Adapting a Novel Lateral Flow Immunoassay to

Rapidly Detect *Burkholderia pseudomallei* in Sarawak, Malaysia

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of
Master of Science in the Duke Global Health Institute
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ABSTRACT

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Abstract

Background

Melioidosis is a neglected tropical disease that is highly prevalent in Southeast Asia. Misdiagnoses are common as the presenting symptoms are similar to other diseases including upper respiratory infections. When not treated with antibiotics, the disease can lead to severe morbidity or death. Current diagnostics in low- and middle-income countries are often not sensitive nor rapid. Point-of-care rapid diagnostic tests (POC-RDTs) are a potential solution. Few studies have compared the accuracy of POC-RDTs and molecular assays against blood culture. The goal of this study was to conduct such comparisons in detecting *Burkholderia pseudomallei* infections among infection-suspected patients in Kapit, Sarawak, Malaysia.

Methods

We used an informed consent process as approved by two institutional review boards. In this cross-sectional study, we engaged patients meeting a melioidosis-like case definition that included classical symptoms such as prolonged fever with joint pain and/or abscess. We studied the patients routinely collected clinical specimens with a POC-RDT (Active Melioidosis *Detect*™) and a

molecular assay compared with the *B. pseudomallei* bacterial culture for isolation of the bacterial organism.

Results

One hundred patients aged 6 months - 79 years from Kapit Hospital were enrolled in the study from June 12, 2018 to January 8, 2019. Of the 100 sera, 97 urine, and 16 bodily fluid samples (total n= 213) tested with the RDT, 23 samples gave positive results (7 sera, 15 urine, and 1 bodily fluids). Compared to the molecular assay, the POC-RDT had a sensitivity of 40% (95% CI, 5%- 85%), specificity of 94% (95% CI, 87% - 98%), and an accuracy of 90% (95 CI, 82% - 95%) for sera; and a sensitivity of 80% (95% CI, 28%- 99%),a specificity of 65% (95% CI, 55% - 75%), and an accuracy of 87% (95 CI, 77% - 94%) for urine; and a sensitivity of 80% (95% CI, 28%- 99%), a specificity of 65% (95% CI, 55% - 75%), and an accuracy of 81% (95 CI, 54% - 96%) for other bodily fluids. Additionally, when compared to the bacterial culture results, the POC-RDT showed a sensitivity of 38% (95% CI, 9%- 76%), specificity of 95% (95% CI, 88% - 99%), and an accuracy of 90% (95 CI, 82% - 95%) for sera; a sensitivity of 88% (95% CI, 47%- 100%), a specificity of 88% (95% CI, 77% - 95%), and an accuracy of 94% (95 CI, 84% - 98%) for urine; and a sensitivity of 25% (95% CI, 1%- 81%), a specificity of

100% (95% CI, 74% - 100%), and an accuracy of 81% (95 CI, 54% - 96%) for other bodily fluids.

Conclusion

While study enrollment will continue, data from the first 100 participants, suggests the POC-RDT had poor sensitivity, good accuracy, and high specificity in detecting *B. pseudomallei* infection. Thus far, the POC-RDT assay seems to work better on urine specimens. Due to low sensitivity, the study data do not support recommending POC-RDT strips as a single diagnostic method. However, as the POC-RDT had high specificity, when the test is positive it seems appropriate for clinicians to assume the patient is infected and to prescribe specific antimicrobial therapy. While more participant data are needed, it seems likely that the POC-RDT could be useful in helping physicians to begin treatment early with the high specificity that the POC-RDT has exhibited. If paired with an RDT with high sensitivity, this POC-RDT would add a great value to infection management.

Dedication

I dedicate this thesis work to my family for their continued support during this journey. I am especially thankful for my little sister, Yujung, for her endless encouragement and faith in me. Thank you, Doongi (Yujung's nickname that is only used by me), for standing next to me every step I took while preparing this thesis draft. You are my light.

Contents

Abstract iv

List of Tables xi

List of Figures.. xiv

Acknowledgements xv

1. Introduction 1

1.1 Melioidosis 2

1.1.1 Transmission 2

1.1.2 Melioidosis symptoms and high-risk populations 3

1.1.3 Melioidosis diagnosis and treatment 4

1.1.4 Melioidosis mortality rate 5

1.2 Description of *B.* pseudomallei 6

1.3 Melioidosis epidemiology in Malaysia 8

1.4 Challenges in diagnosing melioidosis 10

1.5 Gaps in current melioidosis diagnostics in Malaysia 11

1.5.1 Bacterial culture as the gold standard 11

1.5.2 Molecular evaluation for melioidosis diagnostics 12

1.5.3 Active Melioidosis Detect™ rapid testkKit 13

1.6 Study Objectives 15

1.6.1 Assessment of agreements between different diagnostic tools 15

1.6.2 Assessment of risk factors for *B. pseudomallei* infections 16

1.6.3 Long term goal of the study 17

2. Methods 18

2.1 Ethics approval 18

2.2 Research setting 18

2.3 Participant recruitment 199

2.4 Procedures 201

2.4.1 Patient enrollment and sample collection 201

2.4.2 Sample processing 222

2.5 Measures 202

2.5.1 Active Melioidosis Detect™ rapid test kit 223

2.5.2 Molecular Assay 233

2.5.3 Bacterial Culture and Gram Stain 244

2.6 Statistical Analysis 245

3. Results 277

3.1 Concordance assessment 278

3.2 Disease prevalence 33

3.3 Baseline characteristics 34

3.4 Primary clinical presentations and chronic illness status 36

3.5 Behavioral risk factors and *B. pseudomallei* infection status........................... 38

4. Discussion 41

4.1 Sensitivity and specificity of the point-of-care test... 41

4.2 Disagreements between the AMD and bacterial culture results 42

4.3 Disagreements between the qPCR and bacterial culture results 42

4.4 Implications for policy and practice 44

4.5 Implications for further research.. 45

4.6 Study strengths and limitations 46

5. Conclusion 499

Appendix A.. 51

Appendix B 52

Appendix C.. 53

Appendix D.. 54

References 59

List of Tables

Table 1. Methods used to detect *B. pseudomallei* among 100 study participants in Kapit Hospital from June 21, 2018 to January 8, 2019 15

Table 2. Primers and probe used to conduct qPCR for *B. pseudomallei* 24

Table 3. Cohen's kappa interpretation 27

Table 4. Summary of agreement between diagnostic methods, % (95 % CI) 28

Table 5. AMD results against bacterial culture results, % (95 % CI) 29

Table 6. Molecular assay results against bacterial culture results, % (95 % CI) 29

Table 7. AMD results against molecular assay results, % (95 % CI) 30

Table 8. AMD results against clinical diagnosis at discharge, % (95 % CI) 31

Table 9. Molecular assay results against clinical diagnosis at discharge, % (95 % CI) 32

Table 10. Prevalence of *B. pseudomallei* by diagnostic methods among 100 patients in Kapit Hospital from June 21, 2018 to January 8, 2019 33

Table 11. Characteristics of 100 study participants with infection status defined by one or more diagnostic methods in Kapit Hospital from June 21, 2018 to January 8, 2019 35

Table 12. Primary clinical presentations, chronic diseases, and confirmed *B. pseudomallei* infections in Kapit Hospital from June 21, 2018 to January 8, 2019 37

Table 13. Association between behavioral factors and *B. pseudomallei* infections among 100 study participants in Kapit Hospital from June 21, 2018 to January 8, 2019 38

Table 14. Primary clinical presentations, chronic diseases, and confirmed B. pseudomallei infections in Kapit Hospital from June 21, 2018 to January 8, 2019 52

Table 15. Association between behavioral factors and B. pseudomallei infections among 100 study participants in Kapit Hospital from June 21, 2018 to January 8, 2019 53

Table 16. 2x2 contingency table for RDT results vs qPCR results of all sample types...... 54

Table 17. 2x2 contingency table for qPCR results vs bacterial culture results of all sample types 54

Table 18. 2x2 contingency table for RDT results vs bacterial culture results of all sample types 54

Table 19. 2x2 contingency table for RDT results vs bacterial culture results of serum samples 54

Table 20. 2x2 contingency table for RDT results vs bacterial culture results of urine samples 55

Table 21. 2x2 contingency table for RDT results vs qPCR results of other bodily fluid samples 55

Table 22. 2x2 contingency table for qPCR results vs bacterial culture results of serum samples 55

Table 23. 2x2 contingency table for qPCR results vs bacterial culture results of urine samples 55

Table 24. 2x2 contingency table for qPCR results vs bacterial culture results of other bodily fluid samples 55

Table 25. 2x2 contingency table for RDT results vs qPCR results of serum samples........ 56

Table 26. 2x2 contingency table for RDT results vs qPCR results of urine samples 56

Table 27. 2x2 contingency table for RDT results vs qPCR results of other bodily fluid samples 56

Table 28. 2x2 contingency table for RDT results vs clinical diagnosis at discharge of serum samples 56

Table 29. 2x2 contingency table for RDT results vs clinical diagnosis at discharge of urine samples 57

Table 30. 2x2 contingency table for RDT results vs clinical diagnosis at discharge of other bodily fluid samples 57

Table 31. 2x2 contingency table for qPCR results vs clinical diagnosis at discharge of serum samples 57

Table 32. 2x2 contingency table for qPCR results vs clinical diagnosis at discharge of urine samples 57

Table 33. 2x2 contingency table for qPCR results vs clinical diagnosis at discharge of other bodily fluid samples 58

List of Figures

Figure 1. The design of the AMD strips (A) and the interpretations 14

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1. Introduction

Melioidosis, also known as Whitmore disease, is caused by the flagellated bacterium *Burkholderia pseudomallei*, which is found in soil and fresh water (1). The disease has emerged over the past 25 years as a significant cause of human morbidity and mortality in tropical and subtropical regions. Among the tropical regions, Southeast Asia and northern Australia (2) have reported the greatest number of melioidosis cases. Inhabitants of these regions are at high risk of contracting melioidosis because of rice farmers' exposures. Melioidosis has been studied more regularly in Thailand, the world's largest rice exporter. A 2005 report estimated that 20% of Thailand's population has been infected by *B. pseudomallei* (3). In the neighboring country, Singapore, Heng et al. (1998) conducted an epidemiological surveillance study and reported a mean annual incidence rate of 1.7 per 100,000 humans and a fatality rate of 39.5% (4). A more recent surveillance study in Cambodia indicated that 40% of respiratory patients and 32% of patients who died during hospitalization were similarly infected (5). Cases also have been reported in the United States.; however, these cases are largely associated with international travel. In Vietnam, concerns toward melioidosis surfaced during the deployment of the U.S. Armed Forces in the 1960s as melioidosis cases were consistently reported among the American

soldiers (6). Some melioidosis cases were reported after a long period of time of exposure to the source of contamination, leading to the melioidosis nickname 'Vietnam Time Bomb' for its latent nature.

B. pseudomallei can be found in soil worldwide, but typically remains dormant for years. Some studies have reported that the bacteria has been isolated in the rhizosphere of diverse plants (7), including tomatoes (8) and fungi (9). Further, the bacteria have been detected in the roots, xylem, and vascular bundles, as well as the aerial parts of plants, which are thought to contribute to the bacteria's airborne distribution (10). On farms and in home gardens, *B. pseudomallei* may be detected in plants largely because of anthropogenic manipulations of the environment, such as irrigation (11). Irrigation can have adverse effects, such as ground-water pollution, soil salinization, and the creation of swamps and lagoons, all of which can increase health risks for humans due to potential *B. pseudomallei* infections. This pattern is notable, as *B. pseudomallei* is considered an opportunistic pathogen, in that it becomes harmful only when it infects humans and animals (12).

1.1 Melioidosis

1.1.1 Transmission

The bacteria are transmitted to humans primarily through skin contact with contaminated soil or surface water (13). Other routes of transmission include

inhalation and ingestion (14). Animals also are common hosts for *B. pseudomallei*. Melioidosis cases have been reported in various animals including sheep, goats, pigs, cattle, horses (15), dogs, cats (16), birds (17), and rodents (3). Although rare, person-to-person transmission by sexual contact and vertical transmission at childbirth (18, 19) has been reported and animal-to-human transmission via a consumption of the contaminated goat milk (20) has been suspected as well. While melioidosis is transmitted largely by direct contact with contaminated sources, transmission via nosocomial infections and laboratory accidents (21) have also been reported. Melioidosis is strongly associated with rainfall and environmental damage: it spreads via water through endemic areas during the rainy season and through air transport affected by extreme weather events (10).

1.1.2 Melioidosis symptoms and high-risk populations

Infected individuals may manifest a myriad of symptoms, including high fever, headache, and pulmonary infection (1, 2). The disease also has manifestations that range from localized to disseminated abscesses, septicemia, and shock. While melioidosis primarily affects the lungs, causing abscesses and septicemia (22), a severe cutaneous melioidosis case was also previously reported in Singapore (23).

Melioidosis primarily affects susceptible persons who have frequent direct contact with the contaminated wet soil and water—such as those who work in

the rice paddy fields; *B. pseudomallei* has been indicated to cluster together in moist soil, increasing the risk of causing an infection in the individuals exposed to such environments (10). Additionally, immunosuppressed elderly persons are at increased risk of developing the infection. Chronic diseases, including diabetes, kidney complications, and respiratory diseases have also been noted to place patients at high risk of develop severe morbidity when infected with *B. pseduomallei* (1, 24).

1.1.3 Melioidosis diagnosis and treatment

Diagnosis of melioidosis is typically determined via microscopic evaluation of clinical specimens or by isolating the bacterial organism from cultures (25). A diagnosis is confirmed by identifying the *B. pseduomallei* in clinical samples—blood, sputum, pus, urine, and other bodily fluids. The sample is placed on a growing medium and observed for bacterial growth.

The infection can be treated with an antibiotic treatment if detected early. The first line of antibiotics treatment is completed with ceftazidime if patients have no other complications, and in severe melioidosis cases, meropenem is administered (26). In their randomized control trial study, White *et al.* (1989) reported that the mortality rate was reduced by 33% when septicemic melioidosis patients received ceftazidime treatment for a median of eight days. The study also reported that approximately 25% of those patients continued to

show positivity toward the end of the treatment and some patients had relapses of melioidosis, which is an indication of the antibiotic resistance (27).

B. pseudomallei is known to be intrinsically resistant to a wide range of antimicrobials as: 1) the bacterium uses an efflux pump mechanism to flush foreign objects from its system, and 2) the bacteria can develop an outer coating that protects it from antibodies and some antibiotics (28, 29). Consequently, Limmathurotsakul *et al.* (2016) estimated that the treatment with ineffective antimicrobials resulted in 89,000 deaths among 165,000 people who were infected with *B. pseudomallei* globally in 2015 (30).

1.1.4 Melioidosis mortality rate

Melioidosis is known to have high mortality rates, attributable primarily to septicemia. In fact, the disease can cause up to 20% of all community-acquired sepsis in the tropics, including 40% of sepsis-related mortality, as reported in northern Thailand (31). The mortality overall for the disease can be particularly high in certain regions; it has been reported as high as 50% in low-resource settings of northeastern Thailand and up to approximately 20% in northern Australia where modern diagnostics for pathogen detection are more available (24, 31).

Despite the high mortality rates, melioidosis has drawn sparse attention from international health organizations such as the U.S. Centers for Disease

Control and Prevention or the World Health Organization because it has a relatively limited geographical distribution. Currently, there is a severe lack of public education about the disease, no licensed vaccines available to protect humans and animals, and sparse research to develop antimicrobial therapy. While melioidosis commonly is considered to be endemic in tropical regions, nearly 165,000 cases are estimated to occur worldwide each year (32). Further, because of its severe and fatally infectious nature, melioidosis is considered an emerging global threat. In fact, the U.S. military considers *B. pseudomallei* a potential bioweapon, as the disease can be acquired via inhalation and the viable bacteria can persist in the environment for up to six years (33). With increasing international travel and population migration, more melioidosis cases have recently been reported outside of endemic regions (34), rendering the disease an important public health issue today.

1.2 Description of *B. pseudomallei*

The bacterium is a Gram-negative saprophyte that measures 2-5 um in length and 0.4–0.8um in diameter (35) and is a facultative intracellular pathogen that can remain latent for decades (36). Bacterial proliferation occurs optimally at 40˚C in slightly acidic environments (37).

The bacteria can invade and multiply in both phagocytic (e.g., neutrophils) and nonphagocytic (e.g., epithelial) cells (12). When *B. pseudomallei*

enters a nonphagocytic cell, it attaches to the cell using its Type IV pili, which act as a hook by which the bacterium adheres to the cell, and its thin capsular polysaccharide (CPS) layer also helps it attach to the host cell (12). Once the bacterium invades the cells, it leaves the host cell using its Type III secretion system (TTSS), which is a needle-like structure that injects toxins through the walls of the host cell membrane. The toxins injected enable the bacterium to escape and inhibit autophagy. The TTSS is critical for *B. pseudomallei* infection because without it, the bacteria cannot reach the cytosol of a cell, which is where the bacteria gain motility to infect more cells (38). Once released into the cytoplasm, the bacterium assembles an actin tail at one of its poles, which allows it to propel itself to other adjacent cells. In a phagocytic cell, *B. pseudomallei* retains its components by neutralizing the reactive oxygen species and proteases, which are the factors that typically destroy pathogens (39, 40). The bacterium causes further damage in the host cell by destroying the red blood cells with hemolysins that it produces and by hijacking the host cell's iron compounds with its siderophores (12).

According to an *in vivo* study that quantified the presence of *B. pseudomallei* in various parts of the organs, Nualnoi *et al.* (2016) demonstrated that an important component of the pathogen, capsular polysaccharide (CPS), was rapidly cleared from the blood stream, passed through kidneys, and

excreted into urine, making the concentration of CPS the highest in urine (41). Based on this finding, from a pathophysiology perspective, *B. pseudomallei* is likely flushed out of the blood stream and excreted into urine in the infected individuals. Additionally, many reports indicated positive results of bacterial culture from clinical samples such as pus and abscess (42, 43), therefore, *B. pseudomallei* is also expected to be found at high concentrations in the affected organs containing pus or abscesses.

1.2.1 History of *B. pseudomallei*

According to a phylogenomic analysis, the bacterium originated in Australia and migrated to Southeast Asia during the last ice age, approximately 16,000 to 225,000 years ago (44). Interestingly, the bacterium was also found to have evolved in Madagascar approximately 1,500 to 2,000 years ago (45). This evolution is thought to have occurred when travelers migrating from the island of Borneo to Madagascar, transported the bacteria there. Half of the population in Madagascar is of African ancestry, and *B. pseudomallei* is thought then to have spread to the Americas from West Africa during the slave trade of the 1800s. While the bacteria exist in other areas, such as Central and South America (46, 47), the tropical climate of Southeast Asia and northern Australia serve as an ideal environment.

1.3 Melioidosis epidemiology in Malaysia

Melioidosis is endemic in Southeast Asia and northern Australia. The tropical rainforests of Malaysia provide an optimal location for *B. pseudomallei* to grow and proliferate. Each year, approximately one thousand melioidosis cases have been reported across Malaysia (48) and more than two thousand people are estimated to die from melioidosis every year (6). Despite the high frequency of cases reported, melioidosis is still considered a widely unrecognized disease in Malaysia, and its true incidence is unknown due to the variability in case reporting through the country (49). Its incidence seems to vary between, and even within states, and there may be various hotspots. For instance, highly agricultural states, such as Kedah, generally report a greater incidence of melioidosis. The state of Kedah, which borders Thailand and is considered the country's major rice growing region, previously reported an incidence of 16.35 human cases per 100,0000 population per year. However, due to lack of surveillance, the precise disease incidence and epidemiological patterns are unknown (2).

Previous studies in Malaysia have estimated that the mortality rate among *B. pseudomallei*-positive patients in 2016 was 43% (50). In a study of pediatric melioidosis, Mohan *et al.* reported that 10 (23.8%) of 42 children died in Sarawak (51). Transmission via inhalation was also reported in a patient who developed acute, septicemic melioidosis after inhaling infective dust (5). While melioidosis

occurs at any age, including at birth, the greatest incidence in Malaysian cases is reported as between 40 and 60 years of age, which is the age range that the most co-morbid conditions (e.g. pneumonia) develop (50). The majority of cases found were among males. This gender difference may be attributable to the greater numbers of men working in soil- or water-exposed occupations and activities. Most cases have been reported in the population of the Malay ethnicity (49), which may reflect the higher proportion of Malay rice paddy farmers and agricultural employees with the potential to be exposed to contaminated water and soil, or certain states' general preponderance of those of Malay ethnicity.

1.4 Challenges in diagnosing melioidosis

Melioidosis can be difficult to diagnose for two primary reasons. First, its diverse clinical manifestations are easily confused with those of other diseases. The clinical signs that distinguish melioidosis from other diseases are the swelling of lymph nodes and abscesses; however, these are frequently overlooked because of the general lack of awareness of the infection. The similarities in the signs and symptoms present in the infected individuals often leave the patients misdiagnosed, thereby delaying the appropriate treatment.

Second, limited laboratory training and diagnostic techniques contribute to potential misdiagnoses (6, 52) due to the inadequacy of conventional bacterial identification methods in laboratory (25). Diagnosing melioidosis or detecting the

B. pseudomallei requires relatively sophisticated microbiology techniques, such as incubation of the clinical samples, isolation of the bacterial colonies on blood agar or MacConkey agar, and microscopic evaluation. Furthermore, maintaining a sterile environment in the laboratory is critical for accurately identifying the pathogen. Without suitable instruments or laboratory facilities, the required tasks cannot be performed to produce reliable results. Unfortunately, the necessary laboratory facilities are not always available, especially in the resource-limited settings, making it difficult for the clinicians to provide an accurate diagnosis for their patients.

1.5 Gaps in current melioidosis diagnostics in Malaysia

1.5.1 Bacterial culture as the gold standard

Current diagnostics for *B. pseudomallei* in endemic regions such as Malaysia often involve bacterial culture. Such isolation of the pathogen offers the most accurate medical diagnosis. For this reason, bacterial culture method is considered the gold standard. However, bacterial culture may take up to two weeks as it involves multiple steps, including an incubation of the clinical samples, Gram-staining of the bacterial colonies, and microscopic confirmation of the bacterial organism (25). Hence, relying upon bacterial culture often delays diagnosis and subsequent treatment.

Additionally, *B. pseudomallei* is often not readily isolated from clinical specimens because of the antibiotics added to the culture media. For melioidosis diagnostics, the clinical samples are typically incubated in selective culture media—Ashdown's media, which contains gentamicin that prevents the growth of bacteria that are not of interest (25). Gentamicin may inhibit the growth of *B. pseudomallei* and requires at least a 96 hour-long incubation period. Modified media are available, such as *Burkholderia pseudomallei* selective agar, however, this medium is known to yield a decreased growth of the pathogen (25). Francis medium, which is known to result in the highest yield of bacterial growth, still requires at least 18 hours of incubation (53).

Because *B. pseudomallei* infected individuals require early treatment and only an accurate diagnosis can lead to such treatments, relying solely upon bacterial culture method for melioidosis diagnosis will place the patients at higher risk for dying.

1.5.2 Molecular evaluation for melioidosis diagnostics

A molecular technique—real-time polymerase chain reaction (rt-PCR or qPCR)—which measures bacterial DNA in clinical specimens, can identify infection status, sooner and more accurately as it relies on the presence of the bacterial DNA in clinical specimens. The molecular assay employed for this study used the type III secretion 1 open reading frame 2 (TTS1-*orf2*) as the gene

target (54). This gene encodes the open reading frame for the TTS1 proteins, which is the critical for *B. pseudomallei* infectivity. From a validation assessment, TTS1-*orf2* was found to be extremely specific to *B. pseudomallei*. Although this assay is not commercially available, a laboratory evaluation suggested a high sensitivity (80%) and specificity (100%) when fifty bacterial isolates were assessed (54, 55), but an evaluation using clinical samples has not been quantified.

The qPCR method involves an extraction of bacterial DNA and a performance of qPCR assays, which together may take up to five hours from the time that the samples are received in a laboratory to the production of the results. While this method is completed much more rapidly than the bacterial culture, it involves considerable diagnostic delays, making it less ideal in urgent clinical situations. Additionally, qPCR techniques require considerable technical skills and advanced laboratory equipment, making it less ideal in low-resource settings.

1.5.3 Active Melioidosis Detect™ rapid testkKit

To address the delay in diagnosis and treatment, melioidosis diagnostics potentially can be completed by employing a point-of-care (POC) rapid diagnostic tool that provides results more quickly. In this study, a rapid diagnostic tool based on a lateral flow immunoassay (LFI) was used. The test kit

used for the study, Active Melioidosis *Detect*™ rapid test kit (AMD), was developed by the collaborative effort between the research team at the University of Nevada and a biomedical company called InBios, Inc, based in Seattle, WA.

The AMD qualitatively detects the capsular polysaccharide (CPS) produced by *B. pseudomallei*. The strips were developed using a monoclonal antibody (mAb) 3C5 that targets the *B. pseudomallei* CPS. The test line is sprayed with 3C5 and the control line is sprayed with goat anti-chicken IgY. The AMD strip consists of a sample pad, conjugate pad, and a membrane with test line and control line, and absorbent pad (in the direction of capillary flow) (Figure 1A). When a sample is loaded onto the sample pad, the capillary flow moves along the other components of the strip in the respective order stated above. The AMD can be used to test human blood, serum, and other clinical specimens, including urine, pus, sputum, and bacterial colonies. In a presence of the CPS, the strip shows two bands and the test is considered positive; if the membrane shows only one band, the sample is considered negative (Figure 1B).

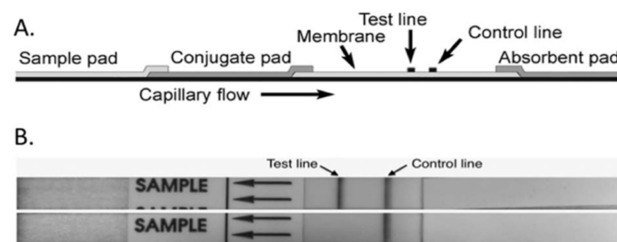

Figure 1. The design of the AMD strips (A) and the interpretations of AMD test results (B) Source: Houghton *et al.* (2016)

Laboratory evaluation of the strips have demonstrated a high sensitivity (>95%) and high specificity (>95%) when they were applied to 77 bacterial isolates (15). However, sensitivity and specificity have not been quantified using clinical samples. The AMD strips are currently only available for research purposes and have not been commercialized.

The various melioidosis diagnostics are summarized in Table 1.

Table 1. Methods used to detect *B. pseudomallei* among 100 study participants in Kapit Hospital from June 21, 2018 to January 8, 2019

Diagnostic Tool	Results Available in	Characteristics
Bacterial culture	4-6 weeks	Gold standard but takes too long; requires specific media that may be unavailable
qPCR	Up to 5 hours	Requires expensive equipment, such as thermal cycler, and appropriate laboratory set up (e.g., to process samples)
AMD LFI	15 minutes	Point-of-care; rapid and simple to use

qPCR: real-time polymerase chain reaction; AMD LFI: Active Melioidosis Detect lateral flow immunoassay

1.6 Study Objectives

1.6.1 Assessment of agreements between different diagnostic tools

The first objective of the study was to assess the agreement between the diagnostic tools, including the AMD strips, qPCR, and bacterial culture. A bacterial culture detects viable *B. pseudomallei* and is therefore considered the gold standard. Based on the laboratory evaluations, I hypothesized that the AMD and qPCR would detect *B. pseudomallei* at comparable sensitivities compared to bacterial culture in evaluating clinical samples.

Using the AMD to detect *B. pseudomallei* is not yet a standard point-of-care in endemic areas. Validating the AMD will improve infection management, particularly in low-resource settings with limited access to accurate and timely diagnostic tools. Once validated, the AMD may replace other time-consuming techniques, such as bacterial culture and qPCR. Additionally, when such rapid testing method is made available, populations living in endemic regions throughout Southeast Asia and northern Australia would benefit from timely diagnosis.

Our research team collaborated with the Kapit Hospital in Kapit, Sarawak, to help the hospital set up a molecular laboratory and supplied the laboratory with a real-time PCR platform. Our introduction of these qPCR assays in Kapit has strengthened the ability of the Kapit medical team to better detect *B. pseudomallei*.

1.6.2 Assessment of risk factors for *B. pseudomallei* infections

The second objective of the study was to examine the association between potential risk factors and the infection status using the questionnaire data. The questionnaires collected information on the demographics, clinical symptoms/chronic conditions, and behaviors. Based on the previous studies conducted, I hypothesized that *B. pseudomallei* infections are positively associated with male individuals in their 40-60s who work mostly outdoors, present

symptoms such as lymph node swelling or abscess, have chronic illness, and who do not practice protective behaviors such as using protective gear during exposure with soil or water.

1.6.3 Long term goal of the study

With this pilot study, the research team hopes to help the medical staff at Kapit Hospital adopt one or more laboratory assays for *B. pseudomallei* to accurately diagnose infections locally.

2. Methods

2.1 Ethics approval

Approval for this study was obtained by the Medical Research and Ethics Committee of Malaysia and the Institutional Review Board at Duke University on April 25, 2018 and May 1, 2018, respectively.

2.2 Research setting

This study was conducted between June 2018 and January 2019 at Kapit Hospital of Kapit District in the state of Sarawak, Malaysia. While the true prevalence or true incidence rates of melioidosis are not available due to poor reporting and large variability within the state, Mohan et al. (2017) did report that an estimated annual incidence rate of 20.2 per 100,000 children for Kapit, which is the highest rate observed among all melioidosis endemic regions (51).

Sarawak, located immediately north of the equator, has a relatively uniform temperature throughout the year with a hot and highly humid climate. The year-long relative humidity exceeds 80% and the air temperature ranges from 23°C to 32°C throughout the day; the water temperature averages 29°C throughout the year. Heavy rains are common between November and February, and annual rainfall averages 3,300 mm to 4,600 mm (56).

Kapit is located south of the Rajang River, the longest river in Malaysia, and is primarily accessible by boat or light aircraft; dense primary forests cover

80% of the city (57). According to the Official Portal of the Sarawak Government, the population of the Kapit district was approximately 56,000 in 2010, while the overall population in the Sarawak state was about 2.5 million. Kapit Hospital is a 134-bed government hospital located in the Kapit district of Sarawak, providing both outpatient and inpatient services. Clinical services available include emergency medicine, pharmacy, pathology, and imaging; the hospital also has diagnostic and health education units. Kapit Hospital's laboratory provides services in pathology, chemistry, hematology, microbiology, and urinalysis.

2.3 Participant recruitment

The eligible participants of the study were patients who were suspected to have melioidosis based on the clinical signs and symptoms. Licensed medical officers (MOs) made clinical assessments of melioidosis-suspected patients. The inclusion and exclusion criteria (Appendix A) of participant eligibility were adapted from the melioidosis case definition provided by the U.S. Centers for Disease Control and Prevention (CDC). All patients who were at least six months of age, admitted to Kapit Hospital, and had prolonged fever accompanied by other melioidosis-like symptoms were eligible to participate in this study. The melioidosis-like symptoms that the MOs looked for included any of the following: enlarged lymph nodes, tender swelling of glands, splenic or liver lesion by bedside scan, joint pain with swelling, purplish vesicle or bullae in

limbs, deep seated abscess or brain abscess, pneumonia not responding to 48 hours of first line of antibiotics, and severe sepsis with or without shock. Patients were excluded from the study if they had a clear alternative diagnosis other than melioidosis provided by a trained healthcare professional at Kapit Hospital, had already begun an antibiotic treatment for melioidosis, or were younger than six months of age.

Sample size calculations were based on a previous melioidosis study conducted by Samy *et al.* (2005) in Thailand, which showed a 20% prevalence of melioidosis (3). From analyses, we assumed an alpha level of α= 0.05, a power level of β= 80%, and that the proportion of the true positive (patients who test positive for *B. pseudomallei* via AMD who indeed have the disease via the bacterial culture—the gold standard) would be 0.95. Mirroring the prevalence in Thailand and assuming that the true melioidosis prevalence in Malaysia is 20%, the study would require a minimum sample size of 284. Based on time and budget constraints, the target sample size was set to 100 participants.

2.4 Procedures

2.4.1 Patient enrollment and sample collection

Before beginning the study, seven medical officers (MOs) received a packet of study-related documents prepared by the Duke One Health study team. The packet included the standardized operating procedures for (a)

enrolling participants based on the inclusion and exclusion criteria, (b) collecting blood, urine, and other bodily fluids, and (c) processing samples.

The MOs received training in enrollment procedures before study participant recruitment began. The MOs administered a questionnaire that captured demographic and potential risk factor data. Then, they collected a single 5.0 ml tube of blood from adults and adolescents aged 12-18; or a 2.0 ml tube of blood from children under 12 years of age. If participants were willing and able, the MOs asked them to provide a urine sample in a sterile urine collection cup. If the MOs deemed it clinically necessary, other bodily fluids such as pus and sputum were also collected and studied.

Informed consent was obtained from enrolled adults more than 18 years of age and from parents or guardians of children under seven years of age. Children between the ages of seven and 18 years provided written assent in addition to the informed consent obtained from a parent or guardian.

The dominant ethnic group in Kapit, Sarawak is Iban. In 2000, approximately 67.4% of the population in Kapit was Iban, with the remaining population of Orang Ulu (18.7%), Chinese (6.7%), Malay (3.4%), and Melanau (1.2%) ethnic groups (3). Considering the large group of Iban residents in Kapit who often do not read English, all study materials (e.g., the patient information

sheet, consent and assent forms, and patient questionnaire) were translated into both Malay and Mandarin.

2.4.2 Sample processing

Sample processing procedures were adapted from the study conducted by Houghton et al. (2014) (15). Upon arriving at the laboratory, blood samples sat upright for up to 60 minutes at room temperature; next, they underwent centrifugation at 2000g for 10 min. The serum separated from the centrifuged tubes was transferred to a 2.0 ml sterile cryovial tube. Urine samples were collected in sterile urine collection cups, and a portion of the sample was subsequently transferred into a clean tube to be centrifuged at 2000g for 10 min. The centrifuged urine specimen with a pellet was transferred to a 2.0 ml sterile cryovial tube. Other bodily fluids such as pus and sputum were collected in sterile specimen collection cups; up to 2.0 ml were transferred to a 2.0 ml sterile cryovial tube. After sample processing, the specimens were immediately used for a point-of-care diagnostic test evaluation. All specimens were stored at -80°C until thawed for the molecular analysis.

2.5 Measures

2.5.1 Active Melioidosis Detect™ rapid test kit

The Active Melioidosis Detect™ (AMD) Rapid Test Kits were supplied by In*Bios*, Inc. For serum, 50ul was combined with 150ul (approximately two drops)

of chase buffer and the solution was then applied to the AMD sample pad. For urine, 50ul was combined with 150ul (approximately two drops) of chase buffer and the solution was then applied to the AMD sample pad. For pus, sputum, and other viscous bodily fluids, 20ul was combined with 100ul of lysis buffer followed by vortexing. The lysate (20ul) was then combined with 150ul (approximately two drops) of chase buffer and the solution was then applied to an AMD sample pad. Each test was allowed to flow for 15 minutes and then a digital image was captured.

2.5.2 Molecular assay

Extraction of bacterial DNA from the stored samples was completed using the Macherey-Nagel Viral RNA Isolation kit (Macherey-Nagel). DNA extracts (70ul) were stored in mini centrifuge tubes at -80°C until ready for molecular analysis.

DNA extracts were examined by a real-time PCR assay (qPCR), using SsoAdvanced Universal Probes Supermix (Bio-Rad, Hercules, CA) for detection of *B. pseudomallei*. The real-time PCR program for *B. pseudomallei* was adapted from Novak et al. (2006) (54) and Kaestli et al. (2012) (55). Primers and probes were identified and validated following a literature review of Novak (2006) (54) and Kaestli (2012) (55). Thermal cycling conditions for the assay were 95°C for 3 min and 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Threshold

cycle (Ct) value— the number of cycles required to detect a PCR product of interest—cut offs were determined based on the literature review (55). Ct values <40 were positive and Ct values >40 were negative. The extracted DNA samples were compared to DNA positive controls and nuclease-free water as negative control.

Table 2. Primers and probe used to conduct qPCR for *B. pseudomallei*

B. pseudomallei	Forward Primer	5′- CGT-CTC-TAT-ACT-GTC-GAG-CAA-TCG -3′
	Reverse Primer	5′- CGT-GCA-CAC-CGG-TCA-GTA-TC -3′
	Probe	5′- FAM-CCG-GAA-TCT-GGA-TCA-CCA-CCA-CTT-TCC-BHQ-1 -3′

2.5.3 Bacterial culture and gram stain

Bacterial culture (gold standard), was performed for each clinical specimen by the Kapit laboratory technicians following their standard procedures, which consisted of isolating the bacterial organism, staining the bacterial colonies, and identifying the bacterial species. The results of bacterial culture, Gram stain, and sensitivity for various antibiotics were collected approximately four weeks after the patient admission date.

2.6 Statistical analysis

Questionnaire data, AMD strip results, real-time PCR results, and bacterial culture/gram stain results were entered into REDCap version 7.0 (58). All data were imported into and all analyses were performed in RStudio version 1.1.447 (RStudio, Inc.). (59)

Kappa statistics analyses and McNemar's tests were conducted to assess agreement between different test methods: AMD vs. bacterial culture, qPCR vs. bacterial culture, AMD vs. qPCR, and AMD vs. clinical diagnosis at discharge, and qPCR vs. clinical diagnosis at discharge. For each comparison, true prevalence, sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were determined.

For the purpose of the risk factor assessement, the prevalence of the infection was determined using the following approach: if any of a pateint's clinical samples (blood, urine, or other bodily fluids) showed evidence of *B. pseudomallei* infection by any of the diagnostic method (AMD, qPCR, or bacterial culture), the patient was considered infected. This number was used to conduct risk factor analysis for the study.

To assess the potential risk factors for *B. pseudomallei* infection, a bivariate analysis was completed to examine the association between potential risk factors and *B. pseudomallei* infection. Potential risk factors included gender, age group, ethnicity, education level, work environment, primary source of water, housing type, clinical symptoms, chronic diseases, and behavioral factors. Fisher's exact tests were used for bivariate work, and odds ratio (OR) with 95% confidence intervals (CI) were calculated.

Based on a previous AMD validation study findings, which speculated that the faint bands observed were not accurate representative of the presence of *B. pseduomallei* (60), any AMD tests that showed faint bands were considered negative, and only the AMD results that showed strong bands were considered positive.

3. Results

From June 21, 2018 to January 8, 2019, we enrolled 100 patients who met the inclusion criteria (see Appendix A) and collected 100 blood samples, 97 urine samples, and 16 other bodily fluid samples. Seven blood samples, 15 urine samples, and one bodily fluid sample were positive for *B. pseudomallei* by the AMD strips. Five blood samples, five urine samples, and five bodily fluid samples were positive for *B. pseudomallei* by the molecular assays. Seven blood samples, one urine sample, and 11 bodily fluid samples were positive for *B. pseudomallei* by the bacterial culture.

3.1 Concordance assessment

The results of the rapid diagnostic tool, molecular assessment, bacterial culture, and the diagnosis made at discharge by the medical officers (MOs) were evaluated for agreement for various sets of pairs with sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). Accuracy was also determined for each comparison. Kappa values were used to determine the strength of the agreement between two diagnostic methods. The interpretation of the kappa values (61) was as follows in Table 3:

Table 3. Cohen's kappa interpretation

Value of Kappa	Level of Agreement
0-0.20	None
0.21-0.39	Minimal

0.40-0.59	Weak
0.60-0.79	Moderate
0.80-0.90	Strong
Above 0.90	Almost Perfect

Table 4 summarizes the kappa analyses, prevalence, sensitivity, specificity, PPV, NPV, and accuracy and their 95% CI ranges of the comparisons between 1) the results of AMD and bacterial culture; 2) the results of qPCR and bacterial culture; and 3) the results of AMD and qPCR.

Table 4. Summary of agreement between diagnostic methods, % (95 % CI)

	AMD vs Bacterial Culture (n= 172)	**qPCR vs Bacterial Culture (n= 171)**	**AMD vs qPCR (n= 213)**
Prevalence	9 (5-15)	9 (5-14)	8 (7-16)
Sensitivity	44 (20-70)	40 (16-68)	38 (15-65)
Specificity	93 (88-96)	96 (91-98)	91 (87-95)
PPV	39 (17-64)	46 (19-75)	26 (10-48)
NPV	94 (89-97)	94 (89-97)	95 (91-97)
Accuracy	87 (82-91)	91 (85-95)	88 (82-93)
Kappa	0.347	0.378	0.240
McNemar	0.82	0.80	0.25

Table 4 shows low sensitivity for each pair of comparisons (38-44%) and high specificity across the three pairs (91-96%). Kappa analysis suggests that the AMD and the bacterial culture and the qPCR and the bacterial culture were in weak agreement; and the AMD and the qPCR were in minimal agreement. To understand which types of the samples were responsible for the observations

above, each comparison set of the diagnostic tests were further investigated by sample types (Table 4-7).

Table 5. AMD results against bacterial culture results, % (95 % CI)

	Sera (n= 93)	Urine (n= 63)	Other bodily fluids (n= 16)
Prevalence	9 (4-16)	12 (5-22)	25 (7-52)
Sensitivity	38 (9-76)	88 (47-100)	25 (1-81)
Specificity	95 (88-99)	88 (77-95)	100 (74-100)
PPV	43 (10-82)	50 (23-77)	100 (3-100)
NPV	94 (87-98)	98 (90-100)	80 (52-96)
Accuracy	90 (82-95)	87 (77-94)	81 (54-96)
Kappa	0.348	0.571	0.333
McNemar	1.00	0.08	0.25

Table 5 summarizes the sensitivity, specificity, PPV and NPV of AMD results against bacterial culture results for sera, urine, and other bodily fluids. While sensitivities for sera (38%) and other bodily fluids (25%) samples were low, with a wide 95% CI, urine samples show higher sensitivity (88%), also with a wide 95% CI. Specificities for all sample types—sera (95%), urine (88%), and other bodily fluid (100%)—were high. The kappa statistics showed that the results of AMD and bacterial culture were in minimal agreement for sera and other bodily fluid samples, and they were in a weak agreement for the urine samples.

Table 6. Molecular assay results against bacterial culture results, % (95 % CI)

	Sera (n= 93)	Urine (n= 62)	Other bodily fluids (n= 16)
Prevalence	9 (4-16)	5 (1-13)	25 (7-52)

Sensitivity	25 (3-65)	33 (1-91)	75 (19-99)
Specificity	96 (90-99)	97 (88-100)	83 (52-98)
PPV	40 (5-85)	33 (1-91)	60 (15-95)
NPV	93 (86-97)	97 (88-100)	91 (59-100)
Accuracy	90 (82-95)	94 (84-98)	81 (54-96)
Kappa	0.259	0.299	0.538
NcNemar	1.00	1.00	1.00

Table 6 summarizes the sensitivity, specificity, PPV and NPV of qPCR results against bacterial culture results for sera, urine, and other bodily fluids. While sensitivities for sera (25%) and urine (33%) samples were low, with a wide 95% CI, bodily fluid samples show higher sensitivity (75%), also with a wide 95% CI. Specificities for all sample types—sera (96%), urine (97%), and other bodily fluid (83%)—were high. The kappa statistics showed that the results of AMD and bacterial culture were in minimal agreement for sera and urine, while for other bodily fluids, there was weak agreement.

Table 7. AMD results against molecular assay results, % (95 % CI)

	Sera (n= 100)	**Urine (n= 97)**	**Other bodily fluids (n= 16)**
Prevalence	5 (2-11)	5 (2-12)	31 (11-59)
Sensitivity	40 (5-85)	80 (28-99)	0 (0-52)
Specificity	94 (87-98)	65 (55-75)	91 (59-100)
PPV	25 (3-65)	11 (3-26)	0 (0-97)
NPV	97 (91-99)	98 (91-100)	67 (38-88)
Accuracy	91 (84-96)	66 (56-75)	63 (35-84)
Kappa	0.262	0.120	-0.116
McNemar	0.51	<0.01	0.22

According to Table 7, sensitivity for sera (40%) and other bodily fluids (0%) samples were notably low, with a wide range of 95% CI. On the contrary, sensitivity for urine samples was relatively high, although this value also presented a wide 95% CI. Specificities for sera (94%) and other bodily fluid (91%) samples were reasonable, however, urine samples showed lower specificity (65%). Kappa analysis suggested that the AMD results and qPCR results have a minimal agreement in sera samples, while they had no agreement in urine or other bodily fluid samples.

At the time of enrollment, the study participants were suspected to be infected with *B. pseudomallei*. At discharge, the MOs provided a clinical diagnosis after reviewing the bacterial culture results, Gram-stain results, and the patients' response to the administered antibiotics. Of the 100 patients enrolled, 47 patients were clinically diagnosed with melioidosis, which exceeded the number of patients (n=26) whose clinical specimens detected *B. pseudomallei* by one or more diagnostics used. To assess the agreement of the AMD results and qPCR results with the clinical diagnosis, concordance analyses were conducted.

Table 8. AMD results against clinical diagnosis at discharge, % (95 % CI)

	Sera (n= 100)	**Urine (n= 97)**	**Other bodily fluids (n= 16)**
Prevalence	47 (37-57)	23 (15-32)	50 (25-75)
Sensitivity	13 (5-26)	45 (24-68)	12 (0-53)
Specificity	98 (90-100)	93 (85-98)	100 (63-100)
PPV	86 (42-100)	67 (38-88)	100 (3-100)

NPV	56 (45-66)	85 (76-92)	53 (27-79)
Accuracy	58 (48-68)	82 (73-89)	56 (30-80)
Kappa	0.114	0.437	0.125
McNemar	<0.01	0.15	0.02

Table 8 summarizes the sensitivity, specificity, PPV and NPV of AMD results against the clinical diagnosis provided by an MO at patient discharge for sera, urine, and other bodily fluids. The sensitivities for sera (13%), urine (45%), and other bodily fluid (12%) samples were remarkably low with wide 95% CI. Specificities for all sample types—sera (98%), urine (93%), and other bodily fluid (100%)—were high. The kappa statistics suggest that the AMD results and clinical diagnoses had no agreement between AMD and sera and other bodily fluid samples, while they had a weak agreement between AMD and urine samples.

Table 9. Molecular assay results against clinical diagnosis at discharge, % (95 % CI)

	Sera (n= 100)	Urine (n= 97)	Other bodily fluids (n= 16)
Prevalence	46 (36-56)	23 (15-32)	50 (25-75)
Sensitivity	9 (2-21)	14 (3-35)	62 (24-91)
Specificity	98 (90-100)	97 (91-100)	100 (63-100)
PPV	80 (28-99)	60 (15-95)	100 (48-100)
NPV	56 (45-66)	79 (70-87)	73 (39-94)
Accuracy	57 (47-67)	78 (69-86)	81 (54-96)
Kappa	0.073	0.151	0.625
McNemar	<0.01	<0.01	0.25

According to Table 9, when qPCR results were compared with the clinical diagnosis made at patient discharge, sensitivity for molecular assays in sera (9%) and urine (14%) samples were notably low, while that of other bodily fluid samples was higher (62%), although this value presented a wide 95% CIs. Specificities for all sample types—sera (98%), urine (97%), and other bodily fluids (100%) samples – were high, but other bodily fluid samples had a wide 95% CI. Kappa analysis suggest that the qPCR results and the clinical diagnosis had no agreement in sera or urine samples, while they have moderate agreement in other bodily fluid samples.

3.2 Disease prevalence

The prevalence of *B. pseudomallei* was defined by using three different methods; 1) bacterial isolation by bacterial culture; 2) bacterial DNA detection by molecular assay; and 3) positivity determined by the AMD strips. For the purpose of the study, if a patient showed any evidence of *B. pseudomallei* infection using any of the three detection methods, the patient was considered *B. pseudomallei* -positive. Table 10 shows the varying prevalence of *B. pseudomallei* across the different detection methods, each with its own sampling strategy.

Table 10. Prevalence of *B. pseudomallei* by diagnostic methods among 100 patients in Kapit Hospital from June 21, 2018 to January 8, 2019

Diagnostic methods	Among 100 participants	Among 26 participants which were positive by any method

	n (%)	n (%)
Any method	26 (26.0)	--
Bacterial culture	16 (16.0)	16 (61.5)
Molecular assay	13 (13.0)	13 (50.0)
Rapid diagnostic test	18 (18.0)	18 (69.2)

Twenty six of 100 patients were found to be infected with *B. pseudomallei* (26%), which was determined if the patient showed any *B. pseudomallei* positivity by one or more diagnostic methods (AMD, qPCR, or bacterial culture). Of the 26 participants whose samples were indicated as infected with *B. pseudomallei*, 16 were determined as infected (61.5%) by bacterial culture; Thirteen by molecular evidence (50%); and 18 by the AMD strips (69.2%).

3.3 Baseline characteristics

Twenty one of 100 patients were children under the age of 5 years (21%); 16 of the patients were of ages 5-15 years (16%); 12 were of ages 16-30 years (12%); 16 were of ages 31-45 years (16%); 16 were of ages 46-60 years (16%) and 19 were over the age of 60 years (19%). Of the enrolled participants, 63 were male (63%) and 37 were female (37%). The majority of the study population was of Iban ethnicity (87%). Thirty-one of 100 patients worked mostly outdoors (31%); thirty-two worked in a mixed outdoor/indoor environment (32%); and 34 worked in indoor settings (34%). Forty-four patients indicated that their primary water source was from pipes (44%), followed by 41 patients who indicated that

they used river water (41%). Of the 100 patients enrolled, 38 patients indicated that they lived in a house (38%) while 55 lived in a longhouse (55%). These values are summarized in Table 11.

Table 11. Characteristics of 100 study participants with infection status defined by one or more diagnostic methods in Kapit Hospital from June 21, 2018 to January 8, 2019

	Patient Population (n=100)		
Variables	***B. pseudomallei* (n=26)**	**No *B. pseudomallei* (n=74)**	**Unadjusted OR (95% CI)**
Gender			
Female	7 (18.4%)	31 (81.6%)	Ref.
Male	19 (30.6%)	43 (69.4%)	1.90 (0.67-6.03)
Age			
<5	3 (14.3%)	18 (85.7%)	0.48 (0.10-1.62)
5-15	5 (31.2%)	11 (68.8%)	0.45 (0.08-2.22)
16-30	7 (58.3%)	5 (41.7%)	2.00 (0.42-10.03)
31-45	6 (37.5%)	10 (62.5%)	Ref.
46-60	2 (12.5%)	14 (87.5%)	0.24 (0.03-1.28)
60+	3 (15.8%)	16 (84.2%)	0.13 (0.01-0.92)
Ethnicity			
Iban	21 (24.1%)	66 (75.9%)	Ref.
Other	5 (38.5%)	8 (61.5%)	0.58 (0.15-2.44)
Education level*			
None	10 (32.3%)	21 (67.7%)	Ref.
Primary	8 (42.1%)	11 (57.9%)	1.11 (0.25-4.55)
Post-Primary	5 (71.4%)	2 (28.6%)	0.54 (0.11-2.31)
Work environment			
Mostly Outdoors	10 (32.3%)	21 (67.7%)	Ref.
Outdoor/Indoor	9 (28.1%)	23 (71.9%)	0.82 (0.28-2.42)
Mostly Indoors	7 (20.6%)	27 (79.4%)	0.54 (0.17-1.66)
Primary Water Source			
Piped	10 (22.7%)	34 (77.3%)	Ref.
River	14 (27.5%)	37 (72.5%)	1.29 (0.51-3.35)
Other	2 (33.3%)	4 (66.7%)	1.70 (0.31-10.14)
Housing Type			
House	11 (28.9%)	27 (71.1%)	Ref.
Longhouse**	13 (23.6%)	42 (76.4%)	0.76 (0.30-1.96)
Other	2 (28.6%)	5 (71.4%)	0.98 (0.11-4.22)

*For assessment of the education level, children aged <5 (n=21) were excluded as they are not expected to be enrolled in school
** Longhouses are infrastructure common in rural parts of Sarawak, Malaysia. These buildings consist of a long, closed hallway that is shared many families share a long, closed hallway. The infrastructure has been considered a public health threat in Malaysia as they place residents at high risk of transmitting infectious diseases.

The association of gender and ethnicity against infection status was examined by performing Fisher's exact tests to obtain odds ratio (ORs). The association of age group, educational level, and housing type against presence of infection was examined by fitting a bivariate logistic regression model to obtain ORs. No predictors had a p-value <0.1 when bivariate analysis was conducted (not shown in table), therefore, no further statistical analysis was performed. According to Table 4, the baseline characteristics are similar between those who were diagnosed with melioidosis and those who were not.

3.4 Primary clinical presentations and chronic illness status

Melioidosis is characterized by a wide range of clinical symptoms. Fever, cough, and headache were the most common symptoms observed in this study. The clincal symptoms were recorded at the time of physical examination by an MO. As summarized in Table 5, of the 26 *B. pseudomallei*-positive patients, all had fever (100%); 16 showed cough (62%); 11 reported headaches (42%); seven reported muscle aches (27%). Many showed inflammatory symptoms as well.

Eight had lymph node swelling or abscess (23%); and two had joint pain and/or swelling (8%) (See Table 12).

Individuals with chronic conditions such as diabetes and kidney complications are also reported to have higher risk of being infected with *B. pseudomallei*. Of the 26 *B. pseudomallei*-positive patients, two had a history of diabetes (8%); two had kidney complications (8%); one had a respiratory disease (4%); no one reported a history of a blood disorder (0%) or cancer (0%).

The possible association of the clinical symptoms and infection status was studied using the Fisher's exact test, which was also used to determine the association of the chronic disease presence and infection status. Based on the odds ratios obtained, none of the clinical symptoms or chronic diseases was statistically significant when compared between the two patient groups (those with diagnosis of *B. pseudomallei* and those without). No further statistical analyses were performed.

Table 12. Primary clinical presentations, chronic diseases, and confirmed *B. pseudomallei* infections in Kapit Hospital from June 21, 2018 to January 8, 2019

	Patient Population (n=100)		
Variables	***B. pseudomallei* (n= 26)**	**No *B. pseudomallei* (n= 74)**	**Unadjusted OR (95% CI)**
Clinical Symptoms			
Fever	26 (26.3%)	73 (73.7%)	Inf (0.06-inf)
Cough	16 (32.7%)	33 (67.3%)	1.97 (0.73-5.57)
Headache	11 (22.4%)	38 (77.6%)	0.70 (0.25-1.87)
Muscle Ache	7 (28.0%)	18 (72.0%)	1.14 (0.35-3.46)

Abdominal Pain	5 (26.3%)	14 (73.7%)	1.02 (0.26-3.48)
Vomiting	6 (20.7%)	23 (79.3%)	0.67 (0.19-2.02)
Lymph Node Swelling/Abscess	8 (29.6%)	19 (70.4%)	1.28 (0.41-3.75)
Joint Pain and/or Swelling	2 (33.3%)	4 (66.7%)	1.45 (0.13-10.89)
Chronic Diseases			
Diabetes	2 (18.2%)	9 (81.8%)	0.60 (0.06-3.23)
Kidney Complications	2 (18.2%)	9 (81.8%)	0.60 (0.06-3.23)
Respiratory Diseases	1 (16.7%)	5 (83.3%)	0.55 (0.01-5.32)
Blood Disorder	0 (0%)	6 (100%)	NA
Cancer	0 (0%)	1 (100%)	NA

3.5 Behavioral risk factors and *B. pseudomallei* infection status

For behavioral risk factor analysis, six behaviors were assessed for their frequency. The six behaviors included using personal protective gear (PPE) during exposure to water, using PPE during exposure to soil, washing after exposure to soil or water, cooking foods with heat before consumption, boiling water before consumption, and having an open wound exposed to contaminated water or soil. Using a 4-point Likert scale, participants indicated the frequency of their behaviors as Never (zero times a week), Rarely (1-3 times a week), Often (4-6 times a week), and Always (7 times a week).

Table 13. Association between behavioral factors and *B. pseudomallei* infections among 100 study participants in Kapit Hospital from June 21, 2018 to January 8, 2019

	Patient Population (n=100)		
Variables	***B. pseudomallei* (n=26)**	**No *B. pseudomallei* (n=74)**	**Unadjusted OR (95% CI)**
Frequency of Using PPE During Exposure to Water			
Never	20	53	Ref.

Rarely	2	3	1.77 (0.22-11.43)
Often	0	2	NA
Always	2	13	0.41 (0.06-1.65)
Frequency of Using PPE During Exposure to Soil			
Never	17	48	Ref.
Rarely	1	4	0.71 (0.03-5.20)
Often	1	1	2.82 (0.11-74.09)
Always	5	20	0.71 (0.21-2.07)
Frequency of Washing After Exposure to Soil/Water			
Never	2	3	3.17 (0.39-21.20)
Rarely	3	4	3.56 (0.64-18.30)
Often	6	9	3.56 (1.01-12.26)
Always	12	57	Ref.
Frequency of Consuming Cooked Foods			
Never	2	1	3.30 (0.38-29.0)
Rarely	0	0	NA
Often	3	6	1.65 (0.33-6.87)
Always	20	67	Ref.
Frequency of Consuming Boiled Water			
Never	2	1	3.09 (0.35-2.70)
Rarely	0	1	NA
Often	2	3	2.06 (0.26-1.32)
Always	22	69	Ref.
Frequency of Direct Contact with Water or Soil to Open Wounds			
Never	14	63	Ref.
Rarely	9	9	4.50 (1.51-13.67)
Often	1	1	4.50 (0.17-118.5)
Always	0	1	NA

4. Discussion

4.1 Sensitivity and specificity of the point-of-care test

Overall, the point-of-care AMD strips exhibited poor sensitivity and high specificity when compared with the bacterial culture results. The sensitivity was considerably high in the urine samples (>80%), which is likely due to the *B. pseudomallei* being readily excreted into urine. The AMD evaluations were dependent on a bacterial concentration in the samples; the AMD has a limit of detection (LOD) of 0.2 ng/ml capsular polysaccharides (CPS) (15). Thus, samples with bacterial load less than this value are not detected by the AMD strips. Serum samples, which exhibited 33% sensitivity when compared with the bacterial culture results, likely did not have sufficient concentration of bacteria as the CPS from internal abscesses are shed into the blood stream and has a short half-life—2.9 to 4.4 hours (41). Consequently, serum samples tend to have a low detection rate. Unlike the molecules produced by similar bacteria such as *C. negoformans* and *B. antracis*, when the CPS leaves the blood stream, it is passed through kidneys and is excreted into the urine, instead of being deposited in other organs. Surprisingly, other bodily fluid samples—pus, sputum, and needle aspirations—in this study exhibited poor sensitivity (25%). While the CPS concentration is expected to be high in these samples, as I would expect the bacteria manifest in the affected organs, the results showed otherwise. While

bodily fluids may be considered the best vehicle to detect bacterial DNA in the body, the AMD strips were found not to be as sensitive as previously reported in other studies. Further investigations would need to be conducted to understand this observation.

4.2 Disagreements between the AMD and bacterial culture results

We observed false-positive AMD results across different sample types when compared to the bacterial culture results. Four serum samples (57.1%) and seven urine samples (70.0%) showed falsely positive results (Appendix D, Tables 4-6). It is unclear why the AMD reactivity was observed in the *B. pseudomallei*-negative individuals, and additional work is needed to elicit an explanation. We speculate that the false-positives may be due to laboratory contamination. It is also possible that these false-positives are truly positive for *B. pseudomallei* but the clinical samples may not have sufficient bacterial concentration; in another study where the patients can be followed over time and the research team is able to document disease development, these patients' clinical samples may show positivity with further disease development. The false-negative AMD results are likely to have resulted from a low initial bacterial load.

4.3 Disagreements between the qPCR and bacterial culture results

The poor detection of the bacterial DNA using the qPCR method may be due to multiple reasons depending on the sample type. Within the serum samples, the bacterial concentration may be too low (62). The qPCR conditions were optimized and evaluated for sensitivity (80%) and specificity (100%) using the bacterial isolates. However, the clinical samples were not as concentrated as the bacterial isolates, which led to a poor sensitivity (25%). In the case with the urine samples, the high concentration of urea in the urine samples may be acting as a major inhibitor in qPCR as it can degrade the polymerase (63). It is also possible that urea in the urine samples inhibited the bacterial polymerase activity, preventing further bacterial DNA amplification, resulting in false-negative result. Other bodily fluid samples such as pus, sputum, and needle aspirations showed higher sensitivity (75%) against the bacterial culture results compared to that of sera and urine samples. These observations were likely due to the generally high bacterial manifestation occurring in these body parts, resulting in a high concentration of the bacteria in the samples, although the AMD results did not agree.

The false-positives observed in the comparisons between the qPCR and bacterial culture may be due to the potential contamination of the samples during the molecular assessment preparations. The molecular laboratory was newly set up in Kapit Hospital during the study even though the hospital was

not adequately equipped to house a molecular laboratory. The facility did not have an appropriate ventilation system nor the working space to prepare the molecular reagents. Additionally, the master mix preparation room and the amplicon room were a significant distance apart with several patient wards in between, therefore, sample contamination may have been unavoidable. Similarly with the false-negative results observed between the AMD strips and bacterial culture, it is possible that the bacterial concentrations in the clinical samples at the time of collection were too low. If the patients whose clinical samples showed false-positive results were followed over time, it is possible that their disease development progression would have resulted in true positive results.

4.4 Implications for policy and practice

With no vaccines available for melioidosis, current diagnostic efforts in melioidosis-endemic regions largely involve the reinforcement of using personal protective equipment while working in or near potentially contaminated soil and water. Until accurate point-of-care diagnostic tools are successfully adapted, residents in the endemic regions would need to rely heavily on continued empiric treatments and prevention measures.

Additionally, the final clinical diagnosis was made at discharge after the MOs reviewed the bacterial culture results and the patients' response to the administered antibiotics. While positive results via bacterial culture are the

strongest evidence that a patient is infected with *B. pseudomallei*, some patients' diagnosis at discharge remained melioidosis if they presented the defined symptoms (e.g. liver or spleen abscess) or if they responded well to the antibiotic treatment. Based on this observation, it is possible that the patients were overtreated with antibiotics, which may contribute to further antibiotic resistance that *B. pseudomallei* is intrinsically prone to (28). Therefore, more sensitive diagnostic methods will benefit human populations at high risk of *B. pseudomallei* infections. While the AMD strips examined in this study did not show desirable sensitivity or specificity when used with the clinical specimens, similar diagnostic tools should be developed to provide adequate care that the infected individuals need.

4.5 Implications for further research

To our knowledge, this is the first attempt to quantify the agreement measures for the AMD strips and qPCR assays using patient clinical samples rather than the bacterial isolates. This study provides a set of referential surveillance data of *B. pseudomallei* infections and validation data for the AMD and qPCR performances within the *B. pseudomallei*-suspected patient population in Kapit, Sarawak, Malaysia. The study findings provide useful an important information about the behavior of the AMD strips in a hot and humid environment. Additionally, because we did not detect the *B. pseudomallei*-

confirmed bacterial culture, we may benefit from genetic analysis to explore if different *B. pseudomallei* strains were causing the infections in the participants, and if and how their genetic makeup differs from the strain that was used as the positive control in the study.

4.6 Study strengths and limitations

Strengths of the study include employing molecular analysis in addition to the bacterial culture evaluation and clinical diagnosis made by the medical staff at Kapit Hospital. The employment of the molecular evaluation not only added another layer of comparisons, examining the performance of the AMD strips against the qPCR performance. Another strength of the study is derived from obtaining duplicates for the molecular assessments and the blinded review of the AMD strip results. The AMD results were assessed by two study members—a Kapit laboratory technician and a Duke One Health study member—to ensure the interpretations were in agreement.

This pilot study had limitations. First, the analyses only include 100 participants and 213 samples—relatively small numbers—that may substantially limit the breadth of the data and, hence, the analyses. Second, there was suboptimal sample integrity. When patient samples were collected, AMD strips were performed immediately. However, the remaining samples were stored at -80°C until molecular evaluation was ready. Prolonged sample storage time at -

80°C may have damaged sample integrity by reducing the concentration of bacterial nucleic acids in the samples to an insufficient amount. Such a reduction can make detection via molecular assays difficult. Additionally, the cross-sectional study design was severely limiting to understand the performance of AMD strips with the disease progression. A prospective study design with a follow-up to verify disease development in patients would have strengthened the study further. Lastly, although bacterial culture method was considered the gold standard in this study, it is still not the perfect gold standard due to low bacterial load in the clinical samples. Tissue biopsies were likely not conducted aggressively in this study setting, limiting the true outcome of the bacterial culture.

Despite our study's limitations, our findings were valuable to the *B. pseudomallei*-infected patient population and to the medical staff. While assays lacked high sensitivity if any single clinical for assay was positive for *B. pseudomallei*, the high specificity of these assays suggested that antimicrobial treatment was appropriate. As we enroll more patients in the study, we will have a better assessment of the usefulness of these particular assays and how they might best fit into clinical care algorithms. We also are sending some positive specimens to the U.S. Center for Disease Control and Prevention for

genome-based intensively study. We trust these additional analyses will also help the hospitals clinical care teams.

5. Conclusion

As melioidosis gains more public attention, this study serves as an important addition to both public health research and public knowledge. As no vaccines against *B. pseudomallei* has not yet been developed; preventive measures, accurate diagnosis, and targeted treatment are the key elements in reducing melioidosis mortality and morbidity.

Based on the findings of this limited pilot study, no major significant risk factors were found to be associated with *B. pseudomallei* infections, although some trends were observed. For example, those who work outdoors were observed to be at higher risk, but the use of protective behaviors can offset or reduce that risk. Additionally, chronic diseases, which are documented as risk factors for *B. pseudomallei* infections, were not observed to follow the general trends noted in literature. Considering the relatively small number of participants thus far (*n*=100), the study may not reflect generally perceived patterns.

The AMD strips, in general, showed poor sensitivity but high specificity when clinical samples were used. Our study findings suggest that the AMD diagnostics should not be recommended as a single diagnostic tool due to the

poor sensitivity. However, with their high specificity, the strips may yet prove useful in combination with another more sensitive assay.

While further research effort is required to identify the challenges for melioidosis diagnostics to ensure the tools can be used in endemic areas, this study provides the characteristics of the AMD strips in a tropical climate such as Malaysia. Future studies should focus on improving the ability of the AMD strips to detect the bacteria at lower antibody concentrations and to identify the factors that lead to false positives resulting from a weak interaction between the AMD strip and the CPS.

Appendix A

Inclusion criteria:

- Admitted to Kapit Hospital
- Fever more than 3 days and one or more of the following:
  - Enlarged cervical, inguinal or axillary lymph nodes (greater than 3 cm in diameter), painless adenopathy or lymphadenitis or lymph node abscesses
  - One or both sides glands involvement such as tender swelling over medial aspect of the lower eyelid of the lacrimal glands; submandibular glands, parotic glands
  - Bedside scan with splenic or liver hypoechoic lesion (less than 2cm in diameter)
  - Pneumonia not responding to 48 hours of first line antibiotics
  - One or more joint pain with swelling
  - Purplish vesicle or bullae mainly over the lower limbs, may resulting in ulcer after rupture
  - Deep seated abscess or brain abscess or genitourinary abscess or osteomyelitis
  - Severe sepsis patient with or without shock and/or with multiorgan involvement
  - Unexplained fever that lasts 7 days or longer

Exclusion criteria:

- Have a clear alternative diagnosis other than melioidosis by a trained health care professional at the Kapit Hospital
- Have already been treated for melioidosis (e.g. received antibiotics before the start of the study)
- Are younger than 6 months of age

Appendix B

Table 14. Primary clinical presentations, chronic diseases, and confirmed B. pseudomallei infections in Kapit Hospital from June 21, 2018 to January 8, 2019

	Patient Population (n=100)		
Variables	***B. pseudomallei* (n= 26)**	**No *B. pseudomallei* (n= 74)**	**Unadjusted OR (95% CI)**
Clinical Symptoms			
Fever	26	73	Inf (0.06-inf)
Cough	16	33	1.97 (0.73-5.57)
Headache	11	38	0.70 (0.25-1.87)
Muscle Ache	7	18	1.14 (0.35-3.46)
Abdominal Pain	5	14	1.02 (0.26-3.48)
Vomiting	6	23	0.67 (0.19-2.02)
Lymph Node Swelling/Abscess	7 (43.8%)	9 (56.2%)	2.63 (0.73-9.21)
Gland Tender Swelling	1 (9.1%)	10 (90.9%)	0.26 (0.01-2.00)
Joint Pain and/or Swelling	2 (33.3%)	4 (66.7%)	1.45 (0.13-10.89)
Chronic Diseases*	5 (19.2%)	21 (80.8%)	0.50 (0.13-1.59)

*chronic diseases include diabetes, kidney complications, respiratory diseases, blood disorder, and cancer

Appendix C

Table 15. Association between behavioral factors and B. pseudomallei infections among 100 study participants in Kapit Hospital from June 21, 2018 to January 8, 2019

	Patient Population (n=100)		
Variables	***B. pseudomallei* (n=26)**	**No *B. pseudomallei* (n=74)**	**Unadjusted OR (95% CI)**
Frequency of Using PPE During Exposure to Water			
Never	20	52	Ref.
Ever	4	18	0.58 (0.13-2.08)
Frequency of Using PPE During Exposure to Soil			
Never	17	47	Ref.
Ever	7	25	0.78 (0.24-2.30)
Frequency of Washing After Exposure to Soil/Water			
Never	2	3	Ref.
Ever	21	69	0.46 (0.05-5.86)
Frequency of Consuming Cooked Foods			
Never	2	2	Ref.
Ever	23	72	0.32 (0.02-4.70)
Frequency of Consuming Boiled Water			
Never	2	2	Ref.
Ever	24	72	0.34 (0.02-4.89)
Frequency of Direct Contact with Water or Soil to Open Wounds			
Never	14	62	Ref.
Ever	10	11	3.96 (1.25-12.73)

Appendix D

Contingency tables for comparing the results of RDT and qPCR, qPCR and bacterial culture results, and RDT and bacterial culture results across all sample types are shown below:

Table 16. 2x2 contingency table for RDT results vs qPCR results of all sample types

	qPCR pos	qPCR neg	Total
RDT pos	6	17	23
RDT neg	10	180	190
Total	16	197	213

Table 17. 2x2 contingency table for qPCR results vs bacterial culture results of all sample types

	Culture pos	Culture neg	Total
qPCR pos	6	7	13
qPCR neg	9	149	158
Total	15	156	171

Table 18. 2x2 contingency table for RDT results vs bacterial culture results of all sample types

	Culture pos	Culture neg	Total
RDT pos	7	11	18
RDT neg	9	145	154
Total	16	156	172

Contingency tables for comparing the results of RDT and bacterial culture, separated by sample type, are shown below:

Table 19. 2x2 contingency table for RDT results vs bacterial culture results of serum samples

	Culture pos	Culture neg	Total
RDT pos	3	4	7

RDT neg	5	81	86
Total	8	85	93

Table 20. 2x2 contingency table for RDT results vs bacterial culture results of urine samples

	Culture pos	Culture neg	Total
RDT pos	3	7	10
RDT neg	1	52	53
Total	4	59	63

Table 21. 2x2 contingency table for RDT results vs qPCR results of other bodily fluid samples

	Culture pos	Culture neg	Total
RDT pos	1	0	1
RDT neg	3	12	15
Total	4	12	16

Contingency tables for comparing the results of qPCR and bacterial culture, separated by sample type, are shown below:

Table 22. 2x2 contingency table for qPCR results vs bacterial culture results of serum samples

	Culture pos	Culture neg	Total
qPCR pos	2	3	5
qPCR neg	6	82	88
Total	8	85	93

Table 23. 2x2 contingency table for qPCR results vs bacterial culture results of urine samples

	Culture pos	Culture neg	Total
qPCR pos	1	2	3
qPCR neg	2	57	59
Total	3	59	62

Table 24. 2x2 contingency table for qPCR results vs bacterial culture results of other bodily fluid samples

	Culture pos	Culture neg	Total

qPCR pos	3	2	5
qPCR neg	1	10	11
Total	4	12	16

Contingency tables for comparing the results of RDT and qPCR, separated by sample type, are shown below:

Table 25. 2x2 contingency table for RDT results vs qPCR results of serum samples

	PCR pos	PCR neg	Total
RDT pos	2	6	8
RDT neg	3	89	92
Total	5	95	100

Table 26. 2x2 contingency table for RDT results vs qPCR results of urine samples

	PCR pos	PCR neg	Total
RDT pos	4	32	36
RDT neg	1	60	61
Total	5	92	97

Table 27. 2x2 contingency table for RDT results vs qPCR results of other bodily fluid samples

	PCR pos	PCR neg	Total
RDT pos	0	1	1
RDT neg	5	10	15
Total	5	11	16

Contingency tables for comparing the results of RDT and clinical diagnosis at discharge, separated by sample type, are shown below:

Table 28. 2x2 contingency table for RDT results vs clinical diagnosis at discharge of serum samples

	Melio diagnosed	No melio diagnosed	Total
RDT pos	6	1	7

RDT neg	41	52	93
Total	47	53	100

Table 29. 2x2 contingency table for RDT results vs clinical diagnosis at discharge of urine samples

	Melio diagnosed	No melio diagnosed	Total
RDT pos	10	5	15
RDT neg	12	70	82
Total	22	75	97

Table 30. 2x2 contingency table for RDT results vs clinical diagnosis at discharge of other bodily fluid samples

	Melio diagnosed	No melio diagnosed	Total
RDT pos	1	0	1
RDT neg	7	8	15
Total	8	8	16

Contingency tables for comparing the results of qPCR and clinical diagnosis at discharge, separated by sample type, are shown below:

Table 31. 2x2 contingency table for qPCR results vs clinical diagnosis at discharge of serum samples

	Melio diagnosed	No melio diagnosed	Total
qPCR pos	4	1	5
qPCR neg	42	53	95
Total	46	54	100

Table 32. 2x2 contingency table for qPCR results vs clinical diagnosis at discharge of urine samples

	Melio diagnosed	No melio diagnosed	Total
qPCR pos	3	2	5
qPCR neg	19	73	92
Total	22	75	97

Table 33. 2x2 contingency table for qPCR results vs clinical diagnosis at discharge of other bodily fluid samples

	Melio diagnosed	No melio diagnosed	Total
qPCR pos	5	0	5
qPCR neg	3	8	11
Total	8	8	16

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