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Development of a PCR system for detection and differentiation of *Burkholderia mallei* and *B. pseudomallei* in clinical and environmental matrices

Mestrado em Biologia Molecular e Genética

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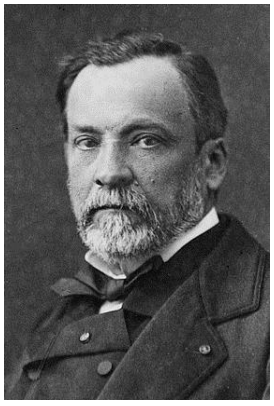

“In the fields of observation chance favors only the prepared mind”

Louis Pasteur

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ABSTRACT

Glanders and melioidosis are two infectious diseases caused, respectively, by the Gram-negative bacteria, *Burkholderia mallei* and *B. pseudomallei*. These species are classified as Class B agents by the Centre of Disease Control (CDC) and as level 3 risk agents by the European Parliament, due to their fast aerosol dissemination, high infectiousness, potential zoonotic capability, absence of vaccines and resistance to a wide variety of antibiotics. The potential use of these microorganisms in biological warfare, already applied in the American Civil War and World Wars I and II, leads to the need of strategic protocols in laboratories of reference to detect and differentiate both agents in a rapid, effective and distinctive way.

A duplex qPCR approach was optimized and evaluated for direct detection and differentiation of *Burkholderia mallei* and *B. pseudomallei* in different matrices. Since in Portugal naturally infected tissues or contaminated material with these agents do not exist, spiked samples were previously prepared. Known concentrations of serial decimal dilutions of *Burkholderia mallei* NCTC 10245 and *B. pseudomallei* NCTC 10276 strains were inoculated in lung tissues and swabs, while soils were spiked only with *B. pseudomallei* NCTC 10276. The duplex qPCR has as targets the *psu* gene that encodes for a putative acetyltransferase specific of *B. pseudomallei* and the transposase of ISBma2, an insertion sequence present in about 48 copies in *B. mallei* genome and in about 6 copies in *B. pseudomallei* genome. Due to the complexity of some matrices that might present PCR inhibitors, giving PCR false negative results, an Internal Amplification Control (IAC) was constructed based on a 125 bp fragment of the m000.5L/R gene of myxoma virus, cloned in the pNZY28 vector.

The duplex qPCR was firstly optimized, evaluated and compared with singlepex qPCR, using purified DNA from strains *B. mallei* NCTC 12938^{T} and *B. pseudomallei* NCTC 12939^{T}. Four hundred nM of each four primers proved to be the best concentration in the duplex reaction, while 200 nM were the appropriated concentration of the two probes targeting both ISBma2 and *psu* gene. The optimal annealing temperature, that gave detection of the target at the lowest quantification cycle (Cq) value, was 58.1 ºC. The limit of detection of the duplex qPCR was 29 fg and 455 fg for, respectively, *B mallei* and *B. pseudomallei*.

The coefficient variance percentages for the repeatability and reproducibility of the duplex qPCR were, respectively, 1.337% and 2.288%, a low variance that indicates high repeatability and reproducibility. The assay was also specific for *B. mallei* and *B. pseudomallei* since it didn't detect DNA from 13 other bacteria, including *Mycobacterium tuberculosis*, *Pseudomonas aeroginosa* and *Burkolderia thailandesis*. This methodology applied to the prepared spiked samples was capable to detect both agents in pulmonary macerates until the less concentrated dilution (10^{-6}) with corresponding Cq values between 15.93 (10^{-1}) and 25.95 (10^{-6}) for *B. mallei* and between 23.44 (10^{-1} – *psu* target) and 38.18

(10^{-6} – *psu* target) for *B. pseudomallei*. For non–enriched swabs, both agents were also detected until the highest dilution 10^{-6}, with Cq values ranging from 20.33 (10^{-1}) to 39.25 (10^{-6}) for *B. mallei* and from 28.98 (10^{-1} – *psu* target) to 38.37 (10^{-6} – *psu* target) for *B. pseudomallei*. Enriched swabs (incubation of swabs in BHIB 48h at 37ºC) but a slightly improvement in the detection of both microorganisms. The alternative approach by performing the qPCR in *B. pseudomallei* isolated colonies showed an increase of sensitivity of the method resulting in Cq values as low as 27.75 for the *psu* target. The "gold standard" culture media method performed in parallel with the qPCR detection, presented some discrepancies mainly for *B. mallei* swabs that showed no growth, probably due to the absence of a specific culture media for this agent proving to be less sensitive than the qPCR.

Keywords: *Burkholderia mallei*; *Burkholderia pseudomallei*; quantitative polymerase chain reaction; Internal Amplification Control; Spiked Samples

RESUMO EM PORTUGUÊS

Mormo e melioidose são patologias causadas pelas bactérias Gram-negativas *Burkholderia mallei* e *B. pseudomallei*, respectivamente. Sendo os equídeos o principal alvo hospedeiro de mormo, cavalos, mulas e burros para exportação necessitam de procedimentos *standard* europeus de despistagem do agente através de ensaios de fixação do complemento pela detecção de anticorpos específicos. Erradicado de Portugal em 1952 e da Europa Ocidental, o mormo é ainda reportado em alguns locais da Ásia, África, Médio Oriente e América do Sul.

Nunca declarada em território português, a melioidose trata-se duma doença endémica em países como Tailândia e norte da Austrália, com expansão em países do continente asiático como as Filipinas, India, Indonésia, Laos, Singapura, Camboja e Vietname, alertando-se também para sua existência em zonas de África e América do Sul.

O potencial zoonótico de *B. mallei* é descrito na literatura, estando identificados como principais grupos de risco investigadores científicos, cujo alvo de estudo implica a manipulação e multiplicação do microorganismo, profissionais de medicina veterinária e funcionários de matadouros. Afectando os animais, o homem e ambiente, a capacidade zoonótica de *B.pseudomallei* não se encontra estabelecida. Contudo, estão declarados inúmeros factores de risco que contribuem para a transmissão da doença no hospedeiro humano, nomeadamente: diabetes, alcoolismo, doenças crónicas renais, hepáticas e pulmonares e terapias imunossupressoras.

As manifestações clínicas de ambas as patologias culminam, geralmente, em vastas complicações a nível pulmonar, podendo levar à morte. As vias de transmissão das duas doenças são principalmente cutânea, através de lesões expostas, inalação e, ocasionalmente, ingestão.

Não existem vacinas ou tratamentos 100% eficientes contra ambas as doenças. Contudo, algumas terapias com base em combinações de diversos antibióticos têm sido estabelecidas mas a sua eficácia depende do progresso de cada patologia e, portanto, qualquer caso de mormo e/ou melioidose deve ser tratado com o máximo de brevidade possível.

Devido à sua rápida disseminação, capacidade de infecção por inoculação e formação de aerossóis, alto factor de contágio e largo espectro de resistência antimicrobiana, *B. mallei* e *B. pseudomallei* foram classificados como agentes de classe B pelo Centre of Disease Control (CDC) e de risco 3 pelo Parlamento Europeu, segundo a Directiva 2000/54/CE.

Numa reunião organizada em conjunto pela Organização Mundial da Saúde (WHO) e a Organização Mundial da Saúde Animal (OIE), especialistas alertaram para o risco eminente em países cujos mecanismos de preparação e prevenção para determinados agentes se encontram inactivos, tornando-os mais susceptíveis à libertação deliberada do agente.

Tendo em conta as declarações acima descritas, protocolos foram estabelecidos para a detecção e diferenciação de *B. mallei* e *B. pseudomallei*, utilizando a metodologia biomolecular através da técnica quantitativa em tempo real de reacção de polimerização em cadeia (qPCR) em matrizes clínicas e ambientais.

Deste modo, e uma vez que não existem amostras clínicas e ambientais de mormo e/ou melioidose em Portugal, três matrizes foram seleccionadas para serem inoculadas com diluições decimais seriadas de *B. mallei* NCTC 10245 e *B. pseudomallei* NCTC 10276*,* de modo a obterem-se amostras experimentalmente infectadas ou *spiked samples*. A escolha das matrizes teve em consideração as amostras comumente recolhidas quando há suspeita de alguma destas infecções: zaragatoas, pois os exsudados ou feridas purulentas são normalmente colhidos com estas ferramentas; macerados pulmonares, visto que ambas as doenças proliferam a nível pulmonar e, no caso específico de melioidose, solos, uma vez que este é o reservatório natural de *B. pseudomallei*.

À excepção dos macerados pulmonares, a identificação de ambos microorganismos nas *spiked samples* foi avaliada em dois tempos diferentes: imediatamente após a infecção, e 48 horas após incubação das matrizes a 37 ºC, comparando a sensibilidade de detecção do método de cultura com a metodologia de qPCR desenvolvida. O isolamento dos agentes através de cultura bacteriana foi realizado utilizando o meio de cultura Agar Ashdown's, específico de *B. pseudomallei*, e o meio de Agar Columbia com 5% de sangue carneiro para *B. mallei*. Enquanto *B. pseudomallei* produz colónias rosas rugosas morfologicamente distinguíveis, as colónias de *B. mallei* não detêm características particulares que permitam a sua diferenciação doutras bactérias.

O duplex qPCR desenvolvido consiste num sistema capaz de identificar e diferenciar os dois microorganismos num só tubo de reacção. Dois alvos foram escolhidos para a detecção e diferenciação de *B. mallei* e *B. pseudomallei*: o gene *psu* que codifica para uma putativa acetiltransferase, pertencente ao *cluster* de genes do sistema tipo III de secreção de *B. pseudomallei* e um gene que codifica uma transposase ISBma2, uma sequência de inserção presente em cerca de 48 cópias e 6 cópias em *B. mallei* e *B. pseudomallei,* respectivamente*.* Deste modo, a amplificação e detecção de sinal por parte das sondas de hidrolisação dos dois genes alvo corresponde à identificação positiva de *B. pseudomallei* enquanto, a amplificação e detecção apenas do gene que codifica a transposase ISBma2 diz respeito a uma amostra positiva para *B.mallei*.

Junto desta plataforma de diagnóstico, foi também construído um controlo interno de amplificação (IAC – Internal Amplification Control), pNZYmyx, clonando o fragmento de 125 pares de base do gene diplóide m000.5 L/R da estirpe Laussane do mixoma vírus no vector pNZY28. A finalidade deste controlo consiste em aferir se a reacção de PCR detém

qualquer factor que resulte na inibição da reacção, afectando a amplificação dos genes alvo.

A adaptação deste sistema de qPCR necessitou de optimização dos oligonucleotídeos necessários à reacção (iniciadores ou primers e sondas) bem como o ajuste da sua temperatura de hibridação (*annealing)* utilizando as estirpes de referência *B. mallei* NCTC 12938^{T} e *B. pseudomallei* NCTC 12939^{T}. Esta optimização de reacção foi executada com os dois alvos em separado (*singleplex*) e em conjunto (*duplex*), seguida de testes de especificidade, sensibilidade, repetibilidade e reprodutibilidade. Em singleplex, a concentração final óptima para cada alvo provou ser 400 nM enquanto que a concentração óptima das sondas de hidrolisação foram 100nM e 300 nM para ISBma2 e *psu*, respectivamente. As concentrações finais óptimas em duplex de primers e sonda para ambos os alvos foram, respectivamente, 400 nM e 200nM e a temperatura de *annealing* que demonstrou o *Cq* (*Quantification Cycle*) mais baixo foi de 58.1 ºC. A especificidade do sistema foi provada testando o qPCR com 17 microorganismos, incluindo *Mycobacterium tuberculosis*, *Pseudomonas aeroginosa* e a espécie geneticamente próxima, *Burkholderia thailandensis*, na qual o sinal de fluorescência foi somente detectado em *B. mallei* e *B. pseudomallei*. O sistema *duplex* qPCR provou ser capaz de detectar 29 fg e 455 fg de DNA de *B. mallei e B. pseudomallei*, respectivamente. O coeficiente de variação calculado para avaliar a repetibilidade e reprodutibilidade obteve valores máximos de 1.337% e de 2.288 %, respectivamente comprovando este sistema ser altamente repetível e reproduzível.

A técnica de qPCR estabelecida foi capaz de identificar e distinguir os dois microorganismos em todas as matrizes inoculadas. No que diz respeito aos macerados pulmonares, o qPCR foi capaz de identificar correctamente os dois microorganismos até à diluição menos concentrada (10^{-6}) detendo valores de *Cq* entre 15.93 (10^{-1}) e 25.95 (10^{-6}) em *B. mallei* e entre 23.44 (10^{-1} – alvo *psu*) e 38.18 (10^{-6} - alvo *psu*) para *B. pseudomallei*. Foi também possível identificar ambos agentes até à diluição menos concentradas para zaragatoas sem o passo de incubação, variando os valores de *Cq* entre 20.33 (10^{-1}) e 39.25 (10^{-6}) for *B. mallei* e entre 28.98 (10^{-1} - alvo *psu*) e 38.37 (10^{-6} - alvo *psu*) para *B. pseudomallei*. A adição prévia do passo de incubação para as zaragotas demonstrou uma variação ligeira indicando com valores de *Cq* inferiores comparativamente às zaragatoas não incubadas. A detecção de *B. pseudomallei* em solos sem incubação prévia foi igualmente possível até à diluição menos concentrada. Porém, a análise de colónias isoladas provou ser altamente sensível, detectando todas as amostras com valores de *Cq* inferiores a 30.

No entanto, o isolamento por cultura bacteriana (*gold standard*) provou ser um método de diagnóstico menos sensível comparando com o sistema de qPCR. A sensibilidade obtida por meio de cultura e qPCR para *B. pseudomallei* foi, respectivamente, 80% e 97%

indicando uma baixa percentagem de falsos negativos para as duas metodologias, contudo, o método de qPCR é mais sensível mostrando ser capaz de identificar amostras consideradas negativas pelo método de cultura. Comparativamente, o método de qPCR para *B.mallei* mostrou ser 100% sensível ao identificar o microorganismo em todas as amostras enquanto que a sensibilidade do método de cultura para a isolação deste agente é significativamente menor, 17%, possivelmente devido à falta de um meio de cultura específico para o isolamento deste microorganismo.

Desta forma, a identificação de *B. mallei* e *B. pseudomallei* por qPCR consiste num teste de diagnóstico sensível, específico, repetível e reprodutível capaz de identificar e diferenciar os dois agentes em amostras previamente inoculadas.

Palavras-chave: *Burkholderia mallei*; *Burkholderia pseudomallei*; duplex qPCR; Spiked Samples; Bioterrorismo

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ABBREVIATIONS

µL - microliter

µm – micrometer

µM – micromolar

A – Adenine

ATCC – American Type Culture Collection

BHIB – Brain Heart Infusion Broth

bp – base pairs

BSL-2 – Biosafety Laboratory Level - 2

BSL-3 – Biosafety Laboratory Level – 3

C – Cytosine

CA/S – Columbia Agar with 5% Sheep Blood

CFT – Complement Fixation Test

CFU - Colony Forming Units

Cq – Quantification Cycle

dsDNA – Double-Stranded DNA

ELISA – Enzyme-Linked Immunosorbent Assay

EPF - End-Point Fluorescence

fg - femtograms

G – Guanine

H - Hours

IAC – Internal Amplification Control

IPTG – Isopropyl-1-thio-β-D-galactoside

IS – Insertion Sequence

LB – Luria Broth

mL - mililiter

mM - milimolar

NCTC – National Collection of Type Cultures

nm - nanometer

nM – nanomolar

NTC – Non-Template Control

OIE - Organisation Mondiale de la Santé Animale/ World Organization of Animal Health

PCR – Polymerase Chain Reaction

PPE – Personal Protective Equipment

qPCR – Real-Time Polymerase Chain Reaction/ quantitative Polymerase Chain Reaction

rDNA – ribossomal Desoxyribonucleic Acid

RFU – Relative Fluorescence Units

T – Thymine

R – Adenine or Guanine

TSA – Tryptic Soy Agar

TSB – Tryptic Soy Broth

TTSS - Type III Secretion System

WGS – Whole-Genome Sequencing

WRAIR - Walter Reed Army Institute of Research

X-Gal - 5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1 - INTRODUCTION

1.0. Genus *Burkholderia*

The β-Proteobacterial *Burkholderia* genus is composed by more than 40 species with wide versatile ecological features. These bacilli shape Gram-negative bacteria ranging from 1–5 μm in length and 0.5–1.0 μm in width were previously classified in the heterogeneous *Pseudomonas* genus. The *Burkholderia* genus was proposed by Yabuushi et al. in 1992 on the basis of 16S ribosomal DNA (rDNA) sequence, DNA–DNA homology values, cellular lipid and fatty acid composition, and phenotypic characteristics [1,2]. This genus includes plant pathogens, microbial biodegradation of pollutants, opportunistic human pathogens (*B. cepacia* complex interacts with cystic fibrosis patients) and primary pathogens *B. mallei* and *B. pseudomallei*, the etiological agents of glanders and melioidosis, respectively, with the ability to infect both humans and animals [3].

Despite glanders being recognized for centuries, multilocus sequence typing (MLST) and whole genome sequencing (WGS) have shown that *B. mallei* is a clone of *B. pseudomallei*, with a considerably smaller genome. The genome reduction resulted in the evolution of *B. pseudomallei* to the mammalian-adapted pathogen *B. mallei*, unable to survive in the environment outside its host [4].

1.1. *Burkholderia mallei*

Burkholderia mallei is the causative agent of glanders, a fatal disease with zoonotic capability [5]. Described as “malis” by Hippocrates in 450 B.C., glanders adopted various names through history e.g. malleus given by Aristotle meaning “depicting a malignant disease”, equinia and droes. The skin form is often described as “farcy”, a designation recognized by the World Organization of Animal Health (OIE) [7,8].

It was first isolated by Friedrich Loeffler and Wilhelm Schütz in 1882 from the infected liver and spleen of a horse and since then, the pathogen has been classified as *Loefflerella mallei*, *Pfeifferella mallei*, *Malleomyces mallei*, *Actinobacillus mallei*, *Corynebacterium mallei*, *Mycobacterium mallei*, *Pseudomonas mallei* and *Bacillus mallei* [9,10].

The high infectiousness, zoonotic capability, aerosol transmission, absence of vaccines and antibiotic resistance characteristics qualifies this agent as a potential biological weapon [7,9,11]. In fact, the use of *B. mallei* as a biological warfare agent during the American Civil War, World Wars I and II and the Russian invasion of Afghanistan has been reported [9].

B. mallei is non-motile, nonsporulating, facultative intracellular and obligate mammalian pathogen. Outside the host, it represents susceptibility to heat, sunlight and common disinfectants. Even so, it can remain viable in water for up to 100 days and at room temperature [5,10].

Blood agar and other nutrient culture media are used for the growth of *B. mallei* since the microorganism has no specific culture media. Colonies become visualized after 48 hours of incubation at 37ºC (See key phenotypical features and growth medium conditions in **Table 1**). The 5.8 Mb genome of NCTC 12938^T strain with G+C content of 68.5 %, contains numerous insertion sequence elements (IS) that mediated extensive deletions and rearrangements. Mutations in pseudogenes linked to flagellum biosynthesis and flagellum motor likely account for *B. mallei* being non-motile and non-flagellated, unlike other closed related species as, for example, *B. pseudomallei* and *B. thailendensis* [12].

1.1.1. Glanders

a) Epidemiology and Clinical Manifestations

With quarantine and veterinary control, glanders has been eradicated from most parts of Western Europe and North America since 1939 [6]. However, sporadic cases still occur in Asia, Africa, the Middle East, and South America. The most recent case of glanders in animals belongs to a dromedary in Baharin in 2011 [13].

Glanders is transmitted by direct invasion of abraded or lacerated skin, inhalation with deep lung deposition and by bacterial invasion of the nasal, oral, and conjunctival mucous membranes [7,11]. Solipeds (e.g. mules, donkeys, horses) are the natural reservoir of *B. mallei* [9,11]. Carnivores can acquire glanders by eating infectious meat while small ruminants will obtain the disease if the contact with the infected horses is persistent [5].

Equine glanders generally takes an acute form in donkeys with high fever and respiratory signs (swollen nostrils, dyspnoea, and pneumonia) and death occurs within a few days. In horses, glanders generally takes a more chronic course with a variety of signs and symptoms dependent on the route of infection including mucopurulent nasal discharge, lung lesions and nodules involving the liver and spleen and horses may survive for several years [5,10]. In the skin form, ”farcy”, lymphatics nodular abscesses are develop, fostering towards suppurative ulcers. It is also stated that vertical transmission from mare to foal and venereal transmission from stallions to mares is possible [10]. Most human cases during the 20th century were occupational infections among laboratory scientists, horse handlers, butchers and veterinarians whose occupation exposes them to infection [6,14]. Human-to-human transmission is rare but it may occur during occupational exposure in medical practice or at autopsies [7].

The mortality rate of human glanders can reach 95% within 3 weeks in untreated acute course. However, survival is possible if the infected person is treated early and aggressively with multiple systemic antibiotic therapies [5].

b) Diagnosis

OIE divides diagnostic techniques for glanders in two groups: Identification of the Agent and Detection of Immune Response.

Serological tests provide information regarding the prevalence of the disease in individuals and communities, contribution to eradication policies. Glanders has been eradicated in several countries due to the international implementation of complement fixation test (CFT) in horses, mules and camels. Immunoblot and Enzyme-Linked Immunosorbent Assay (ELISA) assays have been developed over time but difficulties have been reported in distinguishing *B. mallei* from the close relative species, *B. pseudomallei* [15].

Biochemical tests can be performed (**Table 1.**) but confirmation of the agent by Polymerase Chain Reaction (PCR) is recommended. Numerous PCR strategies have been developed for the identification of *B. mallei* in clinical samples, namely real-time PCR (qPCR) in which the fluorescence is measured alongside with the amplicon production, giving faster results and avoiding the electrophoresis analysis (See **1.4.**).

1.2. *Burkholderia pseudomallei*

In 1912, Whitmore and Krishnaswami described a newly recognized septicemic disease in morphine addicts in Rangoon, Burma. They isolated a bacillus that was similar to *B. mallei* but motile. Whitmore noted the clinical similarity to glanders, and Stanton and Fletcher subsequently proposed the name melioidosis, derived from the Greek melis (distemper of asses) [4]. *Burkholderia pseudomallei* is a Gram-negative soil saprophyte, and its natural reservoir is water and wet soils in rice paddy fields in endemic areas. The bacterium is motile, aerobic, and non-spore-forming. Ashdown's selective medium is commonly used to culture the organism and colonies can take different characteristic intra and inter strains being the most common the pink rough texture (**Table 1.**) [16]. *B. pseudomallei* is a facultative intracellular pathogen that invades and replicates inside polymorphonuclear leukocytes, macrophages, and some epithelial cell lines. Atkins et al. (2004) publicized the complete genome of Bp strain K96243, revealing two circular chromosomes with a total genome length of 7.25 Mb and G+C content of 68.06% [17].

1.2.1. Melioidosis

a) Epidemiology and Clinical Manifestations

Melioidosis is endemic in several parts of Southeast Asia being northern Australia and Thailand the main endemic foci. Melioidosis is also being increasingly reported from many countries across south and east Asia as well as parts of South America, Papua New Guinea and the Caribbean (**Figure 1.**) [6,18]. Sporadic cases were declared during and after World War II, in soldiers fighting in Vietnam, during the war of independence with France and the later conflict involving the USA. Incidence after post-natural disasters has been described [18,19].

Melioidosis is the third most frequent cause of death from infectious diseases in northeast Thailand and is the most common cause of community-acquired bacteraemic pneumonia in parts of northern Australia with mortality rates between 40% and 10% [4,18].

The commonest routes of infection are inoculation, inhalation and ingestion. There is no evidence to support direct human-to-human transmission via the respiratory route and its zoonotic power is still unclear [18]. The clinical spectrum of disease ranges from localized cutaneous infection with no systemic manifestations to overwhelming sepsis and death. The incubation period ranges from 1 to 21 days for acute presentations.

Disease can be remitting and relapsing over months or years has been described and misdiagnosed as tuberculosis [4,6].

With rapid diagnosis, appropriate antibiotics, and state-of-the-art management of sepsis, death from melioidosis in those without identified risk factors such as diabetes, hazardous alcohol use, chronical lung and renal diseases and immunosuppressive therapy, is uncommon [4].

b) Diagnosis

Isolation of *B. pseudomallei* by culture methods is currently the “gold standard” diagnostic. For environmental sampling of *B.pseudomallei* is suggested a collection of 10 grams of soil with 30 cm in depth followed by the enrichment in 10 mL of Ashdown’s broth and incubation at 37 - 42 ºC for 48 hours. Isolated colonies are then obtained by plating the supernatant for another 48 hours [20]. This process can take up to 7 days for culture, even for clinical samples. Serologic testing with indirect hemagglutination or various ELISA without culture confirmation is considered inadequate to confirm a diagnosis due to the background seropositivity rates in those living in endemic locations. Alternatively, flagged blood cultures or bacterial colonies on culture plates can now rapidly and accurately be identified using qPCR targeting the Bp (TTSS) gene cluster [4].

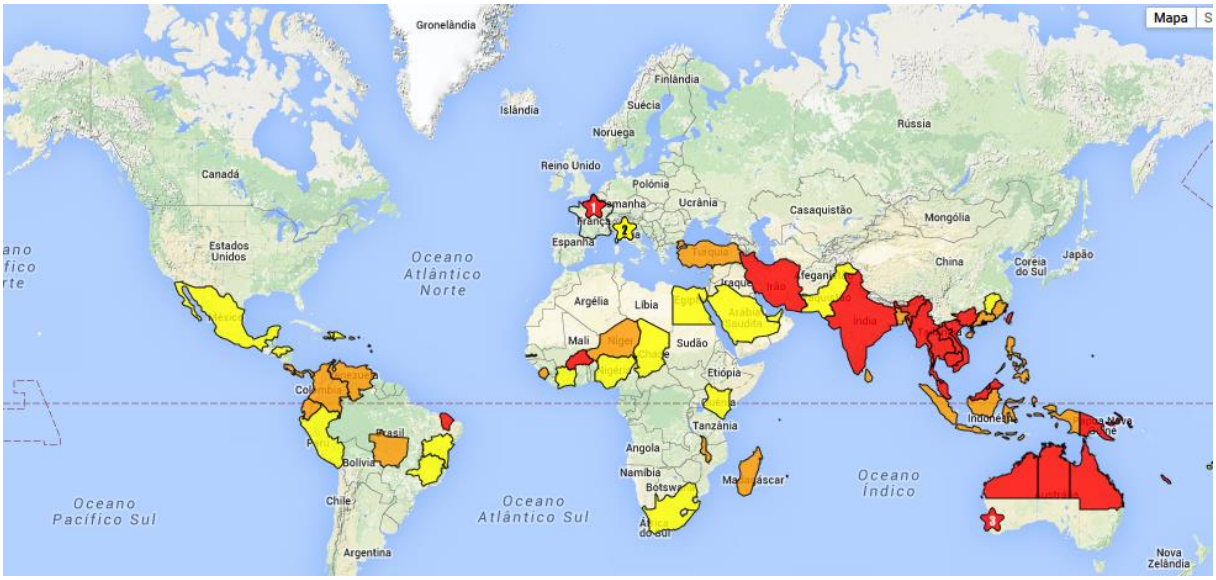

Figure 1. Global distribution of *B. pseudomallei*. Red background represents countries where *B. pseudomallei* has been isolated from soil or water samples and melioidosis clinical reports are documented. Countries with orange background are those where only clinical melioidosis has been reported whilst yellow background countries can't distinguish the isolated organism from other *Burkholderia* spp. and no acquire melioidosis are reported (adapted from [20]).

Table 1. Key phenotypical features of *B. mallei* and *B. pseudomallei*.

Feature	*B. mallei*	*B. pseudomallei*
Gram-stain morphology	Gram-negative coccobacilli	Bipolar Gram-negative bacilli
Growth on medium	Growth on blood agar within 24 to 48 hours	Growth on blood and Ashdown's agar within 24 to 48 hours
Morphology of colonies	Smooth texture with clear or yellow color	Blood Agar: White or yellow, smooth; Ashdown's Agar: Generally, rough and pink colonies
Motility	Non-Motile	Motile
Cytochrome oxidase activity	Variable	Positive
Catalase activity	Positive	Positive
Nitrate reduction to gas	Negative	Positive
Sugar utilization	Non-fermenter	Non-fermenter
Indole production	Negative	Negative

Adapted from Gilad, J. et al. (2007)[6]

1.3. *B. mallei* and *B. pseudomallei* in biological warfare

Awareness of use of microorganisms for hostile proposes has been increasing since World War I catastrophes, by characterization of an infectious agent and preparation of national laboratories for rapid diagnosis tests and hospitals for potential therapeutics.

Three current organizations are updated with the bio warfare problematic worldwide: World Health Organization (WHO), an agency of the United Nations focused on international public health; World Organization for Animal Health (OIE), intergovernmental organisation responsible for improving animal health worldwide; Centre of Disease Control and Prevention (CDC), a United States of America agency to protect public health and safety through the control and prevention of disease, injury, and disability.

By July 2015, OIE together with the WHO hosted the first Global Conference on Biological Threat Reduction reuniting a variety of experts in the fields of public health, animal health, ecosystem health, and security sectors in order to highlight the framework for global preparedness against biological threats, its difficulties and possible solutions as well as sustainable investments in health system. Countries where certain diseases are eradicated or never been declared represent a special risk for the deliberate release of the agent once surveillance and control mechanisms are no longer, or have never been, active [21]. Glanders, has been eradicated from Portugal since 1952 whilst melioidosis, has never been reported [22].

Due to their fast dissemination, ability to infect via inoculation, aerosols and ingestion, high contagiousness between humans, animals as well as the environment and resistance to a wide variety of antibiotics, *B. mallei* and *B. pseudomallei* are classified as Class B agents by the CDC and as level 3 risk agents by the European Parliament [11,23]. The use of these microorganisms for terrorism attacks is not only acknowledgeable but recorded in past civil and world wars, as previously stated. The potential of these microorganisms for biological warfare leads to the need of strategic protocols to detect both agents in a rapid, effective and distinctive way.

1.4. Real time Polymerase Chain Reaction (qPCR)

Given the great impact of PCR as diagnostic technology, improvements have been made in the last few years by developing a new PCR platform called Real-Time PCR (qPCR), capable of monitoring by fluorescence detection, the accumulation of PCR products during cycling steps, eliminating the need of post-PCR procedures e.g. gel electrophoresis.

The detection of fluorescence in qPCR can be produced by two different chemistries: 1) DNA-binding dyes e.g. SYBR® Green, and 2) Fluorescence Resonance Energy Transfer (FRET) probes e.g. TaqMan®. By binding to non-specifically double-stranded DNA (dsDNA), SYBR®

Green allows the performance of a melting-curve at the end of the qPCR run to analyse the specificity of the reaction. SYBR® Green is incapable of performing multiplex reactions where qPCR is optimized to detect more than one target in one tube, whereas this is possible with hydrolysis probes, often referred to Taqman® probes. Hydrolysis probes consist in fluorescence labelled sequence-specific oligonucleotide with FRET chemistry. The probe contains a fluorescence reporter at the 5' end and a quencher at the 3' end of the oligonucleotide. During combined annealing/extension step of the amplification reaction the probe hybridizes the target the 5'→3' exonuclease activity of the DNA polymerase, cleaves the reporter resulting in a fluorescence signal from the freed quencher. Hydrolysis probes assays include many advantages: 1) the use of another sequence-specific oligonucleotide offers a higher specificity; 2) the signal-to-noise ratio and 3) the ability to perform multiplex reactions [24,25].

The qPCR technology permits two forms of data analysis: qualitative and quantitative. Qualitative analysis indicates the presence or absence of the targeting genes, an approach widely use in pathogen diagnostics [26]. The quantitative manner of qPCR allows the user to quantify the results and it can be done in two different ways: Relative quantification, most applied in gene expression case studies, measures the changes in the steady-state levels of a gene of interest relative to a housekeeping gene, using published mathematical equations like the ΔΔCq model and the Pfaffl model [27]. Absolute quantification requires an independent standard curve construction with the known diluted concentrations of the targeting genes in every analysis. Unknown samples are quantified by using the linear equation of the standard curve analysis [25].

1.5. Internal Amplification Control (IAC)

The Minimum Information for Publication of Quantitative Real-time PCR Experiments (MIQE) Guidelines suggests a Non-Template Control (NTC) in every qPCR assay. This NTC can be nuclease-free water and guarantees the reliability of the performance by detecting any source contamination in the sample run. However, it doesn't grant if there is any inhibition factor as malfunction of thermal cycler, incorrect PCR mixture, poor DNA polymerase activity, or presence of inhibitory substances in the sample matrix [26].

A strategy commonly used to certify if the reaction was successful is the implementation of an IAC. An IAC is a non-target DNA sequence co-amplified simultaneously with the target sequence. This strategy can be approached in two ways: 1) In a competitive manner, where the set of primers hybridize with both target sequence and IAC, compromising the efficiency and detection limit of the performance, or 2) Non-competitive manner, in which the target and IAC

are amplified using a different primer set for each. Here, the IAC pair of primers targets a synthetic DNA (e.g., plasmid DNA) or a gene present in any microorganism and in higher copy number than the principal target gene (e.g., encoding rRNA) compromising the amplification of this target if the organism isn't present. By using a non-competitive method, concentrations of primers and probe (if applied) of the IAC must be limited in order to limit the competition between the target for nucleotides and DNA polymerase. The practical advantage of this method is its extensive use of the conceived IAC in different qPCR assays [28].

1.6. qPCR as a diagnostic tool for glanders and melioidosis

Real-time PCR tests have been extensively developed in clinical microbiology laboratories for routine diagnosis of infectious diseases, particularly bacterial diseases [29]. For the identification of glanders and melioidosis etiologic agents, a numerous approaches have been developed through years (**Table 2.**).

Table 2. Resume table of PCR developments towards the identification of *B. mallei* and *B. pseudomallei*

Method	Target DNA/Target bacteria	Reference
Sequencing	23S rRNA gene / Bp and Bm	Bauernfeind et al., 1998, J.Clin. Microbiol
Sequencing	16S rRNA gene / Bp and Bm	Gee et al., 2003, J.Clin. Microbiol
Taqman qPCR	TTSS gene/ Bp and Bm	Thibault, et al., 2004, J.Clin. Microbiol
Taqman qPCR	SNPs/ Bp and Bm	U'Ren, et al., 2005, J.Clin. Microbiol
Multiplex PCR	Repetitive DNA elements/ Bp and Bm	Lee et al, 2005, FEMS Immunol.Med. Microbiol
Taqman qPCR	*bimA(ma)* gene/ Bm	Ulrich, M.P at al., 2006, J. Med. Microbiol.
SYBR Green qPCR	ATP-binding (BPSL1664)/ Bp	Andresen, K., et al., 2009, The Open Pathology Journal
Multiplex qPCR	ISBma2; hypotetical protein genes/ Bp and Bm	Janse, et al., 2013, BMC Infectious Diseases

Bm – *B. mallei*; Bp – *Burkholderia pseudomallei*; Adapted from Botelho, A. in Accreditation of a PCR system for detection and differentiation of *Burkholderia mallei* and *Burkholderia pseudomallei*, IB-BIOALERTNET Conference, July, 2015, Madrid

1.7. Aims

In the frame of the project IB-BIOALERTNET (2013-2015) it was proposed to develop a qPCR that could detect and distinguish *B. mallei* and *B. pseudomallei* in different possible infected matrices, and to standardize and accredited the procedures in order to establish a net of prepared laboratories of bioterrorism alert in case of an emergency and deliberated realise of these agents.

Therefore, to attain these aims the following experimental strategy was implemented:

- Preparation of spiked samples of pulmonary macerates, sterile swabs with *B. mallei* and the above mentioned and soils with *B. pseudomallei* reference strains;
- Development of a qPCR system for the detection and differentiation of *B. mallei* and *B. pseudomallei*, following an adaptation of Janse et al. 2013 work;
- qPCR analysis with the purified DNA extracted from the spiked samples;
- Development of a non-competitive Internal Amplification Control for the qPCR assay;
- Establishment of standard operational protocols of biological alert, to be applied in case of suspicion of *B. mallei* and *B. pseudomallei* release.

CHAPTER 2 – MATERIAL AND METHODS

2.0. Bacterial strains

All procedures regarding the handling of *B. mallei* and *B. pseudomallei* strains were performed in a Biosafety Laboratory Level – 3 (BSL-3) facilities in a Class II biosafety cabinet (BSC II), using the required personal protective equipment (PPE) and following the protocols and Biosafety Manuals accessible on the *Rede Laboratorial Portuguesa de Biossegurança*-LABPTBIONET (http://www.labptbionet.ibmc.up.pt/) such as the Laboratory Biosafety Manual published by WHO (2013) and CDC Biosafety in Microbiological and Biomedical Laboratories (2009).

Two strains of *B. mallei* and two strains of *B. pseudomallei*, acquired to NCTC, United Kingdom (**Table 3.**) and received lyophilized, were reconstituted in Brain Heart Infusion Broth (BHIB) media and inoculated in Tryptone Soy Agar (TSA) plates. After incubation at 37ºC for 48 hours to 5 days, single colonies were inoculated in TSB (Tryptone Soy Broth) aliquots with 30% glycerol, stored at -20 ºC and defrosted when needed. Type strains NCTC 12939^{T} and NCTC 12938^{T} were selected for the optimization of the qPCR (See **2.1.**), while the remaining two strains were used for the spiked sample process (See **2.0.2.**).

2.0.1. Bacterial growth

For *B. pseudomallei* culture, Ashdown's Agar (AA) and Broth (AB) (**Appendix I** – Ashdown's Agar composition) were used. Brain Heart Infusion Agar (BHIA) supplement with 100 U/mL of Penicillin and 1:1000000 Crystal Violet (BHIA+Pen+CV)[30] and Columbia Agar with 5% Sheep Blood (CA/S) were used for *B. mallei* culture.

Table 3. *B. mallei* and *B. pseudomallei* strains used.

	Strains (NCTC reference)	Other References	Characteristics
B. mallei	NCTC 12938^{T}	ATCC 23344	Clinical Isolate, Human, China 1944 [31]
	NCTC 10245	ATCC 10399; CHINA 5	Clinical Isolate, Horse, 1972 [32]
B. pseudomallei	NCTC 12939^{T}	ATCC 23343; WRAIR 286	Clinical Isolate, Human, USA
	NCTC 10276	PRINCE	Clinical Isolate, Human, UK, 1962 [33]

2.0.2. Spiked Samples

B. pseudomallei NCTC 10276 and *B. mallei* NCTC 10245 strains were chosen to spike three different matrices: pulmonary macerates, sterile swabs and soils. Soils were only tested with *B. pseudomallei* NCTC 10276 as *B. mallei* doesn't persist in the environment.

For this procedure, a glycerol stock of each bacterial strain was defrosted and cultured on agar plates followed by incubation at 37ºC for 48h or until visualization of colonies. Single colonies were suspended in BHIB and incubated at 37ºC until the absorbance at 600 nm reached 0.5 (GeneQuant Pro Spectrophotometer, Pharmacia), the equivalent to approximately $1x10^9$ colony forming unites (CFU) per mL [34]. These bacterial suspensions were serial tenfold diluted up to six orders of magnitude to spike the chosen matrices. Optical densities at 600 nm were measured and 10 µL of each dilution were plated in duplicate in the selective medium (See **2.0.1.**).

To evaluate of the performance of the "gold standard" culture method and the molecular qPCR technique and enable comparison between them, the same spiked sample was tested in parallel by each method: plating in specific culture media and inactivation at 99ºC for 60 minutes, for subsequent DNA purification and qPCR analysis, out of the BSL3 facilities(Resume in **Figure A 1. - Appendixes**). For swabs and soils, a previous incubation procedure of the sample at 37ºC for 48 hours in culture media, was evaluated in comparison with no incubation.

a) Swabs

A single sterile swab was immersed in each dilution of each strain for 120 seconds and transferred into a 15 mL disposable tube (Sarstedt™, Germany) supplemented with 2 mL of BHIB. After a brief vortex, 10 µL were spread on the surface of selective medium and one millilitre was immediately inactivated for DNA extraction and purification, naming it Swab Time 0 (S T=0 H). The remaining inoculated broth was subjected to the incubation period (S T=48 H) prior to its plating and inactivation for DNA extraction and purification (See **2.1.**).

b) Pulmonary macerates

Approximately 10 grams of a swine pulmonary tissue (Internal Code: 11251 8-5) were placed into a flask tube and mixed in a Stomacher (Stomacher 400, Colworth) with 8.5 % of sodium chloride saline solution, resulting in the pulmonary macerate.

In a BSC II in a Biosafety Laboratory Level – 2 (BSL-2), 25 mg of macerate were weight in a 2 mL screw cap micro tubes (Sarstedt™, Germany) for the spiked process.

In the BSL3 facilities, 800 µL/g of either *B.mallei* or *B.pseudomallei* culture dilution were inoculated in the respective micro tube. All micro tubes were briefly vortexed for homogenization and 10 µL were spread in the respective selective culture medium (See **2.0.1.**). One hundred

and eighty µL of Digestion Buffer from PureLink® Genomic DNA Mini Kit (Invitrogen™) were added to 1 mL of the spiked pulmonary macerates before inactivation (See **2.1.**).

c) Soils

Soil samples were collected in INIAV, Pólo Benfica (38º.44'55.84N, 9º.11'59.88''O) territory near an orange tree irrigated hours before. To homogenise the collected sample, soil was spread on a bench in a cone shape starting from the centre to the periphery. Five grams of soil were collected from the top and distributed into 50 mL disposable tubes.

For the spiked process, 800 µL/g of each dilution of *B. pseudomallei* were added to the soil matrix. All tubes were vigorously shaken and 5 mL of AB were added. Ten µL were spread on AA and 1 mL of the supernatant was inactivated, defining these samples Soil culture at Time 0 (Sc T=0). The remaining spiked soils followed an incubation period of 48 hours at 37 ºC. One millilitre was withdraw from the incubated sample (Sc T=48) and 10 µL were plated in AA and incubated at 37ºC for 48 h. Typical *B. pseudomallei* colonies from Sc T=48, were selected and transferred to 1 mL of BHIB and inactivated for DNA extraction and purification naming these Soil culture Isolates (Sc I) (See **2.1.**).

2.1. Inactivation of the bacteria, DNA purification and quantification

The samples collected from the spiked process were inactivated in a Labnet AccuBlock™ Digital Dry Bath (Citomed, Portugal) at 99º C for 60 minutes. Efficiency of inactivation was performed by plating 10 µL of the inactivated product into the selected bacteria medium (See **2.0.1.**). Incubation was set at 37 ºC and plates were observed 6 days after, confirming the absence of any CFU, allowing the samples to be transferred to BSL-2 facilities for DNA purification.

DNA purification was performed using PureLink® Genomic DNA mini Kit (Invitrogen™) following the manufacturer's instructions, according to the type of matrix, and Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific™) was used for DNA quantification.

2.2. qPCR system for the detection of *B. mallei* and *B. pseudomallei*

Development of the qPCR system was based on Janse et al. (2013) novel approach using the primers and probes depicted in **Table 4.** and synthetized by NZYTech (Lumiar, Portugal). Probe targeting *psu* gene with fluorophore CF590 was changed to HEX, a fluorophore calibrated for used thermocycler.

2.2.1. *In silico* analysis

The specificity of primers, probes and target sequences depicted in **Table 4.** and **Table 5.** was tested and confirmed using BLASTn software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All oligonucleotides were analysed in Thermo Scientific webtool, Multiple Primer Analyzer, (http://www.thermoscientificbio.com/webtools/multipleprimer/) to check for self-dimer and cross-dimer reactions.

Table 4. Oligonucleotides for the qPCR duplex *Burkholderia* system.

	Oligo Function	Sequence 5'-3'[a]	Amplicon size (bp)	Target	Function
B. pseudomallei	Primer F	GCGCGATCCGTCGAG	123	*psu*	Hypothetical protein
	Primer R	AGCCGCTACGACGATTATG			
	Probe	***HEX**-CCGCGACAATACGAC-CATCC-**BHQ1**			
B. mallei* and *B. pseudomallei	Primer F	GCGGAAGCGGAAAAAGGG	98	IS*Bma2*	ISBma2 transposase
	Primer R	GCGGGTAGTCGAAGCTG			
	Probe	**FAM-**TCRCCAGACGCAGCAGCAT**-BHQ1**			

[a] - Based on Janse et al, 2013 ; *-Modifications made from Janse et al work; Primer F – Primer Forward; Primer R – Primer Reverse

2.2.2. qPCR optimization

B.mallei NCTC 12938^{T} and *B. pseudomallei* NCTC 12939^{T} purified DNA was used as DNA templates for the qPCR optimization. qPCR reactions were carried out in a Bio-Rad CFX96™ Thermal Cycler (Bio-Rad Laboratories Srl, Redmond, USA) using Bio-Rad CFX Manager, version 3.0 software for data analysis. Cycling conditions were adapted from Janse et al. (2013): Enzyme activation at 95 ºC for 5 minutes and 44 cycles of 5 seconds at 95 ºC and 35 seconds at 60ºC. In a reaction volume of 20 µL, 3 µL of DNA template were added in a concentration of 10 ng/µL. For Non-Template Controls (NTC), ultra-pure water was as template in every experimental set, to rule out any source of contamination.

The qPCR system was optimized by testing different final concentrations of primers and probes in singleplex and duplex reactions. Annealing temperature was optimized for the duplex reaction.

a) Primers and probe concentration optimization

SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories Srl, Redmond, USA) was used to test final primer concentrations from 100 nM to 400 nM with 100 nM iterations of each set of primers, separately. Cycling conditions were as described above, adding a final step of 65ºC to 95ºC with increments of 0.5ºC in 5 seconds each for the melting curve analysis in order to exclude primer combinations that produce any inefficient or primer-dimer products.

Final primer concentrations ranging 100 nM to 400 nM and final probe concentrations ranging 100 nM to 300 nM with 100 nM iterations were tested in a duplex reaction using polymerase NZY qPCR Master Mix 2x, NZYTech (Lumiar, Portugal) and the cycling conditions above mentioned.

The combination that exhibits the earliest quantitative cycle (Cq) and the highest end-point fluorescence (EPF) values while minimizing non-specific amplification was chosen as the optimal primer concentrations.

b) Annealing Optimization

Given the optimized concentrations of primers and probes for the duplex reaction, annealing temperature was tested by subjecting qPCR reactions to a gradient of annealing temperatures ranging from 62.9, 62.5, 61.6, 60.0 to 58.1 ºC. The temperature at which the Cq value and EPF gave the highest values, was chosen as the optimal temperature.

c) Evaluation of Cq variance between singleplex and duplex assays

DNA templates in a concentration, respectively, of 2.9 ng/µL and 4.5 ng/µL of per reaction, were used in duplicates to evaluate the optimized singleplex and duplex assays in the same run to determine significate differences between the Cq values of each platform.

2.2.3. Estimation of the limit of detection (LOD), specificity, repeatability and reproducibility

DNA extracted from *B. mallei* NCTC 12938^{T} and *B. pseudomallei* NCTC 12939^{T} cultures were used to determine the linearity and sensibility of singleplex and duplex reactions. Ten-fold dilutions ranging 10^{-0} to 10^{-10} from 10 ng/µL of DNA template were prepared and standard curves were constructed, with the qPCR results from two replicates per dilution, by plotting the Cq values to the logarithm of the DNA concentration per reaction (fg/reaction). Slopes were used to obtain the qPCR efficiency percentage by the following equation: $10^{1/slope}1x\ 100$. Estimation of the limit of detection (LOD), sensitivity, was determined by the lowest concentration of template per reaction that produced positive results in both replicates.

For specificity, purified DNA from *B. mallei*, *B. pseudomallei* and other bacteria were used as DNA templates in the optimized qPCR duplex system.

For the intra-assay (repeatability) and inter-assay (reproducibility) variability of singleplex and duplex assay, 3 dilutions of each *B. mallei* NCTC 12938^{T} and *B. pseudomallei* NCTC 12939^{T} were tested. Each dilution was tested in duplicate and in two independent runs performed in different days by different operators. The mean Cqs values, standard deviation and percent coefficient of variation were calculated independently for each DNA dilution.

2.3. Construction of an Internal Amplification Control (IAC) system for PCR reactions

DNA of myxoma virus Lausanne strain, kindly provided by Doctor Margarida Duarte, (INIAV, IP), was used to construct an IAC by PCR amplification of a 125 bp fragment of the m000.5L/R gene and cloning into pNZY28 vector.

2.3.1. Conventional PCR amplification

Conventional PCR was performed using primers described in Duarte et al (2014)[35] (**Table 5.**). For the master mix reaction, High Fidelity PCR Master Mix (Roche Diagnostics, Indianapolis, IN, USA) was used in the total reaction of 25 µL using 2 µL of DNA template at a concentration of 10ng/µL and a final concentration of 1 µM for each primer. Amplifications were performed with forty cycles of denaturation at 95ºC for 30 seconds, annealing at 50ºC for 45 seconds and extension at 72ºC for 30 seconds, followed by a final step of extension at 72ºC for 5 minutes. Reactions were processed in a MJ Mini™ Personal Thermocycler (Bio-Rad Laboratories Srl, Redmond, USA) and PCR products were subjected to agarose gel electrophoresis on 2% low melting SeaPlaque® GTG® Agarose (FMC® Bioproducts, USA) stained with 2 mg/mL ethidium bromide (UltraPure™ Ethidium Bromide, Invitrogen™) in parallel with 100 bp DNA ladder (Promega).

Table 5. Primers and probes for pNZYmyx.

Microorganism	Oligo Function	Sequence 5'-3'	Amplicon size (bp)
Myxoma Virus	Primer F	CGACGTAGATTTATCGTATACC	125
	Primer R	GTCTGTCTATGTATTCTATCTCC	
	Probe	**[FAM]**TCGGTCTATCCTCGGGCAGAC ATAGA**[TAMRA]**	

Primer F – Primer Forward; Primer R – Primer Reverse

2.3.2. Cloning of the 125 bp fragment of m000.5L/R gene in pNZY28 vector – plasmid pNZYmyx construction

The 125 bp amplicon was excised from the gel with the help of a scalpel under a UV transilluminator (White/UV Transilluminator, UVP). Purification of the amplicon was done following the instructions of the NZY Gelpure kit (NZYTech, Lisbon, Portugal) and DNA quantification was measure in a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific). Cloning procedure was done using the pNYZ28 vector (**Figure A 4. - Appendixes**) of the NZY-A PCR Cloning Kit, NZYTech (Lumiar, Portugal), using 1:3 ratio of vector:insert for the ligation reaction and transformation into *E.Coli* NZYStar Competent Cells (Genotype: *endA1 hsdR17(rk-, mk+) supE44 thi -1 recA1 gyrA96 relA1 lac[F´ proA+B+ lacI^{q}ZΔM15 :Tn10(Tc^{R})]*.

Transformed cells were plated in LB agar plates supplemented with antibiotics (100 µg/mL ampicillin and 15 µg/mL tetracycline) and lactose analogues (100 µg/mL X-Gal and 0.5 mM of IPTG) and incubated overnight at 37 ºC. White colonies with recombinant plasmid (pNZYmyx) were selected, plasmid DNA extracted and, to confirm the effectiveness of the cloning procedure, a conventional PCR targeting the inserted fragment of the plasmid pNZYmyx was performed, using the above primers. The cells confirmed to have the recombinant plasmid (recombinants) were transferred to 10 mL cryotubes with 2 mL LB broth with the antibiotics above mentioned. Ten percent of glycerol (v/v) was added and the cryotubes were stored at -80 ºC and defrosted whenever needed.

2.2.3. qPCR using pNZYmyx as IAC

The qPCR for the pNZYmyx recombinant plasmid was performed following the NZY qPCR Master Mix 2x (Lumiar, Portugal) recommended conditions: enzyme activation step at 95 ºC for 2 minutes, 95 ºC for 10 seconds and annealing with fluorescence measurement at 60 ºC for 20 seconds. Primers and probe concentrations were 0.4 µM and 0.1 µM, respectively, in a final reaction volume of 20 µL with 2 µL of DNA template at a concentration of 10ng/µL.

2.3. Data analysis

Statistical values and graphics were done in GraphPad Prism 5.03 and Microsoft Excel 2007 Office Tool.

Sensitivity and McNemar tests were computed using the clinical research calculators of the online VassarStats software (http://vassarstats.net) and GraphPad online software, QuickCalcs (http://graphpad.com/quickcalcs/).

CHAPTER 3 – RESULTS AND DISCUSSION

3.0. Growth of *B. mallei* and *B. pseudomallei* strains in culture media

Colonies of *B. pseudomallei* NCTC 10276 showed different morphologic aspects inter and intra-species. The most common colony features was its pink colour, irregular form and mucoid texture (**Figure 2. - C**).

Brain Heart Infusion Agar (BHIA) supplement with 100 U/mL of Penicillin and 1:1000000 Crystal Violet (BHIA+Pen+CV) displayed difficulties for the growth of *B. mallei* NCTC 10245. When colonies were present, they assumed a darkish grey colour with smooth texture, forming agglomerates in the periphery of the Petri dish. This fact created an obstacle for the determination of colony forming units (CFU). Therefore, Columbia Agar with 5% Sheep Blood (CA/S) was selected for the isolation of *B. mallei*, where colonies presented two distinctive forms: pin-point clear colonies (**Figure 2. - A**) or opaque yellow with variable dimension (**Figure 2. - B**).

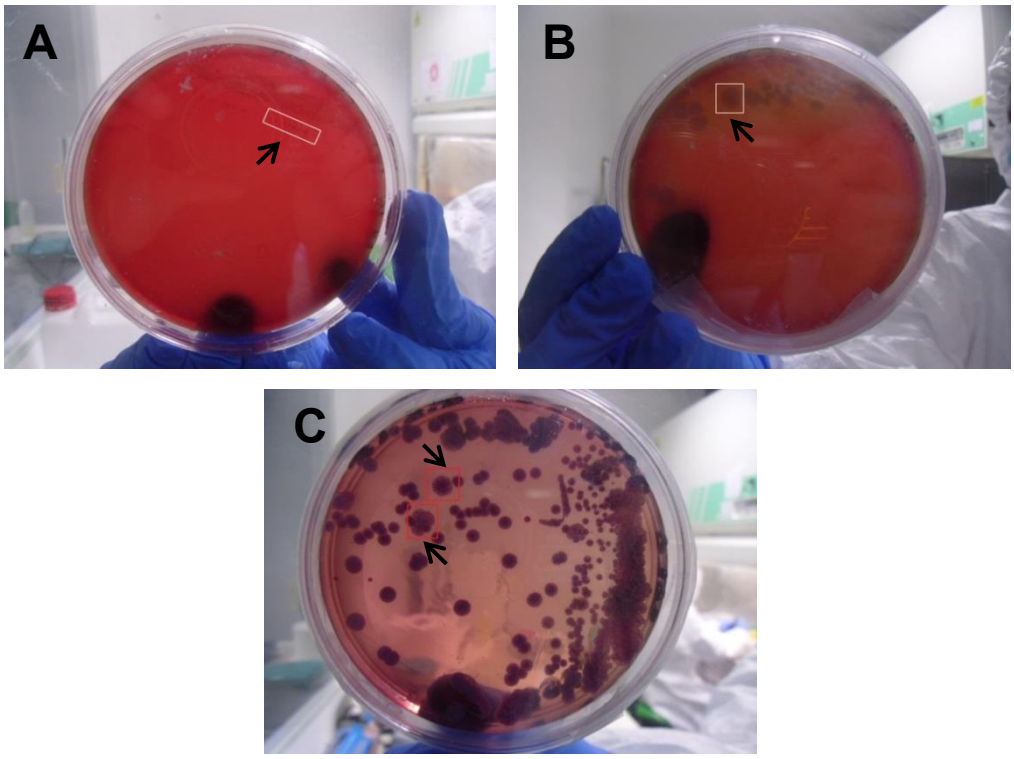

Figure 2. Colonies of *B. pseudomallei* and *B. mallei* in selective media. Characteristic colonies of *B. mallei* 10245 are shown in (A) and (B), while *B. pseudomallei* 10276 most common colony morphology is shown in (C). Translucid pin-point colonies of *B. mallei* are indicated by arrows in plate (A) and irregular shape with yellow color in CA/S plate (B), inoculated with 10 µL of *B. mallei* NCTC 10245. Plate (C) represents colonies of *B. pseudomallei* in Ashdown's media, from a 10^{-2} dilution spiked soil sample.

To evaluate the number of CFU/mL to be inoculated in each spiked matrices and further evaluate the sensitivity of both culture and PCR methods, the absorbance at 600 nm (OD_{600}) was measured for each ten-fold serial dilution of *B. mallei* and *B. pseudomallei* cultures that were, in parallel, plated in agar plates with the respective selective bacterial media and incubated at 37ºC for 48 hours. The number of CFU/mL was calculated by counting the number of CFU in each dilution plate and multiplying by the correspondent inoculum factor.

B. mallei platted dilutions presented growth until the third ten-fold dilution, with an OD_{600} of 0.006 corresponding to 250 CFU/mL (**Table A 1. - Appendixes**). For *B. pseudomallei* fourth ten-fold dilution was established as the limit for CFU visualization (650 CFU/mL), corresponding to an OD_{600} of 0, a value associated with absence of any bacteria in culture. Human manipulation errors or spectrophotometer inaccuracy might explain these incongruences.

3.1. Spiked samples bacteriological culture

Spiked sample process was performed by inoculating each 10^{-1} to 10^{-6} dilution from bacterial cultures corresponding, respectively, to $2.5x10^4$, $4.4x10^3$, $2.5x10^2$ and 0 CFU/mL for *B.mallei* 10245, and $5.9x10^4$, $3.7x10^4$, $2.7x10^4$, $6.5x10^2$ and 0 CFU/mL for *B.pseudomallei* (**Table A 1. - Appendixes**). Ten µL of each swab and soil spiked sample, was plated into selective culture media (See **2.0.1.**) without previous enrichment incubation period and after 48 hours incubation at 37ºC in selective media. No previous enrichment was performed for pulmonary macerates (See **2.0.2.** and **Figure A 1. - Appendixes**) due to the natural presence, in this type of clinical sample, of other bacteria that could easily over grow, competing with *Burkholderia* and unable its detection.

a) Swabs

For *B. pseudomallei* it was possible to detect $4.0x10^2$ CFU/mL for S T=0 and $1.35x10^5$ CFU/mL for S T=48 (**Figure 3. - A** and **B**). Swabs spiked with *B. mallei* showed no growth in any dilution either with (S T=48) or without incubation period (S T=0) (results not shown). The absence of specific and sensitive culture media for isolation of *B.mallei* might have had influence in these results. The plating of 10 µL of suspension instead of the direct smear of the swab in the culture media could also have had influence since the inoculum might have been too less.

b) Pulmonary macerates

For *B. pseudomallei*, the forth ten-fold dilution represents the limit of CFU visualization corresponding to $2.0x10^2$ CFU/mL (**Figure 3. – A - Lungs)**. For *B.mallei* spiked pulmonary macerates lowest dilution bacterial growth detection was the second ten-fold dilution corresponding to $3.3x10^3$ CFU/mL (**Figure 4.**). These results show that the isolation of *B.*

pseudomallei by Ashdown's culture media is more sensitive than the CA/S used for the isolation of *B. mallei* when it comes to pulmonary macerates.

c) Soils

Soil samples spiked only with *B. pseudomallei* without incubation (Sc T=0) and after 48 hours incubation (Sc T=48) presented CFUs, respectively, until dilution 10^{-3} (**Figures 3. - A – Soils)** and dilution 10^{-6} (**Figure 3. - C)**. The limit of detection was, respectively, $1.0x10^2$ and $2.3x10^3$ CFU/mL for Sc T=0 and Sc T=48. Incubation for 48 h allowed a more sensitive detection of *B. pseudomallei* in soils.

B. pseudomallei isolation in selected culture media presented bacterial growth in all three spiked matrices (swab, pulmonary macerates and soil) and in lower concentrations of inoculum, when compared with *B. mallei* growth. In fact, growth of *B. mallei* was only observed in spiked pulmonary macerates (no growth in spiked swabs) and in higher concentrations of inoculum. The lack of *B. mallei* bacterial growth from spiked swabs at S T=0 and S T=48 might be overcome by inoculating the agar directly with the swabs. Since *B. mallei* hasn't a selective media as *B. pseudomallei* has, the selection of specific and characteristic colonies is more difficult and hampered by other bacterial contaminants, eventually present in the sample. Since the swabs were sterile before spiking they don't truly represent a natural matrix from infected wounds, for example, where other ambient bacteria may be present, competing for growth.

Identification of *B. mallei* colonies from lung samples was difficult to achieve due, again, to the absence of a specific and selective media for this bacteria. The overgrowth of other microorganisms, present in the sample, difficult the detection of the agent by cultural growth. On the other hand *B. pseudomallei* colonies were easily identified in mixed cultures in Ashdown's media.

For *B. pseudomallei* soil spiked matrices, the results were unexpected: Sc T=0 samples presented around 20 times more CFUs when compared to Sc T=48. It's important to emphasize the variability of colony dimension between these two types of samples: Sc T=0 showed pin-point colonies, while matrices submitted to incubation, Sc T=48, produced colonies with a much higher diameter. This fact could explain ambiguities in the counting of colonies leading to some discrepancies between Sc T=0 and Sc T=48 results. Soil samples were the more reliable and appropriated for isolation of *B. pseudomallei* and, therefore, recommended as ideal sample in case of suspicion in the environment. Previous soil sample incubation enhances the detection level.

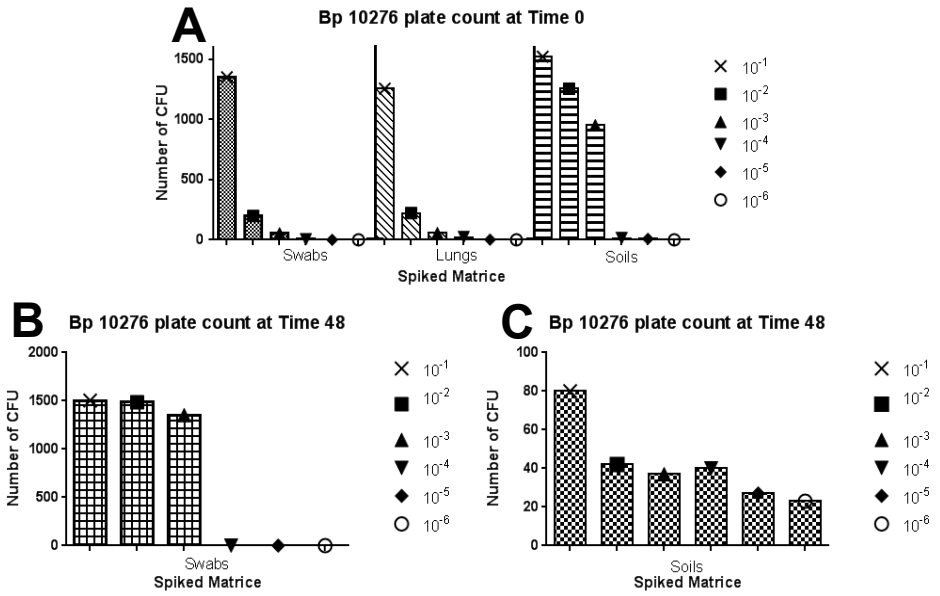

Figure 3. *B. pseudomallei* (Bp) 10276 spiked samples plate count. Bars correspond to the plate count of culture dilutions with which the samples were spiked. Respective dilutions are indicated by symbols. Without previous enrichment/incubation of spiked samples (A) and with previous enrichment/incubation of spiked swabs (B) and soils (C).

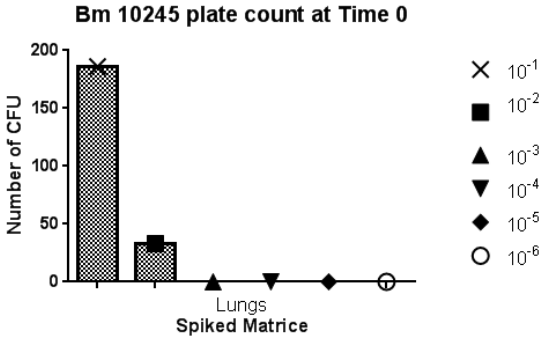

Figure 4. *B. mallei* (Bm) 10245 pulmonary macerates spiked samples. Bars correspond to the plate count of culture dilutions with which the samples were spiked. Respective dilutions are indicated by symbols.

3.2. Optimization and evaluation of singleplex and duplex qPCR

3.2.1 Specificity of primers and probes

Janse et al. (2013) described four pairs of primers and four probes for the detection of *B.mallei* and *B.pseudomallei* in a multiplex platform targeting: *psu* gene (*B. pseudomallei*

species specific), *mau* gene (*B. mallei* species specific), ISBma2 transposase (specific of both genus) and an IAC.

BLASTn results demonstrated the presence of *psu* gene and ISBma2 transposase sequence in 50 complete *B. pseudomallei* genome sequences, while *B. mallei* presented ISBma2 in 12 complete genome sequences. No other organisms presented similar E values and Query Cover, proving the specificity of the target sequences and respective pair of primers and probes. Whilst *psu* gene is present in one copy in the chromosome 2 of all fifty *B. pseudomallei* published genomes, ISBma2 transposase is present in the two chromosomes of both *B. mallei* and *B. pseudomallei*. ISBma2 exists in an average of 37 copies in chromosome 1 and in 11 copies in chromosome 2 of *B. mallei*, whereas for *B. pseudomallei*, it exists in an average of 5 copies in chromosome 1 and in average of one copy in chromosome 2.

The first step towards the optimization of the qPCR was using only primers targeting *mau* and *psu* genes. Further tests of *mau* pair of primers and probe showed difficulties regarding the consistent amplification of NTC with Cq values between 36-38 for negative controls. Numerous attempts were performed to clarify the source of contamination (**Figure A 2. - Appendixes**) but its persistency drove to the exclusion of these primers and probe and so, the optimization of the qPCR was achieved by using the *psu* gene and ISBma2 transposase oligonucleotides previously described (**Table 4.**).

3.2.2 Concentration of primers/probes and annealing temperature

Primers and probe concentrations from 100 nM to 400 nM, with 100 nM iterations, were tested in the reaction. Four hundred nM of each primer proved to be the best concentration for singleplex and duplex reactions. One hundred nM and 300 nM proved to be the best concentration for ISBma2 and *psu* targeting probes, respectively, in singleplex assay, while 200 nM of each probe were suited for the duplex performance (Resume in **Table 6.**).

The annealing temperature was optimized by performing duplex qPCR reactions with *B. pseudomallei* NCTC 12939^{T} and *B. mallei* NCTC 12938^{T} as templates at different temperatures, selecting the one with the higher Cq value and EPF. The temperature of 58.1 ºC presented the highest of these values and therefore, it was selected as the annealing optimal temperature (**Figure A 3. - Appendixes**).

After optimization of the above parameters, the qPCR, was performed under the following conditions: Enzyme activation at 95 ºC for 5 minutes and 44 cycles of 5 seconds at 95 ºC and 35 seconds at 58.1ºC.

Table 6. Primer and probe optimal final reaction concentrations for singleplex and duplex performances.

	Target	Primer concentration (nM)	Probe concentration (nM)
Singleplex	ISBma2	400	100
	psu	400	300
Duplex	ISBma2	400	200
	psu	400	200

3.2.3 Comparison of Singleplex vs Duplex qPCR performance

Singleplex and duplex platforms were performed in duplicates side-by-side in order to compare the Cq values of each assay. Results showed no significant statistical difference (*p-value*>0.005) between the Cq values for the targets ISBma2 and *psu* in each platform, indicating that analysis of duplex qPCR was not affected by the presence of more than one target in the reaction (**Table 7.**).

Table 7. Cq values of singleplex reaction against multiplex reaction.

	Mean ± SD Cq Values		
	ISBma2		***psu***
	B. mallei	*B. pseudomallei*	*B. pseudomallei*
Singleplex	14.07 ± 0.1250	31.99 ± 0.2200	22.42 ± 0.1400
Duplex	14.16 ± 0.1200	26.96 ± 2.715	22.32 ± 0.05000

The data represents the mean and standard deviation of Cq values (duplicates) in each singleplex and duplex reaction. The *t-student test* performed, showed no significant statistical difference (*p-value*>0.005) between the Cq values.

3.2.4 Efficiency, analytical specificity and limit of detection of qPCR

Ten-folds dilutions of extracted and purified DNA from *B. mallei* NCTC 12938^{T} and *B. pseudomallei* NCTC 12939^{T} strains were used to perform standard curves, determine the lowest concentration detected and calculate the efficiency of the performance and standard deviation average (**Table 8.**). Linear range of all standard curves included at least five ten-fold dilutions with the exception of the ISBma2 detection in singleplex for *B. pseudomallei* that could only amplify the three highest concentrated dilutions (10^{0} to 10^{-2} dilutions from the purified DNA templated). For the efficiency analysis, *psu* indicated 0.991 (99.1%) and 0.934 (93.4%) for singleplex and duplex platforms, respectively. As for ISBma2, using *B. mallei* as DNA template, efficiencies were 0.985 (98.5%) in singleplex and 1.016 (101.6%) in duplex performance; whereas for *B. pseudomallei*, were 0.958 (95.8%) and 1.183 (118.3%) for singleplex and duplex,

respectively. The ideal value would be 1.00 (100%) which indicates that the amount of product doubles each cycle.

Analytical specificity, as described in the MIQE Guidelines[26], is the ability of the assay to detect no other templates than the selected targets. Cq values of DNA from several microorganisms used as template in the duplex platform are presented in **Table 9.** As data displays, only *B. mallei* and *B. pseudomallei* strains were amplified; therefore, this assay is specific for these bacteria.

Table 8. Efficiency and Limit of Detection (LOD) of singleplex and duplex systems, for *B.mallei* NCTC 12938^T and *B.pseudomallei* NCTC 12939^T

	Microorganism	Target	E (%)	R^2	Linear Range (fg/reaction)	LOD (fg)	$\overline{SD}$
Singleplex	***B. mallei***	ISBma2	98.5%	0.999	$2.9x10^0$ – $2.9x10^7$	2.9	0.06
	B. pseudomallei	*psu*	99,1%	0.996	$4.55x10^0$ – $4.55x10^6$	4.5	0.11
		ISBma2	95,8%	0.994	$4.55x10^5$ – $4.55x10^7$	$4.6x10^5$	0.25
Duplex	***B. mallei***	ISBma2	101.6%	0.999	$2.9x10^1$ – $2.9x10^7$	29	0.14
	B. pseudomallei	*psu*	93,4%	1.00	$4.55x10^3$ – $4.55x10^7$	455	0.11
		ISBma2	118,3%	0.976	$4.55x10^3$ – $4.55x10^7$	455	0.4

E – Efficiency; R^2 – Correlation coefficient; LOD – Limit of Detection; $\overline{SD}$ – Mean of Standard Deviation

As data shows, the linear range of the constructed standard curves and LOD vary between the ISBma2 and *psu* targets in the two platforms. For the singleplex performance, ISBma2 target could be detected as low as 2.9 fg and $4.6x10^5$ fg, respectively, in *B.mallei* NCTC 12938^T and *B.pseudomallei* NCTC 12939^T. The *psu* target performed in separate was able to detect up to 4.5 fg of *B.pseudomallei* NCTC 12939^T.

The duplex assay offers the advantage of identifying these two close related species in one single tube reaction, on other hand, the sensitivity of these multiplex assays can be affected [26]. LOD of ISBma2 and *psu* targets in the duplex assay decreased by, respectively, one and two

order of magnitude with the exception of the identification of *B.pseudomallei* 12939^{T} by the ISBma2 target which, surprisingly, increased the LOD by two orders of magnitude.

Comparing the optimal qPCR here developed, the multiplex assay published by Janse et al. (2013) declares a higher sensitivity in one order of magnitude but the information of ISBma2 and *psu* targets analysed separately are not provided in their work.

The difference between the sensitivity of both singleplex and duplex performances can influence the choice of platforms according to the type of sample for analysis; e.g. if the sample consists of a soil matrix, *psu* analysis as the only target in the reaction is sensitive and specific enough to identify *B. pseudomallei*, without the need of the less sensitive ISBma2 target.

Table 9. Specificity of the duplex assay.

Microorganism	Strain/Code Name	Cq value	
		ISBma2	*psu*
Burkholderia mallei	NCTC 12938 T	14.24	-
	NCTC 10245	12.88	-
	CIP A 199	25	-
	ATCC 15310	21	
Burkholderia pseudomallei	NCTC 12939 T	18.55	27.32
	NCTC 10276	17.2	20.01
	ATCC 15682	-	29
Burkholderia thailendesis	CIP 106301	-	-
Mycobacterium bovis	VV-E-457	-	-
Mycobacterium avium subsp. *avium*	ATCC 25291	-	-
Mycobacterium tuberculosis	H37Rv	-	-
Mycobacterium avium subsp. *paratuberculosis*	VV-E-523	-	-
Pseudomonas aeroginosa	CIP 100720	-	-
Campylobacter jejuni	N.8 2011	-	-
Campylobacter coli	N.8 2011	-	-
Vibrio vulnificus	655	-	-
Vibrio cholerae	5165	-	-
Escherichia coli	ED647	-	-
Escherichia coli	D2598	-	-
Salmonella serotype Braenderup	H9812	-	-

The specificity of the duplex assay was experimentally tested with different purified DNAs of the listed microorganisms.

3.2.5. Repeatability and Reproducibility

Repeatability, or intra-assay variance, refers to the precision of the assay to produce the same results within replicates. (**Table A 2. - Appendixes**) shows the maximum percentage of coefficient variance is 1.337 %, a very low value suggesting that singleplex and duplex qPCR are highly repeatable [36].

Reproducibility, or inter-assay variance, refers to the variation between results within different runs by different operators. Results show a maximum percentage of inter-assay variance of 2.288 %, a very low value suggesting the singleplex and duplex performance are highly reproducible between runs and different workers [36].

3.3. qPCR analysis of the spiked samples

DNA from spiked samples was purified and analysed with the optimized duplex qPCR (See **3.2.2.** and **Table 6.**).

Table A 3. and **Table A 4.** in **Appendixes** represent the Cq values of each spiked matrix dilution for each target.

a) Swabs

All spiked swab samples were correctly identified in the duplex reaction. Non-incubate swabs with *B. mallei* present Cq values between 20.33 (10^{-1}) and 39.25 (10^{-6}), whilst *B. pseudomallei* between 28.98 (10^{-1}) and 38.37 (10^{-6}) for the *psu* target. Swabs with previous incubation showed a slightly improvement in the detection of both bacteria.

b) Pulmonary macerates

Pulmonary macerates showed positive results in all samples by correctly identifying the presence of each bacterium, proving the sensitivity of the duplex assay in this matrix. Cq values of pulmonary samples showed to be between 15.93 (10^{-1}) and 25.95 (10^{-6}) for *B. mallei* and between 23.44(10^{-1}) and 38.18 (10^{-6}).

c) Soils

As for soils, lower Cq values were obtained when the samples were submitted to an incubation period, followed by the selection of typical colonies for qPCR analysis, presenting Cq values below 27.25(10^{-6} – *psu* target) (**Table A 4. - Appendixes**). The soil samples qPCR analysis can easily be affected by the presence of inhibitors when using the Ashdown's Broth (AB) suspension for DNA extraction and direct detection of *B. pseudomallei* as the result of the less concentrated sample was negative. Therefore, it is suggested performing the qPCR in soil samples with purified DNA from suspicious colonies obtained from the platted the incubated sample.

Although the duplex qPCR was able to detect *B. mallei* and *B. pseudomallei* in swabs and pulmonary macerates without incubation period, incubated swab samples with *B. pseudomallei* presented lower quantification cycles which reflect in the sensitivity of the diagnosis. High quantification cycles (>40) are normally excluded as positive samples as they are associated with degradation of the probe-based fluorophore by cross contamination or by nonspecific amplification of background nucleic acids [37]. The swabs used were sterile and even though results suggest incubation of the swab samples for a more sensitive analysis, the possibility of the overgrowth of other microorganisms present in the nature of the sample that could inhibit *Burkholderia growth* should be considered.

For detection of *B. pseudomallei* in soils, the Limmathurotsakul et al. (2013) recommendations should be applied, by selecting isolated colonies, rather than purifying DNA directly from the Ashdown's Broth (AB) suspension, avoiding impurities and PCR inhibitors that may affect the performance of PCR and therefore, nullifying *B. pseudomallei* detection.

Unlike the *psu* conservative gene that is present in only one copy in chromosome 2 of *B. pseudomallei*, ISBma2 is present multiple copies in chromossomes 1 and 2 of both agents but comparative less in *B. pseudomallei* (See **3.2.1.**). Therefore, the sensitivity of the qPCR assay is greatly enhanced, recommending this target for the detection of both species. However, the capacity of this insertion sequence to be excised and to transpose into different locus of the genome may result in a great variability of ISBma2 copies, making it a rather instable target. In fact, Janse et al (2013) showed that some *B.pseudomallei* strains were only identified by the *psu* target in opposite to *B. mallei*. This can be explained by the abundant presence of this insertion sequence in *B.mallei* genome correlated with evolution in host adaptation and reductive selective pressure, whereas these elements don't contribute equally to *B.pseudomallei* genomic variation and are highly variable intra-species [17,38].

In short, ISBma2 target can be seen as the first line detection method for *B. mallei* and *B. pseudomallei*, in parallel with the *psu* target (**Figure A 1. Appendixes**).

3.4. Sensitivity of the qPCR versus sensitivity of the culture method and significance of difference

The comparison between both molecular and classic microbiology techniques was calculated and results of each species were organized the in a contingency table of 2x2 separately.

The percentage of sensitivity of each methodology was achieved by dividing the number of true positives by the sum of true positives and true negatives and multiplying this result by 100

[38]. qPCR sensitivity for the *B. mallei* spiked samples tested was 100% whereas the culture method calculated sensitivity was significantly lower, 17%. This suggests that the molecular method implemented is more sensitive by correctly identifying all the spiked samples in comparison to the established culture method. Meanwhile, sensitivity of the qPCR for the identification of *B. pseudomallei* in spiked samples was approximately 97% and 80% for the culture method, proving both methodologies correctly detected most spiked samples.

McNemar's test [39] assessed the significance of the difference between the two diagnostic tests. The outcome of *B. mallei* qPCR vs culture methodologies showed to be statistically significant presenting a *p* value below 0.0001 and therefore, the null hypothesis that both methods produce equal outcomes was rejected, assuming both methodologies are statistically different. On other hand, comparison between the qPCR and culture method for *B. pseudomallei* resulted in *p* value higher than 0.0001 (*p* value = 0.2207) and so, both methodologies produce statistically similar results.

This analysis evidences a high difference between each methodology in *B. mallei*, probably due to the sensitivity difference between each other, suggesting that qPCR should be applied for its identification. As for *B.pseudomallei*, both culture and qPCR methods proved a high sensitivity towards the detection and isolation of this agent suggesting both methodologies are highly capable of identifying correctly *B.pseudomallei*.

3.5. pNZYmyx as an IAC

The pNZYmyx plasmid was constructed by cloning the 125 bp fragment of the m00.5L/R gene from myxoma virus into pNZY28 vector.

Although the use pNZYmyx as an IAC for the optimized *B. mallei* and *B. pseudomallei* was excluded due to the presence of FAM dye label probe for two different targets, pNZYmyx and ISBma2. For future remarks, it's recommended the replacement of the dye label choosing one compatible with this qPCR system. However, this cloning product displays a versatile use in every qPCR bacterial diagnostics by amplifying a specific viral gene, uncommon to be present in most bacterial infections.

CONCLUSION

B. pseudomallei and *B. mallei* are two dangerous pathogens with highly infectious ability, capacity of airborne transmission and resistance to a wide spectrum of antibiotic therapy, classifying them as potential bio-weapons. Countries where these agents have never been reported or the mechanisms of response towards these etiologies are no longer active, are more susceptible to hostile attacks.

In this work a duplex qPCR was developed for the detection of both *B. mallei* and *B.pseudomallei* in three possible matrices: swabs, lungs or other tissue and soil. Since naturally infected matrices do not exist in Portugal, spiked samples were prepared to evaluate, for each of them: i) sample preparation for qPCR and cultural analysis; ii) sensitivity, specificity, repeatability and reproducibility of duplex and singleplex qPCR; iii) performance of duplex qPCR in comparison with the "gold standard" bacteriological culture and correlation between both approaches.

Based on the results of the work developed, in case of biological alert due to suspicion of *B. mallei* or *B. pseudomallei* spread infection in animals or humans or realise in the environment, the recommended procedure is depicted:

i) For *B. pseudomallei* detection in **soil samples**, the best results were obtained with a previous incubation step of the sample at 37ºC for 48 h in Ashdown's Broth, inoculation in Ashdown's agar, and isolation and identification of suspected typical pink rough colonies by singleplex qPCR targeting the *psu* gene. By using a selective media prior to qPCR this procedure avoids PCR inhibitors and surpasses the competition with other bacteria present in a complex matrix as soil. The use of singleplex approach with *psu* gene as the only target increases the sensitivity of the analysis. **Swabs** should be incubated at 37 ºC for 48 hours in Ashdown's Broth and Brain Heart Infusion Broth for, respectively, *B. pseudomallei* e *B. mallei* and directly analysed by the established duplex qPCR. However, the swabs used in this work did not represent a natural collected sample from an infected wound and, therefore, the overgrowth of other bacteria that might limit the growth of *B. mallei* or *B. pseudomallei* should be considered. In suspicious **lung infections**, pulmonary macerates should be inactivated and directly analysed by duplex qPCR.

ii) The duplex qPCR showed a limit of detection of 29 and 455 fg, respectively, for *B.mallei* and *B. pseudomallei*, and proved to be highly repeatable and reproducible with coefficients of variation ≤ 2% [36].This assay proved to be specific for *B.mallei* and *B.pseudomallei* by testing

DNA templates from other microorganisms, including *Mycobacterium tuberculosis* and the close species *Burkholderia thailendensis*. The qPCR targeting *psu* gene can be performed in a singleplex system for specific detection of *B. pseudomallei* in soils showing a LOD of 4.5 fg.

iii) qPCR provided a higher sensitivity in comparison to the "gold standard" method, culture media. Both methods proved to be statistical different for *B. mallei* identification, favouring qPCR as the best approach towards the detection of both microorganisms, either directly in the sample or after cultural isolation of the agent as in the case of soils. The comparison of culture results with qPCR for swabs were affected by the lack of growth of *B. mallei* in all spiked swabs, highlighting the need of specific culture media towards the isolation of this agent.

The duplex qPCR presented in this work is capable of identifying and differentiate both *B. mallei* and *B. pseudomallei* in different matrices in one tube reaction, offering a sensitive, specific, highly repeatable and reproducible diagnostic tool in any laboratory of reference.

Standard Operation Procedures (SOPs) were elaborated according to ISO17025 legislation and audits have been squealed for accreditation of methods described in this work. Ring trials, organized by the Europen Reference Laboratory for equine diseases at ANSES, France, were performed for *B. mallei* detection and identification by qPCR, and the results were 100% in agreement.

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Appendixes

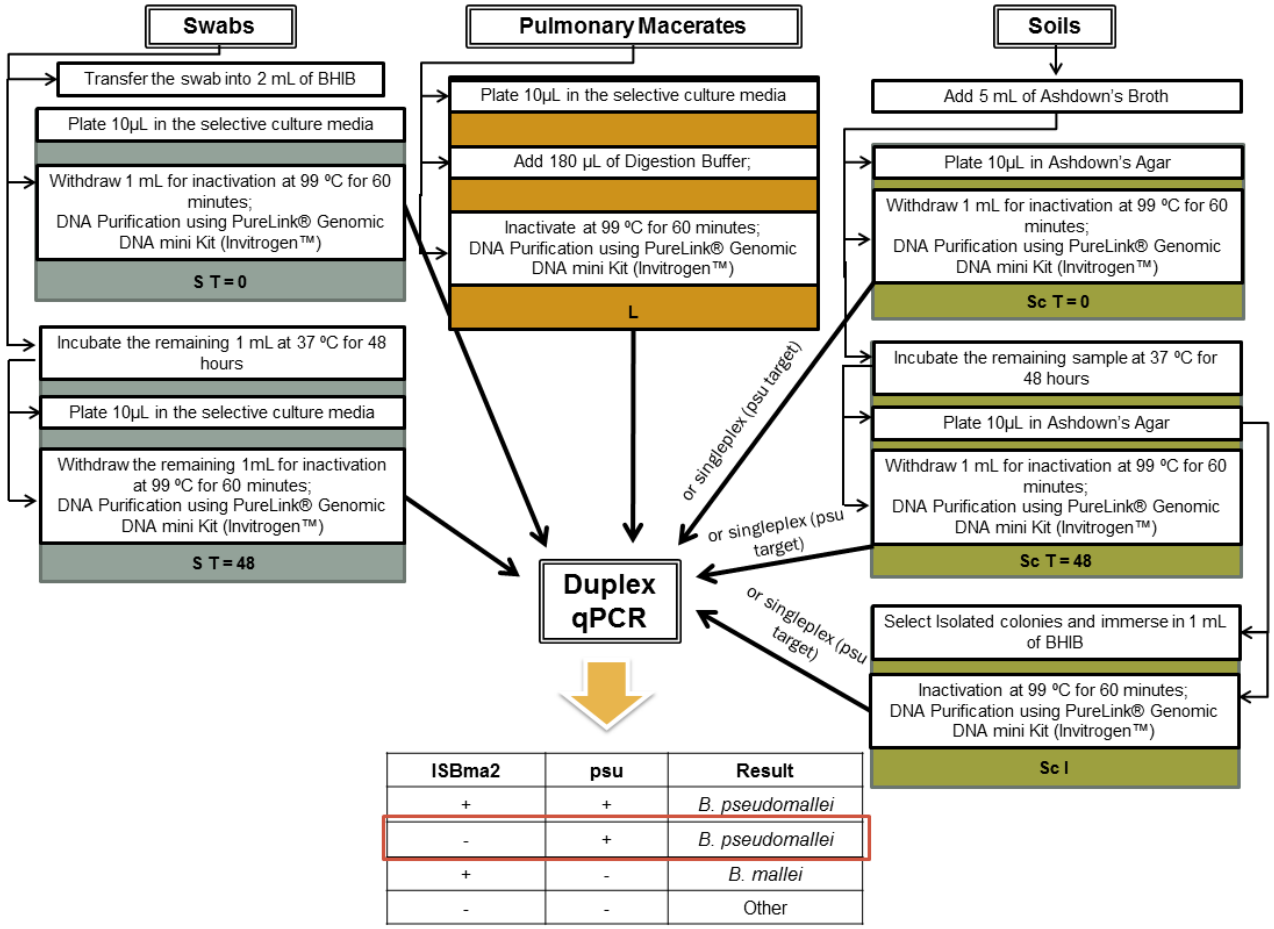
Appendix I: Ashdown's Agar composition (adapted from [11])

Materials

I. 475mL distilled water
II. 7.5g Bacto Agar (BD-214010)
III. 5g Tryptone Soy Broth (Oxoid CM 0129)
IV. 20mL warmed glycerol (Merck 104094)
V. 2.5mL 0.1% crystal violet
VI. 2.5mL 1.0% neutral red (Sigma-Aldrich® N4638 – 1G)
VII. Freshly prepared 100 µg/mL gentamicin solution
VIII. Glass universal containers
IX. 1 litre glass flask
X. Plastic Petri dishes

Method

1. Mix ingredients I to VI in a 1 litre glass flask. Steam to dissolve leaving the caps loose. Autoclave at 15 psi for 15 minutes.
2. Cool down to 56°C and dispense 1mL of 100ug/mL gentamicin on the petri dishes and add 19 mL of warmed agar. Mix carefully.
3. Label plates "ASH" with date of preparation, and store at 4°C for no more than one week.



ISBma2	psu	Result
+	+	*B. pseudomallei*
-	+	*B. pseudomallei*
+	-	*B. mallei*
-	-	Other

Figure A 1. Sample processing with subsequent qPCR analysis. The present data resumes the processing of swabs, pulmonary macerates and soil samples followed by the qPCR analysis and its possible outcomes. *B. pseudomallei* in soils can be performed by targeting the *psu* gene in a singleplex assay. Red box indicates the possibility of some *B. pseudomallei* strains to not amplify ISBma2 target as previously stated by Janse et al. (2013).

Table A 1. OD_{600} values and correspondent CFU/mL mean for each serial dilution of *B.mallei* 10245 and *B.pseudomallei* 10276

	***B. mallei* 10245**		***B. pseudomallei* 10276**	
Dilutions	**OD_{600}**	**Mean CFU/mL**	**OD_{600}**	**Mean CFU/mL**
10^{-1}	0.11	$2.5x10^4$	0.153	$5.9x10^4$
10^{-2}	0.014	$4.4x10^3$	0.019	$3.7x10^4$
10^{-3}	0.006	$2.5x10^2$	0	$2.7x10^4$
10^{-4}	0.01	0	0	$6.5x10^2$
10^{-5}	0.003	0	0	0
10^{-6}	0.004	0	0	0

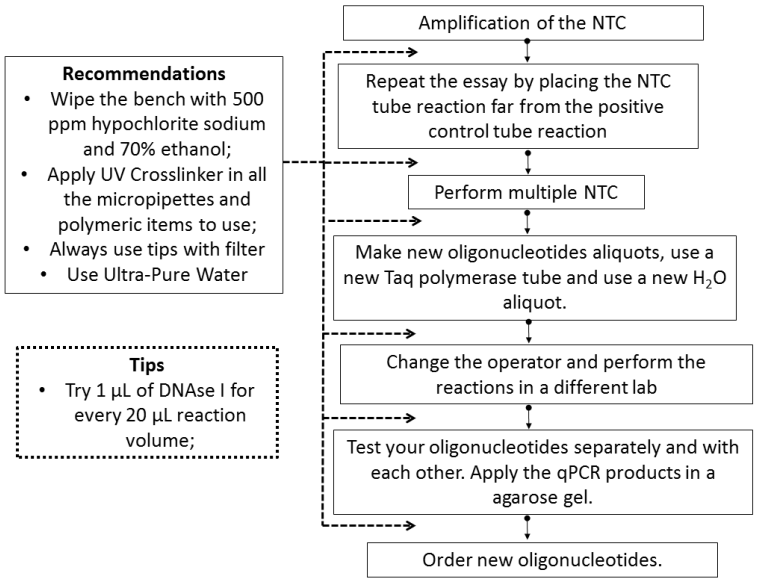


Figure A 2. Decontamination strategies. Every vertical arrow leads to a new strategy followed by the persistency of the NTC amplification. If any step demonstrated to be efficient, no further steps were taken. The dashed arrows represent the recommendations performed before and after any step.

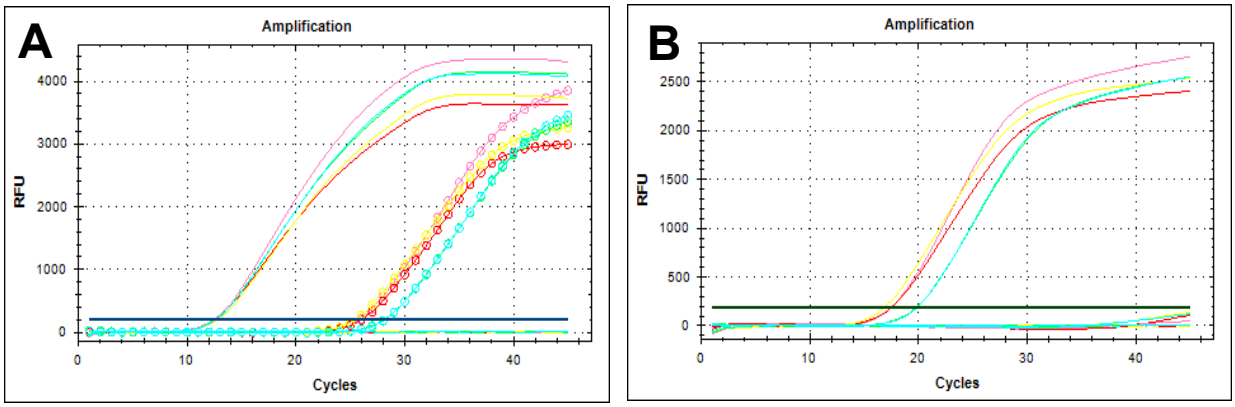


Figure A 3. Optimization of the annealing temperature of duplex qPCR. Annealing was optimized by subjecting the reaction to a range of temperatures: pink lines represent a temperature of 58.1 ºC, bright blue - 60 ºC, bright green – 61.6 ºC, yellow – 62.5 ºC and red 62.9 ºC. This test included a positive control for *B. pseudomallei* and *B. mallei* and a NTC. The amplification of the two targets in the same duplex reaction is shown separately: (A) Detection of FAM (ISBma2 dye label targeting *B. pseudomallei* and *B. mallei*) fluorescence. Straight lines refer to *B. mallei* positive control and doted lines to *B. pseudoamallei* positive control. (B) Detection of HEX (*psu* gene.dye label targeting *B. pseudomallei*) fluorescence.

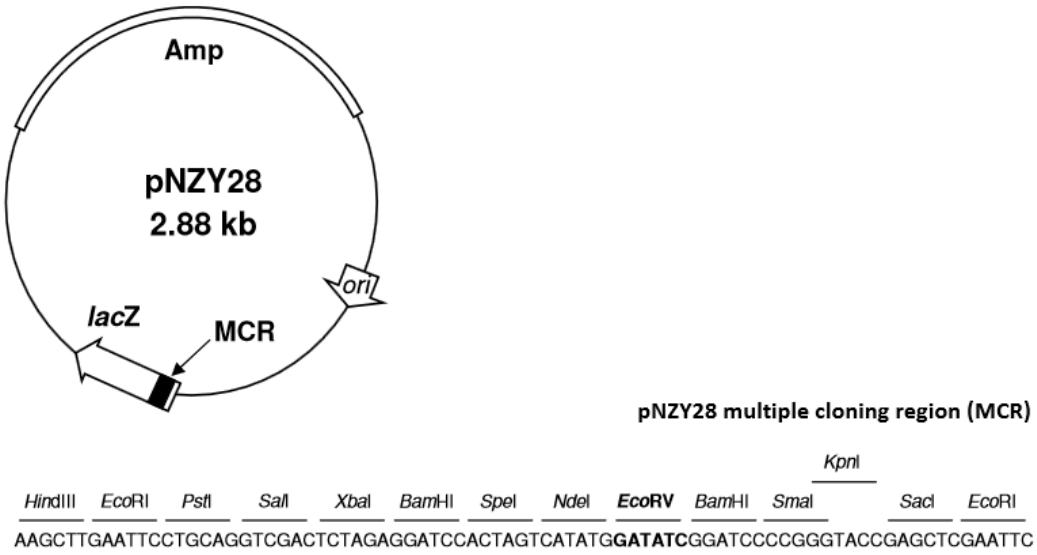


Figure A 4. pNZY28 vector with the multiple cloning region displayed

Table A 2. Repeatability and Reproducibility of the singleplex and duplex assay

	Target	Dilutions	Repeatability			Reproducibility		
			Cq Mean	SD	Coefficient of Variation (%)	Cq Mean	SD	Coefficient of Variation (%)
Singleplex	ISBma2 (Bm)	10^{-1}	14.94	0.050	0.335	16.53	0.378	2.288
		10^{-2}	18.095	0.005	0.028	19.43	0.378	1.947
		10^{-3}	21.055	0.035	0.166	22.7275	0.069	0.303
	ISBma2 (Bp)	10^{0}	30.64	0.160	0.522	30.840	0.200	0.649
		10^{-1}	34.37	0.180	0.524	34.415	0.045	0.131
		10^{-2}	37.5	0.180	0.480	37.990	0.490	1.290
	psu	10^{-1}	22.785	0.105	0.461	22.945	0.0256	0.112
		10^{-2}	25.845	0.035	0.135	25.9825	0.0189	0.073
		10^{-3}	29.36	0.120	0.409	29.335	0.0006	0.002
Duplex	ISBma2 (Bm)	10^{-1}	16.025	0.125	0.780	16.6175	0.2782	1.677
		10^{-2}	19.265	0.095	0.493	19.495	0.3025	1.552
		10^{-3}	22.575	0.185	0.819	22.4625	0	0
	ISBma2 (Bp)	10^{1}	28.455	0.005	0.018	28.7225	0.068906	0.240
		10^{-2}	31.785	0.425	1.337	32.34	0.0169	0.052
		10^{-3}	35.065	0.415	1.184	35.33	0.0225	0.064
	psu	10^{-1}	21.26	0.13	0.611	21.475	0.215	1.001
		10^{-2}	24.74	0.14	0.566	24.828	0.087	0.352
		10^{-3}	28.26	0	0.000	28.428	0.167	0.589

Bm – *B. mallei*; Bp – *B. pseudomallei*; Cq – Quantification Cycle; SD – Standard Deviation

Table A 3. qPCR results for the spiked swabs and pulmonary macerates with *B.mallei* NCTC 10245 and *B.pseudomallei* NCTC 10276

qPCR Results for the spiked samples									
	Spiked Matrix	**Dilution**	**Cq Value**			**Spiked Matrix**	**Dilution**	**Cq Value**	
			ISBma2	*psu*				ISBma2	*psu*
Burkholderia mallei	S T= 0	10^{-1}	20.33	-	***B.pseudomallei***	S T= 0	10^{-1}	26.21	28.98
		10^{-2}	24.79	-			10^{-2}	29.24	32.16
		10^{-3}	27.83	-			10^{-3}	30.20	34.40
		10^{-4}	30.43	-			10^{-4}	31.23	36.87
		10^{-5}	33.80	-			10^{-5}	32.30	39.01
		10^{-6}	39.25	-			10^{-6}	33.34	38.37
	S T=48	10^{-1}	22.31	-		S T=48	10^{-1}	13.51	16.22
		10^{-2}	26.04	-			10^{-2}	12.96	15.63
		10^{-3}	28.43	-			10^{-3}	13.12	15.83
		10^{-4}	31.70	-			10^{-4}	23.37	26.04
		10^{-5}	34.64	-			10^{-5}	16.25	18.80
		10^{-6}	32.46	-			10^{-6}	25.85	28.42
	L	10^{-1}	15.93	-		L	10^{-1}	21.29	23.44
		10^{-2}	20.12	-			10^{-2}	25.02	27.40
		10^{-3}	22.66	-			10^{-3}	27.50	30.52
		10^{-4}	25.27	-			10^{-4}	27.27	33.39
		10^{-5}	25.73	-			10^{-5}	27.39	38.06
		10^{-6}	25.95	-			10^{-6}	26.75	38.18

S T=0 – Swabs without incubation; S T=48 – Swabs with 48 hours of incubation; L – Pulmonary Macerates

Table A 4. qPCR results for the spiked soils with *B.pseudomallei* 10276

	Spiked Matrix	Dilution	Cq Value	
			ISBma2	*psu*
Burkholderia pseudomallei	Sc T= 0	10^{-1}	26.97	29.58
		10^{-2}	27.85	30.43
		10^{-3}	30.75	34.73
		10^{-4}	32.12	38.58
		10^{-5}	31.57	38.73
		10^{-6}	31.08	38.56
	Sc T=48	10^{-1}	27.68	30.74
		10^{-2}	29.14	31.51
		10^{-3}	31.11	34.03
		10^{-4}	28.74	31.76
		10^{-5}	31.42	33.97
		10^{-6}	NA	NA
	Sc I	10^{-1}	12.24	16.79
		10^{-2}	14.60	18.02
		10^{-3}	13.73	17.18
		10^{-4}	24.50	28.11
		10^{-5}	23.74	27.27
		10^{-6}	24.11	27.75

Sc T=0 – Soil cultures without incubation; Sc T=48 – Soil cultures with 48 hours of incubation; Sc I – Soil culture isolated colonies