INVESTIGATION OF POTENTIAL ENVIRONMENTAL RESERVOIRS OF *MYCOBACTERIUM ULCERANS* IN NORTH QUEENSLAND

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DECLARATION

I certify that this thesis is composed of my original work. This thesis does not contain any materials previously submitted for a degree or diploma at any university or other institution of tertiary education.

To best of my knowledge, this thesis does not contain any materials previously published or written by another person. Information obtained from published or unpublished work from others has been properly referenced.

A. Singh

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STATEMENT OF CONTRIBUTION OF OTHERS

Nature of Assistance	Contributions	Names, Titles
Intellectual support	Proposal writing Data analysis Editorial assistance	Prof. John McBride, College of Medicine and Dentistry, James Cook University A/Prof. Brenda Govan, College of Public Health, Medical and Veterinary Sciences, James Cook University Dr. Mark Pearson, Australian Institute of Tropical Health & Medicine, James Cook University
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STATEMENT OF CONTRIBUTIONS TO JOINTLY-AUTHORED WORKS CONTAINED IN THE THESIS

Chapter	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate	I confirm the candidate's contribution to this paper and consent to the inclusion of the paper in this thesis
Two	**Singh A,** McBride WJH, Govan B, Pearson M. Potential Animal Reservoir of *Mycobacterium ulcerans*: A Systematic Review. Tropical medicine and infectious disease. 2018;3 (2):56.	**AS**: Conducted extensive literature search to aggregate literature available on online databases; developed Study protocol; analysed literature, drafted and edited manuscript. **WJHM:** Provided support on literature review, revised and reviewed final manuscript **BG:** Provided support on literature review, reviewed final manuscript **MP:** conducted extensive literature search to aggregate literature available on online databases	**William J. H. McBride** Signature: **Brenda Govan** Signature: **Mark Pearson** Signature:
Four	**Singh A**, McBride WJH, Govan B, Pearson M, Ritchie, SA. A survey for *Mycobacterium ulcerans* in Mosquitoes and March flies captured from endemic areas of Northern Queensland, Australia. Tropical	**AS:** All aspects of study: study design, implementation, sample collection, analysis, interpretation, preparation of manuscript and submission to the journal. **WJHM:** Provided support on conceptualization, revised manuscript for important intellectual	**William J. H. McBride** Signature: **Brenda Govan** Signature: **Mark Pearson** Signature:

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Five	**Singh A,** Mcbride WJH, Govan B, Pearson M. Survey of local fauna from endemic areas of Northern Queensland, Australia for the presence of *Mycobacterium ulcerans*. Int J Mycobacteriol 2019; (In Press)	**AS:** All aspects of study: study design, implementation, sample collection, analysis, interpretation, preparation of manuscript and submission to the journal. **WJHM:** Provided support on conceptualization, revised manuscript for important intellectual content, reviewed final manuscript. **BG:** Provided support on conceptualization, revised manuscript for important intellectual content, reviewed final manuscript. **MP:** Provided support on laboratory analysis of samples	**William J. H. McBride** Signature: **Brenda Govan** Signature: **Mark Pearson** Signature:

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ABSTRACT

Mycobacterium ulcerans is the causative agent of Buruli ulcer (BU). It is a geographically restricted neglected tropical disease characterized by extensive and painless necrosis of the skin and soft tissue with formation of large ulcers, usually on the extremities. This disease has been reported in 33 countries worldwide including Australia. In Australia, BU has been reported in coastal Victoria and the Mossman-Daintree area of north Queensland. The mode of transmission and potential environmental reservoir of the organism causing this disease is not well understood. It appears likely that these factors vary in different geographical locations and epidemiological settings. This dissertation aims to investigate the potential reservoirs of *M. ulcerans* in the BU endemic area of north Queensland, Australia.

In Chapter 2, a systematic review is presented to describe what is known of potential animal reservoirs of *M. ulcerans* in regions throughout the world. The review was conducted using MEDLINE and INFORMIT databases to aggregate published data on this topic. Non-human cases of BU were found only in Australia and small native mammals were identified as potential reservoirs of the infection.

Chapter 3 describes the geospatial analysis of cases of BU reported between 2009 and 2018, in an endemic area of North Queensland, Australia, using ArcMap 10.4.1 software. Hot-spot analysis did not find any statistically significant cluster of cases within the endemic areas. Additionally, an association between the amount of rainfall and month of diagnosis of BU cases was described, supporting other observations about the average incubation period of this disease.

Chapter 4 and 5 detail the survey of local fauna from the same endemic area of north Queensland, Australia, for the presence of *M. ulcerans*. Mosquito and animal traps were set up at endemic sites throughout the study period and mosquitoes and animal faecal samples were collected and tested for the presence of *M. ulcerans* DNA. DNA from *M. ulcerans* was detected from two bandicoot scat samples and one mosquito pool. These samples were collected from sites in close proximity to human cases of BU.

Chapter 6 describes a blood feeding experiment to investigate the role of mosquitoes in transmission of *M. ulcerans*. Batches of mosquitoes were fed with defibrinated sheep blood containing heat-killed *M. ulcerans*. DNA extracted from heads, abdomen and legs of mosquitoes was tested for the presence of *M. ulcerans* DNA. DNA from *M. ulcerans*

was detected from heads of mosquitoes and pools of whole mosquitoes, providing supportive evidence for the role of mosquitoes as a mechanical vector in the transmission of *M. ulcerans.*

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1 Chapter 1- INTRODUCTION

Aims of this chapter

- To introduce *Mycobacterium ulcerans* and disease caused by this organism
- Provide history of *M. ulcerans* and its global distribution and epidemiology
- To present clinical manifestation of the disease
- To discuss on mode of transmission and environmental reservoir of *M. ulcerans*
- To present objective and hypothesis of thesis
- To present conceptual framework to fulfill all objectives

Mycobacterium ulcerans (M. ulcerans) is the causative agent of the Buruli ulcer (BU), also known, in Australia, as Daintree ulcer or Bairnsdale ulcer. This destructive skin disease is characterized by extensive and painless necrosis of the skin and soft tissue with the formation of large ulcers, commonly on the leg or arm (1). To date, 33 countries with tropical, subtropical and temperate climates in Africa, the Americas, Asia and the Western Pacific have reported cases of Buruli Ulcer (2). The disease is rarely fatal, although it may lead to permanent disability and/ or disfigurement if not treated appropriately or in time. It is the third most common mycobacterial infection in the world after tuberculosis and leprosy (1). The precise mode of transmission of *M. ulcerans* is yet to be elucidated. Nevertheless, it is possible that the mode of transmission varies with different geographical areas and epidemiological settings. Though advances has been made in past few years to understand the nature of the disease, the ecological factor and transmission are yet to be studied in detail. The knowledge about the possible route of transmission and potential animal reservoir of *M. ulcerans* is poorly understood and still remains patchy. The following chapter outline the present comprehension of BU and its causative agent, *M. ulcerans* and also uncertain research issue in this field.

1.1 History of *Mycobacterium ulcerans*:

Sir Albert Cook, a British missionary doctor appointed at the Mengo Hospital in Kampala, Uganda first noted the skin ulcer caused by *Mycobacterium ulcerans* in 1896. Later in the late 1930's, two general practitioners, Dr. J. R. Searl and D. G. Alsop,

working in rural Victoria, noticed a group of cases around the town of Bairnsdale with mysterious skin ulcers (3). The cases were not published in the literature at the time and the causative organism was not identified and characterized. Professor Peter MacCallum and his colleagues first provided the detailed description of the disease in 1948 from six patients at Bairnsdale district, near Melbourne, Australia. They were the first to isolate *M. ulcerans* as the causative organism of the mysterious skin ulcer (4). The first large clusters of *M. ulcerans* infection was identified in the Buruli County in Uganda (now called Nakasongola District) in 1960's and the disease was termed as "Buruli Ulcer" thereafter (5).

In the 1980's, Buruli ulcer (BU) emerged as a serious public health problem, mainly in west and central African countries. Cases were reported in the Democratic Republic of the Congo (6, 7), Ghana (8, 9), Uganda (10, 11) and Nigeria (12). Subsequently, several new foci of *M. ulcerans* infections were also reported in Angola (7, 13), Benin (14-16), Toga (17), Côte d'Ivoire (18, 19), Ghana (9, 20) and Cameroon (21, 22). Realizing the burden of disease, and in order to harmonize Global Buruli ulcer research, the World Health Organization (WHO) established the Global Buruli Ulcer Initiative (GUBI) in February 1998 with financial support from the Nippon Foundation, Tokyo, Japan (23). In July 1998, the first international conference on Buruli ulcer was organized by WHO to draw the attention of global researchers on the importance of this disease. The conference led to the "Yamoussoukro Declaration" on Buruli Ulcer. At the conference, the then Director-General of WHO, Dr. Hiroyoshi Nakajima drew the global attention to this neglected tropical disease and stated:

> *"I decided to place emphasis on the fight against Buruli ulcer for the following reasons. In the 21st century, where infectious diseases are concerned, the world will have to find the means both to control major long-standing scourges such as tuberculosis and malaria, and also to deal effectively with emerging diseases such as Buruli ulcer. I am convinced that these two different sorts of challenges will have to be tackled simultaneously. If we fail to do so, the prevalence of infectious diseases as a whole is likely to increase worldwide, and the severity of specific diseases may well increase too."*
>
> *WHO, (23)*

1.2 Epidemiology and distribution round the world:

Till today, 33 countries with tropical, subtropical and temperate climates extending from Africa, the Americas, Asia and the Western Pacific have reported the cases of Buruli ulcer (24). Fifteen out of thirty-three countries reported the data on Buruli ulcer to World Health Organization in 2015. Following is the list of 33 countries with the report of Buruli ulcer:

Table 1: List of countries with cases of Buruli ulcer (Source: (24)

Africa	Angola, Benin, Burkina Faso, Cameroon, Congo, Ivory Coast, Democratic Republic of the Congo (former Zaire), Equatorial Guinea, Gabon, Ghana, Guinea, Kenya, Liberia, Malawi, Nigeria, Sierra Leone, Sudan, Togo and Uganda
Latin America	Brazil, French Guiana, Mexico, Peru, Suriname
South- East Asia	Indonesia, Sri Lanka
Western Pacific	Australia, Kiribati, Papua New Guinea, Malaysia, China and Japan

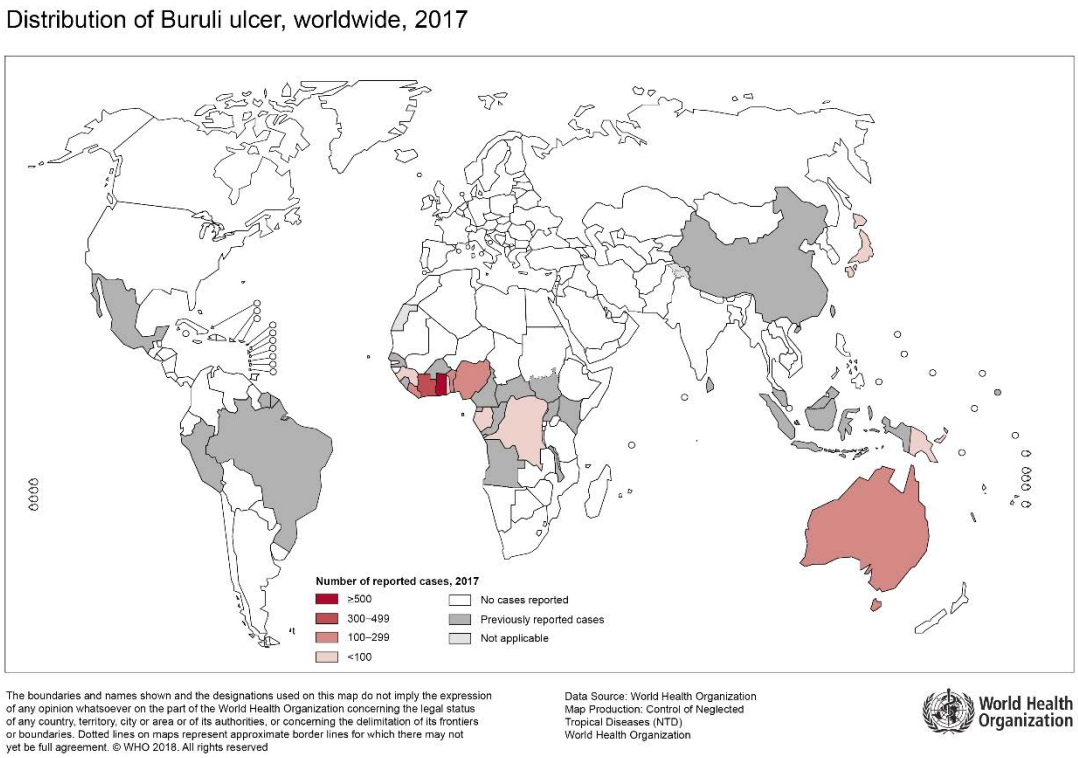


Figure 1. Distribution of Buruli ulcer worldwide, 2017.

The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city, or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. Dotted lines on maps represent approximate border lines for which there may not yet be full agreement. Data source: World Health Organization. Map production: Control of Neglected Tropical Diseases. Data source: World Health Organization. Map production: Control of Neglected Tropical Diseases (25)

The overall distribution of Buruli ulcer is not focal however, distinct foci of disease may exist within endemic regions. The fact that majority of cases are prevalent in West African countries, it become challenging to get precise burden due to poor reporting system of disease in these countries. In 2014, highest number of new cases were reported from Côte d'Ivoire (827 cases) followed by Ghana (443 cases) and Benin (443 cases). Altogether, 2,251 new cases were recorded in 2014 (26).

1.3 The organism:

Taxonomically, *M. ulcerans* is classified as follows (27):

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actinobacteria

Order: Actinomycetales

Family: MycobacteriaceaeGenus: *Mycobacterium*

Species: *ulcerans*

M. ulcerans is an atypical mycobacterial species. It does not cause tuberculosis and is usually unresponsive to first line anti tuberculosis drugs.

The classification of atypical *Mycobacterium* species is shown in the table below:

Table 2: Classification of a small section of atypical Mycobacterium species Source: Laboratory diagnosis of nontuberculous mycobacteria (28).

Slow Growing	Rapid Growing
Established pathogens	Established pathogens
M. avium-intracellulare complex	
M. haemophilum	*M. abscessus*
M. kansasii	*M. chelonae*
M. leprae	*M. fortuitum*
M. malmoense	
M. marinum	Newly discovered or emerging mycobacteria
M. scrofulaceum	
M. simiae	*M. agri*
M. szulgae	*M. alvei*
M. ulcerans	*M. bonickei*
M. xenopi	*M. brumae*
	M. chitae
Newly discovered or emerging mycobacteria	*M. confluentis*
	M. fortuitum biovariant subtypes
M. bohemicum	*M. hassiacum*
M. branderi	*M. houstonense*
M. celatum	*M. immunogenum*
M. conspicuum	*M. mageritense*
M. genavense	*M. mucogenicum*
M. heciceshornense	*M. novocastrense*
M. heidelbergense	*M. porcinum*
M. interjectum	*M. senegalense*
M. intermedium	*M. septicum*
M. kubiacae	*M. smegmatis* group
M. lentijlavum	*M. goodii*
M. triplex	*M. peregrinum*
M. tusciae	*M. smegmatis (sensu stricto)*
	M. wolinskyi/

Similar to other *Mycobacterium* species, *M. ulcerans* has a relatively high G+C content (65%) DNA. It has a unique cell wall with a lipid-rich layer outside the peptodiglycan

layer. *M. ulcerans* has a long generation time compared to other *Mycobacterium* species. The experiment conducted by Hidetake Yaoi and his colleague compared the generation time of *M. ulcerans* and *M. tuberculosis*. They found the generation time for *M. ulcerans* was 42 hours at 33 degrees Celsius and 56 hours at 37 degrees Celsius while thegeneration time for *M. tuberculosis* was 36 hours at 32 degrees Celsius and 25 hours at 37 degrees Celsius (29). A separate study conducted by Hidetake Yaoi and his colleague found that the degree of acid fastness of *M. ulcerans* matched with other pathogenic mycobacteria however acid fastness was relatively strong compared to nonpathogenic *Mycobacterium* species (30).

1.4 Taxonomy, Phylogeny, and Genetics:

The genus *Mycobacteria* consists of groups of pathogenic and non-pathogenic organisms (Figure 2). Some of the organism of this genus such as *M. tuberculosis* and *M. leprae*, have significant public health importance.

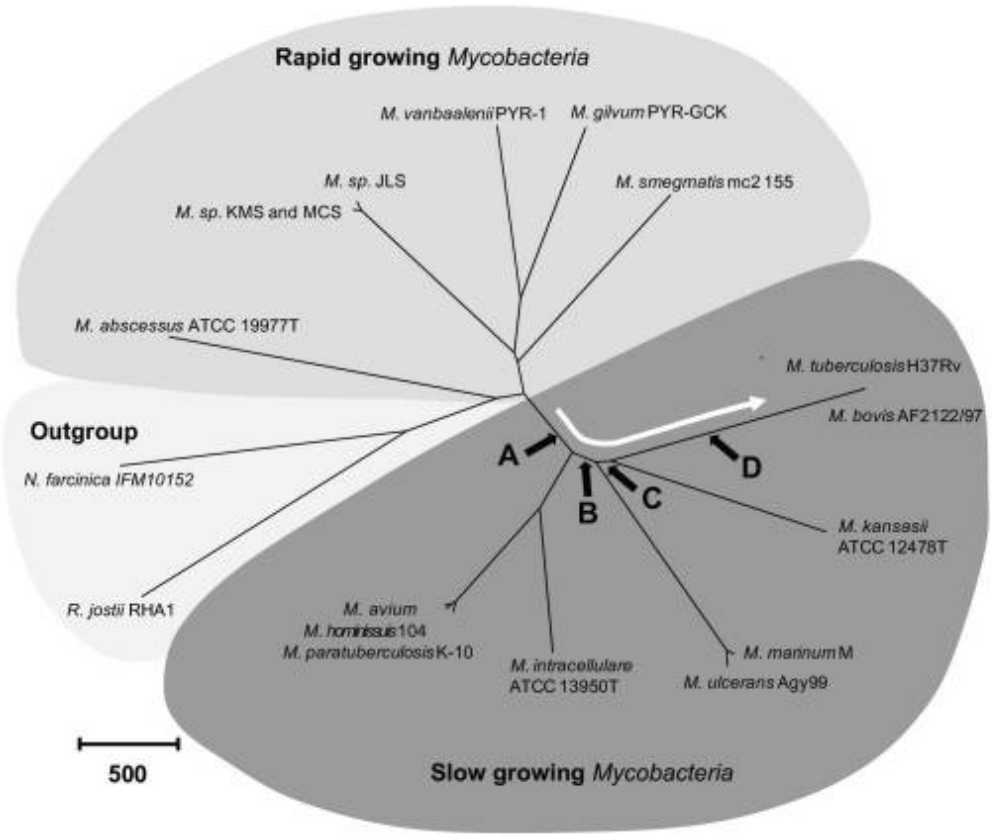


Figure 2: Phylogeny of Mycobacterium genus. Linkage indicated based on growth rate of organism and scale represents the number of amino acid difference.Adapted from (31)

On the basis of the universal molecular clock rate (32), *M. ulcerans* is believed to have evolved from a common ancestor, *Mycobacterium marinum* through a series of genetic divergence approximately one million years ago, by acquisition of circular plasmid named pMUM001, which encodes the enzyme required for the production of a toxin named mycolactone (32, 33). However, there are some closely related mycolactone producing mycobacteria (MPM) namely *M. shinshuense, M. marinum, M. pseudoshottsii* and *M. liflandii* which produce mycolactone but are not associated with BU (34-36). Genetic analyses of these MPM shows that they are genetically coherent and are derived from a common ancestor (37). Therefore, it has been proposed to rename all MPM as *M. ulcerans (37, 38)*. *M. ulcerans* diverged into two principal linkages: ancestral and classical after the acquisition of pMUM and insertion sequence IS*2404*, IS*2606* and KR by extensive gene loss for adaptation to a new environment. The classical linkage consists of most pathogenic genotypes originating from Africa, Australia and South East Asia and ancestral linkage consists of strain from Asia, South America and Mexico (39). The ancestral linkage is further subdivided into Linkage 1 consisting of human isolates from South America and worldwide fish and frog isolates and Linkage 2 consisting of isolates from Japan. It has been suggested that "each of the *M. ulcerans* lineages probably represents different ecotypes, reflecting adaptation to related but distinct niche environments" and should be considered as *M. ulcerans* ecovars (37).

1.5 Clinical manifestations of Buruli ulcer:

Once the organism successfully enters into the skin, it confines itself and multiplies in subcutaneous tissue and overlying skin. Buruli ulcer initially manifests as painless nodules under the skin or as a papule. This form of disease is mostly observed in the patients of Australia (40). The nodules gradually worsen to form ulcers with necrosis. If not treated well and on time, permanent disability occurs due to self-healing process and extensive scar formation. World Health Organization (WHO) suggests about 85% of the lesions occur on the limbs (41). The table below demonstrates the location of lesions of Buruli ulcer at different endemic regions:

Table 1: Location of Lesion of Buruli Ulcer, Source: Source: Treatment of *Mycobacterium ulcerans* disease (Buruli Ulcer): Guidance for health workers (42).

	Upper limb	Lower limb	Other parts of the body
Africa	25%	63%	11%
Australia	31%	64%	5%
Japan	50%	38%	13%

Disease is prevalent regardless of age group and gender, however almost 50% of cases in Africa are among children below 15 years of age (24). Buruli ulcer may appear as either an ulcerative or non-ulcerative form.

Non-ulcerative forms may present as (1):

i. Papule: usually painless, raised skin lesion, less than 1 cm in diameter. This form of disease is more common in Australia (Figure 3A).
ii. Nodule: Lesion extends from the skin into the subcutaneous tissue. Lesion is usually painless but sometimes it may be itchy. It is 1–2 cm in diameter. This form of lesion is more common in Africa (Figure 3 B).
iii. Plaque: This is a firm, painless, elevated, well demarcated lesion more than 2 cm in diameter. The skin over the lesion is either reddened or discolored (Figure 3C).
iv. Oedematous form: There is a diffuse, extensive, usually non-pitting swelling. Affected area has an ill-defined margin and lesion is firm and painless. Sometimes, fever may be noticed (Figure 3 D).

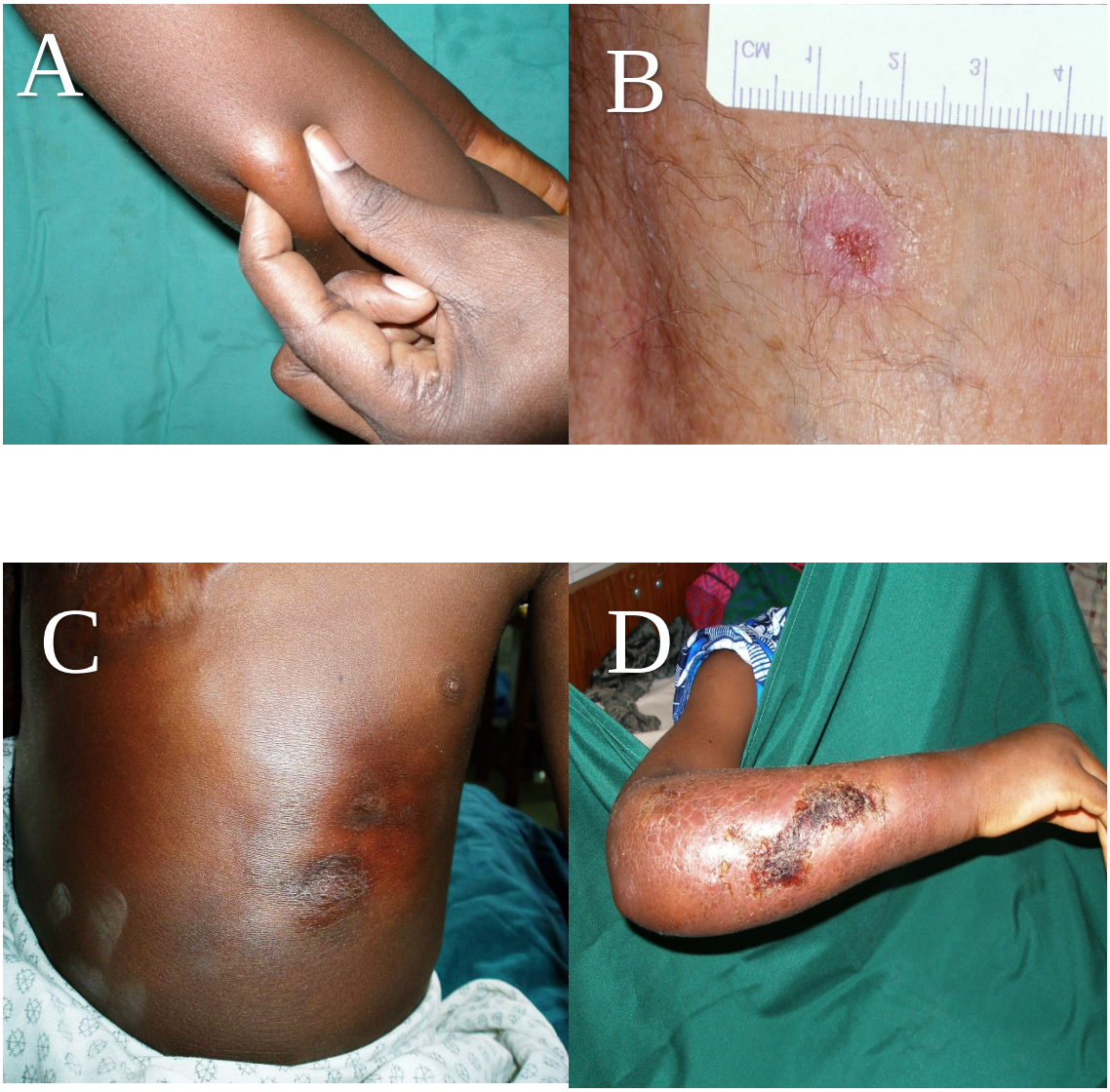


Figure 3: Non-ulcerative form of Buruli ulcer: Stages: Source: Buruli ulcer: Photo Library (43)

Legends: A: Nodule; B: Papule; C: Plaque; D: Oedematous form

Ulcerative forms of the disease may appear when the disease is fully developed. An ulcer with undermined edges and peripheral induration is noticed. The floor of the ulcer may have a white cotton wool-like appearance from necrotic slough (1). Ulcers are usually painless. However, some case studies have reported that the patients experienced pain, sometime very considerable, from their ulcers (44-47).

Figure 4: Ulcerative form of Buruli ulcer, Buruli ulcer: Photo Library (43)

On the basis of size of lesion, WHO has classified the Buruli ulcer in three categories: Category-I: a single lesion of less than 5 cm in diameter; Category-II: a single lesion of 5-15 cm in diameter and Category-III: a single lesion of more than 15 cm in diameter and additional lesion at critical sites and osteomyelitis. Category-III is further divided into three sub-categories namely: IIIa with single lesion of more than 15 cm in diameter and osteomyelitis; IIIb with lesions at critical sites such as eye, breast, genitalia and IIIc with small multiple lesions (42).

1.6 Mode of transmission and environmental reservoirs of *Mycobacterium ulcerans*

The precise mode of transmission of *M. ulcerans* is yet to be elucidated. However, it is evident that the mode of transmission differs with different geographical areas and epidemiological settings. Interaction among agent, host and environment is essential for the onset of infectious diseases. The majority of emerging or remerging infectious disease in human populations are spread by animals: either wildlife, livestock or pets (48-50). Animals may act as hosts or reservoirs and subsequently spread the organism to the

environment or directly to the human population. The reservoirs play a major role in maintenance of the organism in the environment, and in the mode of transmission. This remains valid for *M. ulcerans*.

Australia is the only developed country where substantial transmission of *M. ulcerans* has been recorded. Foci of BU infection have been found in tropical Far North Queensland (51), the Capricorn Coast region of central Queensland (52), the Northern Territory (53) and temperate coastal Victoria (54). In Australia, cases of Buruli ulcer have also been recorded in animals including koalas (*Phascolarctos cinereus*) (55), common ringtail possums (*Pseudocheirus peregrinus*), common brushtail possum *(Trichosurus vulpecula)* (56), horses (57), dogs (58), an alpaca (59) and a cat (60). All of these recordings were located in the vicinity of human cases of BU. Unlike Australia, not a single study in Africa has reported cases in non-human species or the presence of *M. ulcerans* positive DNA in animals, suggesting that transmission dynamics may be different in Africa and southern Australia or, alternatively, that a host animal is yet to be identified in Africa. A study conducted by Fyfe and colleagues, between 2007-2009 in an endemic area of BU in Australia, found 38% of ring tail possums and 24% of brush tail possums with laboratory confirmed *M. ulcerans* lesions DNA (56). Together, the evidence was proposed to support a link with mosquitoes and native mammals in the ecology of BU in Victoria (54, 61).

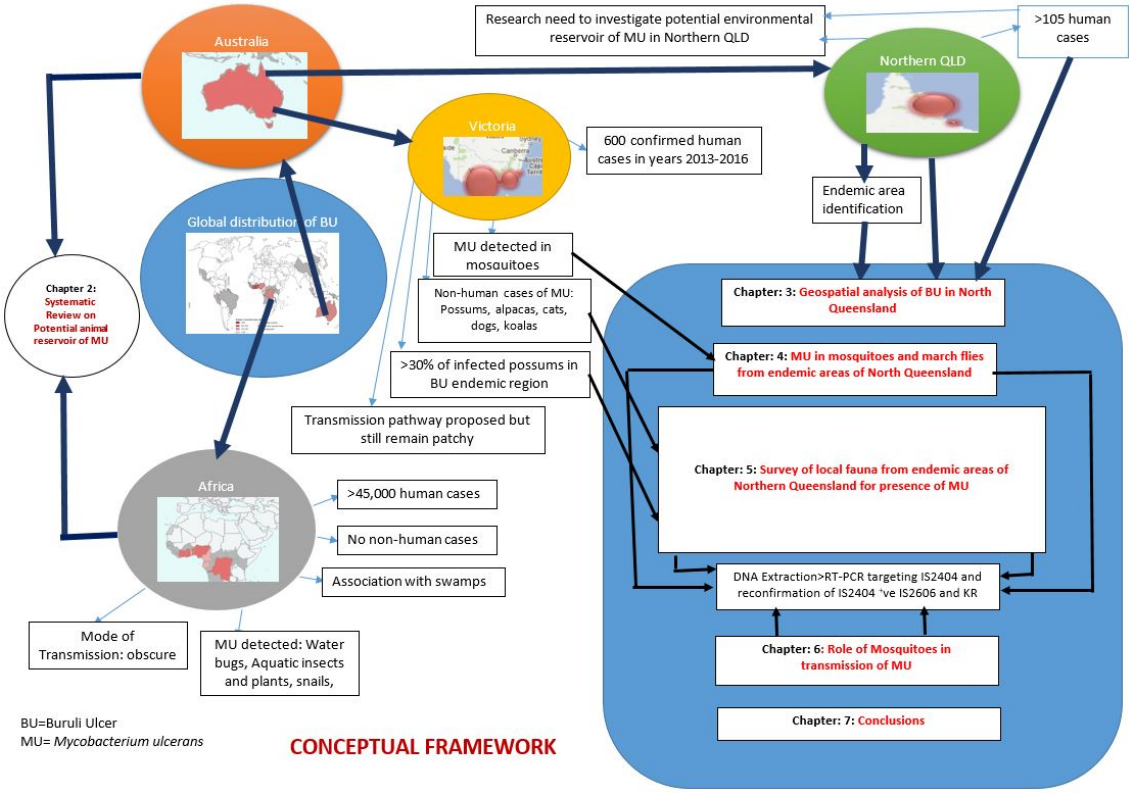
Another endemic area for *M. ulcerans* in Australia is far north Queensland in an area extending from the Daintree river and Forest Creek in the north to Mossman in the south (51). Recently, there was a report of the presence *M. ulcerans* in two bandicoot *(Isoodon macrourus*) scat samples collected in this region (62). The same study also found one sample of an individual mosquito and one pool of two mosquitoes out of 35 insect/insects pool positive for *IS2404*. However, the *IS2404* positive mosquito pool contained DNA of a closely related *M. ulcerans* subspecies that had a low copy number for *IS2606* and that does not commonly cause disease in human. This study highlighted a need to examine a larger sample size to gauge the significance of the role of mosquitoes and native mammals in the ecology of BU in Northern Queensland (62). An additional suggestion put forward by the local population (including people with a history of BU) was that March flies (Tabanidae) might have a role in transmission. We therefore aimed, in this study to investigate epidemiology of *M. ulcerans* in the BU endemic area of Northern Queensland.

1.7 Objectives and Hypothesis:

In this thesis I investigate the epidemiology of *M. ulcerans* in the BU endemic area of Northern Queensland. I hypothesize that the small mammals such as bandicoots and possums act as reservoirs of infection of *M. ulcerans* and play a major role in environmental persistence and insect vectors such as mosquitoes and march flies transmit infection from small mammal reservoirs to humans in north Queensland. This thesis aims to:

- ✓ Generate a Geographic Information System (GIS) map of the human cases at the study area
- ✓ Survey environmental samples such as mosquitoes, march flies, and scats of bandicoots and other native small mammals collected from the NQ region for the presence of *M. ulcerans*
- ✓ Examine swabs from animals collected by local Veterinary practices with suspicious lesions for the presence of *M. ulcerans*.
- ✓ Conduct transmission experiments to identify potential of mosquitoes as transmission vectors

The overall aim of this thesis can be visualized in the conceptual framework given below:



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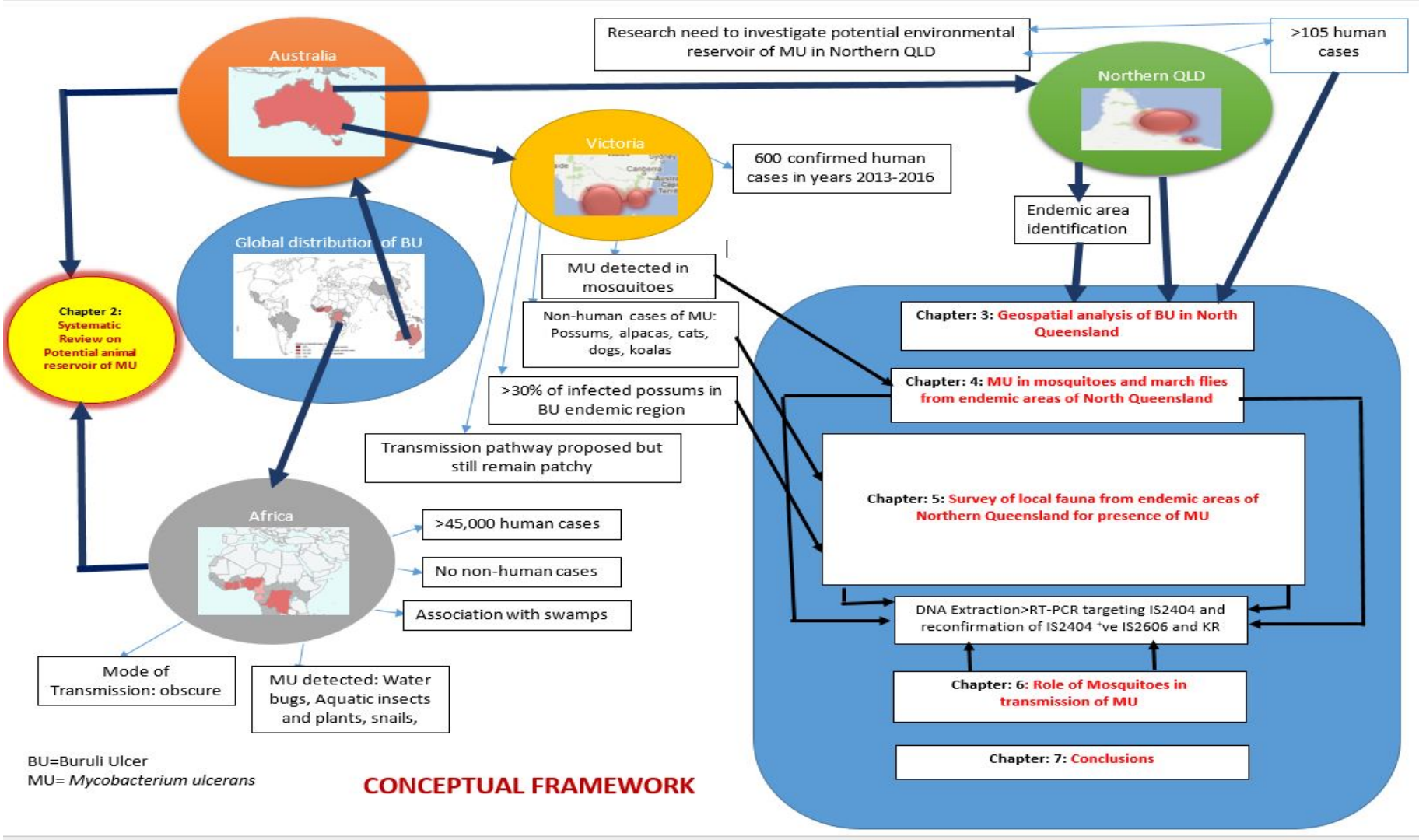
Australia
Research need to investigate potential environmental reservoir of MU in Northern QLD
>105 human cases
Northern QLD
Victoria
600 confirmed human cases in years 2013-2016
Endemic area identification
Global distribution of BU
MU detected in mosquitoes
Chapter 2: Systematic Review on Potential animal reservoir of MU
Non-human cases of MU: Possums, alpacas, cats, dogs, koalas
>30% of infected possums in BU endemic region
Transmission pathway proposed but still remain patchy
Chapter: 3: Geospatial analysis of BU in North Queensland
Chapter: 4: MU in mosquitoes and march flies from endemic areas of North Queensland
Chapter: 5: Survey of local fauna from endemic areas of Northern Queensland for presence of MU
DNA Extraction>RT-PCR targeting IS2404 and reconfirmation of IS2404 +ve IS2606 and KR
Chapter: 6: Role of Mosquitoes in transmission of MU
Chapter: 7: Conclusions
Africa
>45,000 human cases
No non-human cases
Association with swamps
Mode of Transmission: obscure
MU detected: Water bugs, Aquatic insects and plants, snails,
BU=Buruli Ulcer
MU= Mycobacterium ulcerans
CONCEPTUAL FRAMEWORK

2 Chapter-2: POTENTIAL ANIMAL RESERVOIR OF *MYCOBACTERIUM ULCERANS*: A SYSTEMATIC REVIEW

This article has been published (May 30, 2018) in an international, peer reviewed MDPI journal: Tropical Medicine and Infectious Disease (ISSN 2414-6366).

Aim of this chapter

To describe what is known of potential animal reservoirs of *M. ulcerans* in regions throughout the world.

I was the main author of this peer-reviewed manuscript. My principal contribution to this manuscript are as follows:

- I conducted an extensive literature search to aggregate literatures available on online database
- I developed the study protocol to achieve the aim of this study
- I registered the study protocol to PROSPERO: available online: https://www.crd.york.ac.uk/prospero/display_record.php?RecordID=85484
- I conducted a systematic review
- I drafted the manuscript and submitted to journal. The manuscript was accepted for publication in Tropical Medicine and Infectious Disease (see below).

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Tropical Medicine and Infectious Disease

MDPI

Review

Potential Animal Reservoir of *Mycobacterium ulcerans*: A Systematic Review

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Abstract: *Mycobacterium ulcerans* is the causative agent of Buruli ulcer, also known in Australia as

Daintree ulcer or Bairnsdale ulcer. This destructive skin disease is characterized by extensive and painless necrosis of the skin and soft tissue with the formation of large ulcers, commonly on the leg or arm. To date, 33 countries with tropical, subtropical and temperate climates in Africa, the Americas, Asia and the Western Pacific have reported cases of Buruli ulcer. The disease is rarely fatal, although it may lead to permanent disability and/or disfigurement if not treated appropriately or in time. It is the third most common mycobacterial infection in the world after tuberculosis and leprosy. The precise mode of transmission of *M. ulcerans* is yet to be elucidated. Nevertheless, it is possible that the mode of transmission varies with different geographical areas and epidemiological settings. The knowledge about the possible routes of transmission and potential animal reservoirs of *M. ulcerans* is poorly understood and still remains patchy. Infectious diseases arise from the interaction of agent, host and environment. The majority of emerging or remerging infectious disease in human populations is spread by animals: either wildlife, livestock or pets. Animals may act as hosts or reservoirs and subsequently spread the organism to the environment or directly to the human population.

The reservoirs may or may not be the direct source of infection for the hosts; however, they play a major role in maintenance of the organism in the environment, and in the mode of transmission. This remains valid for *M. ulcerans*. Possums have been suggested as one of the reservoir of *M. ulcerans* in south-eastern Australia, where possums ingest *M. ulcerans* from the environment, amplify them and shed the organism through their faeces. We conducted a systematic review with selected key words on PubMed and INFORMIT databases to aggregate available published data on animal reservoirs of *M. ulcerans* around the world. After certain inclusion and exclusion criteria were implemented, a total of 17 studies was included in the review. A variety of animals around the world e.g., rodents, shrews, possums (ringtail and brushtail), horses, dogs, alpacas, koalas and Indian flap-shelled turtles have been recorded as being infected with *M. ulcerans*. The majority of studies included in this review identified animal reservoirs as predisposing to the emergence and reemergence of *M. ulcerans* infection. Taken together, from the selected studies in this systematic review, it is clear that exotic wildlife and native mammals play a significant role as reservoirs for *M. ulcerans.*

Keywords: *Mycobacterium ulcerans*; animal reservoir; transmission

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2.1 1. Introduction

Sir Albert Cook, a British missionary doctor appointed at the Mengo Hospital in Kampala, Uganda, first noted the skin ulcer caused by *Mycobacterium ulcerans* in 1896. Later, in the late 1930s, two general practitioners, Drs. J. R. Searl and D. G. Alsop, working in rural Victoria, Australia, noticed a group of cases of mysterious skin ulcers around the town of Bairnsdale [1]. The cases were not published in the literature at the time and the causative organism was not identified or characterized. Professor Peter MacCallum and his colleagues first provided the detailed description of the disease in 1948, using presentation data of six patients in the Bairnsdale district, near Melbourne. They were the first to isolate *M. ulcerans* as the causative organism of the mysterious

skin ulcer [2]. The first large cluster of *M. ulcerans* infection was identified in the Buruli County of Uganda (now called Nakasongola District) in the 1960s and the disease was termed 'Buruli ulcer' (BU) thereafter [3].

There have been several known outbreaks of Buruli ulcer around the world and each outbreak has its own unique characteristics in terms of epidemiology and the animals reported to be involved in transmission [4,5]. The World Health Organization (WHO) has classified BU as a neglected tropical disease [6]. Presently, BU has been reported (but not always microbiologically confirmed) in more than 30 countries spread over Africa, the Americas, Asia, and Oceania [7]. Australia is the only developed country with significant local transmission of BU, with foci of infection in tropical Far North Queensland [8,9], the Capricorn Coast region of central Queensland [10], the Northern

Territory [11] and temperate coastal Victoria [10]. Non-human cases of *M. ulcerans* are prevalent in Australia only, where several cases of BU have been described in both native wildlife and domestic mammal species such as koalas (*Phascolarctos cinereus*) [12,13], common ringtail possums (*Pseudocheirus peregrinus*) [14,15], a mountain brushtail possum (*Trichosurus cunninghami*) [5,14,15], two horses [16], an alpaca [17], four dogs [18] and a cat [19]. Recent research in Victoria, Australia, has suggested the transmission of infection by mosquitoes, and possums with chronic BU as an important environmental reservoir of *M. ulcerans* in Victoria [14].

2.2 2. Materials and Methods

The PRISMA guidelines developed by the Centre for Review Dissemination (CRD) were used as the methodology for the systematic review [20]. A review protocol was registered with PROSPERO international prospective register of systematic reviews, which can be viewed online [21]. The systematic literature review was conducted using online databases MEDLINE and INFORMIT to aggregate all the published literature. Initially, MEDLINE was used to retrieve all the scientific information concerning the research topic. INFORMIT was searched with same search strategies adopted for MEDLINE. The following key words were chosen after a series of trial searches in order to ensure an adequate number of relevant articles were reviewed: (Buruli OR '*Mycobacterium ulcerans*') AND (Host OR Vector OR Reservoir OR Animal), accessed on 6 May 2018. The title and abstract of each of the articles were initially scanned to ensure that the

included articles met the aim and scope of the systematic review. Articles that were deemed irrelevant to the aim of this systematic review or out of the research scope were excluded. For those articles that were not clear by the title and abstract, the full text was retrieved and further analyzed in order to determine if they met the inclusion and exclusion criteria below. The studies that reported only experimental or laboratory exposure of *M. ulcerans* in animals were excluded. The search strategy exclusively focused on potential animal reservoirs, not the vectors. The detection of the causative agent had to be confirmed by culture of bacteria and/or PCR. To be considered positive a sample needed to be confirmed either by culture of bacteria or positive for IS 2404 and reconfirmed by KR and IS 2606. Undoubtedly, PCR targeting IS 2404 is highly specific for detecting *M. ulcerans* in clinical specimen [22]. However, for detecting *M. ulcerans* from environmental samples, confirmatory PCR targeting two additional insertion sequences, IS 2606 and the ketoreductase B domain (KR), is essential to differentiate *M. ulcerans* from other environmental mycobacteria that may carry IS 2404 and other non-mycolactone-producing mycobacteria [22]. Thus, IS 2404-PCR used in conjunction with IS 2606 and KR-PCR confirms that the detected organism is *M.*

ulcerans. There were no language restrictions. Risk of bias was assessed by one reviewer on the basis of independent factors such as sample size, location and nature of infection.

2.3 3. Results

2.3.1 3.1. Results of the Literature Search and Method of Inclusion

The total number of discovered articles in MEDLINE database was 351. Three hundred and fourteen articles were excluded after reading the title and abstracts as they were not relevant to the research question. Full texts of thirty-seven studies were retrieved in portable document format (PDF) for further analysis. Of these remaining 37 studies, 19 were excluded as they clearly did not meet inclusion criteria (i.e., they were review articles, focused on vectors rather than on animal reservoirs, or pertained to laboratory or experimental exposure). One additional duplicate article was excluded as well. The remaining 17 studies from the PubMed database were included for systematic review. There were no additional articles in INFORMIT that did not appear in the initial

MEDLINE search results. The flow chart for study selection process is shown in Figure 1.

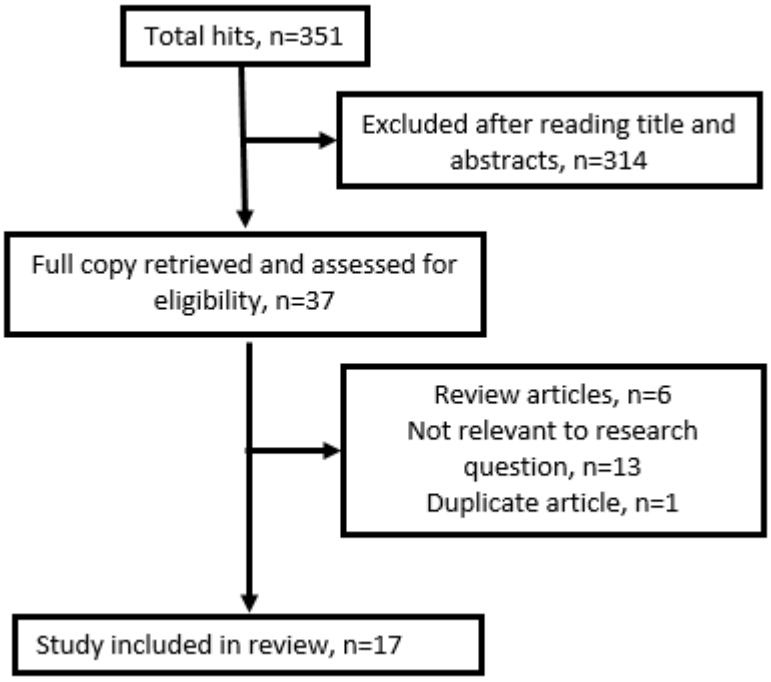

Figure 1. Flow chart of study selection process.

2.3.2 3.2. Basic Characteristics of Selected Studies

Out of the 17 included studies, ten were conducted in Australia, two in Ghana and one was conducted in each of Ivory Coast, North America, United States, Benin and Japan. The basic characteristics of selected studies for review are shown in Table 1 below.

Table 1. Basic characteristics of selected studies on occurrence of *Mycobacterium ulcerans*.

Author and Year	Sample and Sample Size	Collection Year, Location and Setting	Detection Method, Result or *M. ulcerans* Positive Signal
Roltgen, Pluschke, Johnson, & Fyfe, 2017 [9]	102 environmental samples: 55 from soil/vegetation; 35 from insects or small insects pool and 12 from animal excreta	September 2013 Northern Queensland, Australia	RT-PCR IS 2404 positive: 1 soil specimen: 2 bandicoot faeces, one individual mosquito and 1 pool of 2 mosquitoes IS 2606 and KR (ketoreductase) positive: 2 bandicoot faeces and pool of two mosquitoes
Tobias et al., 2016 [23]	180 faecal specimens from dominant domestic animals (ovine, porcine, avian, reptiles, canine)	September 2013 4 BU-endemic and one non-endemic villages of Ghana, West Africa	RT-PCR IS 2404 positive: 2/86 ovine; 1/69 avian: 1/16 reptiles IS 2606 and KR: all negative
Tian, Niamke, Tissot-Dupont, & Drancourt, 2016 [24]	496 environmental samples: 100 from soil (endemic *n* = 50 and non-endemic *n* = 50); 200 from stagnant water (endemic *n* = 100 and non-endemic *n* = 100); 100 from plants (endemic *n* = 50 and non-endemic *n* = 50) and 96 animal faeces (*Thryonomys swinderianus* (agouti) stools) (endemic *n* = 48 and non-endemic *n* = 48)	June–October 2014 Ivory Coast, West Africa	RT-PCR 43 samples with at least one positive IS 2404 and KR Out of 43, only 10 positive for both IS2404 and KR, IS 2606 not performed: 7 water specimen; 2 *T. swinderianus* (agouti) faeces and one soil specimen
Carson et al., 2014 [5]	Fecal sample: 216 common ringtail possums and 6 common brushtail possums	Southeast Australia, State Victoria	RT-PCR targeting IS 2404, IS 2606 and KR 20 common ringtail possums and 4 common brushtail possums
O'Brien et al., 2014 [15]	69 possums (ringtail and brushtail) trapped at Point Lonsdale: Faecal samples: 57; blood samples: 63; buccal swab: 67; urine sample: 16; pouch swab: 15; cloacal swab: 20 69 fecal	1998–2011 Victoria, Australia	RT-PCR targeting IS 2404, IS 2606 and KR Point Lonsdale: Positive: faecal sample: 12 (25%); blood sample: 0; buccal swab: 7 (16%); urine sample: 0; pouch swab: 3 (20%) Bellbird Creek:

	samples from 15 mountain brushtail possums		Positive: 4 mountain brushtail possums (27%)
C. O'Brien et al., 2013 [17]	Case report: two alpacas *(Vicugna pacos)* ulcerated tissue	Case 1: September 1997 Case 2: May 2011 Victoria, Australia	RT-PCR targeting IS 2404, IS 2606 and KR positive
Willson et al., 2013 [25]	587 fish representing 13 genera and 17 species and 351 amphibians representing 10 genera: external swab	2008–2009 Ghana, West Africa	RT-PCR targeting IS 2606 and KR not performed. Not confirmed
C. R. O'Brien et al., 2011 [18]	Case report: Case 1: 14 months old female kelpie Case 2: 3 years old female kelpie Case 3: 6 years old male whippet Case 4: 3 years old male koolie	2011 Victoria, Australia	RT-PCR targeting IS 2404, IS 2606 and KR All 4 dogs positive for *M. ulcerans*
Sakaguchi et al., 2011 [26]	Case report; Indian flap-shelled turtle, *Lissemys punctata punctata*	Imported from India to aquarium in Japan	PCR assays targeting the *rpoβ* gene: unable to differentiate *M. ulcerans* from mycolactone-producing *M. marinum* (MPMM)

Table 1. *Cont.*

Author and Year	Sample and Sample Size	Collection Year, Location and Setting	Detection Method, Result or *M. ulcerans* Positive Signal
Fyfe et al., 2010 [14]	589 fecal samples from ringtail possums and 250 samples from brushtail possums. Live trapping: 42 ringtail possums and 21 brushtail possums	2007–2009 Victoria, Australia	RT-PCR targeting IS 2404, IS 2606 and KR *M. ulcerans* DNA detected in 43% of ringtail possum and 29% of brushtail possum faecal samples. 38% ringtail possum have *M. ulcerans* lesion and/or positive faeces Lower in brushtail possums: 1 with *M. ulcerans* lesion and/or positive faeces and 4 with no lesions and low *M. ulcerans* DNA in faeces.
Durnez et al., 2010 [27]	565 small mammals: 326 rodents and 222 shrews	2006 Benin, West Africa	RT-PCR: No *M. ulcerans* specific DNA detected
Van Zyl et al., 2010 [16]	2 horses: Case report Case 1: 21-year-old quarterhorse-cross Case 2: 32-year-old standard bredgelding	Case 1: May 2006 Case 2: October 2006 Southeastern Australia	RT-PCR *M. ulcerans* specific DNA detected from both horses
Elsner et al., 2008 [19]	Cat: Case report 10-year-old castrated male domestic cat	2006 Victoria, Australia	RT-PCR *M. ulcerans* specific DNA detected
Appleyard & Clark, 2002 [28]	Case report: three cats Case 1: An 8-year-old spayed female shorthair Case 2: 6-year-old spayed female shorthair Case 3: 11-year-old domestic longhair cat	2002 North America	PCR Could not differentiate *M. ulcerans* from other *Mycobacterium* spp. (a new *Mycobacterial* spp. namely '*Mycobacterium visibilis*' suggested)
Heckert, Elankumaran, Milani, &Baya, 2001 [29]	60 wild striped bass: Swab from external ulcerative dermatitis and granulomatous-like lesions in the internal organs	1997 Chesapeake Bay, USA	PCR No *M. ulcerans* specific DNA detected (a new mycobacterial spp. suggested)

Mitchell, McOrist, &Bilney, 1987 [13]	36 male and 51 female adult koalas captured	1980–1985 Raymond Island, Southeastern Australia	Pathological and bacteriological examination 18 out of 87 captured koalas had skin wound 11 koalas were found positive for *M. ulcerans*
McOrist, Jerrett, Anderson, & Hayman, 1985 [12]	Case study: 2 koalas: one male and one female Ulcerated tissue	1982 Raymond Island, Southeastern Australia	Pathological and bacteriological examination Both koalas suggested positive for *M. ulcerans*

2.4 4. Discussion on Possible Reservoirs and Vectors of *Mycobacterium ulcerans* by Country

This systematic review assessed the potential animal reservoir of *M. ulcerans* around the world recorded to date. This is essential for understanding the epidemiology and mode of transmission of the disease, which subsequently aids in prevention, control and elimination strategies.

2.4.1 4.1. Australia

Out of 17 studies included in this review, 10 were conducted in Australia. In Australia, the disease is more prevalent in the southeastern state of Victoria and in Far North Queensland. After the detection of *M. ulcerans* infection in four koalas in 1980 at Raymond Island, Australia [13], the entire island was searched for koalas in the following year. Thirty-six male and 51 female koalas were captured and examined. Of these, 18 out of 87 animals had skin wounds and 11 were found positive for *M. ulcerans*. Diagnosis was made on pathological and bacteriological examination; the PCR-based method used for the identification of *M. ulcerans* from clinical and environmental samples was only implemented in 1996 [30]. Non-human cases of *M. ulcerans* in Australia have been reported in marsupial species such as koalas [13], ringtail and brushtail possums [14,15,31], horses [16], alpacas [17], dogs [18] and cats [19]. A study conducted by Fyfe and colleagues between 2007–2009, at Point Lonsdale, a small coastal town south east of Melbourne, Australia, which is also endemic for BU, found that 43% of ringtail possum and 29% of brushtail possum faecal samples were positive for *M. ulcerans* DNA [14]. Only 1% of faecal samples from non-endemic area possums were positive for *M. ulcerans* DNA in this study, suggesting terrestrial mammals such as possums are potential reservoirs of *M. ulcerans* in southeast Australia. Several studies have identified possums (both ringtail and brushtail) as potential reservoirs since then [5,15]. In Australia, other than the southeastern state of Victoria, BU is also prevalent in Far North Queensland [8]. Inspired by the evidence of possums as potential reservoirs of *M. ulcerans* in Victoria, a study conducted by Roltgen and colleagues (2013) in northern Queensland, Australia, detected *M. ulcerans* DNA from two bandicoot faecal samples, suggesting the possibility that bandicoots are a potential reservoir of *M. ulcerans* in Far North Queensland [9].

2.4.2 4.2. Africa

Out of the 17 studies included in this review, four were conducted in West African countries: two in Ghana [23,25], one in the Ivory Coast [24] and one in Benin [27]. Durnez and colleagues (2006) caught 326 rodents and 222 shrews from endemic and non-endemic villages of Benin and tested for *M. ulcerans,* but no specific DNA was detected from any of their samples [27]. Despite their results, they suggested the necessity of more intensive research focusing on small mammals in Africa. Willson reported positive PCR with IS 2404 only from tadpoles and fishes from Ghana [25]. Similarly, two faecal specimens from *Thryonomys swinderianus* (agouti) were reported positive for *M. ulcerans* in a study conducted by Bi Diangoné Tian and colleagues (2014) from the Ivory Coast [24]. They suggested agouti, which are closely related to Australian possums, could be a potential reservoir of *M. ulcerans* in Africa. However, RT-PCR targeting IS 2606 was not conducted to confirm *M. ulcerans*. A faecal survey of domestic animals in rural Ghana for *M. ulcerans* conducted by Tobias and associates suggested no evidence of association between domestic animals and *M. ulcerans* in endemic and non-endemic villages in Ghana [23]. Unlike Australia, not a single study in Africa has reported the presence of *M. ulcerans*-positive DNA or cases in non-human species, suggesting that transmission dynamics may be different in Africa and Australia or, alternatively, a host animal is yet to be identified in Africa.

2.4.3 4.3. Other Countries

No study has reported *M. ulcerans* DNA or cases in non-human species in any country other than Australia. A study conducted by Heckert in 1997 at Chesapeake Bay, USA detected a new *Mycobacterium* species from wild striped bass [29]. This new isolate was closely related to *M. marinum*, *M. ulcerans*, and *M. tuberculosis*. Similarly, Sakaguchi and associates reported an atypical mycobacterial infection in an Indian flap-shelled turtle (*Lissemys punctata punctata)*, imported from India to Japan in an aquarium [26]. A PCR assay targeting the *rpoB* gene revealed the isolate had 89–100% homology to *M. ulcerans* and *M. marinum*. Again, this study could not differentiate *M. ulcerans* from mycolactone-producing *M. marinum* (MPMM). Appleyard and Clark (2002) reported a new *Mycobacterial* species, namely '*Mycobacterium visibilis*' from three cats initially suspected of having *M. ulcerans* infection [28].

2.5 5. Conclusions

Human cases of BU have been reported in more than 30 countries from Africa, America, Asia and Oceania. Since the implementation of PCR-based methods for the detection and identification of *M. ulcerans* from clinical and environmental samples, there has been a significant increase in overall knowledge of BU. There is no record of direct human-to-human transmission of *M. ulcerans*, unlike tuberculosis and leprosy. Australia is the only country where non-human cases of BU have been identified, with small mammals, especially possums and, to some extent, bandicoots, being implicated as potential reservoirs of *M. ulcerans*. Despite there having been several outbreaks in African countries, no non-human cases have been recorded so far and there is no evidence of any animal acting as a potential reservoir for this organism. None of the studies included in this review discussed strain variation of *M. ulcerans* in different geographical regions leading to an increase or decrease in susceptibility among animal or human population. Compared to other mycobacteria, such as *M. tuberculosis*, there is very little genetic diversity among isolates of *M. ulcerans*. Some variation among the strains of *M. ulcerans* from Africa, the Americas, Asia and the Western Pacific has been recorded; however, the linkage between these various strains and virulence in human or animal population has not been recognized so far. Remarkable differences in the type of mycolactone produced by *M. ulcerans* in different geographical location has been recorded. African strains produce more mycolactone variant A and B, whereas strains from Australia produce more mycolactone variant C. However, this variation has nothing to do with host susceptibility to *M. ulcerans*; rather, it determines cytopathogenecity and thus clinical presentation of disease.

This systematic review suggests the need for extensive laboratory and field research focusing on domestic animals and wildlife to elucidate their roles in BU-endemic countries.

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Conflicts of Interest: The authors declare no conflict of interest.

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2.7 Exegesis

At the time of submission there had been no reports of the detection of *M. ulcerans* in any domestic animals from Africa. Subsequently, in 2018, Djouaka *et. al.* reported two *M. ulcerans* lesions in domestic animals from an endemic region in Africa (1).

Fyfe *et. al.*, in 2010, proposed a hypothetical pathway for the amplification of *M. ulcerans* in possums and detected *M. ulcerans* DNA from possum faeces in endemic areas. The authors did not demonstrate that the bacteria in the faeces were viable (2).

Tian *et. al* in 2014 reported two *M. ulcerans* positive faecal samples from *Thryonomys swinderianus* in Africa. *(3). T. swinderianus* is commonly known as the greater cane rat but is referred to as an agouti in the systematic review. We are now aware that this name is more commonly applied to a South American rodent. Although, possums and the African greater cane rat have been represented as closely related species in this review, it should be noted that possums are marsupials and the African greater cane rat is a rodent.

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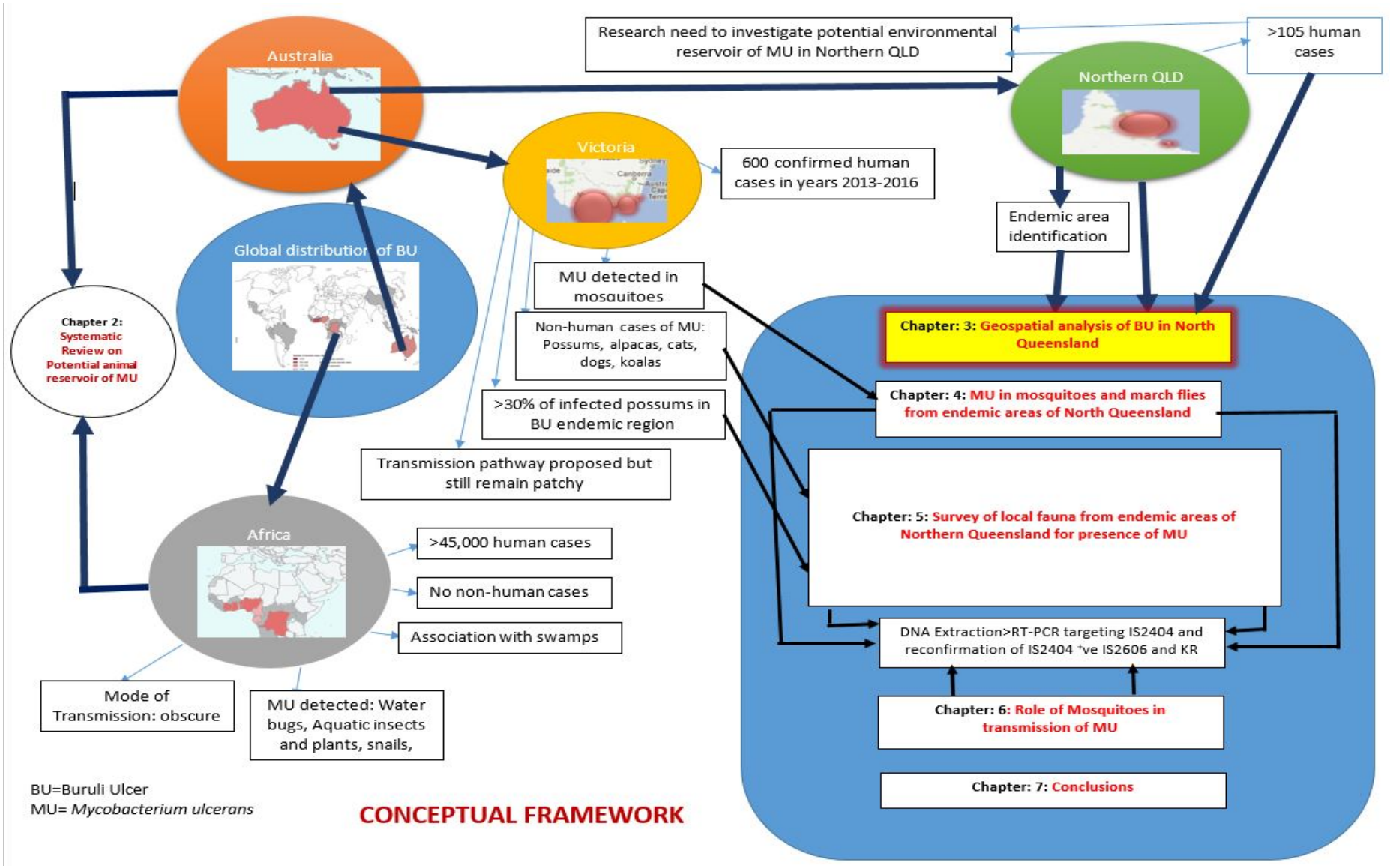

Australia
Research need to investigate potential environmental reservoir of MU in Northern QLD
>105 human cases
Northern QLD
Victoria
600 confirmed human cases in years 2013-2016
Endemic area identification
Global distribution of BU
MU detected in mosquitoes
Chapter 2: Systematic Review on Potential animal reservoir of MU
Non-human cases of MU: Possums, alpacas, cats, dogs, koalas
Chapter: 3: Geospatial analysis of BU in North Queensland
>30% of infected possums in BU endemic region
Chapter: 4: MU in mosquitoes and march flies from endemic areas of North Queensland
Transmission pathway proposed but still remain patchy
Chapter: 5: Survey of local fauna from endemic areas of Northern Queensland for presence of MU
Africa
>45,000 human cases
No non-human cases
Association with swamps
DNA Extraction>RT-PCR targeting IS2404 and reconfirmation of IS2404 +ve IS2606 and KR
Mode of Transmission: obscure
MU detected: Water bugs, Aquatic insects and plants, snails,
Chapter: 6: Role of Mosquitoes in transmission of MU
Chapter: 7: Conclusions
BU=Buruli Ulcer
MU= Mycobacterium ulcerans
CONCEPTUAL FRAMEWORK

3 Chapter-3: GEOSPATIAL ANALYSIS OF BURULI ULCER REPORTED IN AN ENDEMIC AREA OF NORTH QUEENSLAND, AUSTRALIA

Aim of this chapter

To incorporate GIS technology to understand and visualise the spatial distribution and hot-spots of Buruli ulcer in northern Queensland, Australia.

I prepared a manuscript based on this chapter and intend to submit this to a special issue on "Spatial Epidemiology of Neglected Tropical Diseases (NTDs)" of a MDPI journal: Tropical Medicine and Infectious Disease.

I am the main author of this manuscript. My principal contribution to this manuscript are as follows:

- I conducted the literature search to aggregate the published literatures available
- I developed the study design
- I enrolled in a basic GIS training module offered by JCU to familiarise myself with GIS techniques.
- I conducted analysis of the data on ArcMap 10.4.1 software
- I drafted the manuscript which is to be submitted to MDPI journal (see below).

Geospatial analysis of Buruli ulcer reported in an endemic area of north Queensland, Australia

3.1 Introduction:

Buruli ulcer (BU) is caused by a slow growing environmental pathogen *Mycobacterium ulcerans* (1, 2). It is a neglected tropical disease and recognized as the third most common mycobacterial infection of humans around the world, next to tuberculosis and leprosy (3). Currently, this disease has been reported in more than 33 countries with tropical, subtropical and temperate climates (4). The disease is geographically restricted with the majority of foci in West Africa (5) with other foci in Australia (6, 7) Peru (8), Papua New Guinea (9) and Japan (10). Australia is the only resource rich country with a significant foci of transmission. In Australia, this disease has been recorded in tropical far north Queensland (11), the Capricorn Coast region of central Queensland (12), the Northern Territory (13) and temperate coastal Victoria (6). Local names for Buruli ulcer in Australia are "Daintree ulcer" in northern Queensland and "Bairnsdale ulcer" in Victoria.

Buruli ulcer (BU), is a non-tuberculous infection of the skin caused by *Mycobacterium ulcerans*. The disease is rarely fatal if diagnosed and treated with appropriate antibiotics in a timely fashion. Any delay in treatment may lead to the requirement for surgical intervention. In Victoria, Australia, a marked increase in incidence of Buruli ulcer has been observed in recent years. More than 600 cases were recorded during 2011-2016 in Victoria (14). At the same time fewer human cases of BU ulcer have been recorded in northern Queensland. The largest outbreak of BU in north Queensland was recorded in 2011-2012 with more than 60 cases (7). The average reported rate of BU in Northern Queensland over the period of 15 years from 2002-2016 is 0.2 cases/100,000 population per year (15). In north Queensland, cases of BU are largely restricted to the Douglas Shire (11, 12). The total population of the Douglas shire is around 11,000 and covers an area of 2,455 sq. Kms. Outbreaks of human cases of BU in north Queensland have been linked with heavy rainfall and flooding (11).

Distribution of Buruli ulcer cases in northern Queensland is geographically restricted to between Forest Creek in the north to Mossman in the south (11). The discrete focal nature of occurrence of the disease in the endemic area suggests a spatial correlation with geographical factors associated with infection. Spatial epidemiology is defined as the "*description and analysis of geographically indexed health data with respect to*

demographic, environmental, behavioral, socioeconomic, genetic, and infectious risk factors"(16). It is an important tool to understand the geographical distribution of diseases while investigating the risk factor for the occurrence of diseases (16-18). Geographic Information Systems (GIS) technology has been widely used to study and analyses special distribution of the diseases. This technology offers a unique perspective in the study of the epidemiology of diseases over large or small geographical regions and identification of hot-spots of the diseases. The results obtained from spatial analysis of diseases can aid stakeholders to implement surveillance and control strategies to eradicate the diseases. At the same time, it can provide unique opportunities for the researchers to understand the ecology of emerging diseases like Buruli ulcer. There is a lack of detailed information on the spatial distribution of BU in northern Queensland, Australia. The aim of the current study was to incorporate GIS technology to understand and visualise the spatial distribution and hot-spots of BU in northern Queensland, Australia.

3.2 Material and Methods:

Ethical approval was obtained from Cairns and Hinterland Hospital and Health Service Human Research Ethics Committee (Ethics approval number: HREC/13/ QCH/94-859LR). The data set consisted of prospectively recorded data from patients referred to one of the authors, cases seen by other local medical practitioners and retrospective data from patients' medical records. Place of residence and year of diagnosis were recorded in an Excel spreadsheet.

3.3 Description of the study area:

The study area is located in the tropics of Australia in a coastal region north of Cairns, located within Douglas Shire. The Douglas shire covers an area of 2,445 km^2. Total population of the area is about 11,000 and 70% of population resides in two major towns: Port Douglas and Mossman. The occurrence of BU is geographically restricted within a specific area of the Douglas shire. The BU endemic region of North Queensland comprises an area extending from the Forest Creek in the North, Wonga Beach in the East, Stewart Creek to the west and Whyanbeel in the south located around the base of the Dagmar range (Fig 1 & 2). In north Queensland, the Daintree River arises in the mountainous rainforest region northwest of the town of Mossman and flows into the sea north of Wonga Beach. The majority of cases have been reported from Wonga Beach and

Daintree village. These two places are urban centers within the shire which is otherwise sparsely populated. Australian Bureau of Statistics 2016 census recorded the population of Daintree village and Wonga beach to be 129 and 975 respectively (19). The outbreak of BU in the study area is thought to be linked with rainfall (11). In the study area, Daintree River accumulates its water from the mountainous rainforest and flows into the sea at Cape Tribulation.

Fig 1: View of BU endemic area of North Queensland, Australia (Source: Tourism Tropical North Queensland)

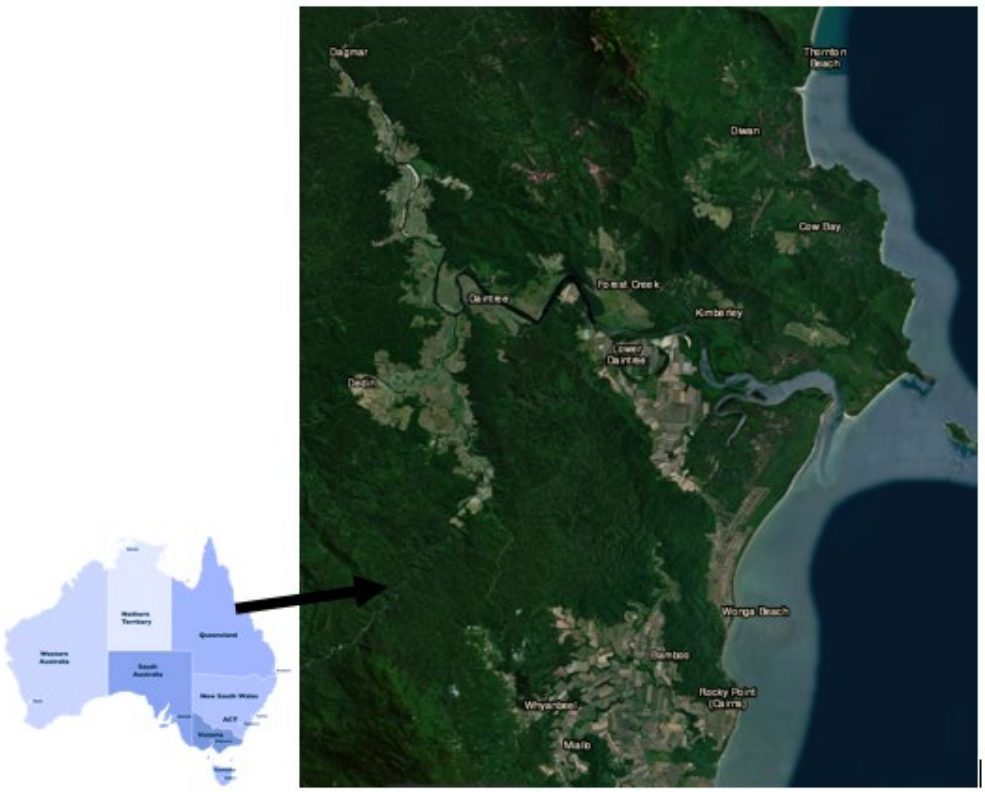


Fig 2: BU endemic region of North Queensland, Australia. This figure was created using base layer obtained from https://landsatlook.usgs.gov/

3.4 GIS Mapping:

The latitude and longitude coordinates of each case of BU reported between years 2009-2018 were obtained using the residential addresses of each patient in the study area. ArcMap 10.4.1 software was used visualise the spatial distribution and hot-spots of BU in north Queensland, Australia. ArcMap is one of the major software tools provided by Environmental Systems Research Institute (ESRI) for the geospatial analysis of the data. It is one component of ArcGIS. GIS maps were generated showing distribution of BU cases within the study area. Hot-spot analysis was done to examine the significance of clusters of BU cases within the endemic area. This analysis uses vectors to identify the locations of statistically significant hot spots and cold spots in the data by accumulating points. Hot spot analysis has been used by many researchers globally to identify the hot

spots for diseases (20-22). A buffer zone of 1.5 kms was created around each feature for proximity analysis of each case of BU in the study area. Monthly rainfall records of Whyanbeel valley (Bureau of Meteorology station number: 031062) were obtained from year 2009-2018 from Bureau of Meteorology, Climate data online (23). Rainfall data was plotted against the number of cases of BU reported each year from 2009-2018.

3.5 Results and Discussion:

The majority of BU cases (64) was reported in the largest epidemic in 2011. Since 2011, there has been a drastic decrease in the number of cases of BU in north Queensland, with less than 10 cases per year. Although, the cases of BU are geographically restricted, our hot-spot analysis of BU cases within endemic areas of north Queensland using ArcMap 10.4.1, did not identified any statistically significant clusters of the reported cases within the endemic areas (Fig 3). One plausible reason behind this could be the low number of cases within the endemic area during the time period anlaysed.

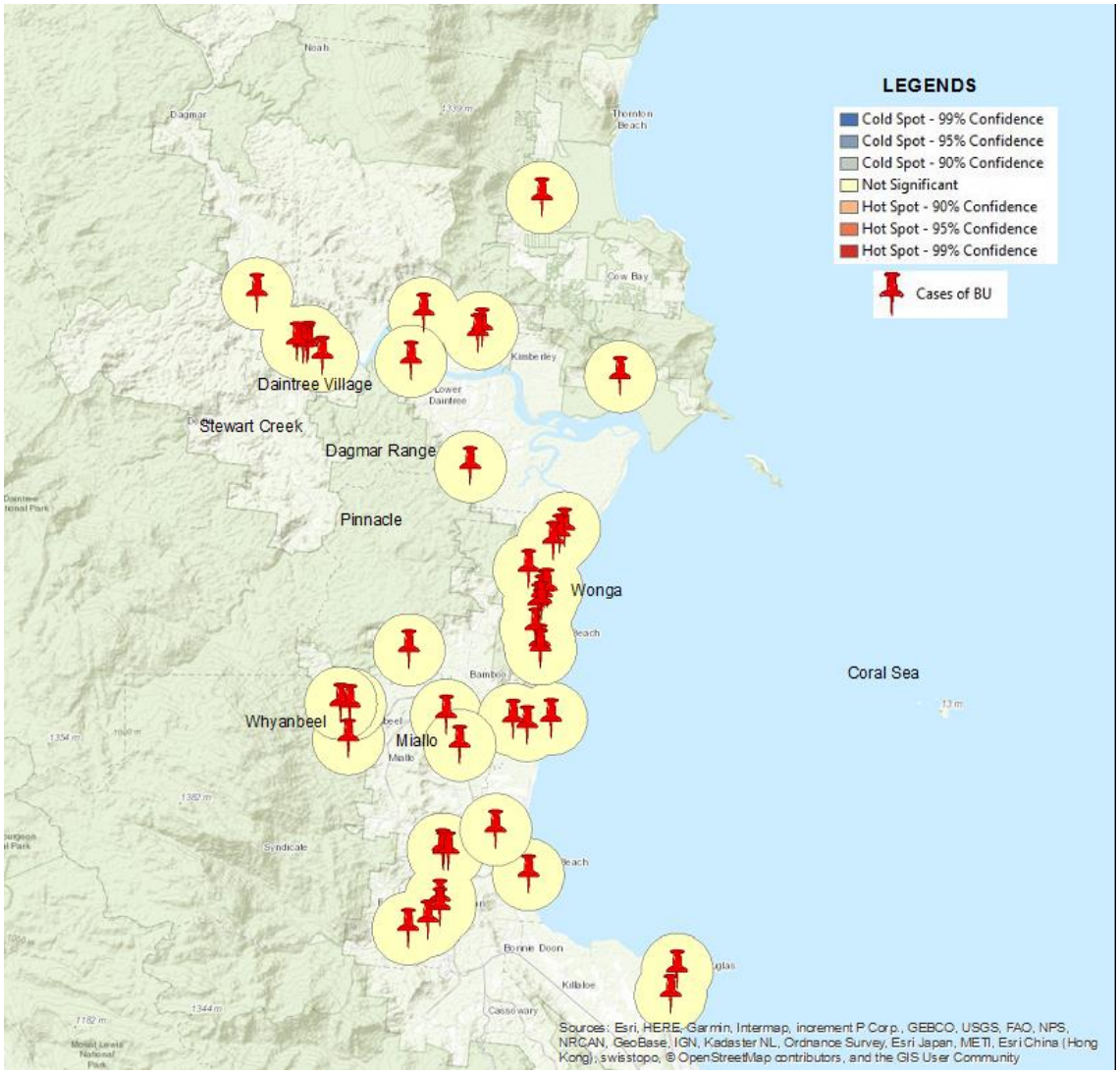


Fig 3: Hot-spot analysis of Buruli ulcer cases in the endemic area of north Queensland showing no statistically significant clustering of the disease.

A 0.5 km and 1 km buffer around each case of BU was created for proximity analysis of each case. The 1 km buffers were further categorised to value field: location to identify the spatial features around each case of BU and to determine proximity of the cases within the certain locality (Fig 4). All of the cases in each locality fell within 0.5 km buffer zone. However, our hot-spot analysis has shown that there was no statistically significant clustering of these cases. No specific spatial features were identified within the buffer zone. Nevertheless, a 1 km buffer zone of the majority of cases had water bodies, either a creek or river in close proximity

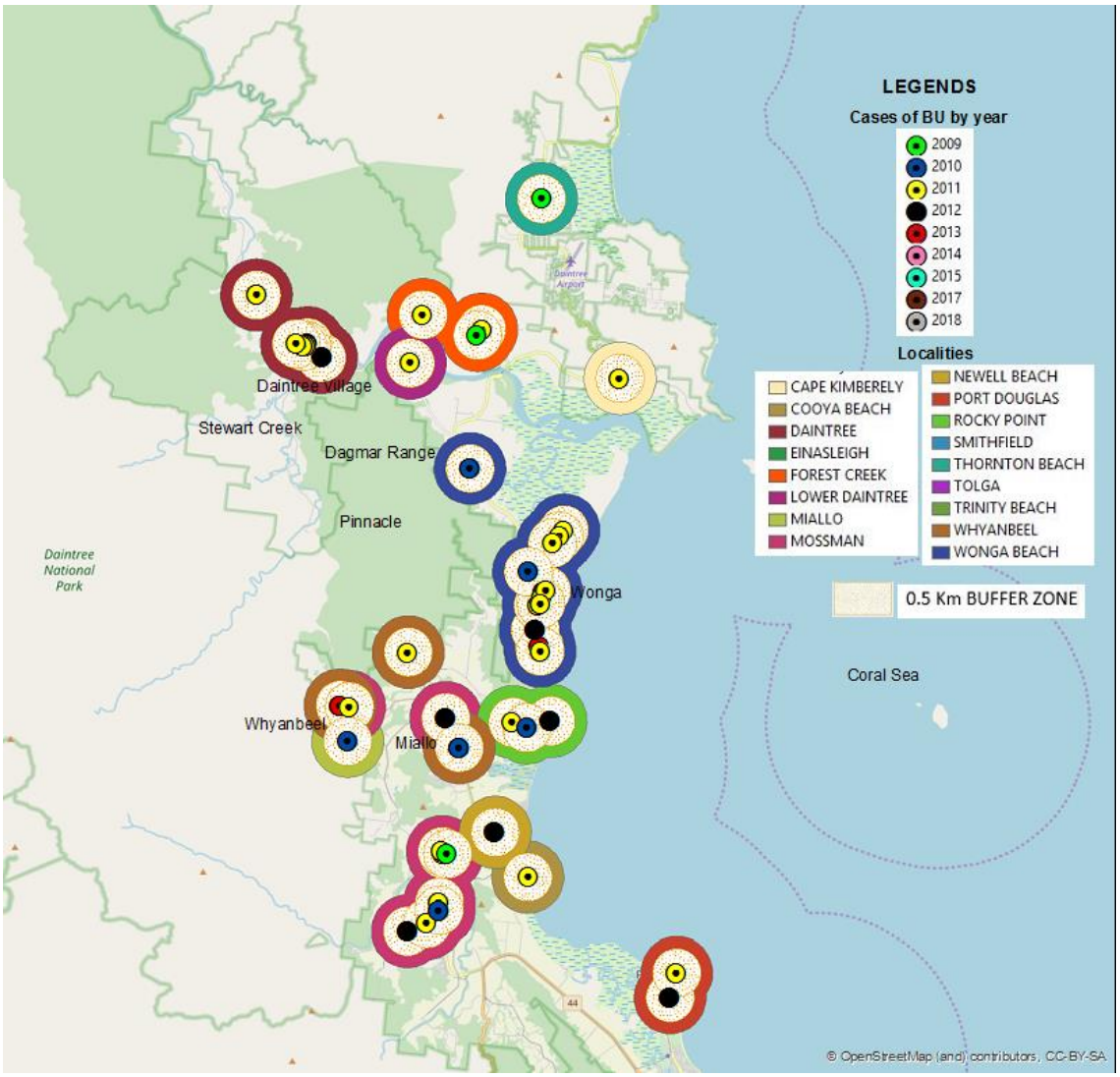
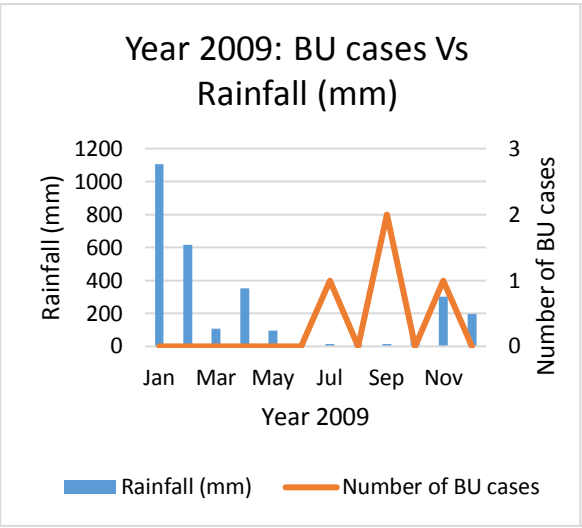
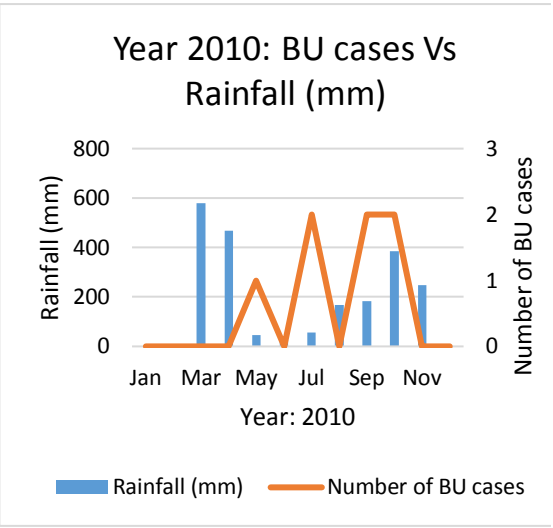
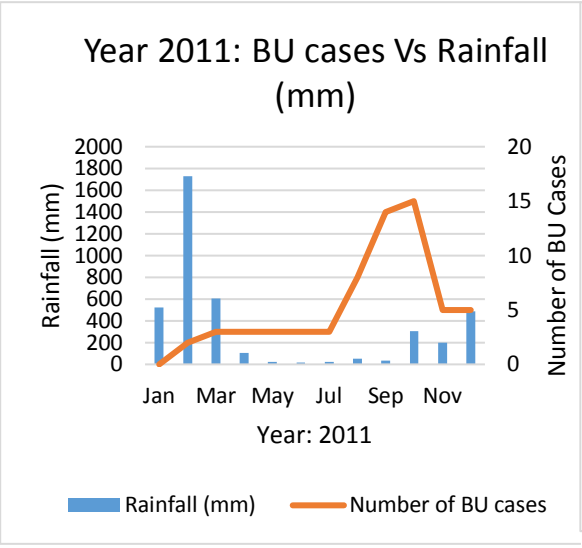


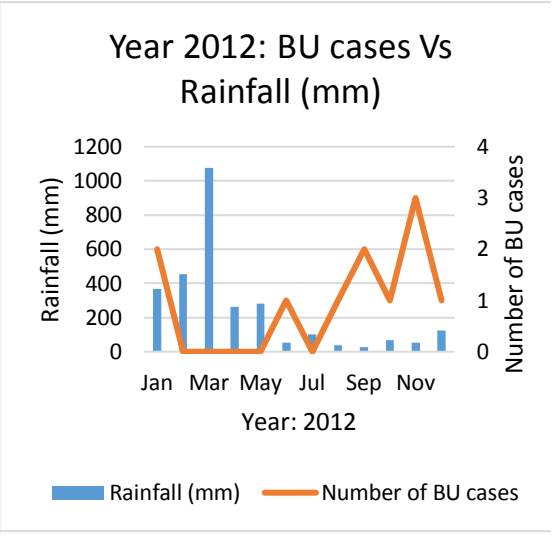
Fig 4: A 0.5 Km and 1 Km buffer around each case of BU in the endemic area of north Queensland. A 1 Km buffer was been further categorised based on locality of each case.

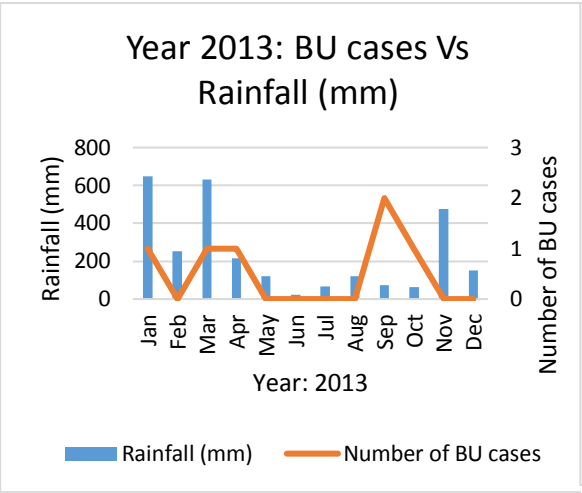
The number of reported cases of BU in north Queensland has significantly fallen since the largest outbreak in 2011 (7). This outbreak was assumed to be linked with rainfall and flooding. No cases of BU were reported in 2016. The monthly rainfall data was plotted against the number of cases of BU reported each year from 2009-2018 to analyse the association between the amount of rain that fell each month and the number of BU cases (Fig 5).

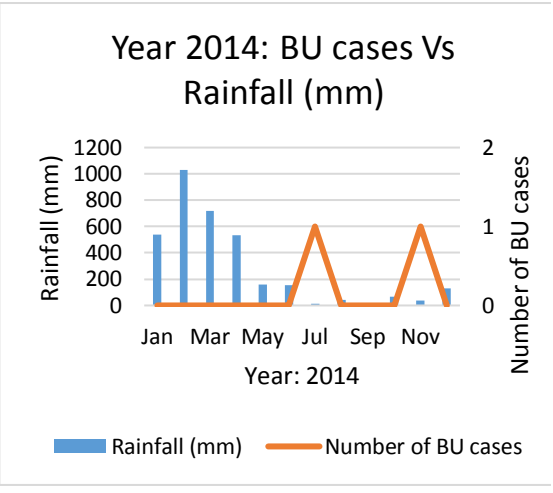

Year 2009: BU cases Vs Rainfall (mm)
Rainfall (mm)
Number of BU cases
Jan Mar May Jul Sep Nov
Year 2009
Rainfall (mm)
Number of BU cases


Year 2010: BU cases Vs Rainfall (mm)
Rainfall (mm)
Number of BU cases
Jan Mar May Jul Sep Nov
Year: 2010
Rainfall (mm)
Number of BU cases


Year 2011: BU cases Vs Rainfall (mm)
Rainfall (mm)
Number of BU Cases
Jan Mar May Jul Sep Nov
Year: 2011
Rainfall (mm)
Number of BU cases


Year 2012: BU cases Vs Rainfall (mm)
Rainfall (mm)
Number of BU cases
Jan Mar May Jul Sep Nov
Year: 2012
Rainfall (mm)
Number of BU cases


Year 2013: BU cases Vs Rainfall (mm)
Rainfall (mm)
Number of BU cases
Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec
Year: 2013
Rainfall (mm)
Number of BU cases


Year 2014: BU cases Vs Rainfall (mm)
Rainfall (mm)
Number of BU cases
Jan Mar May Jul Sep Nov
Year: 2014
Rainfall (mm)
Number of BU cases

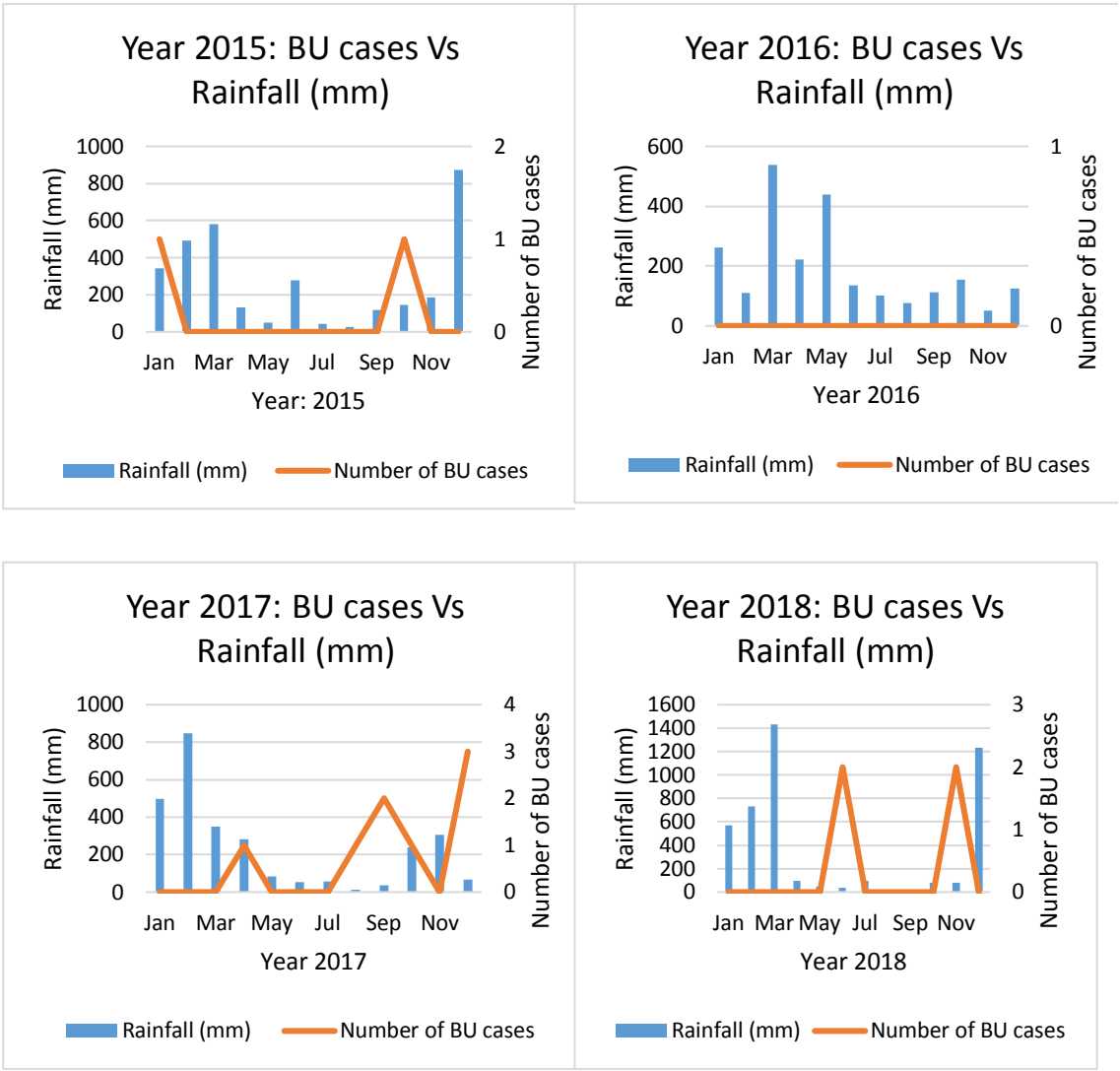


Fig 5: Number of Buruli ulcer cases plotted against monthly rainfall in 2009-2018

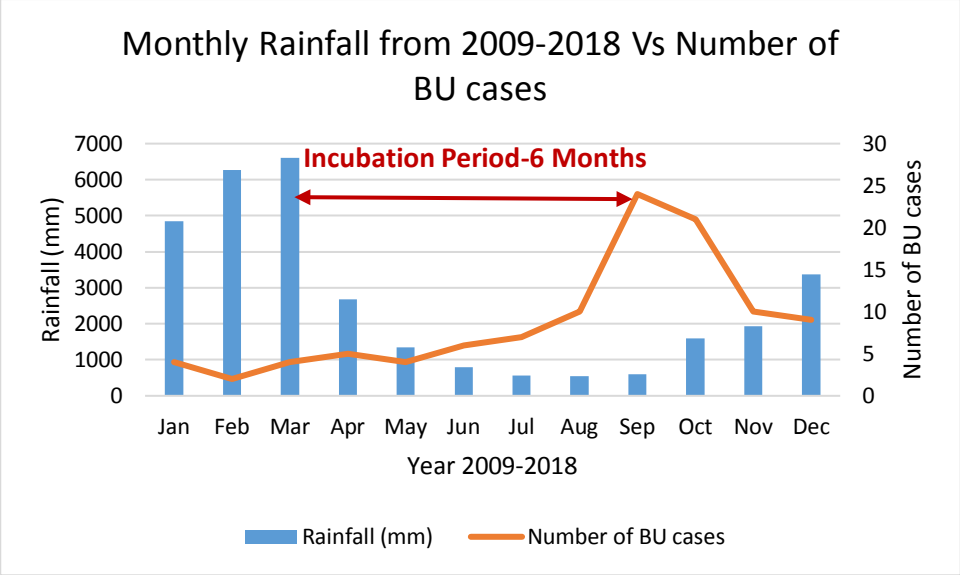


Fig 6: Number of BU cases plotted against sum of monthly rainfall from 2009-2018

The wet season in north Queensland normally starts from November/December and continues up until April. Rainfall patterns plotted against month of diagnosis in Fig 5 & 6 show that the majority of cases of BU were diagnosed in the dry season. It is assumed, but not proven that the transmission of *M. ulcerans* occurs in the wet season. However, the small number of cases occurring in some of these years may have influenced the analysis. Sums of monthly rainfall from year 2009-2018 plotted against number of BU cases diagnosed each month shows that the peak number of cases were diagnosed around 5-6 months after the peak rainfall (Fig 6). This is consistent with another estimate of the average incubation period of BU of 4.8 months (24).

3.6 Conclusion:

The aim of this study was to analyse the spatial distribution of BU in the endemic area of north Queensland. The geographic distribution of the cases within the endemic areas show the geographically restricted and clustered nature of distribution of the disease. However, hotspot analysis on ArcMap did not confirmed any statistically significant pattern of clusters. All the cases of BU in endemic areas were in close proximity to each other and water bodies were featured within the buffer zone of each cases. Our analysis on rainfall pattern in north Queensland in the years 2009-2018 and the time of the year of the peak number of BU cases diagnosed, revealed that peak number of cases were diagnosed around 5-6 months after the peak rainfall.

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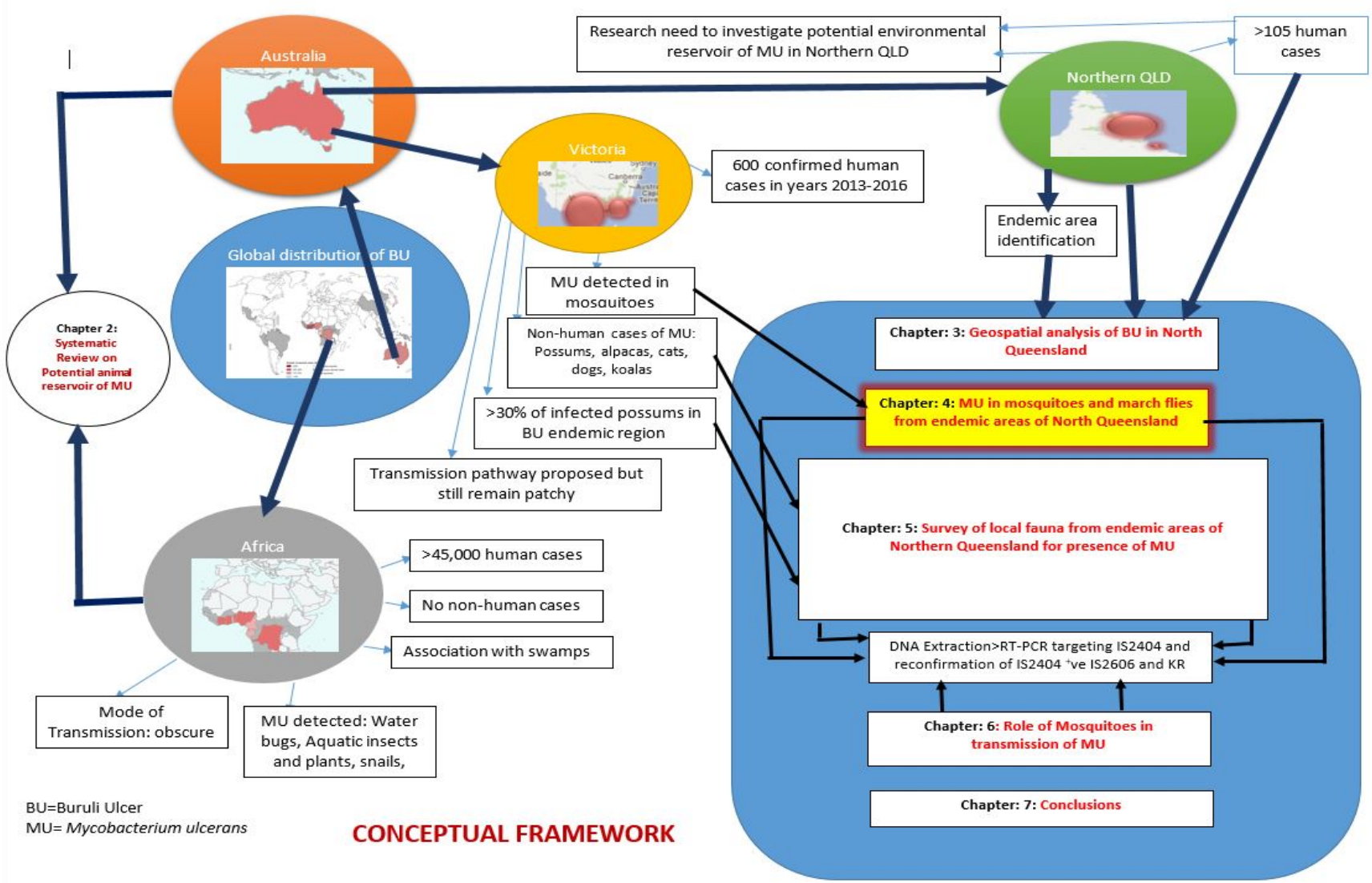
Australia
Research need to investigate potential environmental reservoir of MU in Northern QLD
>105 human cases
Northern QLD
Victoria
600 confirmed human cases in years 2013-2016
Endemic area identification
Global distribution of BU
MU detected in mosquitoes
Chapter 2: Systematic Review on Potential animal reservoir of MU
Non-human cases of MU: Possums, alpacas, cats, dogs, koalas
Chapter: 3: Geospatial analysis of BU in North Queensland
>30% of infected possums in BU endemic region
Chapter: 4: MU in mosquitoes and march flies from endemic areas of North Queensland
Transmission pathway proposed but still remain patchy
Chapter: 5: Survey of local fauna from endemic areas of Northern Queensland for presence of MU
Africa
>45,000 human cases
No non-human cases
Association with swamps
DNA Extraction>RT-PCR targeting IS2404 and reconfirmation of IS2404 +ve IS2606 and KR
Mode of Transmission: obscure
MU detected: Water bugs, Aquatic insects and plants, snails,
Chapter: 6: Role of Mosquitoes in transmission of MU
Chapter: 7: Conclusions
BU=Buruli Ulcer
MU= Mycobacterium ulcerans
CONCEPTUAL FRAMEWORK

4 Chapter-4: A SURVEY FOR *MYCOBACTERIUM ULCERANS* IN MOSQUITOES AND MARCH FLIES CAPTURED FROM ENDEMIC AREAS OF NORTHERN QUEENSLAND, AUSTRALIA

Aim of this chapter

To capture and screen mosquitoes and March flies for the presence of *M. ulcerans* DNA in the BU endemic area of north Queensland.

This chapter is presented in the form of manuscript submitted to international Peer-reviewed journal *PLOS Neglected Tropical Diseases*. This article is published in *PLOS Neglected Tropical Diseases.*

I was the main author of this peer-reviewed manuscript. My principal contribution to this manuscript are as follows:

- I led almost all aspect of study: study design, implementation, sample collection, analysis, interpretation, preparation of manuscript and submission to the journal.

The citation for this manuscript is:

Singh A, McBride WJH, Govan B, Pearson M, Ritchie SA. A survey on *Mycobacterium ulcerans* in Mosquitoes and March flies captured from endemic areas of Northern Queensland, Australia. PLoS Neglected Tropical Diseases. 2019; 13(2):e0006745.

A survey for *Mycobacterium ulcerans* in Mosquitoes and March flies captured from endemic areas of Northern Queensland, Australia

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4.1 Abstract:

Mycobacterium ulcerans is the causative agent of Buruli ulcer (BU). This nontuberculous mycobacterial infection has been reported in 34 countries worldwide. In Australia, the majority of cases of BU have been recorded in coastal Victoria and the Mossman-Daintree areas of north Queensland. Mosquitoes have been postulated as a vector of *M. ulcerans* in Victoria, however the specific mode of transmission of this disease is still far from being well understood. In the current study, we trapped and analysed 16,900 (allocated to 845 pools) mosquitoes and 296 March flies from the endemic areas of north Queensland to examine for the presence of *M. ulcerans* DNA by polymerase chain reaction. Seven of 845 pools of mosquitoes were positive on screening using the IS*2404* PCR target (maximum likelihood estimate 0.4/1,000). *M. ulcerans* DNA was detected from one pool of mosquitoes from which all three PCR targets: IS*2404*, IS*2606* and the ketoreductase B domain of mycolactone polyketide synthase gene were detected. None of the March fly samples were positive for the presence of *M. ulcerans* DNA.

4.2 Author Summary:

The causative agent of Buruli ulcer is *Mycobacterium ulcerans.* This destructive skin disease is characterized by extensive and painless necrosis of skin and underlying tissues usually on extremities of body due to production of toxin named mycolactone. The disease is prevalent in Africa and coastal Australia. The exact mode of transmission and potential environmental reservoir for the pathogen still remain obscure. Aquatic and biting insects have been identified as potential niche in transmission and maintenance of pathogen in the environment. In this study we screened mosquitoes and march flies captured from endemic areas of northern Queensland for the presence of *M. ulcerans* DNA. We found seven pools of mosquito out of 845 pools positive for IS*2404.* In only one of the seven samples were the additional targets IS*2606* and KR detected. None of the March fly samples were positive. The results could indicate a low burden of the bacteria in the environment coinciding with a comparatively low number of human cases of *M. ulcerans* infection seen during the trapping period of the study.

4.3 Introduction:

Buruli ulcer (BU), also known regionally as Daintree ulcer in north Queensland, Australia or Bairnsdale ulcer in Victoria, Australia, is an emerging disease of skin and underlying tissue, with a potential to lead to permanent disability, particularly if treatment is inadequate or delayed. The causative agent of this disease, *M. ulcerans* secretes a polyketide exotoxin, mycolactone, the production of which requires expression of a series of contiguous genes on the large pMUM001 plasmid. This exotoxin is the main virulence determinant of the bacteria (1). The outbreaks of BU have been consistently linked with wetland or coastal regions (2). Environmental samples such as water, aquatic plants, soil at endemic areas has been found PCR-positive for *M. ulcerans* DNA (3, 4). Insects such as mosquitoes and aquatic bugs have been proposed as a vital ecological niche for the maintenance of pathogen in environment (5, 6).The detection of *M. ulcerans* DNA in insects does not prove their ability to transmit *M. ulcerans* but could indicate a potential to act as either a biological or mechanical vector. A study conducted by Marsollier and his colleagues provided evidence of the presence of *M. ulcerans* DNA in the salivary glands of wild caught Naucoridae (aquatic bug). They successfully isolated the pathogen by culture from the salivary glands of aquatic bugs and suggested aquatic insects as having an important ecological niche in the maintenance of the organism in the environment. They were also able to demonstrate transmission to mice in a laboratory environment (6). Similarly, a study conducted by Wallace *et al*. provided evidence of the ability of mosquitoes to act as a mechanical vector of *M. ulcerans* (7). Studies conducted in endemic areas of Africa suggest that conducting farming activities close to rivers (8) and swimming in rivers located in endemic areas (9) are risk factors for exposure to *M. ulcerans.*

In Australia, foci of BU infection have been found in tropical north Queensland (10, 11), the Capricorn Coast region of central Queensland (10), the Northern Territory (12) and temperate coastal Victoria (5). In Queensland, Australia, cases of Daintree ulcer have been reported primarily in the Douglas Shire, exclusively in the vicinity of Wonga, Miallo and Daintree (10, 11). A few cases have also been reported from Capricorn Coast region of central Queensland (10). The Douglas Shire covers an area of 2,445 sq. Kms and the total population is around 11,000. A majority of the population (around 70%) reside in Port Douglas and Mossman. Thus, the Daintree ulcer endemic area in north Queensland is sparsely populated. There has been a significant decrease in human cases of BU in

north Queensland, since a large outbreak in 2011-2012, when more than 60 cases were reported. This outbreak occurred after prolonged and heavy rainfall in 2010-2011 (11). The average reported rate over fifteen years period from 2002-2016 was 0.2 cases/100,000 population per year (13).

Victorian researchers detected the presence of *M. ulcerans* DNA in five different species of mosquito during a BU outbreak in an endemic area of Victoria, Australia. They demonstrated the absence of *M. ulcerans* in a neighboring area, where BU did not occur (5). Together, the evidence was proposed to support a link with mosquitoes in the ecology of BU in Victoria (5, 14). More recently, a small study conducted in the BU endemic region of north Queensland, found that of 35 insect/insects pools, one sample of an individual mosquito and one pool of two mosquitoes were positive for IS*2404*. The IS*2404* positive mosquito pool contained DNA of a closely related *M. ulcerans* subspecies that had a low copy number for IS*2606* which does not commonly cause disease in humans. The individual mosquito had insufficient DNA for detection of the additional gene targets. The study highlighted a need to examine a larger sample size to gauge the significance of the role of mosquitoes in the ecology of BU in Northern Queensland (15). An additional suggestion proposed by the local population (including people with a history of BU) was that March flies (Tabanidae) might have a role in transmission. We therefore aimed, in this study to capture and screen mosquitoes and March flies for the presence of *M. ulcerans* DNA in the BU endemic area of northern Queensland.

4.4 Material and Methodology:

Selection of the study site was based on GIS mapping of human cases of BU in Northern Queensland (16). We divided the endemic area of northern Queensland into three regions: Region-1: extending from Miallo to lower Daintree including Wonga/Wonga Beach area, Region-2: Forest Creek area and Region-3: Upper Daintree area (Fig. 1).

Fig. 1: BU endemic areas of Northern Queensland, Australia and Mosquito trapping regions. This figure was created using base layer obtained from **https://landsatlook.usgs.gov/**

4.4.1 Trapping of Mosquitoes:

Mosquitoes were captured using a model 512 "CDC miniature light trap" (John W. Hock Company, Gainesville Florida USA) baited with 1 kg of dry ice as the source of CO_2. This trap is the most reliable, efficient and portable device for trapping mosquitoes and sand flies (17). This trap consists of an electric light and fan just over the collection container and is operated by a 12V battery. A two litre insulated container was used to hold dry ice and a pipe was attached to release CO_2 over the trap to attract mosquitoes (Fig. 2). Thirty overnight trapping sessions were conducted starting from September 2016 through to February 2018, with at least 4 CDC traps placed within a 1 kilometer radius of each-other. Of the 30 trapping sessions, 14 were conducted at eight different sites

within region-1, nine at six different sites within region-2 and seven at five different sites of region-3 (Fig. 1). Traps were placed at different sites after obtaining permission to access properties from the owners and selection of sites was based on history of BU cases in humans in nearby households. Geographical Information System (GIS) coordinates of each trap was recorded. On each occasion, traps were set before dusk and checked for mosquitoes after dawn the next morning. After each occasion of trapping, catches were transported to the Mosquito Research Facility, Australian Institute of Tropical Health and Medicine (AITHM), James Cook University, Cairns, Australia where they were counted, sorted and pooled by genus, with each pool containing ≤ 20 mosquitoes of same genus and collected from the same site. The key of Russell was used to identify the genus of mosquitoes trapped (18).

Fig. 2. CDC miniature light trap baited with dry ice

4.5 Trapping of March Flies:

Several attempts were made to trap march flies from endemic areas with an investigator wearing dark clothes to attract them, or with the use of an insect net sprayed with

insecticide. These attempts occurred from February 2016 through September 2016. The yield from these attempts were very low. A request was made to residents of region-1 through the local State School to collect March flies. This effort was successful and large numbers of March flies of genus *Tabanus* were collected by the local community. The addresses of properties from which March flies were collected were recorded. Sampling of March flies was restricted to region-1.

4.5.1 Molecular analyses:

The molecular analyses were performed using the protocol available on given link: dx.doi.org/10.17504/protocols.io.vqbe5sn

4.5.2 Screening of Mosquitoes and March Flies for MU DNA by PCR:

DNA was extracted from each pool of ≤ 20 mosquitoes of the same genus by using the FastPrep Instrument (MP Biomedicals, Solon, OH, USA) as per manufacturer's instruction with FastDNA Kit (MP Biomedicals). Using the same instrument, DNA from individual March flies was extracted with FastDNA Spin Kit (MP Biomedicals). One sterile water sample in each batch of extractions was used as a negative control to identify the possible contamination during the process of extraction of DNA. Extracted DNA was stored at -20 ^{o}C. The extracted DNA samples were screened for the presence of *M. ulcerans* DNA by using a semi-quantitative real-time PCR adapted from a method for the detection of *M. ulcerans* DNA from environmental samples (19). To rule-out the possibility of contamination, three negative controls (double deionized water, MilliQ) and three positive controls (purified *M. ulcerans* DNA obtained from Victorian Infectious Disease Reference Laboratory) were used during each qPCR assay run. All of the extracted DNA samples were initially screened for the *M. ulcerans* insertion sequence (IS) element IS*2404*. Samples positive for IS*2404* were re-analyzed by a second real-time PCR for the detection of two additional regions in the genome of *M. ulcerans*: IS*2606* and ketoreductase B domain (KR). This screening process has been validated by Fyfe *et al*. to differentiate *M. ulcerans* from other mycolactone producing mycobacteria (MPM) (19). They suggested that the difference in real-time PCR cycle thresholds (Ct) between IS*2606* and IS*2404* (ΔCt [IS*2606* – IS*2404*]) allows for the differentiation of *M. ulcerans* strains commonly causing disease in human from other MPM (which are also considered members of the species *M. ulcerans*) that contain IS*2404* but which have fewer copy

numbers of IS*2606*. Samples containing all three independent DNA sequences and with expected Ct values were considered positive for *M. ulcerans* DNA. The software recommended by Centers of Disease Control and Prevention (Atlanta, GA, USA) was used to calculate the maximum likelihood estimate (MLE) per 1,000 mosquitoes tested (bias corrected MLE) (20).

4.5.3 Accession numbers

The Genebank accession number of nucleotide sequence on *M. ulcerans* gene *IS2404*, *IS2606* and KR have been allocated as BX649209, BX649209 and BX649209 respectively.

4.6 Results:

4.6.1 Screening of Mosquitoes:

A total of 16,900 mosquitoes were captured over the course of the study from 30 occasions of trapping at three different regions of northern Queensland. Total mosquitoes captured from region-1, region-2 and region-3 were 7880, 5100, and 3920, respectively. The majority of captured mosquitos belonged to the *Verrallina* genus (specifically *Verrallina lineata*) 82%, followed by *Coquillettidia* (9%) and *Mansonia* (3%). The remaining 6% consisted of seven other genera that were classified as “other” for screening. See Figure 3 below.

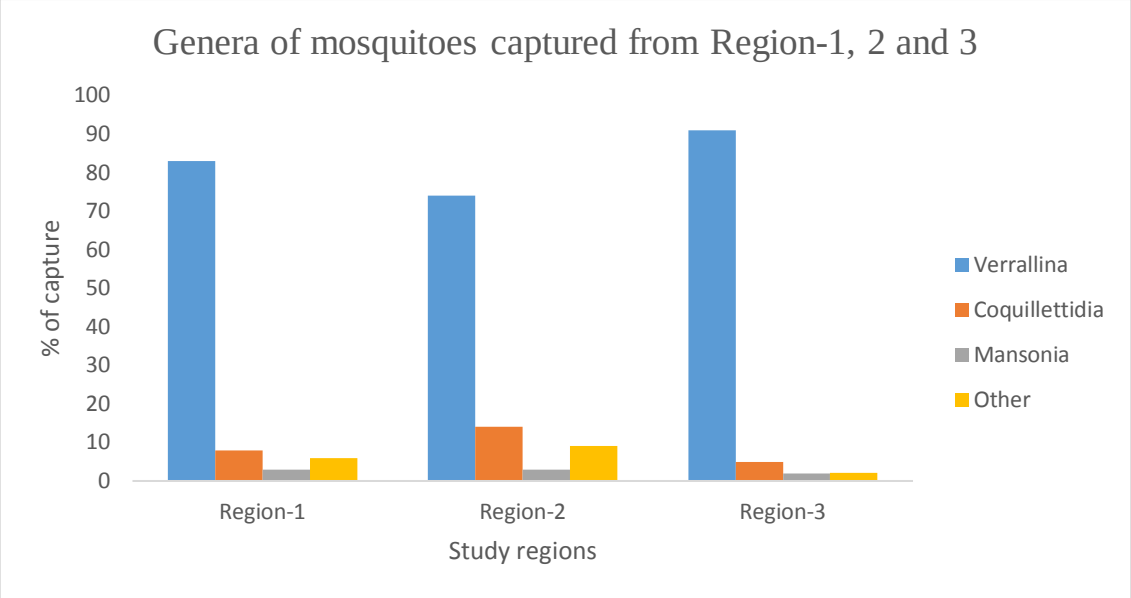

Fig. 3. Genera of mosquitoes captured from three different regions: Region-1 comprising 83% of *Verrallina sp.*, 8% of *Coquillettidia sp.*, 3% of *Mansonia sp.* and 6% of others; Region-2 comprising 74% of *Verrallina sp.*, 14% of *Coquillettidia sp.*, 3% of *Mansonia sp.* and 9% of others and Region-3 comprising 91% of *Verrallina sp.*, 5% of *Coquillettidia sp.*, 2% of *Mansonia sp.* and 2% of others of total catches.

Of a total of 16,900 mosquitoes screened (845 pools), seven pools were positive for IS*2404*. Three of those seven pools were *Verrallina sp.* from region-1, two pools were *Coquillettidia sp.* one each from capture region-1 and 3 and the remaining two pools were *Mansonia sp.* from region-1. Of the seven pools positive for IS*2404*, two pools had high cycle threshold (Ct) values for IS*2404* but did not contain a sufficient amount of DNA to detect IS*2606* and KR. IS*2606* was not detected from four pools, despite having desired Ct values for IS*2404*. All three targets were detected from the remaining pool. Thirty pools of mosquitoes which were negative for IS*2404* were tested for IS*2606* and KR. None of them were positive for these probes signifying the dependent nature of existence of IS*2606* and KR with IS*2404*. Similar findings were reported during the Victorian outbreak (5). The bias corrected MLE value for all mosquitoes collected from study site (region-1, region-2 and region-3) was 0.06 *M. ulcerans* PCR-positive mosquitoes per 1,000 tested (95% confidence interval, 0.00-0.29). Only Region-1 had *M. ulcerans* PCR-positive mosquitoes and calculated MLE value was 0.13 (95% confidence interval,0.01-0.61)/1,000 mosquitoes tested.

Table 1. Ct values on qPCR analysis of mosquito pools

Samples	Species	Location and collection	qPCR analysis			
			IS*2404*	IS*2606*	IS*2404*-IS*2606*	KR
Mosquito Pool-1	*Verrallina sp.*	Region-1; Feb 2017	31.1	32.9	1.8	27.6
Mosquito Pool-2	*Verrallina sp.*	Region-1; March 2017	31.3	ND	ND	ND
Mosquito Pool-3	*Verrallina sp.*	Region-1;Aug 2017	36.1	ND	ND	ND
Mosquito Pool-4	*Coquillettidia sp*	Region-1; Feb 2017	31.2	ND	ND	ND
Mosquito Pool-5	*Coquillettidia sp*	Region-3; Sep 2017	30.4	ND	ND	ND
Mosquito Pool-6	*Mansonia sp.*	Region-1; Feb 2017	32.6	ND	ND	ND
Mosquito Pool-7	*Mansonia sp.*	Region-1; Aug 2017	38.2	ND	ND	ND

ND = not determined

4.7 Screening of March flies:

DNA extracts of 296 March flies were screened for IS*2404*. None of the samples were positive for this probe. Twenty-four randomly selected IS*2404* negative samples were tested for IS*2606* and KR and none were positive.

4.8 Discussion:

Mosquitoes serve as important biological vectors for a variety of pathogens. The movement of pathogens from the gastro-intestinal tract of mosquitoes after a blood meal to the salivary glands for subsequent transmission is well documented for many diseases. However, this phenomenon has not been demonstrated for *M. ulcerans*. A study conducted by Wallace and colleagues (2010) provided evidence for the maintenance of *M. ulcerans* throughout larval development without further passage of the organisms into pupa or adult mosquitoes (21). They concluded that mosquitoes were an unlikely biological vector of *M. ulcerans*. Wallace *et al* (2017) subsequently provided evidence of

mechanical transmission of *M. ulcerans* via anthropogenic skin puncture or mosquito bites (7).

For mechanical transmission, insect vectors such as mosquitoes must acquire the pathogen either from the environment or an infected host. For this to occur efficiently, the organism must be abundantly present in the environment. A survey in Victoria, Australia has confirmed a strong correlation between mosquitoes found to test positive for carrying *M. ulcerans* DNA and the number of human cases of BU occurring (5, 22). The group found a significantly higher number of mosquitoes screened positive for *M. ulcerans* DNA during an intense outbreak of BU in endemic areas, in comparison to areas with a lower incidence of human cases.

The number of human cases of BU has decreased in Northern Queensland, Australia since the largest recorded outbreak in 2011 (> 60 cases). The majority of the cases during the 2011 outbreak were from Wonga and the Wonga Beach area, referred as region-1 in the study by Steffen and Freeborn (2018) (23). Out of 394 pools collected in region 1, six pools were positive for IS*2404* DNA in this study. Interestingly, three of these positive pools were trapped in the backyard of a property in Wonga Beach area (region-1) where two human cases of BU were confirmed in 2017. All other pools of mosquitoes and March flies collected from that property were negative for *M. ulcerans* DNA.

As shown in the result, seven pools of mosquitoes were positive for IS*2404*. However, all three targets with expected Ct value were detected from only one of these seven pools. Samples that were positive for only IS*2404* were not considered further.

In north Queensland, the Daintree River arises in the mountainous rainforest region northwest of the town of Mossman and flows into the sea north of Wonga Beach. The wet season starts normally from November/December and continues up to April, and the dry season starts from May and continues up to October/November. Outbreaks of human cases of BU in north Queensland have been linked with heavy rainfall and flooding. This survey was conducted from September 2016 through to February 2018, when dryer environmental conditions prevailed. Out of seven *M. ulcerans* DNA positive pools of mosquitoes, five were collected in the wet season and two were collected in the dry season. A majority of cases of Daintree ulcer are reported some months after the rainy season ends (13). The estimated mean incubation period of Daintree ulcer is 4.5 months (24). Thus, it is more likely that the transmission occurs in the wet season which justifies

the detection of *M. ulcerans* DNA from the pools of mosquitoes that were captured in wet season in this study.

In a separate study conducted in north Queensland, Australia, one sample of a single mosquito and one pool of two mosquitoes was found positive for IS*2404*.(15). However, it must be noted that this study was conducted soon after 2011 which raises the possibility that sampling should occur as close as possible in time to when transmission is thought to be occurring.

M. ulcerans is an environmental pathogen and detection of *M. ulcerans* DNA positive mosquitoes may only be an indicator for the presence of the organism in the environment. A significant decrease in human cases of BU in northern Queensland in recent years could be due to a lower load of bacteria in the environment. This may explain the low detection of *M. ulcerans* DNA positive mosquitoes and March fly populations in the study sites. However, the detection of *M. ulcerans* DNA even in a single pool of mosquitoes from the endemic areas of Northern Queensland is significant, as it corroborates findings in Victoria where five different species of mosquitoes captured from BU-endemic regions during human outbreaks were positive for *M. ulcerans*.

Our detection of *M. ulcerans* DNA in mosquitoes in northern Queensland does support the earlier report from Victoria in Australia (5). The Victorian study provides evidence for high detection rates of *M. ulcerans* positive mosquitoes if captured during peak times of outbreaks. Our study found that it is less likely to find *M. ulcerans* positive mosquitoes if they are trapped from areas where human incidence of BU is currently low. We hypothesise that mosquitoes and perhaps other biting insects, such as March flies may have a significant role in the ecology and transmission of *M. ulcerans* in endemic areas during outbreaks and that the level of detection of *M. ulcerans* positive mosquitoes in the environment could be an indicator for disease outbreaks.

4.9 Conclusion:

Our study confirms the presence of *M. ulcerans* DNA in the mosquitoes samples captured from the BU-endemic regions of north Queensland, Australia. Lower detection of *M. ulcerans* positive mosquitoes in BU-endemic areas in North Queensland may partially explain low endemicity of the disease.

4.10 Acknowledgements:

We thank laboratory staff of Mosquito Research Facility, AITHM for their technical advice trapping of mosquitoes and designing blood feeding experiments and Janet Fyfe from Mycobacterium Reference Laboratory at VIDRL for her technical advice in analyzing samples. We are grateful to Wonga Beach State School for their assistance in collection of March flies. We thank Hendrik Weimar and local community for their continuous support and assistance in arranging access to the sites for setting traps.

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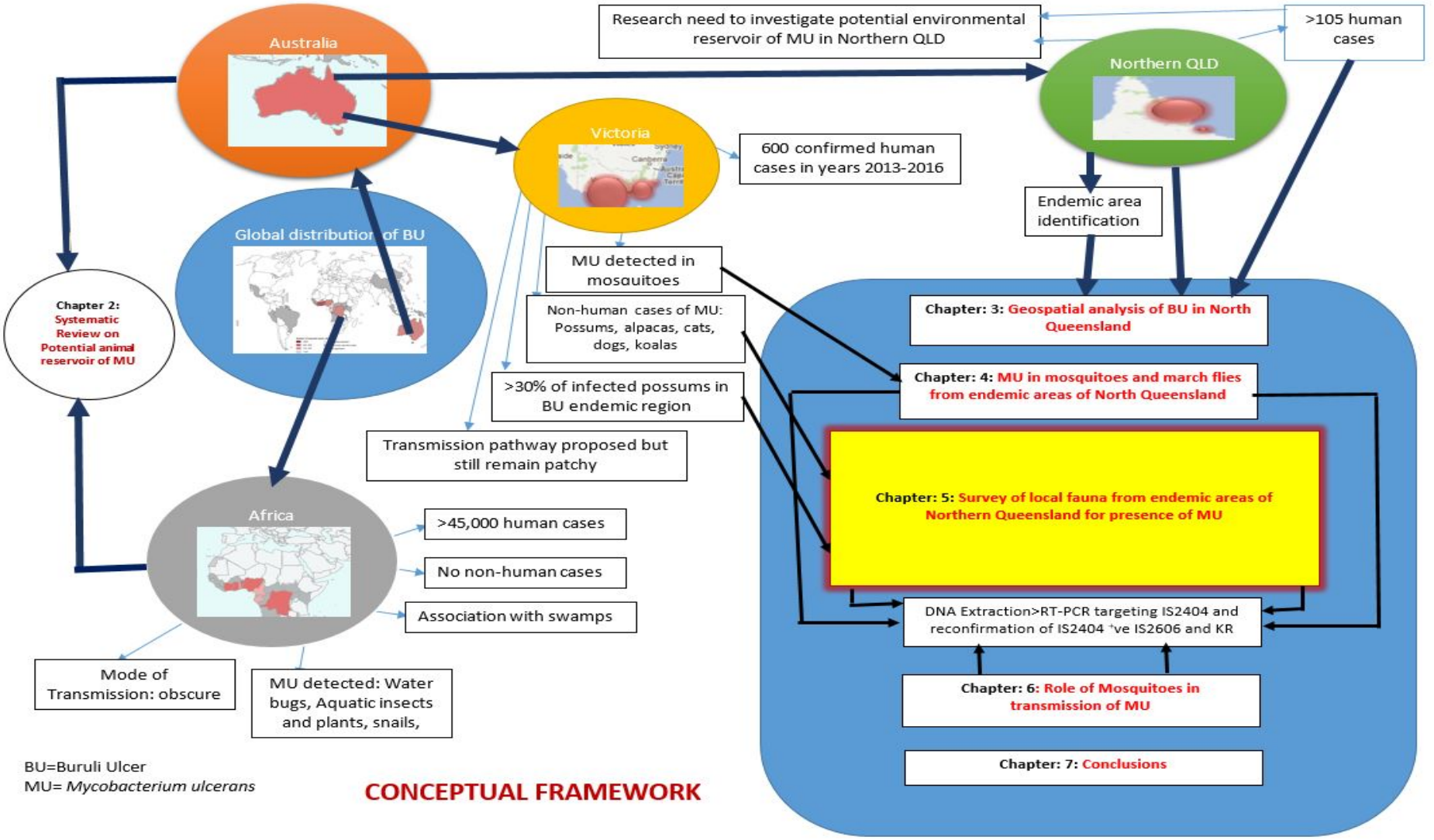
4.12 Exegesis:

This article was published in PLOS Neglected Tropical Diseases. Minor spelling and grammatical errors have been corrected for this thesis.

In 2007, Johnson *et. al.* reported the presence of *M. ulcerans* DNA from the endemic areas of South-eastern Australia. We note that the time of detection of *M. ulcerans* positive mosquitoes did not correspond to the time of the outbreak of human cases of the disease. *M. ulcerans* DNA was detected from the mosquito samples collected in September to February whereas lesions were reported in March to August. The estimated incubation period of M. ulcerans is 4.5 months. The detection of M. ulcerans positive mosquitoes captured in wet season in this study is consistent with observation that the majority of cases of Daintree ulcer in FNQ have been reported after the end of rainy season.


Australia
Research need to investigate potential environmental reservoir of MU in Northern QLD
>105 human cases
Northern QLD
Victoria
600 confirmed human cases in years 2013-2016
Endemic area identification
Global distribution of BU
MU detected in mosquitoes
Chapter 2: Systematic Review on Potential animal reservoir of MU
Non-human cases of MU: Possums, alpacas, cats, dogs, koalas
Chapter: 3: Geospatial analysis of BU in North Queensland
>30% of infected possums in BU endemic region
Chapter: 4: MU in mosquitoes and march flies from endemic areas of North Queensland
Transmission pathway proposed but still remain patchy
Chapter: 5: Survey of local fauna from endemic areas of Northern Queensland for presence of MU
Africa
>45,000 human cases
No non-human cases
Association with swamps
DNA Extraction>RT-PCR targeting IS2404 and reconfirmation of IS2404 +ve IS2606 and KR
Mode of Transmission: obscure
MU detected: Water bugs, Aquatic insects and plants, snails,
Chapter: 6: Role of Mosquitoes in transmission of MU
Chapter: 7: Conclusions
BU=Buruli Ulcer
MU= Mycobacterium ulcerans
CONCEPTUAL FRAMEWORK

5 Chapter 5: SURVEY OF LOCAL FAUNA FROM ENDEMIC AREAS OF NORTHERN QUEENSLAND, AUSTRALIA FOR THE PRESENCE OF *MYCOBACTERIUM ULCERANS*

Aim of this chapter

To survey local fauna from endemic areas of Northern Queensland, Australia for the presence of *M. ulcerans* DNA.

This chapter is presented in the form of manuscript submitted to the international Peer-reviewed journal *International Journal of Mycobacteriology*. This article is published in *International Journal of Mycobacteriology.*

I was the main author of this peer-reviewed manuscript. My principal contribution to this manuscript are as follows:

- I led almost all aspect of study: study design, implementation, sample collection, analysis, interpretation, preparation of manuscript and submission to the journal.

This manuscript can be cited as:

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Original Article

Survey of Local Fauna from Endemic Areas of Northern Queensland, Australia for the Presence of *Mycobacterium ulcerans*

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Abstract

Background: Buruli ulcer (BU), regionally known as the Daintree ulcer or Bairnsdale ulcer is caused by the environmental pathogen *Mycobacterium ulcerans* (MU). This disease is characterized by extensive and painless necrosis of skin and soft tissue with the formation of large ulcers and has been reported in >33 countries worldwide. This organism is geographically restricted and in Australia, the disease has been reported primarily in coastal Victoria and the Mossman–Daintree areas of northern Queensland. Australia is the only country where nonhuman cases of BU have been confirmed. The common ringtail possums and mountain brushtail possums have been suggested as potential animal reservoirs of MU in coastal Victoria, Australia. The exact mode of transmission of this disease remains unknown. **Methods:** In this study, we surveyed local fauna from endemic areas of northern Queensland, Australia, for the presence of MU in scat samples. We collected 140 bandicoot, four white-tailed rats, and two possum scat samples from 56 overnight trapping sessions. Samples were examined for the presence of MU DNA by the polymerase chain reaction. **Results:** Two out of five samples did not contain a sufficient amount of DNA to detect IS2606 and the ketoreductase B (KR) domain of the mycolactone polyketide synthase gene, which is represented by higher cycle threshold (Ct) values for IS2404 shown in table below. Despite of having desired Ct values for IS2404, one IS2404 positive sample possibly contained DNA of closely related M. ulcerans subspecies with lower copy number of IS2606 that do not commonly cause disease in human. All three targets: IS2404, IS2606 and KR were detected from the remaining two scat samples. **Conclusion:** We confirm the presence of M. ulcerans DNA in the scat samples collected from a Buruli ulcer endemic region of Northern Queensland, Australia.

Keywords: Australia, *Mycobacterium ulcerans*, native mammals, northern Queensland

Introduction

Buruli ulcer (BU), locally known as Daintree ulcer in northern Queensland, Australia, is a nontuberculous infection of the skin caused by *Mycobacterium ulcerans* (MU). The disease is rarely fatal if diagnosed and treated with appropriate antibiotics in a timely fashion. Any delay in treatment may lead to the requirement for surgical intervention. Till date, the disease has been reported from >33 countries in Africa, the Americas, Asia, and the Western Pacific.[1] The majority of foci of BU are located in West Africa[2] with other foci in Australia,[3,4] Peru,[5] Papua New Guinea,[6] and Japan.[7] Within these locations, the disease is geographically restricted.

Australia is the only developed country where substantial transmission of MU has been recorded. Foci of BU infection have been found in the tropical Far North Queensland,[8] the Capricorn coast region of central Queensland,[9] the Northern Territory,[10] and temperate coastal Victoria.[3] In Australia, the cases of BU have also been recorded in animals, including koalas (*Phascolarctos cinereus*),[11] common ringtail possums (*Pseudocheirus peregrinus*), mountain brushtail possum (*Trichosurus cunninghami*),[12] horses,[13] dogs,[14] an alpaca,[15] and a cat.[16] All of these recordings were located in the vicinity of human cases of BU. Unlike Australia, not a single study in Africa has reported the cases in nonhuman species or the presence of MU-positive DNA in animals,

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suggesting that transmission dynamics may be different in Africa and Southern Australia or, alternatively, a host animal is yet to be identified in Africa. A study conducted by Fyfe *et al*., between 2007 and 2009 in an endemic area of BU in Australia, found 38% of ringtail possums and 24% of brush tail possums with laboratory-confirmed MU lesions DNA.[12] However, only 1% of possums' samples from nonendemic areas were positive for MU DNA. They suggested terrestrial mammals such as the possums may be potential reservoirs of MU in endemic areas of Victoria, Australia. A similar study conducted in BU endemic villages of Ghana has ruled out the possibility of domestic animals as a reservoir for MU in endemic regions of West Africa.[17] Another endemic area for MU in Australia is far north Queensland in an area extending from the Daintree River and Forest Creek in the north to Mossman in the south.[8] Recently, there was a report of the presence MU in two bandicoot *(Isoodon macrourus*) scat samples collected in this region.[18] The isolated detection of MU in a tropical endemic region in Australia highlighted the need to examine a larger sample size to gauge the significance of the role of native terrestrial mammals in the ecology of BU in northern Queensland. The aim of the current study was to survey samples from local fauna from endemic areas of northern Queensland, Australia, for the presence of MU DNA.

Methods

• Study site and sample collection

Geographic Information System mapping of human cases of BU in northern Queensland from 2009 to 2013 was used as the basis for the selection of study sites. For ease of sampling and analysis, BU endemic areas of northern Queensland were allocated into Region-1 covering the areas of Miallo to lower Daintree, including Wonga/Wonga Beach area, Region-2 covering Forest Creek area, and Region-3 covering upper Daintree area. Figure 1 represents the distribution of human cases of BU in northern Queensland from 2009 to 2013 and the sites from which samples were collected.

• Trapping and sampling of bandicoots

Animal ethics approval was obtained from the Animal Ethics Committee of JCU (Ethics approval number: A2193). A permit to trap native animals for scientific research and educational purposes were obtained from the Department of Environment and Heritage Protection, Queensland, Australia (Permit number: WISP16539915). Cage traps, especially designed for small native mammals, baited with balls of rolled oats and peanut butter were used for trapping animals. Fifty-six overnight trapping sessions, with each session utilizing at least eight traps, were conducted from March 2016 to February 2018. Of the 56 trapping sessions, 22 were conducted at eight sites within Region-1, 16 at sites within Region-2, and 18 at sites of Region-3. All traps were numbered, flagged, and recorded with the global positioning system coordinates to avoid misplacement. Animal traps were set around 2 h before nightfall in each site and checked at first light for trapped animals. Once captured, animals were transferred into a cloth bag for sample collection. In situations where animals passed scat in the trap, this was collected otherwise a cloacal swab was collected. Trapped animals were examined for external lesions and swabs were obtained from the lesions, if found. The captured animals were released on the same day, and at the same location once the samples and data were collected. To identify any event of recapture, fur clipping at the base of the tail was performed. Surrounding areas were screened for additional scats and collected. Scats were identified by visual identification and with the aid of a scat identification manual.[19]

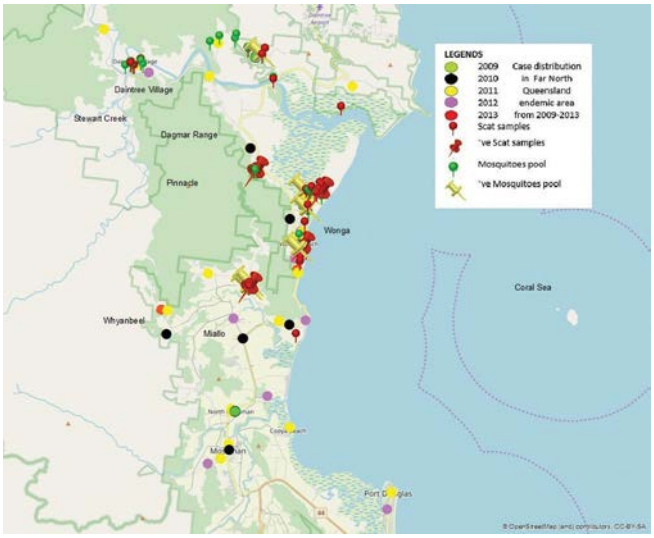

Figure 1: Distribution of human cases of buruli ulcer in buruli ulcer endemic areas of northern Queensland, Australia and location of animal traps. This figure was created using base layer obtained from OpenStreetMap. https://www.openstreetmap.org

• DNA extraction

DNA was extracted from samples using the FastPrep Instrument (MP Biomedicals, Solon, OH, USA) as per the manufacturer's instruction with FastDNA SPIN Kit for Soil (MP Biomedicals). Extracted DNA was stored at −20°C.

• Detection of *Mycobacterium ulcerans* DNA

Two prevalidated semi-quantitative real-time polymerase chain reaction (PCR) assays targeting the insertion sequences IS2404, IS2606 and a sequence encoding the ketoreductase (KR) B domain, KR were used to assess DNA extracts for the presence of MU DNA.[20] During each PCR run, three negative controls (double-deionized water and MilliQ) and three positive controls (purified MU DNA obtained from the Victorian Infectious Disease Reference Laboratory) were used to ensure assay validity. All of the extracted DNA samples were initially screened for the MU insertion sequence element IS2404. Samples positive for IS 2404 were reanalyzed by a second quantitative PCR for the detection of two additional regions, namely, IS2606 and KR B domain in the genome of MU. This screening process has been validated for environmental samples by Fyfe *et al*. and differentiates MU from other *Mycobacteria* that encode mycolactone based on the difference in Ct values

between IS2606 and IS2404 (⊗Ct [IS 2606– IS 2404]).[20] The presence of MU DNA in the samples was confirmed if all three targets (IS 2404, IS 2606, and KR) with expected ⊗Ct values were detected.

Results

- Trapping and sampling of bandicoots

From 56 overnight trapping sessions, each session having at least eight traps, a total of 92 animals were trapped. Of these, 86 were identified as bandicoot *(I. macrourmus)*, four were white-tailed rats *(Uromys caudimaculatus)*, and two common ringtail possums *(P. peregrinus)*. Scat samples were collected from all trapped animals. An additional 54 bandicoot scat samples were collected after screening the proximity of the study site, providing a total of 140 bandicoot scat samples, four white-tailed rat scat samples, and two possums scat samples. One bandicoot trapped at Region-1 near (near South Wonga) had a visible ulcer on the back and nose [Figure 2]. Ulcer swabs and scat specimen were collected and tested for the presence of MU. Those samples were negative for MU. The samples were subsequently transported to the MU reference laboratory at the Victorian Infectious Diseases Reference Laboratory for culture and reanalysis. None of the samples from the bandicoot ulcer were positive for MU DNA. No other animals were found to have ulcers.

Out of 146 scat samples, five bandicoot samples were positive for IS2404 [Table 1]. All of the positive scats were from Region-1. Of the five scats positive for IS 2404, three scats did not contain sufficient DNA to detect IS2606 and KR, which require lower cycle threshold values than IS 2404 [Table 1]. All three targets were detected from the remaining two scats.

Figure 2: Bandicoot with ulcer trapped from South Wonga

Table 1: Polymerase chain reaction analysis of bandicoot scat for *Mycobacterium ulcerans* collected from endemic areas of northern Queensland, Australia

Samples	qPCR analysis Ct values			
	IS2404	IS2606	IS2606-2404	KR
Bandicoot scat[1]	38.6	ND	ND	ND
Bandicoot scat[2]	30.0	ND	ND	ND
Bandicoot scat[3]	31.3	32.9	1.6	27.6
Bandicoot scat[4]	36.1	ND	ND	ND
Bandicoot scat[5]	31.0	32.3	1.3	32.4

[1 and 2]Collected in February 2017, [3]Collected in March 2017, [4 and 5]Collected in August 2017. ND: Not detected, qPCR: Quantitative polymerase chain reaction, Ct: Cycle threshold, KR: Ketoreductase

Discussion

Identification of transmission pathway (s) and potential environmental reservoirs of MU is essential for effective surveillance and control of BU. The occurrence of disease and the geographical distribution of cases have been clearly linked with the aquatic ecosystems.[21,22] Nevertheless, the exact mode of transmission of MU still remains unknown. With the previously recorded detection of MU DNA in the scat and laboratory-confirmed MU skin lesions in ringtail and brushtail possums trapped from high- and low-BU endemic regions in Southern Australia[12,23] and the detection of MU DNA in bandicoot scat in a BU endemic area of northern Queensland,[18] we conducted an extensive survey of local fauna from the main endemic area of north Queensland, Australia, for the presence of MU.

A survey in Victoria, Australia, has led to the suggestion that MU-infected possums are a potential animal reservoir of MU. These animals may also play a role in the maintenance of the organism in the environment of BU-endemic regions for the onset of human cases of BU.[12] Fyfe *et al*. found a strong correlation between BU endemicity of a region and detection of MU DNA in possums feces.[12] An environmental study conducted in Benin has shown a similar correlation, where the proportion of MU DNA in aquatic insects reflected the endemicity of human cases of BU in the same region.[24] Similarly, recent work conducted by our team in the study site has shown a low level of MU DNA in the mosquito populations (unpublished data). The low levels may reflect the decrease of incidence of human cases of BU in the region at the time of sampling.

There has been a low incidence of human cases of BU in northern Queensland, Australia, since the largest recorded outbreak in 2011–2012, where >60 cases were reported.[4] The average reported rate over the 15 years period from 2002 to 2016 was 0.2 cases/100,000 population per year.[25]

The wet season in northern Queensland occurs from November to December and continues up to April, and the dry season starts from May and continues up to October to November. It is well-described that outbreaks of human cases of BU in northern Queensland are linked with heavy rainfall and flooding. The current survey was conducted from March 2016 to February 2018, covering different seasonal conditions with 56 overnight trapping sessions. The rainfall was average during the sampling period. Out of five MU DNA-positive bandicoot scats, three scats were collected during the wet season, and the remaining two were collected in the dry season. Most of the cases of Daintree ulcer in northern Queensland occur at the end of wet season.[25] The estimated mean incubation period of Daintree ulcer is 4.8 months,[26] making it more likely that transmission occurs in the wet season and the disease is evident once the wet season ends.

Despite the large-scale trapping of native mammals and testing of bandicoot feces in endemic areas of northern Queensland over both wet and dry seasons, low number of bandicoot feces was found positive for MU DNA. None of the trapped animals had any laboratory confirmed MU lesions. Conversation with local veterinary practices revealed that none had seen any small animals with any sort of suspicious visible ulcer in their practice in this region. All these findings indicate the presence of only a low amount of the pathogen in the environment, which is reflected by the low numbers of human cases of BU in northern Queensland in recent years.[4] A finding by Roltgen *et al.* in northern Queensland, Australia, of two MU positive bandicoot scats, involved samples that were collected soon after 2011–2012 outbreak, when the transmission was thought to be occurring and the pathogen may have been more prevalent in the environment.[18]

A study conducted by Steffen and Freeborn reported that most of the cases during the 2011–2012 outbreak in northern Queensland were from Wonga and the Wonga beach area, referred to as Region-1 in the study.[4] Out of 146 scat samples collected in the current study, five MU positive samples were from this region. In a separate study conducted by our team, we found seven pools of mosquitoes positive for MU DNA collected from the same study site (unpublished data).

Detection of MU DNA in bandicoot scat in northern Queensland in this study supports earlier reports from northern Queensland and Victoria in Australia.[12,18] Both studies suggest the likelihood of detection of MU positive samples if samples are collected during an epidemic period. Because outbreaks of BU are linked with aquatic ecosystems, we suggest future studies in this region should include sampling of the aquatic environment.

Conclusion

This study confirms the presence of MU in the scat samples collected from a BU endemic region of northern Queensland, Australia. We suggest that there is higher possibility of detection of MU positive scats if the samples are collected soon before the peak endemic, when the transmission cycle is occurring and the organisms are maintaining their existence in the environment.

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Conflicts of interest

There are no conflicts of interest.

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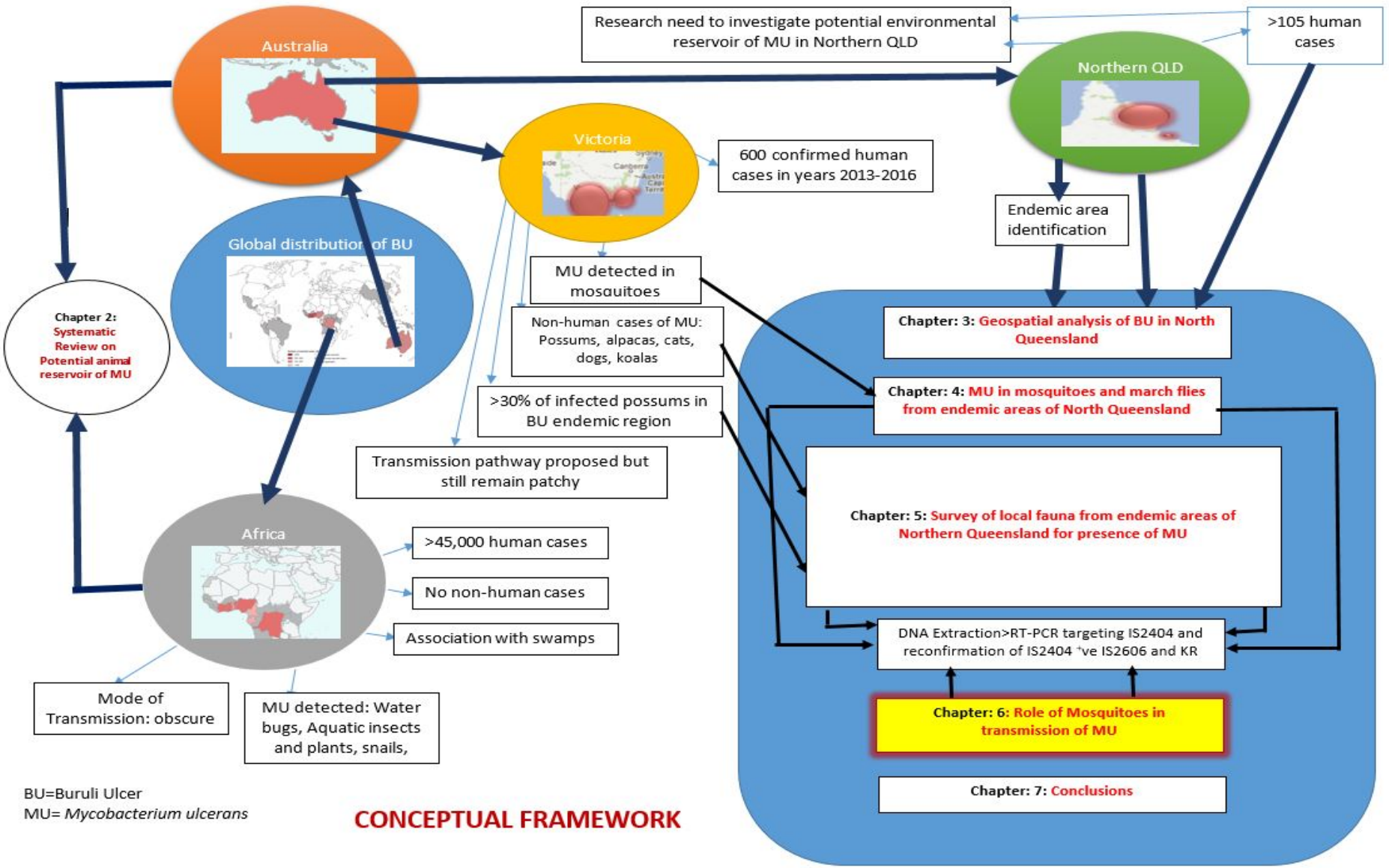
Australia
Research need to investigate potential environmental reservoir of MU in Northern QLD
>105 human cases
Northern QLD
Victoria
600 confirmed human cases in years 2013-2016
Endemic area identification
Global distribution of BU
MU detected in mosquitoes
Chapter 2: Systematic Review on Potential animal reservoir of MU
Non-human cases of MU: Possums, alpacas, cats, dogs, koalas
Chapter: 3: Geospatial analysis of BU in North Queensland
>30% of infected possums in BU endemic region
Chapter: 4: MU in mosquitoes and march flies from endemic areas of North Queensland
Transmission pathway proposed but still remain patchy
Chapter: 5: Survey of local fauna from endemic areas of Northern Queensland for presence of MU
Africa
>45,000 human cases
No non-human cases
Association with swamps
DNA Extraction>RT-PCR targeting IS2404 and reconfirmation of IS2404 +ve IS2606 and KR
Mode of Transmission: obscure
MU detected: Water bugs, Aquatic insects and plants, snails,
Chapter: 6: Role of Mosquitoes in transmission of MU
Chapter: 7: Conclusions
BU=Buruli Ulcer
MU= Mycobacterium ulcerans
CONCEPTUAL FRAMEWORK

6 Chapter-6: ROLE OF MOSQUITOES IN TRANSMISSION OF *MYCOBACTERIUM ULCERANS*: MURINE-MODEL EXPERIMENT

Aim of this chapter

To study the role of mosquitoes in the transmission of *M. ulcerans*

There is lack of knowledge on possible route of transmission of *Mycobacterium ulcerans* and its potential environmental reservoirs (1, 2). A study conducted by Marsollier and his colleagues in 2002 provided evidence for the presence of *M. ulcerans* in the salivary glands of wild caught Naucoridae (aquatic insect). *M. ulcerans* were successfully isolated and cultured from the salivary glands of aquatic insects and suggested they have an important ecological niche in the maintenance of the organism in the environment. (3, 4). Similarly, a study conducted by Wallace *et al*. provided evidence of the ability of mosquitoes to act as a mechanical vector of *M. ulcerans* (5). Although, *M. ulcerans* DNA has been detected from mosquitoes and aquatic insects and these insects have been proposed as important ecological niche for maintenance of this organism in the environment, the detection of DNA does not essentially advocate the abilities of these insects in the transmission of *M. ulcerans*. These insects may act as either biological or mechanical vector or both.

Addressing the transmission pathway of this neglected disease is among one of the research priorities set by WHO. Researchers in Australia have validated evidence-implicating mosquitoes in the transmission of *M. ulcerans (3)*.

This chapter aimed to experimentally demonstrate the transmission of *M. ulcerans* and to provide laboratory evidence for the mechanical transmission of *M. ulcerans* to a mammalian host by blood feeding mosquitoes. A laboratory experiment for a transmission study was planned but did not occur for biosafety reasons. The original experimental plan and subsequent modifications are presented.

6.1 Materials and methods:

6.1.1 Bacterial isolates and culture conditions:

A well-characterised pathogenic strain of *M. ulcerans* from a clinical disease in north Queensland, obtained from Queensland Mycobacterium Reference Laboratory (QMRL) Australia, was sub-cultured onto Lowenstein-Jensen (LJ) slants and liquid Middlebrook 7H9 media supplemented with 10% oleic acid-albumin-dextrose enrichment (OADC). The media was incubated at 30°C to acheive an exponential phase of growth. Spot plating technique was used for colony counts. Five serial 10-fold dilutions (10^{-1} to10^{-5}) were prepared and 3µl of liquid cultures were spotted on 5x5 grid marked agar plates. Spots were allowed to dry in the laminar air flow for 4-6 mins. Once dried, the plates were wrapped in plastic bags and incubated for 10 weeks. Bacterial colonies were counted from the plates.

6.2 Experimental animals:

It was proposed to obtain six BALB/c mice from the Queensland Tropical Health Alliance laboratory at JCU, Cairns. Four of these mice were to be used for this inoculation experiment. Ethical approval was to be obtained from animal ethics committee (AEC) of the James Cook University beforehand. Four pools of 20 adult, female *Aedes aegypti* were to be obtained from Australian Institute of Tropical Health and medicine Mosquito Research Facility, JCU.

6.2.1 Mosquito-mouse transmission experiments:

Experiment 1: Can mosquitoes acquire *M. ulcerans* by feeding on ulcerated tissue?

The purpose of this experiment was to provide laboratory evidence on role of mosquitoes in transmission of *M. ulcerans.* The tails of four mice were to be cleaned using a sterile alcohol wipe. Tails of each mice were to be inoculated with 0.03 ml of sterile water containing ~10^6 CFU/mL of *M. ulcerans.* Mice were to be monitored every two weeks for the development of ulcers. Once an ulcer was observed, mice were to be be humanely killed by cervical dislocation. The ulcerated tail of each mouse would be exposed in a small transparent jar with mesh lid containing 20 female *Aedes aegypti* for a period of 30 minutes. DNA will be extracted from pool of 10 mosquitoes from each jar, each pool containing 10 mosquitoes. The remaining 10 mosquitoes will be used for experiment 2.

The extracted DNA will be screened for the presence of *M. ulcerans* DNA by validated qPCR targeting three independent regions in the *M. ulcerans* genome: IS2404, IS2606 and KR.

Experiment 2: Can mosquitoes exposed to MU ulcers transmit MU?

A healthy mice will be kept in a fresh plastic jar with mesh lid containing 10 female *Aedes aegypti* previously exposed with *M. ulcerans* infected mice for 48 hours. Mice will be monitored daily for signs of pain/distress and ulcer development. The ulcer, once developed, will be swabbed and DNA from the ulcerated tissue will be extracted and screened for the presence of *M. ulcerans.*

We were unable to conduct this experiment for biosafety reasons. Laboratories at James Cook University (both Cairns and Townsville campus) were not equipped to place infected mice and infected mosquitoes in the same settings. Consequently the research plan was adapted and a mosquito artificial blood feeding experiment was conducted to demonstrate an *in vitro* basis for mechanical transmission of *M. ulcerans*.

6.3 Mosquito artificial blood feeding experiment:

6.3.1 Materials and methods:

6.3.1.1 Bacterial isolates and extraction of DNA:

A well-characterised pathogenic strain of *M. ulcerans* from clinical disease in North Queensland, obtained from Queensland Mycobacterium Reference Laboratory (QMRL), Australia was used for this experiment. *M. ulcerans* was confirmed via PCR analysis. Isolates of *M. ulcerans* were subjected to UV light at 250 nanometers for 10 seconds to kill the pathogen. To confirm the sterility of the isolates, an aliquot was sub-cultured onto Lowenstein-Jensen (LJ) slants and liquid Middlebrook 7H9 media supplemented with 10% oleic acid-albumin-dextrose enrichment (OADC). Inoculated media was kept at 31°C in 25cm^2 tissue culture flasks and observed for 8 weeks for growth. No growth was observed confirming the absence of live bacteria. The killed isolates were subjected to qPCR targeting IS2404, IS2606 and KR as described above. qPCR analysis confirmed the presence of *M. ulcerans* DNA.

6.3.1.2 Defibrinated sheep blood:

500 ml PVC pack of sterile defibrinated sheep blood was obtained from Applied Biological Products Management-Australia (Product code: SHBD 0500).

6.3.1.3 Artificial blood feeding method: Simple membrane:

An artificial blood feeding method (simple membrane method) described by Finlayson et al. with some modification was used in this study (6). This procedure is a simple and affordable alternative for direct host feeding (DHF). The method involved pouring warmed defibrinated sheep blood into the indented base on the underside of a plastic container and then covering it with a stretched collagen membrane secured by a rubber band. The container was then turned upside down, filled with warm water and covered by a lid. The feeder was then placed on the mesh side of the cage, allowing the mosquitoes to pierce the collagen membrane to access the blood.

The experiment was conducted using wild type *Aedes aegypti* hatched and reared in the same batch, sorted as pupae into four cages containing 30 female mosquitoes in each. One out of four cages was used as a control (Cage-D) where only defibrinated sheep blood was used as feed and in the remaining three cages (Cage-A, B and C), defibrinated sheep blood mixed with killed *M. ulcerans* isolates (~10^6 CFU/ml) was used. All four cages were exposed to blood for 2 hours. (Figure 6.3) Fully blood fed mosquitoes from each cage were aspirated separately and killed by freezing. Pools of mosquitoes from cage A, and B were dissected, separating the head, abdomen and legs of each insect by sterile fine forceps to avoid contamination during dissections. DNA from the head, abdomen and legs (pooled separately) from the mosquitoes from cage A and B and whole mosquitoes from cage C and D were extracted using FastPrep Instrument (MP Biomedicals, Solon, OH, USA) as per manufacturer's instruction with FastDNA Kit (MP Biomedicals). All the extracted DNA were initially screened for IS*2404* and IS*2404*-positive samples were re-analyzed for IS*2606* and KR with qPCR assay.

Fig 1: Artificial blood feeding experiment

6.3.2 Results and Discussion:

There were a total of seven samples: 2 pools of heads, 2 pools of abdomens, 2 pools of legs (from cage A and B) and 1 pool of whole mosquitoes (Cage C). DNA extracted from pools of heads and abdomens of mosquitoes from cage A and B and the pool of whole mosquitoes from Cage C were positive for *IS2404*. Confirmatory assays targeting *IS2606* and KR revealed that three samples: DNA extracted from the head of mosquitoes from cage A and B and the pool of mosquitoes from cage C were positive for *M. ulcerans* DNA. Controls (a pool of mosquitoes from cage D) were negative for all three targets: *IS2404*, *IS2606* and KR. The detected Ct values of each samples is presented in table 6.1 below.

Table 6.1: Polymerase chain reaction analysis of samples for *Mycobacterium ulcerans*

Samples	**qPCR analysis: Ct values**			
	IS*2404*	**IS*2606***	**IS*2404*-IS*2606***	**KR**
Cage-A: Mosquito head	31.3	32.8	1.5	27.4
Cage-B: Mosquito head	31.2	32.4	1.2	27.8
Cage-A: Mosquito abdomens	37.4	ND	ND	ND

Cage-B: Mosquito abdomens	36.2	ND	ND	ND
Cage-A: Mosquito legs	ND	ND	ND	ND
Cage-B: Mosquito legs	ND	ND	ND	ND
Cage-C: Whole Mosquitoes	31.0	32.6	1.6	28.1
Cage-D Control mosquitoes	ND	ND	ND	ND

*ND: Not detected, Ct: Cycle threshold, KR: Ketoreductase

Detection of *M. ulcerans* from pools of the heads of mosquitoes in the mosquito artificial blood feeding experiments indicate a potential for mosquitoes act as an agent for mechanical transmission of *M. ulcerans.* However, the mosquito artificial blood feeding experiment had some limitations. We were not able to conduct experiments to verify whether *M. ulcerans* positive mosquitoes transmit the pathogen to healthy animals or not. Only proboscises of mosquitoes were in direct contact with blood containing killed *M. ulcerans. M. ulcerans* was only detected from the abdomen of mosquitoes using the *IS2404* target. This might have been due to an insufficient amount of DNA to identify IS*2606* and KR as indicated by a higher Ct value (37.4) for IS*2404.*

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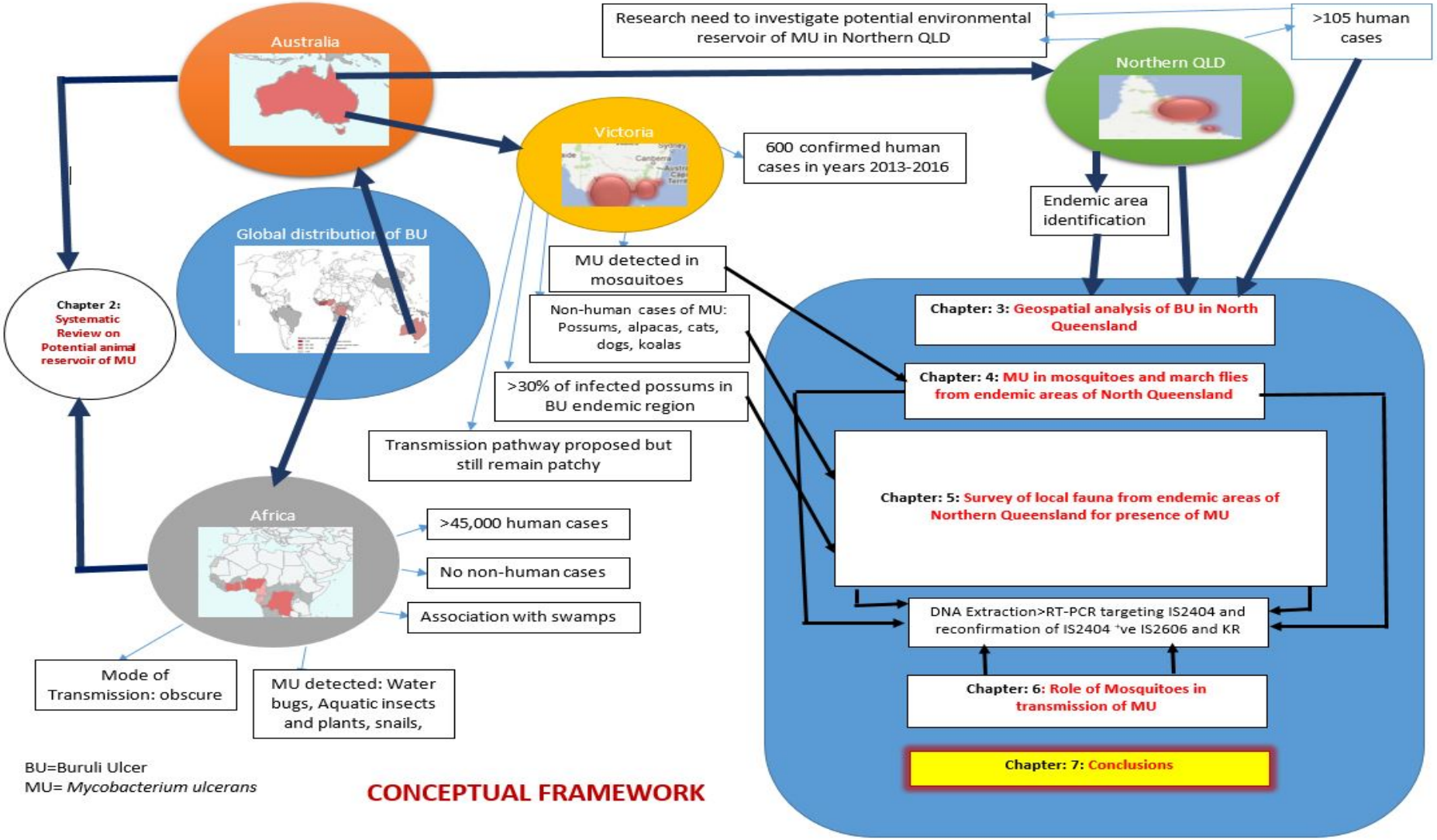
Australia
Research need to investigate potential environmental reservoir of MU in Northern QLD
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Northern QLD
Victoria
600 confirmed human cases in years 2013-2016
Endemic area identification
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No non-human cases
Association with swamps
Mode of Transmission: obscure
MU detected: Water bugs, Aquatic insects and plants, snails,
BU=Buruli Ulcer
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CONCEPTUAL FRAMEWORK

7 Chapter 7: CONCLUSION

The overarching aim of this thesis was to investigate the potential environmental reservoirs of *M. ulcerans* in north Queensland, Australia. Although *M. ulcerans* infection causing Buruli ulcer has been recorded in 34 countries worldwide, Australia is the only country where non-human cases of this infection have been reported. In Australia, *M. ulcerans* infection has been reported from a variety of animals including koalas (*Phascolarctos cinereus*) (1), common ringtail possums (*Pseudocheirus peregrinus*), mountain brushtail possum (*Trichosurus cunninghami*) (2), horses (3), dogs (4), an alpaca (5) and a cat (6). Despite being a global hotspot for BU in humans, Africa has only published one report of non-human cases of this disease (one dog and one goat) and this report postdates my systematic review (7). This suggests the difference in transmission dynamics of the organism may vary geographically. A systematic review conducted as a part of this thesis found research documenting strain variation in the *M. ulcerans* isolates from Africa, The Americas, Asia and the Western pacific. There is no evidence, however, that strains of *M. ulcerans* influence their infectivity for specific hosts.

In Australia, BU cases have been reported in far north Queensland (8), the Capricorn Coast region of central Queensland (9), the Northern Territory (10) and temperate coastal Victoria (11). In Victoria, there has been a marked increase in incidence of BU. More than 600 cases have been recorded during 2011-2016 in Victoria (12). However, there has been a significant decrease in cases of Buruli ulcer in north Queensland. Since the largest outbreak in 2011, when more than 60 cases were recorded, the number of cases has decreased to less than 10 per year. The majority of cases are reported from the Wonga Beach area. The cases in BU areas are geographically restricted and commonly clustered within the endemic areas.

Mosquitoes have been identified as important vectors for a variety of diseases. Mosquitoes may act as either biological or mechanical vectors or perhaps both. No evidence of mosquitoes being biological vectors for *M. ulcerans* has been reported so far. There is evidence of maintenance of *M. ulcerans* throughout the larval developmental cycle without further passage of the organism to pupa or adult mosquitoes (13). Evidence of mosquitoes being mechanical vectors is well established and mechanical transmission of *M. ulcerans* via mosquito bites has been demonstrated in the laboratory (14). Only one

pool of mosquitoes caught in the field were found to be positive for *M. ulcerans* DNA in this study. It must be noted that this study was conducted when there was low case numbers of BU in north Queensland. A Victorian study provides evidence for high detection rates of *M. ulcerans* positive mosquitoes if captured during peak times of transmission (11). As *M. ulcerans* is an environmental pathogen, the level of detection of *M. ulcerans* DNA in mosquitoes could serve as proxy indictor for the burden of the organism in the environment. Lower rates of detection of *M. ulcerans* DNA in mosquitoes might equate with a lower load of organisms in the environment which manifests as a decrease in incidence of the disease. It is increasingly well established that, in Victoria, mosquitoes play a significant role in the ecology and transmission of *M. ulcerans* and my thesis provides support for this statement in the north Queensland endemic area. The artificial blood feeding experiment sheds some light on role of mosquitoes as vectors for mechanical transmission of *M. ulcerans*.

Similar to mosquitoes, only a very low number of scat samples collected from BU endemic areas of north Queensland were found positive for *M. ulcerans* DNA. Findings of this study support earlier reports from northern Queensland and Victoria in Australia (2, 15). These observations suggest the likelihood of detection of *M. ulcerans* positive samples if samples are collected during an active transmission period. In a Victorian study, 38% of ring tail possums and 24% of brush tail possums had laboratory confirmed *M. ulcerans* lesions DNA in endemic areas, however, only 1% of possum samples were positive for *M. ulcerans* DNA in non-endemic areas (2).

M. ulcerans DNA was detected from the native mammals and mosquitoes collected from the BU endemic areas of North Queensland. The level of detection was low which might be due to a lower load of bacteria in the environment. This study suggests that there might be an increased possibility of detection of *M. ulcerans* DNA from environmental samples if the samples are collected at the time of peak transmission, when the “load” of organism in the environment is high. The observations of a 5-6 month incubation period presented in Chapter 5 could suggest the best time for environmental sampling.

It is understood that the transmission of *M. ulcerans* from the environment to the human population can occur through a variety of environmental pathways, each pathway having its own local drivers. In Africa, aquatic insects have been associated in the transmission of *M. ulcerans* and these insects are considered potential vectors for the disease (16-18). *M. ulcerans* DNA has been detected from aquatic insects and molluscs collected from

BU endemic areas of African countries (16-19). A study conducted in a BU endemic area of Benin detected *M. ulcerans* DNA from 8.7% of aquatic insects, however no *M. ulcerans* DNA was detected from mosquitoes and other flying insects (20). In contrast, transmission dynamics in Victoria, Australia is unique and has its own local drivers for the transmission of *M. ulcerans* from the environment to the human population. Several studies have detected *M. ulcerans* DNA from mosquitoes implicating mosquitoes as a vector for the transmission of *M. ulcerans* (11, 21). Furthermore, *M. ulcerans* DNA has been detected from the possums in endemic areas of Victoria, Australia (2). These animals have been established as a potential animal reservoir for *M. ulcerans* in Victoria, Australia. With the detection of *M. ulcerans* DNA from mosquitoes and bandicoots scat samples collected from BU endemic areas in north Queensland, this study suggests the transmission dynamics of BU in north Queensland may resemble those in Victoria, Australia.

Cases of Buruli ulcer are geographically restricted. However, some shifts in geographical location have been observed in endemic areas of Victoria. Almost all cases currently occur on the Mornington or Bellarine Peninsulas in Victoria, Australia, having been observed originally in the Bairnsdale region. Similarly, expansion of the geographical area of human cases of M. ulcerans has been observed in endemic areas of FNQ. Two new human cases (unpublished) of M. ulcerans have been reported in Julatten in the last year. This rural area on the Atherton tablelands is around 40 Kms southwest of the established endemic area of Wonga beach. The reasons for the restriction of pathogen within the endemic areas of far north Queensland is not understood. Particularly intriguing is the absence of cases in the nearby tourist town of Port Douglas. The phenomena is guided by several biotic and abiotic factors. The restriction of *M. ulcerans* within certain geographical areas could be guided by factors within the areas including soil type, rainfall, temperature, the water ecosystem, population density in addition to the fauna and flora of the area. An understanding of the location of the bacteria in the environment of endemic regions is a necessary prerequisite to any understanding of future patterns of disease transmission or changes in geographical extent of disease.

In Victoria, Australia at least four endemic areas of BU have been identified: Bellarine Peninsula, Mornington Peninsula, Frankston region, and the southeastern Bayside suburbs of Melbourne (12). The combined rate of BU per 100,000 population between 2001 and 2016 in: Bellarine Peninsula (7.6), Mornington Peninsula (3.1), Frankston

region (1.1), and the southeastern Bayside suburb (0.6) (12). However, it must be noted that these geographical regions are densely populated. For instance, the Mornington Peninsula area covers 723 Km^2 and has more than 150,000 residents (22). In contrast, the BU endemic areas in North Queensland are restricted within sparsely populated area of Douglas Shire. The majority of cases are geographically restricted to Wonga beach area, Daintree village and Forest Creek within the Douglas Shire. The Douglas shire covers an area of 2,445 km^2 with total population about 11,000 (23). Australian Bureau of Statistics 2016 census recorded the population of Daintree village and Wonga beach to be 129 and 975 respectively (24). Therefore, whilst there is a low average reported case rate of BU in Queensland (0.2 cases/100,000 population per year) (25), it is actually more likely that people residing or visiting BU endemic areas of north Queensland will get infected with *M. ulcerans* than those residing or visiting BU endemic areas of Victoria, Australia.

The following recommendations are suggested for future research to understand the ecology of *M. ulcerans* in north Queensland:

a. Sampling and analysis during peak transmission to observe the level of detection of *M. ulcerans* DNA in environmental samples
b. Conduct mosquito transmission experiments in animal models with isolates from north Queensland
c. Further surveys of the aquatic ecosystem and water bodies in BU endemic areas of north Queensland for the presence of *M. ulcerans* DNA.
d. Sampling and analysis of the samples collected from non-endemic areas of north Queensland for the presence of *M. ulcerans*.

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8 APPENDICES:

Appendix-1: March Fly sampling request from Wonga Beach State School

WONGA BEACH STATE SCHOOL
Snapper Island Drive
WONGA BEACH QLD 4873
PHONE (07) 4099 9777 FAX (07) 4099 9700
Office Mobile: 0447799270

Dear Parents/ Guardians
I am helping a doctoral student with a study into how Daintree Ulcers are spread in the area around the Daintree River. As part of his thesis Avishek Singh is investigating where the bacteria live in our environment and how they are picked up by humans. He is spending around 18 months in our area researching this and would love to have as many people as possible helping him collect specimens from the environment.
The first stage is to collect march flies so they can be tested for exposure to the bacterium. As they are most prevalent in the hotter months they need to be collected in summer.

March Fly Sampling.

- Catch the march fly by hand or with a swatter.
- Place one specimen in the clip lock bag.
- Write your **name**, **place** caught and **date** on the bag.
- Seal the bag and place in the freezer.
- Keep frozen until collection date.

March flies are most common around dusk and are attracted by mammals so around your dog in the late afternoon is a good place to look for them. They also tend to like creeks.
The Daintree Ulcer has affected a great many people in our area and it would be a wonderful breakthrough to find out how it is transmitted. The results of this research could help many people as the Buruli Ulcer as it is known in Africa has a terrible effect on many people's lives in countries where treatment is not readily available.
Over the coming year more notes will come out asking for assistance with specimen collecting to assist Avishek in his research.
Rick Weimar

✂----------

PERMISSION NOTE

I _____parent/guardian give permission to my child_____
(Please Print) (Please Print)
to bring home bags from school for march fly collecting.

Parent/Guardian's Signature: _____ Date: _____

Appendix-2: Scat sampling request

Daintree Ulcer Research

Wanted

Bandicoot Poo or Bandicoots

Avishek Singh has been doing research on where the Daintree Ulcer bacteria live in our local environment and how it is transmitted to humans. He is currently collecting bandicoot droppings and taking swabs from live bandicoots to see if they come in contact with the bacteria while digging in the soil or by eating grubs, worms and insect larvae that live in the soil. In this way the bandicoots can be an indicator of the bacteria being present in the soil.

How you can help

Scats (Poo)

Collect bandicoot droppings from around your property or local area. Put them in plastic bags and put them in a container for later collection. Instructions and all the bags, gloves and plastic container needed for the task will be provided.

Bandicoots

Avishek has a permit to trap and release bandicoots using small animal cage traps. The trapped bandicoots are swabbed and then released unharmed. If you would like to help with bandicoot trapping, traps can be provided or you may use your own if it is appropriate. The traps must be checked in the morning and Avishek will need to be contacted by phone or text so he can swab and release them that morning.

If you have a bandicoot population and would allow Avishek to set traps on your property, this would also help.

The trapping has to be organised around Avishek's time in Wonga so if you tick this option you will receive another sheet to put your contact details on.

.....

Tick whichever applies.

- I would like to help with Daintree ulcer research by **collecting bandicoot droppings**.
- I would like to help with the bandicoot trapping.
- I have a property with bandicoots where Avishek can set traps.

I give permission for my child ..... to collect a sample kit for bandicoot scat collecting and/or a contact sheet for bandicoot trapping.

Signed..... Date / /

Appendix-3: Protocol for *Mycobacterium ulcerans* DNA detection in mosquitoes using qPCR

This protocol is registered with protocols.io and can be available from following website:

dx.doi.org/10.17504/protocols.io.vqbe5sn

Protocol for *Mycobacterium ulcerans* DNA detection in mosquitoes using qPCR

Extraction of DNA

DNA was extracted from each pools of ≤ 20 mosquitoes of the same genus by using the FastPrep Instrument (MP Biomedicals, Solon, OH, USA) as per manufacturer's instruction with FastDNA Kit (MP Biomedicals).

Samples (each pools of ≤ 20 mosquitoes of the same genus) was added to Lysing matrix A tube.

1 ml of Cell Lysis Solution-TC were added. Samples were homogenized in the FastPrep® Instrument for 40 seconds at a speed setting of 6.0 and directly centrifuged at 14000xg for 10 minutes to pellet debris.

800 µl of supernatant was transferred to 2.0 ml microcentrifuge tube and add an equal volume of Binding Matrix was added and tubes were inverted to mix followed by incubation at room temperature for 5 min with gentle agitation on rotator.

Centrifugation was performed at 14,000 x g for 1 minute to pellet the Binding Matrix and supernatant was discarded. 500 µl prepared SEWS-M was added using the force of the liquid from the pipet tip for gently resuspended.

Centrifugation was performed at 14,000 x g for 1 minute and supernatant was discarded. Centrifugation was repeated second time at 14,000 x g for 1 minute and residual liquid was removed with small pipet tip.

DNA was eluted by gently resuspending Binding Matrix in 100 µl of DES and Incubate for 5 minutes at 55˚C in a water bath. Centrifugation was performed at 14,000 x g for 1 minute and eluted DNA was transferred to clean microcentrifuge tube. DNA was stored at -20°C fur further use.

The detailed steps for the extraction of DNA could be found at https://www.mpbio.com/includes/technical/fastdna.pdf

One sterile water in each batch of extraction was used as negative control to identify the possible contamination during the process of extraction of DNA.

qPCR Analysis

The extracted DNA samples were screened for the presence of *M. ulcerans* DNA by using a semi-quantitative real-time PCR adapted from a method for the detection of *M. ulcerans* DNA from environmental samples (1).

Three pairs of primers with probes targeting Insertion sequence IS*2404*, IS*2606* and Ketereductase B-domain gene (KR-B) located on the *M. ulcerans* virulence plasmid pMUM001 were used. The used primers and probe sequences are listed in Table-1 below.

Primers and Probes [a]	Sequences (5'-3')
***IS2404* TF**	AAAGCACCACGCAGCATCT
***IS2404* TR**	AGCGACCCCAGTGGATTG
***IS2404* TP**	**6 FAM**-CGTCCAACGCGATC-MGBNFQ
***IS2606* TF**	CCGTCACAGACCAGGAAGAAG
***IS2606* TR**	TGCTGACGGAGTTGAAAAACC
IS*2606* TP	**VIC**-TGTCGGCCACGCCG-MGBNFQ
***KR* TF**	TCACGGCCTGCGATATCA
***KR* TR**	TTGTGTGGGCACTGAATTGAC
***KR* TP**	**6 FAM**-ACCCCGAAGCACTG-MGBNFQ

[a] TF TaqMan forward primer, TR TaqMan reverse primer, TP TaqMan probe

Table-1. List of Primers and Probes

Three consecutive qPCR were run to conform the presence of *M. ulcerans* DNA in the sample. Preparation of Master-mix is listed in table-2 below:

Reagent	Volume/reaction	
	IS*2404*/IPC* assay	**IS*2606*/KR assay**
2× TaqMan Universal PCR mastermix	1.25 µl	12.5 µl
Primer IS *2404* TF (18 µM)	1.25 µl	-
Primer IS *2404* TF (18 µM)	1.25 µl	-
Primer IS *2404* TR (18 µM)	1.25 µl	-
Probe IS *2404* (5 µM)	1.25 µl	1.25 µl
Primer IS *2606* (18 µM)	-	1.25 µl
Primer IS *2606* TR (18 µM)	-	1.25 µl
Probe IS *2606* TP (5 µM)	-	1.25 µl
Primer KRTF (18 m M)	-	1.25 µl
Primer KRTR (18 µM)	-	1.25 µl
Primer KRTP (5 µM)	-	1.25 µl
50× ExoIPC DNA	0.5 µl	-
10× ExoIPC Mix 2.5 m l –	2.5 µl	-
Nuclease-free water (NFW)	4.75 µl	4 µl
Total	24 µl	24 µl

*IPC internal positive control

Table-2. Preparation of mastermix for *M. ulcerans* real-time PCR assays

qPCR Assay:

24 µl of mastermix was added to each well. 1 µl of NFW was added to NTC well, 1 µl of *M. ulcerans* DNA was added to positive control well and 1 µl of sample DNA was added to sample well. PCR plate was transferred to qPCR instrument. The reaction was run in the following conditions: 50°C for 2 minutes, 95°C for 15 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Samples tested positive for IS*2404* were further tested for IS*2606* and KR using above mentioned mastermix. Samples positive for IS*2404* and IS*2606* were tested for KR-B. All samples were tested in duplicate.

The difference in real-time PCR cycle thresholds (Ct) between IS *2606* and IS*2404* (ΔCt [IS *2606*– IS *2404*]) allows for the differentiation of *M. ulcerans* from other MPM that contain IS *2404* but which have fewer copy numbers of IS*2606*

Reference:

1. Fyfe JA, Lavender CJ, Johnson PD, Globan M, Sievers A, Azuolas J, et al. Development and application of two multiplex real-time PCR assays for the detection of Mycobacterium ulcerans in clinical and environmental samples. Applied and environmental microbiology. 2007;73(15):4733-40.

Appendix 4 -: Scientific Purpose permit from Department of Environment and Heritage Protection, Queensland Government

This administrative form
has been removed

Appendix 5 -: Ethical approval from JCU Animal Ethics Committee

This administrative form
has been removed