yes** 2 hours	**Yes** 45 mins
Total measurement time* (The measurement time depended on the number of samples)	At least 4 hours	At least 1 hours 45 mins

Performance of modified indirect hemagglutination test

The serums were diluted two-fold, ranging from 1:20 to 1:10,240, to evaluate the titers of patient serum against *B. pseudomallei* antigen. Twenty-five microliters (µL) of each diluted serum were added to a 96-well plate. Then, 25 µL of 1% sensitized chicken red blood cells were added to the 96-well plate. Next, the mixtures were incubated for 45 minutes at room temperature (optimized time from validation assay). The reaction titer was the last dilution that presented 50% agglutination. Non-sensitized red cells were used as negative controls. A titer of ≥1:160 was considered positive.

Statistical analysis

Cohen's kappa was used to test the measure of inter-rater reliability between the commercial IHA kit (provided by the National Institute of Health, Department of Medical Science, Thailand) and the modified IHA assay**.** A $p<0.05$ was considered statistically significant. All statistical analyses were performed using STATA version 16 (STATA Corp., Texas, USA).

Results

Validation of modified IHA assay in the diagnosis of melioidosis

The modified IHA assay offers a reduced measurement time, providing reliable readings at 45 mins post-incubation, unlike the commercial IHA kit, which requires a complete 2-hr incubation for accurate results (Figure 2). In validation tests, the modified IHA assay achieved a 100% positive detection rate (titer ≥1:160) for all known positive samples (54 out of 54). Additionally, the assay demonstrated 100% specificity by accurately yielding negative results for all known negative samples (54 out of 54), as summarized in Table 2.

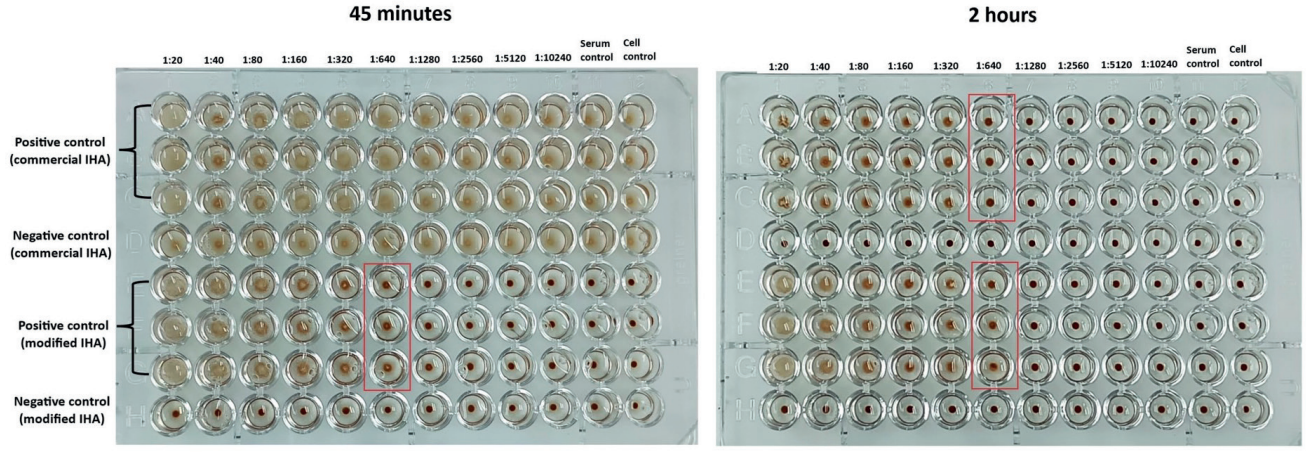

Figure 2. *Comparison of measurement time between the commercial IHA kit and the modified IHA assay.*

Table 2. Comparison of indirect hemagglutination results between the commercial IHA kit and modified IHA assay using known negative and positive samples.

Validation of samples	Modified indirect hemagglutination assay		Total
	Negative	Positive	
Known negative	54	0	54
Known positive	0	54	54
Total	**54**	**54**	**108**

Diagnostic samples

Among the 368 patients, 86.96% (320 out of 368) were found negative for melioidosis, and 13.04% (48 out of 368) were found positive using the commercial IHA kit. The titers of the 320 negative samples were distributed as follows: <1:20 (56.00%), 1:20 (29.50%), 1:40 (12.00%), and 1:80 (2.50%). Among the 48 positive samples, the titers were distributed as follows: 1:160 (18.75%), 1:320 (18.75%), 1:640 (25.00%), 1:1280 (16.67%), 1:2560 (10.42%), 1:5120 (6.25%), and 1:10240 (4.16%).

Using the modified IHA assay, 86.41% (318 out of 368) were classified as negative, and 13.59% (50 out of 368) were classified as positive for melioidosis. The titers of the 318 negative samples were distributed as follows: <1:20 (55.60%), 1:20 (32.40%), 1:40 (10.12%), and 1:80 (1.88%). Among the 50 positive samples, the titers were distributed as follows: 1:160 (20.00%), 1:320 (26.00%), 1:640 (32.00%), 1:1280 (8.00%), 1:2560 (8.00%), 1:5120 (4.00%), and 1:10240 (2.00%) (Figure 3).

The concordance between the commercial IHA kit and the modified IHA assay was 99.46%, with a kappa value of 0.98, indicating excellent agreement (Table 3). However, there were two discordant results between the two assays. Two patients tested positive using the modified IHA assay, with titers of 1:160, while the commercial IHA kit classified these samples as negative with titers of 1:40 and 1:80, respectively (Table 4).

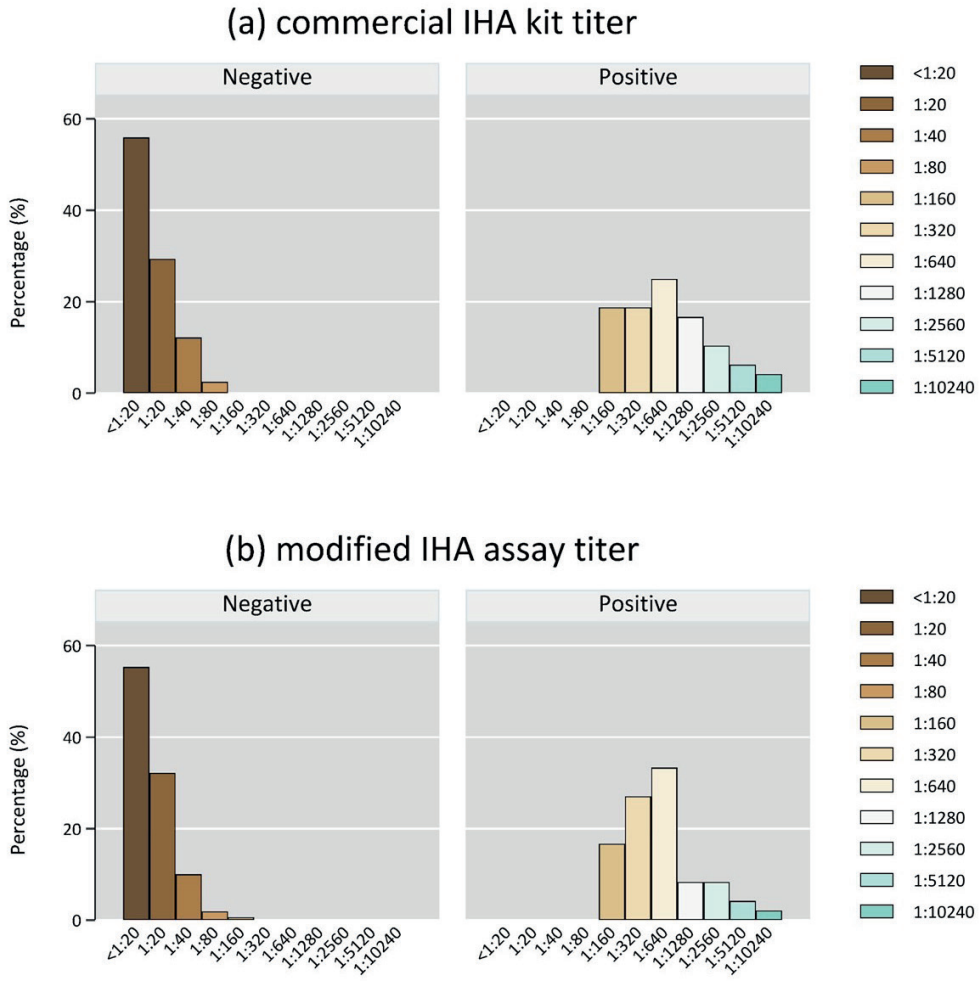

Figure 3*. The proportion of each IHA titer level for each method stratified by IHA positive status.*

Table 3. Comparison of indirect hemagglutination from the commercial IHA kit and modified IHA assay using diagnostic samples.

Commercial IHA kit	Modified IHA assay		Total
	Negative	Positive	
Negative	318	2	320
Positive	0	48	48
Total	318	50	368

Table 4. Clinical relevance of discordance results between commercial IHA kit and the modified-IHA assay.

Discordant case	Commercial IHA kit	Dilution	Modified IHA assay	Dilution	Diagnosis
Case 1	Negative	1:40	Positive	1:160	Afebrile
Case 2	Negative	1:80	Positive	1:160	*Klebsiella pneumoniae* mixed *Streptococcus* spp. infection

Discussion

The IHA is one of the most widely used methods for diagnosing melioidosis in disease-endemic countries, particularly Thailand.[3] It has remained unchanged since it was first described over 50 years ago due to its low cost and simplicity. However, despite its longevity and cost-effectiveness, the method remains time-consuming, with detection times exceeding 4 hours when using sheep red blood cells, as seen in various commercial IHA kits currently available in Thailand. In contrast, chicken red blood cells are commonly used in virology tests such as hemagglutination assay and hemagglutination inhibition tests for quantifying influenza-specific antibody titers. Using chicken red blood cells, the measurement time for HA and HI protocols is less than 2 hours.[13,14] This improved efficiency is attributed to the unique properties of chicken red blood cells, including reduced non-specific binding due to lower cross-reactivity with human antibodies compared to mammalian red blood cells.[11,12] Chicken red blood cells have a shorter lifespan of approximately 1-2 months[17] than sheep red blood cells, which can last 3-6 months[18] due to differences in metabolic activity and cell structure. Despite the shorter lifespan, the reduced detection time and efficiency associated with chicken red blood cells present a potential advantage for improving the IHA method for melioidosis diagnosis.

This study adapted the IHA protocol using chicken red blood cells to detect melioidosis, showing excellent agreement with the commercial IHA kit. Notably, the modified protocol reduced measurement time, improving diagnostic workflow efficiency in clinical settings. This advancement facilitates faster decision-making and potentially enhances patient outcomes, particularly in resource-limited environments. In contrast, a small false positive rate of 0.63% (2 out of 320) was observed in the modified IHA assay compared to the commercial IHA kit. Despite this, the results suggest that the modified IHA assay could be viable for routine melioidosis diagnosis. However, the small sample size in this study underscores the need for further prospective studies with larger clinical cohorts. Additionally, exploring the heat inactivation of serum in future research may help reduce false positives further.

The limitations of this study include the variation in the strains (typically local clinical strains) used for crude antigen preparation compared to the commercial IHA kit. The uncharacterized nature of the antigenic epitopes and antibodies might contribute to the false positive observed. False positives can occur due to high background seroconversion rates in endemic areas, where titers of 1:160 to >1:1,280 have been reported in non-melioidosis patients at rates of 33% and in afebrile patients at rates of 60%.[15] In Thailand, a titer of 1:160 is generally considered positive, with the IHA sensitivity for melioidosis at admission reported as 73% and a specificity as 64%.[10] The unstandardized antigen content and varying host responses may further complicate interpretation. Addressing these issues through additional measures, such as heat inactivation of sera, could help mitigate false positive results.

Bacterial culture remains the gold standard for diagnosing melioidosis, though it can sometimes be misidentified or dismissed as contamination. Notably, *B. pseudomallei* is classified as a hazard group 3 pathogen globally and in Thailand (https://tbrcnetwork.org/riskgroup.php?riskgroup_h=3), handling this bacterium requires strict biosafety measures and regular training.[16]

Despite the limitations, the modified IHA method improves by reducing measurement time to less than two hours while maintaining comparable sensitivity and specificity to the commercial IHA kit. Furthermore, the cost of the modified IHA is approximately 3.5 times lower than the commercial assay (10 Baht/test vs. 35 Baht/test). These findings suggest that the modified IHA assay has the potential to enhance routine diagnostics, particularly in resource-limited settings. Future studies with larger clinical sample sizes will be essential to validate this method's robustness and clinical utility.

Limitation

One limitation of this study is using local clinical strains of *B. pseudomallei* to prepare crude antigens, contrasting with the standardized commercial IHA kit. The uncharacterized nature of the antigenic epitopes and corresponding antibodies may have contributed to the variability in results. Additionally, the impact of heat

inactivation of sera should be further explored in future studies to reduce false positives.

Conclusion

The modified IHA assay using chicken red blood cells offers advantages such as cost-effectiveness and improved laboratory detection time while maintaining sensitivity and specificity comparable to those of a commercial IHA kit for melioidosis. However, further studies with larger clinical sample sizes are needed to validate these findings and confirm the method's utility in routine diagnostics.

Conflict of interest

The authors declare no conflict of interest.

Funding

No funding was received to conduct this study.

Ethical approval

This study was approved by the institutional ethics committee of the Faculty of Medicine, Chiang Mai University (Research ID: 8830/ Study code: PAT-2565-08830). Additionally, this study was approved for biosafety (Research ID: CMUIBC02023/2564) and use of animals (Protocol number: 30/2565) by the institutional biosafety committee and the institutional animal care and use committee of the Faculty of Medicine, Chiang Mai University, respectively.

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