Research Article

Duplex TaqMan Hydrolysis Probe-Based Molecular Assay for Simultaneous Detection and Differentiation of Burkholderia pseudomallei and Leptospira spp. DNA

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Melioidosis and leptospirosis, caused by two different bacteria, Burkholderia pseudomallei and Leptospira spp., are potentially fatal infections that share a very similar spectrum of clinical features and cause significant mortality and morbidity in humans and livestock. Early detection is important for better clinical consequences. To our knowledge, there is no diagnostic tool available to simultaneously detect and differentiate melioidosis and leptospirosis in humans and animals. In this study, we described a duplex TaqMan probe-based qPCR for the detection of B. pseudomallei and Leptospira spp. DNA. The performance of the assay was evaluated on 20 B. pseudomallei isolates, 23 Leptospira strains, and 39 other microorganisms, as well as two sets of serially diluted reference strains. The duplex qPCR assay was able to detect 0.02 pg (~ 4 copies) Leptospira spp. DNA and 0.2 pg (~ 25.6 copies) B. pseudomallei DNA. No undesired amplification was observed in other microorganisms. In conclusion, the duplex qPCR assay was sensitive and specific for the detection of B. pseudomallei & Leptospira spp. DNA and is suitable for further analytical and clinical evaluation.

1. Introduction

Burkholderia pseudomallei and Leptospira are two important infectious agents for melioidosis and leptospirosis, respectively [1–3]. The Gram-negative B. pseudomallei is recognized as CDC Tier 1 select agent and a Category B Priority Pathogen by the National Institute of Allergy and Infectious Diseases (NIAID), in addition to leptospirosis, which has been added to the Emerging Infectious Diseases category (https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens). Both organisms are normally found in the soil and freshwater environment [4, 5].
In addition to their ubiquitous habitats, these organisms routinely infect animals, such as cattle, sheep, and horses. Certain animal classes such as rats may asymptptomatically carry *Leptospira*. It is generally accepted that animals are responsible for shedding and maintenance of *Leptospira* and *B. pseudomallei* in the environments, through their urines and faeces [5–7]. Human cases are usually associated with interactions with the contaminated environments [6, 8]. To date, increasing cases of melioidosis and leptospirosis have been reported worldwide, especially in the tropical and subtropical regions [1, 6].

Infections by *B. pseudomallei* and *Leptospira* portray a very similar spectrum of nonspecific clinical presentations including fever, headache, myalgia, and pneumonia [4, 8]. In animals, *B. pseudomallei* infections cause pneumonia with lung abscesses, anorexia, and encephalitis [9]. Meanwhile, animal leptospirosis is characterized by abortion, jaundice, and infertility [10]. Several factors, such as bacterial load, underlying medical conditions, and serotypes increase hosts susceptibility to melioidosis and leptospirosis [1, 11–13]. Furthermore, the risk of dual infection is apparent, as several incidences of melioidosis-leptospirosis coinfections were reported previously [14, 15]. It is possible that many cases may be underdiagnosed when only one between the two tests is considered or available [16].

Early detection of melioidosis and leptospirosis could significantly increase the chances of survival and reduce potential economic loss [17]. Current gold standard for detecting *B. pseudomallei* is by the culture method which requires 2–7 days to grow [18]. Meanwhile, leptospiral antibody titer is detected by the microscopic agglutination test (MAT) that usually requires paired sera and is less useful during acute infection [5]. As both diagnostic methods are time-consuming, a more rapid laboratory assay is urgently needed. To date, several molecular assays have been described for detection of individual *B. pseudomallei* and *Leptospira* from the clinical specimens [5, 18]. However, to our knowledge, none of the reported assays is able to simultaneously detect and distinguish *B. pseudomallei* and *Leptospira* within the same reaction tube. In this study, we developed a duplex qPCR that can detect *B. pseudomallei* and *Leptospira* DNA and evaluated the assay on selected clinical and environmental isolates.

2. Materials and Methods

2.1. Microorganism Strains and Growth Conditions. A total of 20 *B. pseudomallei* strains, 23 *Leptospira* strains and 39 other microorganisms isolated from human clinical samples and ATCC strains were used in this study (Table 1). These microorganisms were provided by the Department of Medical Microbiology & Parasitology, School of Medical Sciences, Universiti Sains Malaysia; Makmal Kesihatan Awam Kota Bharu; Universiti Putra Malaysia; and Institute for Medical Research. The bacteria were cultured aerobically in nutrient broth overnight at 37°C on a rotating platform of 180 rpm. Meanwhile, *Leptospira* strains were maintained in EMJH media, incubated at 30°C on rotating platform of 40 rpm, overnight. *Entamoeba histolytica* DNA was obtained directly from School of Health Sciences, Universiti Sains Malaysia.

2.2. Isolation of Genomic DNA. DNA was extracted from pure bacterial culture using NucleoSpin® Tissue DNA Extraction kit (MACHEREY-NAGEL GmbH & Co. KG, Germany). The extraction procedure was carried out according to the manufacturer instructions with a minor modification on the final elution step, in which the column was incubated at room temperature for 10 minutes prior to centrifugation at 11,000 × g. Total DNA was quantified using the Eppendorf BioPhotometer (Eppendorf Scientific, Inc., New York, United States) and stored at -20°C until use.

2.3. Duplex Real-Time PCR Parameters. The PCR reaction was prepared in a total volume of 20 μL, containing 10 μL 2× SsoAdvanced™ Universal Probes Supermix, 1 μL PCR grade distilled water, 0.2 μM primers, 0.1 μM probes, and 8 μL DNA template. Sequences of oligonucleotides used are listed in Table 2. The oligonucleotides were designed for amplification of the orf2 region of *B. pseudomallei* type III secretion system (T3SS) and the rrs gene of *Leptospira*. Amplifications were conducted using Biorad CFX96 Touch Real-Time PCR Detection System. Thermal cycling condition included an initial denaturation at 95°C for 5 minutes, followed by 50 cycles of 95°C for 30 seconds and 61.3°C for 30 seconds. Baseline threshold for the postamplification analysis was set at 50 (for *B. pseudomallei*) and 25 (for *Leptospira*). Any Cq value ≤40 is considered positive. All the amplification in this study was carried out in triplicate, unless specified otherwise.

2.4. Analytical Sensitivity and Specificity. The analytical sensitivity of the assay was carried out using extracted *B. pseudomallei* and *L. interrogans* gDNA, diluted 10-fold ranging from 10 ng/μL to 1 fg/μL. Two microliters of each diluted gDNA were used in the duplex qPCR. Amount of bacterial DNA in each reaction was calculated based on a formula previously described by Aghamollaei et al. (2015) [21]. Meanwhile, the assay analytical specificity was determined using 2 μL extracted DNA from other organisms (non-*Leptospira* DNAs and non-*B. pseudomallei*), as listed in Table 1. DNA were extracted using NucleoSpin® Tissue extraction kit.

3. Results and Discussions

Despite the availability of several TaqMan hydrolysis probe-based assays for the detection of either *Leptospira* spp. or *B. pseudomallei*, none of the reported assays are able to simultaneously detect both organisms within the same reaction [18, 22]. Availability of such diagnostic tool that is able to detect and differentiate *B. pseudomallei* or *Leptospira* spp. is crucial as both infections portray similar clinical features and yet require different clinical management. In this study, a duplex qPCR for detection of *B. pseudomallei* and *Leptospira* spp. DNA was evaluated. As shown in Table 3, the developed qPCR was able to amplify 0.02 pg (~4 copies) *Leptospira* spp. DNA and 0.2 pg (~25.6 copies) *B. pseudomallei* DNA, respectively. The sensitivity of the duplex assay for detection of *Leptospira* DNA is comparable to other reported leptospiral probe-based assays that detected between 1 and 20 DNA
| Organism                             | Source            | No. tested (n) | Results in duplex qPCR |
|-------------------------------------|-------------------|----------------|------------------------|
| Aspergillus fumigatus               | USM, Malaysia     | 1              | Negative               |
| Bacillus subtilis                   | USM, Malaysia     | 1              | Negative               |
| Burkholderia cepacia                | USM, Malaysia     | 6              | Negative               |
| Burkholderia pseudomallei           | USM, Malaysia     | 20             | Positive               |
| Burkholderia thailandensis         | USM, Malaysia     | 1              | Negative               |
| Campylobacter jejuni                | USM, Malaysia     | 1              | Negative               |
| Candida albicans                    | USM, Malaysia     | 1              | Negative               |
| Citrobacter freundii                | USM, Malaysia     | 1              | Negative               |
| Entamoeba histolytica              | UNAM, Mexico      | 1              | Negative               |
| Enterococcus faecalis               | USM, Malaysia     | 1              | Negative               |
| Klebsiella pneumoniae              | USM, Malaysia     | 1              | Negative               |
| Leptospira biflexa serovar Patoc   | IMR, Malaysia     | 1              | Positive               |
| Leptospira biflexa serovar Patoc   | UPM, Malaysia     | 1              | Positive               |
| Leptospira borgpetersenii Celledoni| IMR, Malaysia     | 1              | Positive               |
| Leptospira borgpetersenii serovar Ballum | UPM, Malaysia   | 1              | Positive               |
| Leptospira fainei serovar Hurtsbridge | IMR, Malaysia | 1              | Positive               |
| Leptospira fainei serovar Hurtsbridge | UPM, Malaysia   | 1              | Positive               |
| Leptospira interrogans serovar Australis | UPM, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Autumnalis | IMR, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Bataviae | IMR, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Bataviae | UPM, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Canicola | UPM, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Copenhageni | IMR, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Hebdomadis | UPM, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Icterohaemorrhagiae RGA | UPM, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Javanica | IMR, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Pomona | IMR, Malaysia   | 1              | Positive               |
| Leptospira interrogans serovar Pomona | UPM, Malaysia   | 1              | Positive               |
| Leptospira interrogans serovar Pyrogenes | IMR, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Pyrogenes | UPM, Malaysia | 1              | Positive               |
| Leptospira interrogans serovar Tarassovi | IMR, Malaysia | 1              | Positive               |
| Leptospira licerasiae serovar Varillal | IMR, Malaysia | 1              | Positive               |
| Leptospira meyeri serovar Semaranga | IMR, Malaysia     | 1              | Positive               |
| Leptospira wolfii                   | IMR, Malaysia     | 1              | Positive               |
| Plasmodium falciparum              | MKA Kota Bharu    | 5              | Negative               |
| Plasmodium knowlesi                | MKA Kota Bharu    | 5              | Negative               |
| Plasmodium vivax                   | MKA Kota Bharu    | 5              | Negative               |
| Proteus mirabilis                  | USM, Malaysia     | 1              | Negative               |
| Proteus vulgaris                   | USM, Malaysia     | 1              | Negative               |
| Salmonella Paratyphi A (ATCC 9150) | ATCC, USA         | 1              | Negative               |
| Salmonella Paratyphi B (ATCC BAA 1250) | ATCC, USA        | 1              | Negative               |
| Salmonella Paratyphi C (ATCC 9068) | ATCC, USA         | 1              | Negative               |
| Salmonella Typhi (ATCC 7251)       | ATCC, USA         | 1              | Negative               |
| Salmonella Typhimurium (ATCC 14028) | ATCC, USA         | 1              | Negative               |
| Staphylococcus aureus              | USM, Malaysia     | 1              | Negative               |
| Staphylococcus saprophyticus       | USM, Malaysia     | 1              | Negative               |
Table 2: List of primers and probes used in this study.

| Target         | Type       | Sequence (5’→3’)                        | Source |
|----------------|------------|-----------------------------------------|--------|
| *Leptospira*   | Forward primer | ACTGAGACACGGTCCATACT                     | [19]   |
|                | Reverse primer | TAGTTAGCGYGTGCTTTAGGYA                   |        |
|                | Probe        | FAM-ACGGGAGGCAGC-ZEN-AGTTAAGATCTTGC-IBFQ|        |
| *B. pseudomallei* | Forward primer | CCTGGGAGAGCGAGATGTT                     | [20]   |
|                 | Reverse primer | GCTGGATGAGAAGAAAGTCC                    |        |
|                 | Probe        | TexRed-CCACGCACGGCGAGATTCT-IBRQ        |        |

Table 3: Analytical sensitivity of the duplex qPCR assays.

| Copies number | Amount (pg) | Mean Cq | SD | CV (%) | Mean Cq | SD | CV (%) |
|---------------|-------------|---------|----|--------|---------|----|--------|
| 25600000      | 20000       | 18.41   | 0.05 | 0.26   | 4000000 | 2000 | 19.3   | 0.17 | 0.86 |
| 256000        | 2000        | 21.75   | 0.31 | 1.4    | 400000  | 2000 | 23.09  | 0.16 | 0.67 |
| 25600         | 200         | 25.34   | 0.12 | 0.46   | 40000   | 200  | 27.23  | 0.04 | 0.16 |
| 2560          | 20          | 28.97   | 0.13 | 0.44   | 400     | 2   | 30.67  | 0.32 | 1.05 |
| 25.6          | 2           | 32.8    | 0.14 | 0.44   | 400     | 2   | 34.63  | 0.08 | 0.24 |
| 2.56          | 0.02        | 36.96   | 1.06 | 2.88   | 40      | 0.2 | 36.34  | 0.5  | 1.37 |
| 0.256         | 0.002       | -       | -   | -      | 0.4     | 0.02| 38.59  | 1.49 | 3.87 |

Table 4: PCR efficiency and linearity of the duplex qPCR assays.

| Parameter   | Target          | Leptospira spp. | B. pseudomallei |
|-------------|-----------------|-----------------|-----------------|
| Slope       | -3.2776         | -3.7006         |
| Efficiency  | 101.9%          | 86.3%           |
| Linearity, R² | 0.9837         | 0.9987          |

copies per reaction [23–25]. Meanwhile, for the B. pseudomallei detection, the sensitivity was slightly lower than the previously reported assays that amplified 5 and 10 DNA copies per reaction [26–28]. In comparison to the corresponding monoplex assay, the duplex assay had comparable sensitivity for *Leptospira*, but had a reduced sensitivity for B. pseudomallei target (0.2 pg in duplex versus 0.02 pg in monoplex). Reduced performance of multiplex assay as compared to the monoplex assay has been observed in other molecular studies which are associated with primers competition, primer cross hybridization, and template mispriming [29, 30]. When tested on other microorganisms, no cross amplification was observed (Table 1). The *orf2* region is selected because it is only present in B. pseudomallei [31]. Meanwhile, for the leptospiral target, the *rrs* gene is used because the gene is present in multiple copies per *Leptospira* genome [32]. As the current panel included limited coverage of organisms, further validation should include *Burkholderia mallei* and other *Burkholderia cepacia* complex (BCC).

As listed in Table 4, the duplex assay had an efficiency of 101.9% for the detection of *Leptospira* DNA, comparable to the monoplex assay (100.5%). However, for the detection of B. pseudomallei DNA, the duplex assay had an efficiency of 86.3%, lower than the monoplex assay (95.9%). The suboptimal efficiency may be attributed to the decreased sensitivity of the assay on B. pseudomallei target. In an ideal condition, PCR efficiency should be 90% and above [33]. Further optimization is necessary in order to increase the assay efficiency, especially for the B. pseudomallei target. Meanwhile, in terms of linearity, the duplex assays (for *Leptospira* and B. pseudomallei DNA detection) had R² values of close to 1. Noticeably, at low copy number, the CV values ranged between 2.8 and 3.8% (Table 3).

Overall, the establishment of a duplex qPCR assay that can detect and differentiate B. pseudomallei and *Leptospira* spp. may help the diagnosis of melioidosis and leptospirosis. However, prior to clinical evaluation, further analytical validation, such as intra- and interassay variation, a wider spectrum of microorganisms for specificity testing, higher number of replicates, and optimization of assays are necessary. In addition, incorporation of internal amplification control should be considered because certain types of clinical samples such as whole blood and urine may cause PCR inhibition.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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Supplementary Materials

The standard curves of the duplex qPCR assays for the detection of *B. pseudomallei* DNA and *Leptospira* spp. DNA are illustrated in Figure S1 in Supplementary Material. (Supplementary Materials)

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