KWAME NKRUMAH UNIVERSITY OF SCIENCE AND TECHNOLOGY

COLLEGE OF HEALTH SCIENCES

SCHOOL OF MEDICAL SCIENCES

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COMPARATIVE DETECTION OF *MYCOBACTERIUM ULCERANS* DNA BY PCR FROM FTA CARDS, CELL LYSIS SOLUTION AND RNA PROTECT SOLUTION

A THESIS SUBMITTED TO THE DEPARTMENT OF MOLECULAR MEDICINE,

IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF PHILOSOPHY, IMMUNOLOGY

BY

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DECLARATION

The experimental study described in this thesis was carried out at the department of Molecular Medicine, KNUST by me. It has not been submitted for any other degree.

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DEDICATION

I dedicate this work to family and friends who supported and encouraged me through this study.

I will forever be indebted to you.

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ABSTRACT

Background: Buruli ulcer is a necrotizing skin disease caused by *Mycobacterium ulcerans*. It occurs in more than 33 countries with tropical and subtropical conditions. Samples for diagnosis and confirmation are usually transported in liquid media including cell lysis solution (CLS), PANTA, and RNA protect solution from the disease centers to reference laboratories. FTA cards have been developed to store, preserve and protect nucleic acids stored on it. It serves as a transport medium for samples requiring the use of nucleic acids and reduces storage space. Diagnosis of Buruli ulcer requires transporting samples from the remote communities to the laboratories as well as for storage and sample collection. The usage of FTA cards has however not been explored in the field of Buruli ulcer and in Ghana. This study therefore sought to evaluate the use of FTA for obtaining DNA samples and transporting of *M. ulcerans* to the laboratories and to also establish its detection limit.

Methods: Known concentrations of *M. ulcerans* suspensions were spotted on the FTA cards and allowed to air dry. Two millimeters of discs were cut out and DNA was extracted using the in-house extraction procedure to optimize the cards and determine the detection limit of the cards. Swab samples from ulcerative lesions and FNA samples from pre-ulcerative lesions were

taken from 53 suspected Buruli ulcer patients and distributed into CLS, RNA protect solutions and spotted on the FTA cards. DNA was extracted from the samples and amplified using the Dry Reagent Based PCR for the CLS and FTA card samples and qPCR for RNA protect solution samples. Amplicons were viewed on agarose gel and results recorded as positive, negative or inhibited. Samples were also smeared on glass slides for the Zeihl Neelson stain for acid fast bacilli. Samples stored on the FTA cards were kept over a period of six months and extraction done on monthly basis to detect the preservative ability of the card. Comparisons were made and results analyzed using GraphPad prism version 5.0 and Stata version12.0.

Result: From the optimization test, it was observed that in absolute numbers, 1 bacterium in a concentration of 10 bacteria/ml spotted on the card can be detected. The sensitivity of the FTA cards using patient samples was recorded as 58% and a specificity of 70% whiles that of qPCR was 94% sensitivity and 67% for specificity when compared with the gold standard, the DRB PCR. When compared with the smear for microscopy, the sensitivity for FTA cards was 80% and a specificity of 48%. Samples stored over a period of two months had the highest positivity ratio of 100% followed by those stored for five months with 58.8% and 55%, 50%, 44%, and 27% for four months, one month, three months and six months respectively. inter-assay agreement between FTA PCR and standard CLS PCR was 50% and a p-value of 0.58 for swab samples and 67.7%, p value of 0.22 for FNA samples.

Conclusion: FTA cards serve as a good storage and transport medium However because of low sensitivity and low inter-assay agreement rate, it cannot be used as a replacement for the CLS. It may however be used in addition to the smear for microscopy in areas where access to the reference laboratories is difficult.

TABLE OF CONTENTS

KNUST

KNUST

LIST OF TABLES

LIST OF FIGURES

KNUST

LIST OF ABBREVIATIONS

AFB	acid fast bacilli
BU	Buruli ulcer
CI	confidence interval
CLS	cell lysis solution
DNA	deoxyribonucleic acid
DRB	dry reagent based
FNA	fine needle aspiration
FTA	flinders technology and associates
MU	*Mycobacterium ulcerans*
NFSTC	National Forensic Science Technology Center
PANTA	polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin Dubos broth base and Dubos medium albumin
PCR	polymerase chain reaction
PPS	protein precipitate solution
qPCR	quantitative polymerase chain reaction (realtime polymerase chain reaction)
RNA	ribonucleic acid
RP	RNA protect
TEB	Tris EDTA buffer
WHO	World Health Organization
ZN	Ziehl Neelsen

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CHAPTER 1

INTRODUCTION

1.1 Background

Buruli ulcer is the third most common mycobacterial disease after tuberculosis and leprosy and is caused by *Mycobacterium ulcerans.* It is a chronic necrotizing infection of subcutaneous tissue that may affect bones (Eddyani *et al.*, 2009; Yeboah-Manu *et al.*, 2011a). It is found in 33 tropical and subtropical countries in the world especially in poor communities of these areas (Eddyani *et al.*, 2009; Herbinger *et al.*, 2010; Yeboah-Manu *et al.*, 2011a).

In Africa, nearly 50% of those affected are children under 15 years of age. In contrast, most of the patients in Australia and Japan are adults' whiles children under 15 years constitute less than 20% (Vincent *et al.*, 2014). The disease affects persons who have little or no access to health care facilities and their activities may predispose them to the disease since *M. ulcerans* is an environmental organism.

If treatment is delayed, the ulcer leaves scars and contractures that limit movement of limbs and disrupt economic activities and education in children. Early detection and treatment is a priority for the WHO. To achieve this, effective diagnostic methods that speeds up confirmation of suspected *M. ulcerans* infections is required. Current methods for laboratory confirmation include microscopy for acid fast bacilli, culture for viable mycobacteria, histopathology and polymerase chain reaction (PCR) targeting the *IS2404* repeat sequence of *M. ulcerans* genome. PCR is the gold standard diagnostic test for *M. ulcerans* infection at present because of its high sensitivity of 98% and specificity of 100% (Herbinger *et al.*, 2010).

In the past, when surgery was the mainstay of treatment, excised tissue as well as swabs and punch biopsies were used as means of sample collection for laboratory confirmation. Currently

WHO recommends the use of fine needle aspirates (FNA) which is a less invasive approach to obtaining patient specimen (Phillips *et al.*, 2009b; Herbinger *et al.*, 2010). FNA is normally obtained from non-ulcerative lesions such as nodules, plaques or oedemas where samples are obtained from the central point of the lesion with lots of organisms. Swabs are taken from undermined edges of an ulcerated lesion where the organisms are likely to be found. Samples obtained have to be transported to the laboratory in various transport media including PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin

Dubos broth base and Dubos medium albumin) media and cell lysis solution (CLS). The PANTA media keeps the cells alive and at a favorable temperature as well as preventing the growth of other bacteria which usually overgrow the Mycobacterium, which is a slowgrowing organism (Yeboah-Manu *et al.*, 2011b; Agbavor *et al.*, 2012; Bratschi *et al.*, 2014).

Flinders Technology Associates (FTA®) filter papers (FTA cards) are sample collecting cards designed for nucleic acid analysis. It is based on the concept of applying a weak base, chelating agent anionic surfactant or detergent and uric acid or a urate salt to a cellulose based matrix which is the filter paper (Moscoso *et al.*, 2004; NFSTC, 2005). Applying a biological sample containing DNA to the treated filter paper then preserves and stores it for a long time. Samples such as blood and saliva get attached to the paper through the mechanism of entanglement allowing the mixture of chemicals to lyse and denature the proteins (NFSTC, 2005). EDTA and uric acid are important components that maintain long term DNA stability (Saieg *et al.*, 2012) and prevent bacterial growth. The design of FTA cards makes it a suitable device for long term commercial storage of samples and makes transport and storage space easily accessible (Heim *et al.*, 2012).

Nucleic acids could be purified on the FTA Card in three simple steps, all in a single tube at room temperature. DNA remains immobilized on the matrix, and ready for PCR or other amplification techniques. There is a possibility of automation to speed the handling of multiple

FTA punches and to standardize DNA purification. Punches can easily be washed and prepared for PCR on a variety of liquid handling instruments(GE Healthcare, 2011). Buruli ulcer is endemic in rural and remote communities that lack appropriate health care facilities and relevant diagnostic centers, it is imperative that samples are transported to reference laboratories for analysis in a medium that will preserve the integrity of the DNA. It is against this background that this study sought to evaluate FTA cards for its preservative ability.

1.2 Problem Statement and Justification

Detection of nucleic acid targets is an important aspect in molecular diagnostics. The detection of the *IS2404* insertion sequence by PCR is the gold standard for *M. ulcerans* detection. The accuracy of the result depends on the integrity of the DNA and the sensitivity of the PCR. Current methods of sample collection for PCR such as FNA and swab require storage in transport media, and it is hypothesized that the type of transport media used has an effect on the sensitivity of the PCR. *M. ulcerans* infection is endemic in rural and remote communities that lack appropriate storage facilities, and requires samples to be transported to reference laboratories for analysis. The introduction of FTA cards would therefore help in resolving many issues concerning sample collection, storage, transport and hence DNA integrity for molecular analysis. This novel invention has however not been explored in Ghana with regard to *M. ulcerans* disease. It is in view of this that the current study sought to evaluate the use of the FTA cards for obtaining and transporting DNA samples from *M. ulcerans* infected individuals for PCR and to compare it to the current methods and also seeks to establish its detection limit for the *M. ulcerans.*

1.3.1 Objective of Study

The main aim of the study is to evaluate FTA cards for sample collection and determine the detection limit for *M. ulcerans.*

1.3.2 Specific Objectives

1. To determine the preservative ability of *M. ulcerans* DNA on FTA cards.
2. To determine the detection limit of PCR for *M. ulcerans* using FTA cards.
3. To compare PCR results using samples stored on FTA cards and in RNA Protect solution and cell lysis solution (CLS).
4. To compare the results of FTA PCR to that of smear microscopy for acid fast bacilli.

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CHAPTER 2

LITERATURE REVIEW

2.1 Historical facts and epidemiology

Buruli ulcer, also known as Bairnsdale disease is a necrotizing skin disease that is caused by *Mycobacterium ulcerans*. It is a neglected tropical disease and the third most common mycobacteria infections after tuberculosis and leprosy (Yeboah-Manu *et al.*, 2013). In some countries however, it is the second common mycobacterium infection after tuberculosis (Merritt *et al.*, 2010; Yeboah-Manu *et al.*, 2011a).

It was first discovered by a British physician, Sir Albert Cook in 1897 in Uganda and later in the 1920s by Kleinchdt in the north eastern Congo (Johnson *et al.*, 2005b). However, it was described in details by McCallum and his colleagues in Australia and published in 1948 after cases of an unusual skin infections believed to be caused by a mycobacterium were reported (Johnson *et al.*, 2005b; Yeboah-Manu, 2006a). New cases of the disease were later reported in the Congo County called Burundi now known as Nakasongola, between the 1960s and the 1970s. This resulted in the name Buruli for the disease (Clancey, 1964). In recent years, the disease has been reported in more than 30 countries around the world mainly within the tropical countries especially in West Africa.

The disease is mostly endemic in poor communities surrounded by water bodies. People involved in activities in these environments are mostly at risk (Asiedu and Etuaful, 1998; Rondini *et al.*, 2003). In Benin however, studies have shown that altitude may play a role in its focal distribution(Sopoh *et al.*, 2011).

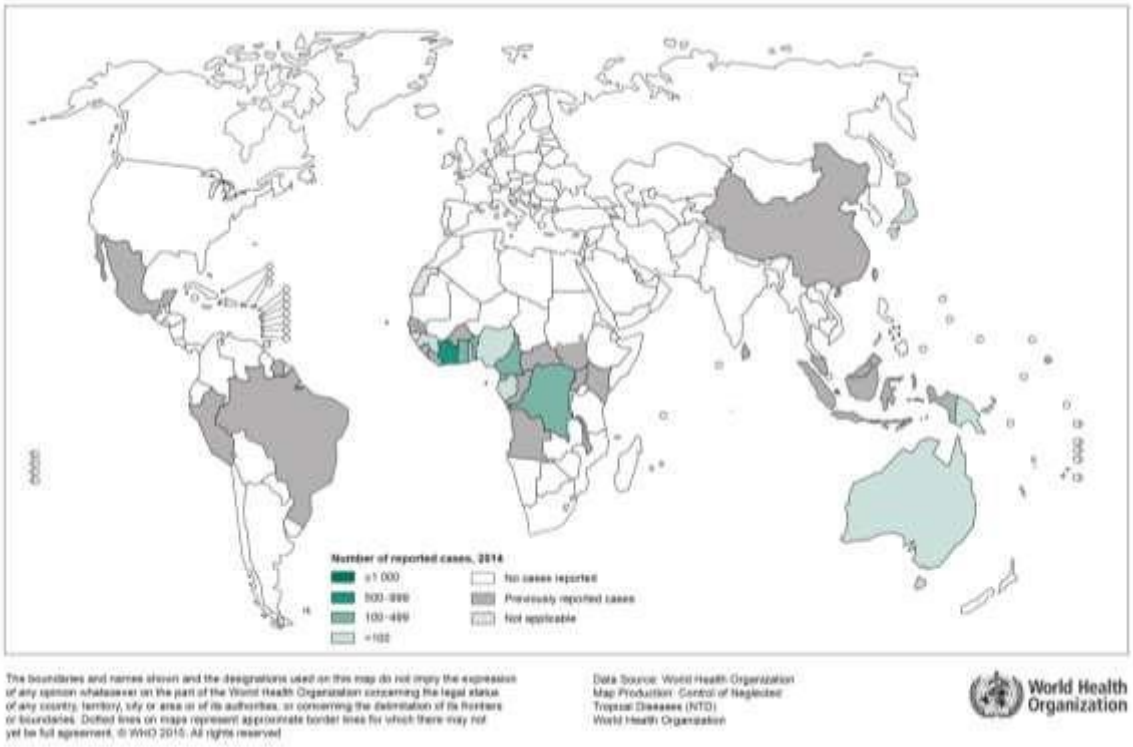

Figure 1: Distribution of Buruli ulcer, worldwide, 2014.

The disease affects people of all ages and sex but children are more affected than adults with an average age of 14. There is no difference in the infection rates between sex (Amofah *et al.*, 1998). The disease affects any part of the body but the limbs are mostly affected about 80% of the time, where if not treated early leads to contractures and even amputation(Asiedu and Etuaful, 1998; Merritt *et al.*, 2010)

2.2 Buruli ulcer in Ghana.

Buruli was first identified in Ghana in the 1970s around the Densu river in the greater Accra Region. Subsequent cases were later reported in the Asante Akim North district of the Ashanti Region in 1989. Other cases were later reported in the Amansie West district in the Ashanti region, which became one of the endemic districts in the country where many researches into the disease were conducted (Van der Werf *et al.*, 1999). This led to the construction of a hospital to serve the community. More cases of the disease were later reported

across the country. In 1999 Amofah et. al. conducted a survey which showed that the prevalence rate in Ghana was 20.7 per every 100 000 and also identified that more women above the age of 20 were infected as against more boys than girls below the age of 20 years (Amofah *et al.,* 2002). Buruli ulcer disease has been on the increase in the country especially in the remote villages where access to medical care was difficult and around water bodies hence activities around these water bodies such as farming and illegal mining popularly known as galamsey posed as risk factors (Merritt *et al.,* 2010).

2.3 Pathogenesis and clinical Presentation

2.3.1 Causative organism

M. ulcerans is an acid fast bacilli belonging to the family mycobacteriaceae, a slow growing environmental organism. It can be cultured from human samples on Loewenstein Jensen media suitable for the growth of mycobacterium. It is however cultured at temperatures lower than that of tuberculosis which is between 29-33 degrees Celsius. It takes between six to eight weeks and even beyond to grow. Subcultures however grow faster within three to four weeks at same temperature (Palomino and Portaels, 1998; Eddyani *et al.*, 2008; Portaels and WHO, 2014). It is a free moving environmental organism, and occupies a specific niche in the environment particularly aquatic organisms.

2.3.2 Clinical Presentation

The disease presents in many clinical forms. It is categorized into pre ulcerative and ulcerative forms (WHO, 2013). The preulcerative forms include the nodules, plaques, edema and papules.

The early stage of the disease is usually characterized by a firm painless mobile swelling in the skin which extends into the subcutaneous tissue. This is known as the nodule which is common in Africa (Portaels *et al.,* 2001; Boleira *et al.,* 2010). In Australia, the early stage swelling is different from the African nodule, where it is only attached to the skin and does not involve the

subcutaneous tissue. The surrounding skin is reddened and may be confused with an insect bite (WHO, 2012). Another form of the pre ulcerative / non ulcerative form is the plaque which is characterized by a larger area of indurated skin. It is painless and appears as a raised portion of the skin with a diameter which is usually greater than three centimeters (Van der Werf *et al.*, 1999). The last of the pre ulcerative form is the edema. It is the more severe form of the disease and starts as a diffuse swelling of a part or all of the limbs. The swelling may occur at other parts of the body too but this is not common. It could be painful but mostly not painful and accompanied by a mild fever. There may be colour change around the area of infection (Johnson *et al.*, 2005a; WHO, 2012).

If not treated early, these may ulcerate into painless lesions with undermined edges. Sloughing occurs as a result of the necrosis of the subcutaneous fat. This may extend beyond where the bacteria are located. It usually becomes painful when secondary infection sets in and may extend to the bones. When healed it may leave a scar and sometimes lead to amputation when the bone is involved (Phillips *et al.*, 2009a; Yeboah-Manu *et al.*, 2013).

Apart from this method of classification of BU, the lesions are also grouped into categories depending on the severity of the lesion. To this we have:

Category I lesions: Includes a single lesion with a diameter less than 5cm. These lesions usually heal faster when treated with antibiotics and do not require surgery. They normally will not leave scars and if any, may not be visible (WHO, 2012).

Category II lesions are single lesions with diameters greater than 5cm but less than 15cm. Some of these lesions heal with only antibiotics and fast but some may take a longer time to heal whiles some require surgery to heal completely in addition to the antibiotics (WHO, 2012).

Category III lesions are characterized by larger lesions with diameters greater than 15cm as well as multiple lesions either at the same site or different sites. These usually take a longer

time to heal and may require in addition to the antibiotics treatment a surgery (WHO, 2012). This classification is based on the rate of healing and treatment method of the various forms of the disease. According to the centers for disease control and prevention, the WHO also classifies the disease into two categories; the active form made up of the nodules or papule, plaque, edema and ulcer. The inactive form is shown by the presence of a scar with or without a sequel. (http://www.cdc 2005)

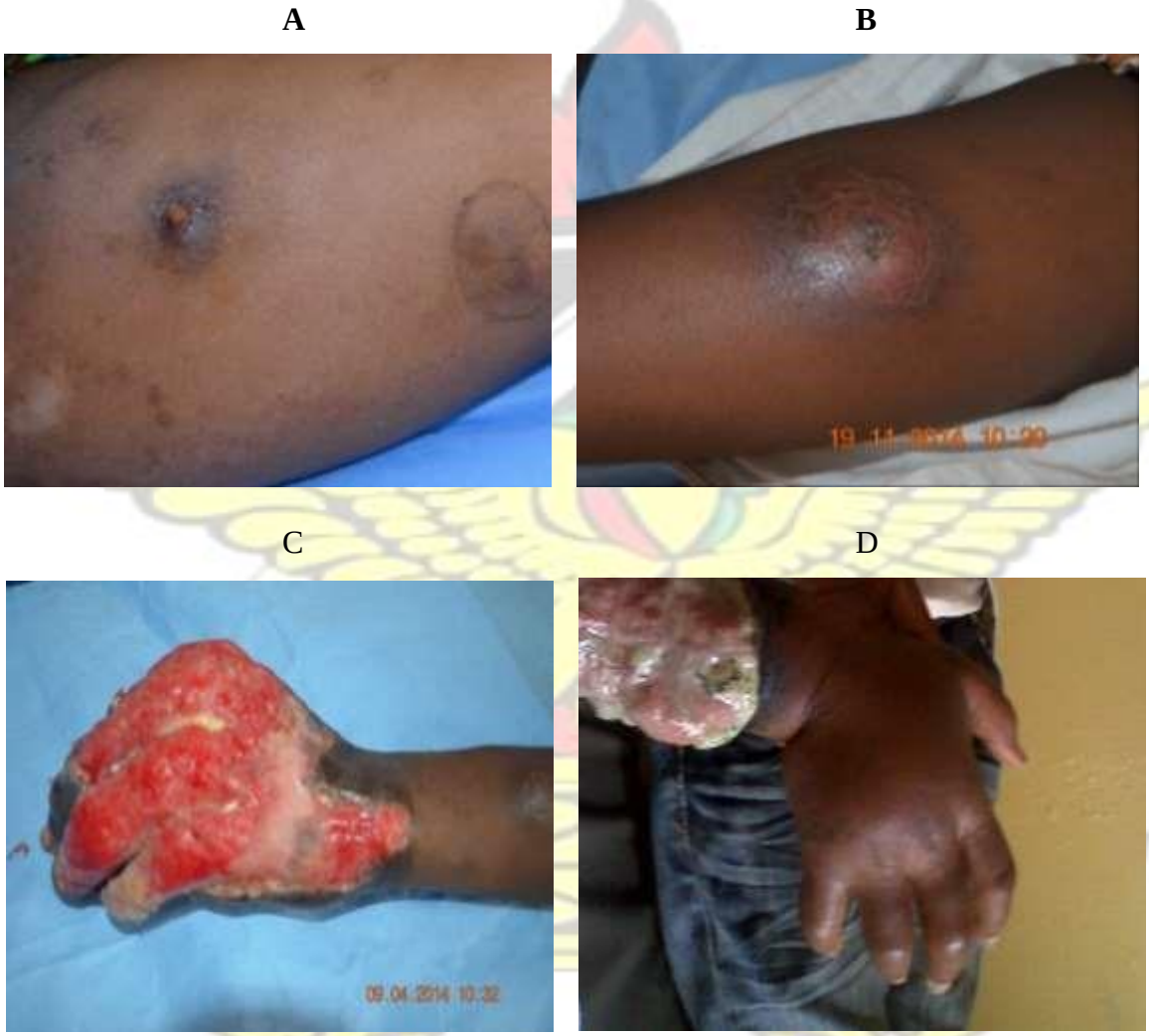

Figure 2: Clinical presentations of Buruli ulcer; A: Nodule B: Plaque C: Ulcer D:

Ulcerated edema

2.3.3 Mycolactone; the virulent factor of *M. ulcerans*

M. ulcerans produces a toxin called mycolactone which is a requirement for the necrosis and the ulceration of the tissues. It is a polyketide immuno suppressive macrolide that plays a role in the virulence of the mycobacterium. Studies with mouse models have shown that the toxin diffuses into peripheral blood and causes cell death resulting in the characteristic features of the disease (Sarfo *et al.,* 2011). Mycolactone is said to be the promoter of the initial swelling that is seen with the disease. Its production is however thought to be affected by antibiotic treatment (Sarfo *et al.,* 2013).

2.4 Transmission

M. ulcerans is an environmental organism and reviews have shown that several routes of transmission may be involved depending on geographical settings and locations (Merritt *et al.,* 2010). Currently, the mode of transmission of BU is still a mystery even though several hypotheses and studies have shown that aquatic environments harbor the organism. Wetlands with humid and hot temperatures are associated with the endemic regions (Debacker *et al.,* 2006; Merritt *et al.,* 2010). In Africa, high prevalence has been recorded in countries like Uganda, Benin, Ivory Coast and Ghana in communities surrounded by water bodies (Van der Werf *et al.,* 1999; Raghunathan *et al.,* 2005). Activities around these water bodies therefore pose as risk factors for the disease (Pouillot *et al.,* 2007; Agbenorku *et al.,* 2012). Studies in Benin however indicated that altitude can also play a role in disease transmissions (Sopoh *et al.,* 2011). Some other studies have also identified aquatic organisms like fish and insects like mosquito as possible carriers or reservoirs for the bacterium (Eddyani *et al.,* 2004; Johnson *et al.,* 2007a). some other organisms including opossums and water bugs may also serve as carriers of *M. ulcerans* in their natural habitat and hence may also play a role in its transmission (Van der Werf *et al.,* 1999; Johnson *et al.,* 2005a)

2.5 Diagnosis

In endemic areas, it is easier for the clinician or the health worker to diagnose Buruli ulcer based on its distinctive clinical presentations. It usually presents with an undermined ulcer or a firm swelling on the part of the body, usually on the limbs, whereas in areas of low endemicity, further confirmation has to be done in the laboratory after clinical diagnosis. However, according to the WHO, all clinically suspected cases should be confirmed by at least two of the four Laboratory methods (Johnson *et al.*, 2007b; Herbinger *et al.*, 2009). Confirmation helps in identifying Buruli ulcer and ruling out other tropical diseases that have similar symptoms and presentations such as cutaneous leishmaniasis, malignant ulcer and yaws (Guarner *et al.*, 2003; Phillips *et al.*, 2005). Laboratory confirmation also helps in determining the prevalence and incidence of the disease in a new endemic area. There are currently four laboratory confirmation methods which are:

a. Microscopy (direct smear for acid fast bacilli)
b. Histopathology
c. In vitro culture
d. Polymerase chain reaction (Amplification of the *IS2404* sequence of the mycobacterium DNA (Guarner *et al.*, 2003; Herbinger *et al.*, 2009).

All four methods have varying sensitivities and specificities with PCR having the highest sensitivity and specificity of 98% to 100% followed by histopathology of 82%. Microscopy and culture have the lowest sensitivities of 42% and 49% respectively (Herbinger *et al.*, 2009; Phillips *et al.*, 2009b).

2.5.1 Sample collection and transport

When surgery was the recommended treatment, samples taken for confirmation were tissue specimen from excised tissues or punch biopsies and swab samples for all four types of confirmation methods. The biopsy is a more painful and an invasive method hence the WHO

recommended the use of Fine Needle Aspiration (FNA) which was considered less invasive and less painful based on studies by Phillips et al in 2009, (Eddyani *et al.*, 2009). They also confirmed its sensitivity when used for PCR as higher compared to the punch biopsies. Samples for confirmation (diagnosis) are taken based on the clinical form of the lesion presented. FNA is taken when the lesion presents in the pre-ulcerative forms and a swab sample taken from the undermined edges of the ulcerative lesions. Tissue samples are taken for histopathology tests either from excised tissues from surgery or punch biopsies in some cases. The samples are then transported in various transport media depending on the method being used to the laboratories (Portaels and WHO, 2014).

2.5.2 Direct Smear Examination

Also known as the microscopy method, the direct smear examination uses the Ziehl Neelsen stain for acid fast bacilli to stain the mycobacterium. It however has the lowest sensitivity. Since other bacteria or artifacts may be present on the slide and also pick up the carbol fuscin stain; it is difficult to use for *M. ulcerans* diagnoses (Yeboah-Manu, 2006b). It is however the cheapest and easiest method of diagnosis as it can be done at the health centers without necessarily transporting samples to referenced laboratories (Yeboah-Manu *et al.*, 2011a; Frimpong *et al.*, 2015).

2.5.3 In vitro Culture

In vitro culture is one method that enables the isolation of viable mycobacteria in vitro, an important aspect in the laboratory diagnosis of *M. ulcerans* infection (Palomino and Portaels, 1998). Its sensitivity as stated by many studies is very low but could be used as a confirmatory test and within control trials (Herbinger *et al.*, 2009). The incubation period of *M. ulcerans* ranges from a period of 6 weeks to12 months (Portaels and WHO, 2014) and this could delay diagnosis and treatment. The organism is grown on egg proteins, the most common one being Löwenstein-Jensen (L-J) slants (Eddyani *et al.*, 2008). Due to its slow growing nature,

many other fast growing bacteria and fungus outgrow it, the reason why samples from the field need decontamination before culturing (Stienstra *et al.*, 2003). Some of the decontamination methods are however harsh to the *M. ulcerans* while some are not able to remove the unwanted organisms (Portaels *et al.*, 1997).

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2.5.4 Histopathology

Though very rigid, histopathology has a low detection rate, with sensitivity lower than PCR but higher than microscopy and culture (Stienstra *et al.*, 2003). It has characteristic features that can be used to describe the various stages of Buruli ulcer disease, from the pre-ulcerative stage to the ulcerative stage. These characteristics are however not specific through the progression period and depends on the quality of specimen taken (Guarner *et al.*, 2003; Herbinger *et al.*, 2009; Portaels and WHO, 2014). One other disadvantage of using this method is the use of invasive procedures in getting samples (punch biopsies) especially for the pre-ulcerative lesions. For the ulcers, surgically excised tissues are used taken from the edges of the entire thickness of the skin (Portaels *et al.*, 1997).

2.5.5 Detection of *M. ulcerans* DNA by PCR

Of the four diagnostic methods available, detection of *M. ulcerans* DNA by PCR gives the highest sensitivity of about 98% and specificity of 100% (Phillips *et al.*, 2005; Herbinger *et al.*, 2010). It has therefore become the gold standard in the diagnosis of Buruli ulcer (YeboahManu *et al.*, 2011a). It was developed based on the identification of some sequences which include *IS2404* insertion sequence, the 16SrRNA gene and the hsp65 gene (Stienstra *et al.*, 2003). But the most common targeted is the *IS2404* repeat sequence in the *M. ulcerans* DNA

(Stinear *et al.*, 1999) and it occurs 249 times in the genome (Phillips *et al.*, 2005), which increases the sensitivity. Because the cell wall of the mycobacterium is very hard, various rigorous methods of DNA extraction are employed commercially and in house; automated and semi-

automated (Durnez *et al.*, 2009; Affolabi *et al.*, 2012). Care is however taken not to destroy the DNA before its use in amplification. Though this method has the highest sensitivity and specificity, it is very expensive to run and requires sophisticated equipment and laboratory as well as highly trained staff. It has therefore not been used as a routine confirmatory test in endemic areas, but as a research tool and a means of confirming otherwise difficult or confusing lesions (Yeboah-Manu *et al.*, 2011a).

2.6 Treatment

There are currently two treatment methods for Buruli ulcer disease, surgical excision mostly for extensive lesions together with antibiotic treatment, for the pre-ulcerative lesions.

2.6.1 Surgery

Not until 2003, surgery was the recommended treatment for Buruli ulcer disease. This is achieved by excising all the affected tissue surrounding the lesion and skin grafting. There was however a high recurrent rate ranging from 16% - 28% as indicated by studies (Debacker *et al.*, 2005; Kibadi *et al.*, 2009)It was also considered an invasive procedure.

In addition, the cost of treatment using the surgery was very high as most patients required more than one surgery for complete healing (Agbenorku *et al.*, 2012). With the introduction of the drug therapy, surgical excision and skin grafting have only become alternatives for extensive lesions (Phillips *et al.*, 2009a).

2.6.2 Antibiotics Treatment

Over the years, major advances had been made in the development of antibiotics for the treatment of Buruli ulcer disease (Etuaful *et al.*, 2005; Phillips *et al.*, 2009a). In 2004, the WHO recommended antibiotics treatment with the combination of rifampicin and an aminoglycoside (streptomycin or amikacin) to be taken for a period of 4–12weeks (WHO, 2013). This was seen to reduce the cost of surgical treatment and reduce the length of stay in the

hospitals (Yeboah-Manu *et al.*, 2013). Initial studies tested various antibiotic combinations including clofazamine and cotrimoxazole but these were not effective against the ulcers. Another combination that was tried was rifampin and dapsone which had little effect on the ulcers. Further studies later shown that a combination of rifampin and the aminoglycosides, macrolides and quinolones were effective against *M. ulcerans*. Some were bactericidal while some were bacteriostatic (Etuaful *et al.*, 2005). Rifampin and streptomycin when administered for about 4 weeks was found to reduce lesion surface area by about 50% thereby reducing rate of surgical excision (Chauty *et al.*, 2007). From 2006, WHO had recommended an 8 week course of rifampicin and streptomycin after other studies showed that *M. ulcerans* could not be cultured from the lesions after the start of the drug treatment (WHO, 2012; Yeboah-Manu *et al.*, 2013). Currently the recommended drug course for the treatment of Buruli ulcer disease is 8 weeks of rifampincin and streptomycin or rifampin and clarithromycin with skin grafting in the case of extensive lesions (Phillips *et al.*, 2009a; WHO, 2012).

2.7 FTA Technology

Flinders Technology and Associates (FTA)® cards, also known as Fast Technology for analysis of nucleic acids cards was designed at Flinders University in Australia by Burgoyne and Fowler (NFSTC, 2005). The main aim for developing the card was to protect nucleic acids from degradation. The idea was based on coating the card with chemicals that will denature proteins as well as nuclease the main degraders of nucleic acids as well as inhibitors (Whatman, 2009). The chemical composition includes a weak base, a chelating agent, anionic surfactant or detergent and uric acid or a urate salt which are applied to a cellulose based matrix which is the filter paper (NFSTC, 2005; Miles and Saul, 2011). The role of these chemicals is to lyse the cells of the applied samples upon contact with the card, denature proteins, destroy nucleases, remove contaminants and inhibit fungal growth and other microorganisms (NFSTC, 2005; Sudhakaran *et al.*, 2009; Whatman, 2009; GE Healthcare, 2010). Other forms of

protection offered by the cards are from damage from oxidation and ultraviolet light (Rajendram et al., 2006; Sudhakaran et al., 2009). The inactivation and removal of nucleases results in the stability of the DNA after drying samples properly (NFSTC, 2005). Biological samples that can be applied to the card include saliva, blood, buccal cells, plant materials, bacteria, cultured cells and plasmids as well as solid tissues (GE Healthcare, 2011). Through a mechanism of entanglement, the lysed DNA is captured into the matrix upon application of the samples. This adhesion is strong enough to make samples captured on classic cards purified while the DNA is still bound to the matrix (Moscoso et al., 2004; Miles and Saul, 2011). There are different types of FTA cards with specific uses, which are suitable for specific applications. Some designs change color upon application of the samples and sample specific cards (GE Healthcare, 2011).

2.7.1 Features of FTA cards

The FTA cards are characterized by special features which are:

i. The ability to store nucleic acid samples at room temperature and keep them stabilized to yield quality DNA for analysis and archiving for a long time (Saieg *et al.*, 2012; Santos *et al.*, 2012).

ii. Simplicity of sample collection that enables its use in the laboratory and in the field without the need for trained persons (Subrungruang *et al.*, 2004; de Bie *et al.*, 2011; Saieg *et al.*, 2012).

iii. Its ability to be automated speeds up punching and subsequent purification (Aye *et al.*, 2011) (Vitha and Yoder, 2005).

iv. Its small size enables packaging and transport of samples over distances (de Bie *et al.*, 2011; Linhares *et al.*, 2012).

v. Ability to lyse cells upon application and drying them rendering them non- hazardous to transport and work with (Moscoso *et al.*, 2004; GE Healthcare, 2011; Al-Kzayer *et al.*, 2012).

vi. The versatility of the chemical composition allows for different sample types to be stored on it (Rajendram *et al.*, 2006; Whatman, 2009).

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2.7.2 Applications and challenges

Due to these features of the FTA card, they have been explored in many fields and they yield good results. Some of the applications include the use in medical diagnostics where diagnosis is based on molecular techniques. The presence or absence of the disease is tested by the detection of the genomic material of the causative organism (Aye *et al.*, 2011; Braae *et al.*, 2013; Geraets *et al.*, 2013). Another area of application is genetic identification of organisms including plants and animals as well as microorganisms (Heim *et al.*, 2012; Yee *et al.*, 2013). In addition to these, the cards are used for storage and archiving of nucleic acids for future use and transport to research laboratories especially in deprived areas (Al-Kzayer *et al.*, 2012; Gonzalez *et al.*, 2012; Saieg *et al.*, 2012). The card has also been used to extract some RNA materials but it cannot be used for culture or cultivating viable organisms (Rajendram *et al.*, 2006). Though the card has been explored for all its good qualities, it has its own challenges that have to be addressed. Due to the entanglement and the adhesion of the nucleic acid to the matrix of the card, downstream analysis is done whiles the sample is still attached to the card. This is however not suitable in analysis requiring the elution of DNA. Some methods use local extraction methods to elute the DNA but the yields are usually too small (GE Healthcare, 2010; Stangegaard *et al.*, 2011). Improvements have been made to increase the yields and the quality of DNA eluted from the cards which include the use of in house extraction procedures and other vigorous procedures as well as repeated extractions (Johanson *et al.*, 2009; Whatman,

2009; Borman *et al.*, 2010; Stangegaard *et al.*, 2011). Other kits have been produced to help in these procedures including Chelex and prepGEM (GE

Healthcare, 2010; Ahmed *et al.*, 2011; Miles and Saul, 2011). The introduction of proteinase K treatment has also been used to increase the yields of DNA (Johanson *et al.*, 2009). This sometimes makes the use of the cards more laborious.

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2.7.3 DNA Integrity

Detection of nucleic acid materials has become an important tool in many medical practices and scientific studies (Roder *et al.*, 2010). This requires the use of quality samples for good results especially in its use for diagnostics. Samples are usually frozen for long term preservation. But this is sometimes affected by freeze and thaw during sample retrievals and unstable power supplies. In most developing countries, access to storage instruments is very difficult and samples are mostly transported to reference laboratories far from the field in liquid media. These pose as biohazards and temperature fluctuations during transport may affect the integrity of the nucleic acids. These challenges can be avoided by the use of FTA cards that have been designed to protect and stabilize nucleic acids at ambient temperature over a long period of time.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Design

A cross-sectional study was conducted from October 2014 to March 2015 in four endemic sites. Study specific data collection forms as well as standard Buruli Ulcer treatment forms (BU01) were used for collecting demographic data.

Ethical clearance was obtained from the Committee for Human Research and Publications and Ethics (CHRPE), School of Medical Sciences, KNUST / Komfo Anokye Teaching Hospital as part of a larger study on the "Pathogenesis and management of *M. ulcerans* disease" (CHRPE/AP/229/12). Informed consent was sought from the eligible patients and all data collected was treated as confidential.

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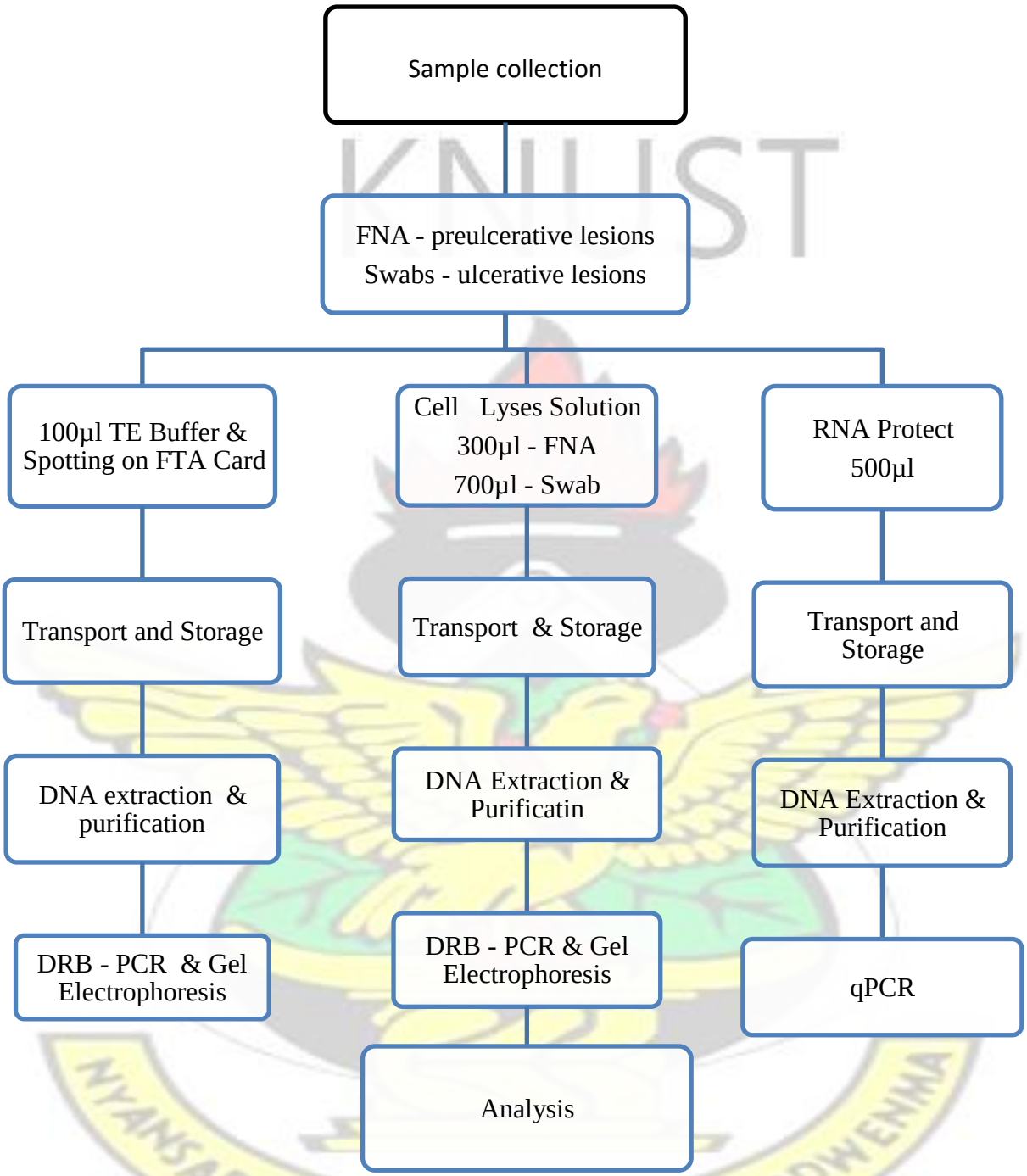
3.2 Study Sites

The study was conducted at four main BU treatment centers namely: Agogo Presbyterian Hospital in the Asante Akim North District of the Ashanti region, Tepa Government hospital in the Ahafo Ano North district of the Ashanti Region; Nkawie Government Hospital, in the Atwima Mponua district of the Ashanti region and the Dunkwa Government Hospital in the Central region. These centers serve surrounding villages where Buruli ulcer is most endemic.

3.3 Study Population

The study included patients that reported to the Buruli treatment centers with clinically suspected lesions, mostly from the endemic communities. There was no age limit to recruitment since routinely any clinically suspected lesions must be confirmed in the laboratory in order for treatment to begin.

Figures 3: Flow chart to study procedure.



3.4.1 Sample Collection.

For every patient reporting to the clinic, a PCR confirmatory test was required to confirm *M. ulcerans* infection. Samples were taken based on the clinical form presented. Duplicate sample

specimen was obtained by fine needle aspirate for the pre-ulcerative forms and swab sample for ulcerative lesions. As recommended by the WHO, at least two confirmatory tests were done,- microscopy method after slides were stained by the Ziehl Neelsen technique for acid fast bacteria and PCR for the detection of the insertion sequence *IS2404* as well as qPCR for the same insertion sequence.

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3.4.2 FNA Samples

For nodules, plaques and oedema which form the pre-ulcerative forms of BU, an FNA was taken using a 5ml syringe and a 21 gauge needle. The lesion site was cleaned with gauze or cotton dipped in alcohol to disinfect the site. The lesion was palpated and the center of the lesion was determined. This was where the needle was inserted targeting the subcutaneous tissue with the adipose tissue location in mind since these are places the organism was likely to be found. Suction was applied and with the needle adjusted in three different angles, fluid was drawn from the lesion. Care was taken not to draw blood into the syringe. The needle was flushed into 100ul of TE buffer (1mM EDTA in 10 mM Tris-HCl, pH 8.0), in sterile 2ml skirted tubes with an O ring (Sarstedt, United Kingdom), for spotting on to the FTA card. A new syringe was used for another sample following the same procedure and dispensed into another tube containing 300ul of cell lysis solution (CLS) and a third containing 500ul of RNA protect for conventional PCR and qPCR respectively. A slide was also prepared for microscopy by smearing the sample on a glass slide before washing the needle in the transport media and the frosted end of the slide was labeled with pencil.

3.4.3 Swab Samples

As *M .ulcerans* are typically found in the undermined edges of ulcerative lesions, specimen for laboratory confirmation was taken by swabbing these areas using sterile cotton swab. This was done by rotating the swab in a clockwise and a downward manner and cleaning the tissues

within the undermined edges of the ulcer. Four swabs were taken and distributed in sterile 2-ml skirted tubes with an O ring (Sarstedt, United Kingdom). The tubes contained 100ul of TE buffer for spotting on the FTA card, 700ul cell lysis solution for conversional PCR and 500ul of RNA Protect solution for qPCr. The last one was smeared on a slide for microscopy staining.

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3.4.4 Sample transport and storage

The samples were transported to the Kumasi center for Collaborative Research in Tropical medicine, (KCCR), KNUST where they were processed for storage and further processing. CLS samples were inactivated at 95^{o}C for 15 minutes and stored at 4^{o} C for further processing.

The FTA samples were spotted on the card with their identification numbers and allowed to dry for 5 minutes at ambient temperature to prevent fungus growth. Since each card stored two patient samples, care was taken not to cross contaminate the samples and fingerprint the card. The dried cards were kept in tightly closed zip lock bags.

Samples transported in RNA Protect were stored at -80^{o}C after they were incubated at ambient temperature and vortexed for 10 seconds every minute for 5 minutes. They were then centrifuged at 5000g for 7 minutes and the supernatant poured away leaving the pellet containing the DNA.

3.5 DNA Extraction

3.5.1 FTA CARDS

Spotted areas of the cards were sectioned into four for FNA samples and two for swab samples. A section was cut out of the card using scalpel on a Harris Micro-Punch™ and transferred into labeled 2ml reaction tubes containing 200µl of TE buffer in duplicates. The scalpel was

disinfected between the cuts using 70% alcohol in Alprep alcohol swabs to prevent cross contamination. DNA was eluted from the card by adding 15µl lysozyme (10mg/ml) to each sample, incubated and vortexed for 10seconds every minute for 5 minutes at ambient temperature. The samples were then transferred into a themomixer set at 37oC and incubated for 1 hour at high speed (800rpm). After this 10µl Proteinase K (20mg/ml) was added and the sample was vortexed for 15 seconds a minute for 5 minutes, incubated in the thermomixer at 55oC for four hours (or overnight) until complete cell lyses and DNA elution. The proteinase K was inactivated by incubating in the thermomixer at 80oC for 20 minutes and samples cooled down to room temperature. Samples were cooled further on ice for 5 minutes after which 230µl of Protein Precipitation Solution (PPS) (Qiagen, USA) was added to each sample and vortexed vigorously at high speed for 20 seconds to ensure uniform mixing with the PPS. They were then placed on ice for 5 minutes and centrifuged at 13000rpm for 5 minutes. During the centrifugation, 2ml reaction tubes containing 700µl of isopropanol and 2µl of glycogen (20mg/ml) (Qiagen, USA) were prepared for the respective samples. The supernatant of the lysate + PPS containing the eluted DNA was poured into the tube with the isopropanol leaving behind any protein pellet that was precipitated. The samples were then mixed thoroughly by inverting the tubes gently 50 times and then centrifuged at 13000rpm for 5 minutes. The supernatant was poured off and 700µl of 70% ethanol was added to the pellet and inverted about 5 times and then centrifuged at 13000 rpm for five minutes. The supernatant was poured off leaving behind the pelleted DNA which was drained on an absorbent paper towel and air dried for 1 hour. The DNA was then hydrated by adding 200µl of hydration solution (Qiagen, USA) and carefully pipetting up and down for

20 times and incubated in a themomixer at 65oC for 1 hour. After hydration it was stored at 4-8^{o}C until PCR was set up.

3.5.2 CLS Samples

CLS with FNAs were treated differently from those with swabs. Swabs were treated as the FTA cards thus cells were lysed by the addition of 15µl lysozyme and incubated for 1 hour at 37^{o}C without the vortexing after which the 10µl of proteinase K was added and incubated at 55^{o}C for 4 hours (or overnight) at low speed. FNAs however were lysed by the addition of the Proteinase K first for 4 hours without vortexing and at lower speed in the thermomixer (Eppendorf AG thermomixer compact) before the addition of the lysozyme. PPS was added in the volumes of 230µl for swabs and 100µl for FNAs. The proceeding steps were followed as done for the FTA. During hydration, 50µl was added to the FNAs whiles the swabs took 200µl.

3.5.3 RNA Protect Samples.

Frozen pellets were thawed on ice and 100µl CLS + Lysozyme buffer was added as well as 20µl Proteinase K to each pellet on ice and vortexed for 15 seconds. Samples were incubated at ambient temperature for 5 minutes and vortexed 15 seconds every minute, and later incubated in a thermomixer at 45^{o}C for 5 minutes at high speed. Buffer RLT Plus containing β- Mecapto Ethanol (Qiagen, Hilden, Germany, ref# 80284) was then added in 350µl and vortexed vigorously for 15 seconds. Lysate was then transferred to labeled Allprep qiashredder and centrifuged at high speed for 2 minutes to homogenise the lysate. The homogenized lysate was transferred from the collection tube into labeled AllPrep DNA spin columns (Qiagen, Hilden, Germany, ref# 80284) in a labeled 2ml collection tube. This was then centrifuged at 9000g for 30 seconds at ambient temperature to allow all liquid to flow through the membrane into the collection tube. The spin columns were then transferred to new collection tubes and 500µl Buffer AW1 (Qiagen, Hilden, Germany, ref# 80284) was added, centrifuged at 9000g for 15 seconds and flow through discarded. Spin columns were placed in new tubes and 500µl of buffer AW2 (Qiagen, Hilden, Germany, ref# 80284) was added and centrifuged at 9000g for 2 minutes. Flow through was discarded and spin column was centrifuged again at 9000g for 1

minute to ensure complete drying. Spin columns were placed in labeled 1.5ml reaction tubes and 50µl of buffer EB (Qiagen, Hilden, Germany, ref# 80284) preheated to 70^{o}C was added directly onto the spin column membrane. These were incubated at room temperature for 2 minutes and centrifuged at 9000g for 1 minute, the spin columns were then discarded leaving the eluent which contained the DNA. The samples were then stored at 4 - 8°C for subsequent *IS2404* qPCR.

3.6.1 Dry –Reagent Based PCR for *IS2404*

For the DRB – PCR, oligonucleotides MU5 (5' aaagcaccacgcagcatct 3') (TibMolbiol, Berlin, Germany) and MU6 (5' cggtgatcaagcgttcacga 3')(TibMolbiol, Berlin, Germany) were lyophilized in 200ul reaction tubes. Lyophilisation was carried out in an RCV 2-25 vacuum concentrator (Christ, Osterode, Germany).

PuReTaq Ready-To-Go PCR Beads (GE Healthcare life Sciences, Freiburg, Germany) were added and dissolved in 22.5µl DNAse free water in the reaction tubes with the lyophilized primers. 2.5µl of extracted DNA was added to make the total volume of the reaction mixture 25µl. The PCR also included negative extraction control which monitored the extraction process, a positive control, a negative PCR control and inhibition controls which checked false negative results due to inhibition. Inhibition control samples were prepared by adding 1.25µl of patient sample and 1.25µl of a known positive sample to the reaction mix. Thermal cycling was done in Eppendoff Mastercycler (Applied Biosystems, USA) using the protocol as follows: 94^{o}C for 10 min, followed by 40 cycles at 94^{o}C for 10 s, 58^{o}C for 10 s, and 72^{o}C for 30 s, with a final cycle at 72^{o}C for 15 min. The amplification products were held at 4^{o}C until they were processed further by agarose gel electrophoresis.(Siegmund *et al.*, 2007).

3.6.2 Agarose gel Electrophoresis

A 1.5% agarose gel was prepared by bringing to boil 1.8g of agarose in 120ml of 0.5x Tris

Boric EDTA (TBE) (Tris 53g, boric acid 27.5g, 0.5M EDTA 20ml pH 8.0 in 1liter water) in a 500ml conical flask placed in a microwave oven for 4 minutes at 750 watt. It was then cooled to about 50^{o}C and 10ul gel red was added and mixed gently and carefully to avoid bubbles. The electrophoresis tray was sealed with a sealing tape to prepare it for the liquid and spacer combs placed at their location on the tray. The gel was poured into the tray gently to avoid bubbles and if any it was removed using pipette tips, it was then allowed to solidify at room temperature. The combs were removed and the tray with the gel was placed in the electrophoresis tank already containing about 800ml 0.5x TBE. The PCR product was loaded after adding 3µl of loading dye to 15µl of the product into the respective sample slots. A 6µl of 100base pair DNA ladder (Invitrogen USA) was then loaded into the first slot that was reserved for it per every row. The negative, positive and inhibition controls were also loaded and the tank connected to power supply (50 Watts) for 45 minutes at 100 volts. The DNAs were visualized under UV light and photographed using Infinity VX2, a 2 megapixel image software with an exposure time of 15 seconds. The bands corresponding to the patients sample were compared with the positive control which produces a band of 492 bp length; a negative reaction produces no band. Results of diagnostic samples were viewed in combination with the respective inhibition controls. Results were recorded as positive, negative or inhibited.

3.6.3 *IS2404* qPCR

For the qPCR , the following primers were used in the preparation of the reaction mix; Primer *IS2404* TF1: 5' aaa gca cca cgc agc atc t'3 (TibMolBiol, Berlin, Germany) ,- Primer *IS2404* TR1: 5' agc gac ccc agt gga ttg'3 (TibMolBiol), - Probe *IS2404* TP2: 5' FAM-ccg tcc aac gcg atc ggc a-BBQ'3 (TibMolBiol), 5x HOT FIREPol® Probe qPCR Mix Plus (no ROX), 1 ml (SolisBioDyne, Tartu, Estonia, ref# 08- 15-00001).

The reagents were thawed and a master mix of all reagents prepared. A TaqMan exogenous internal positive control (IPC) (VIC, 200 rct. Invitrogen, Karlsruhe, Germany) was used in each

amplification reaction to exclude false negative results due to inhibition. Cloned *IS2404* standards were used as positive run controls for the *IS2404* assay. To exclude contaminations with *M. ulcerans* DNA no template controls (containing H2O instead of template; one control for each assay) and negative extraction controls (*IS2404* qPCR) were processed in the same way like the samples. The Master Mix was prepared in a 1.5 ml reaction tube for each assay. Exo IPC DNA was added to the Master Mix in the DNA extraction laboratory. PCR tubes were filled with 18µl of the reaction mix according to the sample size (including all controls) and 2µl of template DNA was added to give a total reaction volume of 20µl and centrifuged at 5000 x g for 2 min. A BioRad CFX96 was programmed according to the manufacturer's specifications using the following settings: Initial Denaturation at 95°C for 15 minutes in 1cycle, Denaturation at 95°C for 15 seconds and Annealing & Extension at 60°C for 60 seconds all in 40cycles. The detection of channels VIC and FAM was activated for all wells following each cycle after annealing and extension. The PCR tubes were transferred to the BioRad CFX96 and the program launched. The results were read from the FAM signal, a positive result gave a threshold cycle of less than 40.0.

3.7 Preparation of slides for microscopy

The smears were heat fixed on the slide by passing a lighted cotton swab soaked in drops of 70% ethanol while the slides were placed on staining racks. They were then flooded with Ziehl Neelsen carbol-fuchsin that had been filtered through a funnel with a filter paper prior to use. The slides were heated using ignited cotton swab until steaming, and allowed to stand for 5 minutes. The stain was rinsed gently under clean running water and the slides flooded again with a decolorizer (20% H_2SO_4) for 3 minutes and rinsed thoroughly with water. A secondary stain (0.1% methylene blue chloride was used to flood the slides and left for 2 minutes, rinsed

with water and air dried. The slides were examined under the x100 oil immersion objective. Positive slides had red colored rod-like bacilli.

3.8 Determination of the Detection limit of PCR for *M. ulcerans* using FTA cards. 100µl of *M. ulcerans* suspension of known concentrations were spotted onto the FTA cards and left to dry at room temperature for two hours. The bacteria suspensions were in TE-buffer with the following concentrations:

i. 10^5bacteria/ml ii. 10^4bacteria/ml

iii. 10^3baceriat/ml

iv. 10^2 bacteria/ml

v. 10 bacteria/ml vi. TE-Buffer(with no bacterium/ml) Table 1 shows the absolute number of bacteria spotted and examined by PCR

Table 1: Absolute number of bacteria spotted and examined by PCR

Dilution	No. Bacteria/ 100µl	No. Bact/1/8 of spotted area
10,000 bact/ml	1000	125
1,000 bact/ml	100	13
100 bact/ml	10	1
10 bact/ml	1	0

0 bact/ml	0	0

The spotted areas were marked out with lead pencils and divided into sections of eight and treated as follows:

A) Direct amplification

A section (1/8 of spotted area), corresponding to 1250 bacteria for 10,000 bacteria / 100µl, 125 bacteria for 1000 /100µl, 13 bacteria for 100 /100µl and 1bacterium for 10 bacteria /100µl of each concentration was placed directly into the DRB- PCR master mix without any purification process. Negative control was set up as well with the card spotted with only 100µl TE buffer without any sample added. Its absolute number of bacteria was estimated to be zero. Amplification was done according to the DRB-PCR protocol but the volume of DNAse free water was increased to 25µl since the sample in this case was not liquid and enough to keep the card in solution. Gel electrophoresis was done using 15µl of solution around the card.

B) In- house extraction and purification

A portion of the sectioned areas ($1/8^{th}$ of the area) of each concentration was placed in a 2ml reaction tube containing 100µl of TE buffer, and 15µl of lysozyme added, incubated in a thermomixer for 1 hour at 37^{o} C, Proteinase K was then added and incubated for 4 hours at 55^{o}C, inactivated at 80^{o}C for 20 minutes to complete the cell lyses and DNA elution process. The purification process was followed as described in section 3.5.1 for the samples as well as for the PCR and the gel electrophoresis. Results were recorded as the presence and absence of a band against a positive control and bacteria numbers extrapolated for the least number that can be amplified from a card.

3.9 Statistical analysis

Statistical analysis was done using Microsoft Excel, Stata version12.0 software and GraphPad prism 5 software programs. Descriptive statistics was used for analysis of median values. Fisher's Exact test was used for comparison between two proportions, determination of sensitivity and specificity.

P value <0.05 was considered significant.

CHAPTER 4

RESULTS

4.1 Demographics of study participants

The demographic characteristics of 53 suspected Buruli ulcer study participants are shown in Table 2. There were 25 (47%) males and 28 (53%) females. 26 (49%) patients had preulcerative lesions and 27 (51%) had ulcerative lesions. The pre-ulcerative lesions comprised 17 (65.4%) nodules, 3 (12%) edema and 6 (23.1%) plaques.

The median age was 14years ranging from 2years to 87years. There was no significant difference in the age at presentation when ulcerative lesions were compared with preulcerative lesions (p value <0.305). For the different forms of lesions, the median age was 13 years for nodular forms but 15 years for edema, plaque and ulcer forms (range 2-67years)

Category I lesions had the highest representation of 34 (64%) when compared with 16 (30%) for category II and 3 (5.7%) for category III. Lesions on the lower limb 38 (72%) were more common than those on the upper limb 12 (23%).

2 : Demographics of study participants

Characteristic	No. (%) of Preulcerative Lesions n=26	No. (%) of Ulcerative Lesions n=27	No. (%)Total lesions n=53	P value
SEX				
Male	15(60)	10 (40)	25 (47.2)	0.132*
Female	11(39)	17 (61)	28 (52.8)	
Age in years				
Median (Range)	13(3 -85)	15(2-67)	14 (2 - 85)	0.305**
Lesion Category				
I	18 (53)	16(47)	34	
II	8(50)	8(50)	16	0.212
III	0(0)	3(100)	3 (5.7)	
Lesion Site				
LL[a]	17 (44.7)	21(55.2)	38 (71.7)	
UL[b]	8 (66.7)	4(33.3)	12 (22.6)	0.355
Others[c]	1 (33.3)	2(66.7)	3(5.7)	
Lesion Type				
Nodules	17(100)	0(0)	17 (32.1)	
Edema	3(100)	0(0)	3(5.7)	NA
Plaque	6(100)	0(0)	6 (11.3)	
Ulcer	0(0)	27 (100.0)	27 (50.9)	

*Mann Witney Test; **fisher exact test; [a]Lower Limb; [b]Upper Limb; [c]Other parts of the body

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Table

4.2 Determination of the detection limit of *IS2404* PCR for samples transported on FTA cards

Initial experiments focused on the determination of the detection limit of *M. ulcerans* transported on FTA cards by spiking the cards with known quantities of bacteria ranging from 1 to 125. After proteinase K treatment and DNA extraction, 2.5µl of sample was amplified in DRB PCR.

Table 3 shows that, 10, 100 and 1000 bacteria could be detected when spiked onto FTA cards and 1/8 of the spiked area extracted and run in DRB PCR. One bacterium in 1ml of solution placed on the cards could not be detected when 1/8 of the FTA spiked area was run in PCR. This suggests that in absolute numbers, 1 bacterium in a solution of 10 bacteria/ml 13, bacteria and 125 bacteria could be detected in DRB PCR. The result was negative when a negative control FTA card with no bacteria was amplified. This showed that the minimum detection limit was one bacterium.

3: Determination of the detection limit of PCR using FTA cards

Dilution	No. of Bacteria/ 100µl TEB*	Estimated No. Bacteria amplified in PCR**	PCR result
10,000 bacteria/ml	1,000	125	Positive
1,000 bacteria/ml	100	13	Positive
100 bacteria /ml	10	1	Positive
10 bacteria /ml	1	0	Negative

0 bacteria /ml	0	0	Negative

*--TEB –Tris EDTA Buffer; ** 1/8 of spotted area

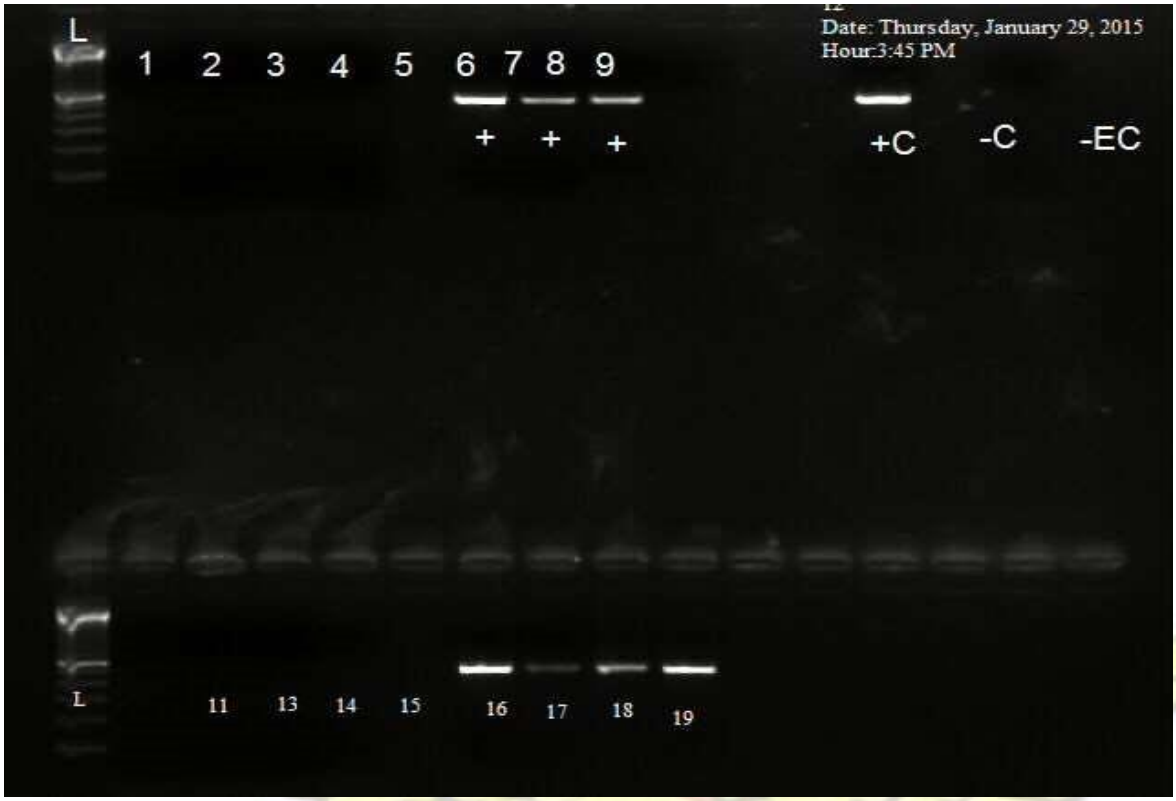

Figure 4: PCR Results for optimization test

4.3 The sensitivity of PCR for DNA templates from FTA cards, Cell Lysis Solution and RNA protect solution.

Samples for PCR are usually transported either in Cell Lysis Solution (CLS) or RNA protect solution. The sensitivity PCR of clinical samples obtained on FTA cards was compared with those obtained from CLS and RNA protect solution. To achieve this, clinical samples, FNAs or swabs were placed in cell lysis solution, RNA protect and FTA cards. After proteinase K treatment and DNA extraction, samples placed in cell lysis solution and FTA cards were

Table

amplified by standard PCR and those obtained from RNA protect buffer extracted using the RNA/DNA QIAGEN kit were amplified by real time quantitative PCR.

Table 4 shows a comparison of the sensitivity of PCR for samples obtained by FTA, cards, RNA protect buffer and CLS. FTA PCR had a sensitivity of 58% (95% CI 42.1% - 73.0%) by contrast to 94% (95% CI 82.3% - 99.4%) of RNA protect qPCR when compared with the standard CLS- DRB- PCR. The Specificity (95% confidence interval) for FTA PCR was 70% (34.8%- 93.3%) and that of qPCR was 67% (95% CI 22.3 - 95.7) when compared with CLS DRB PCR.

There were 44 patient samples for the RNA Protect qPCR as a result of unavailable samples for 9 patients.

4: Sensitivity of FTA PCR and RNA protect qPCR compared with CLS – DRB – PCR as the gold standard

FTA - DRB -PCR	CLS - DRB -PCR		TOTAL	SENSITIVITY% (95% CI)	Specificity% (95% CI)
	POSITIVE	NEGATIVE			
POSITIVE	25	3	28		
NEGATIVE	18	7	25	58 (42.1 -73.0)	70** (34.8 - 98.3)
TOTAL	43	10	53		

RNA Protect qPCR					
POSITIVE	36	2	38	94 (82.3 - 99.4)	67 (22.3 - 95.7)
NEGATIVE	2	4	6		
TOTAL	38	6	44*		

* Samples for 44 patients were run for qPCR, samples were not available for 9 patients; ** determined using Fisher's Exact test

4.4.1 Determination of sensitivity of FTA PCR with smear microscopy for Acid Fast Bacilli as the standard.

With the FTA DRB PCR showing a low sensitivity compared with the CLS-DRB PCR, the FTA PCR was compared with smear microscopy for Acid Fast Bacilli to determine its usefulness. This was achieved by determining the sensitivity after DNA extraction and DRB PCR of samples stored on the FTA cards and Ziehl Neelsen staining for acid fast bacilli for the smears as described in the methods (Section 3.7) for 39 patient samples.

Table 5 shows the sensitivity of FTA DRB PCR was 80 percent (95% CI 44.4% - 97.4%) and the specificity was 48% (95% CI 29.4% – 67.5%).when compared with Ziehl Neelsen microscopy for acid fast bacilli for 39 patients.

For 24 FNA samples obtained on the FTA cards, the sensitivity was higher 100% (95% CI 59.1% - 100%) when compared with those of swabs 33% (95% CI 0.84% - 91.0%). The specificity was similar for swabs 42% (95% CI 15.2% - 72.3%) and FNA 53% (95% CI

Table

28.0% - 77.2%) when compared with Ziehl Neelsen microscopy for acid fast bacilli.

The table shows that, 15 of 29 (51.7%) participants that had negative results with ZN microscopy were positive for FTA DRB PCR suggesting a higher sensitivity for FTA PCR. However, there were 2 of 10 participants that were positive for ZN microscopy but negative for FTA DRB PCR.

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5 Sensitivity of FTA DRB PCR against Smear microscopy for Acid Fast Bacilli and per sample type.

FTA - DRB – PCR	ZN- MICROSCOPY for AFB		TOTAL	SENSITIVITY % [95% CI]	Specificity% (95% CI)
	POSITIVE	NEGATIVE			
POSITIVE	8	15	23	80 (44.4 -97.4)a	48 (29.4-67.5)*
NEGATIVE	2	14	16		
TOTAL	10	29	39		
FTA- DRB PCR SWAB					
POSITIVE	1	7	8		
	2	5	7		
NEGATIVE				33 (0.84 - 91.0)	42 (15.2 - 72.3)
	3	12	15		
TOTAL					

FTA- DRB PCR FNA					
POSITIVE	7 0	8 9	15 9	100 (59.1 - 100)	53 (27.8 - 77.2
NEGATIVE					
TOTAL	7	17	24		

*determined using Fisher's exact test.

4.4.2 A comparison of FTA PCR and smear microscopy for Acid Fast Bacilli with standard CLS DRB PCR

The sensitivity of samples confirmed by the FTA DRB PCR and smear microscopy methods were compared with the CLS DRB PCR which is the gold standard for the laboratory diagnosis of Buruli ulcer. This was done by extracting the DNA from 39 samples made up of both swabs and FNA stored on the FTA cards and CLS after proteinase K treatment and amplified by the Dry Reagent Based standard PCR. The smears were stained by the Ziehl Neelsen method for acid fast bacilli and examined under x100 oil immersion objective.

Table 6 shows the sensitivity of the FTA DRB PCR and ZN microscopy compared with the standard CLS DRB PCR. Comparatively, FTA PCR had a higher sensitivity of 58% (95% CI 42.1% - 73.0% and a specificity of 70%, (95% confidence interval of 34.8% - 98.3%) whilst the microscopy had a sensitivity of 29% (95% CI 15.1% - 47.5%) and a specificity of 100% (95% confidence interval of 47.8% - 100%).

This suggests that though the microscopy had a lower sensitivity, it had a higher specificity.

6 Sensitivity of FTA PCR and Smear microscopy against the standard CLS PCR.

Table

FTA - DRB -PCR	CLS - DRB -PCR		TOTAL	SENSITIVITY % [95% CI]	Specificity% (95% CI)
	POSITIVE	NEGATIVE			
POSITIVE	25	3	28		
NEGATIVE	18	7	25	58 (42.1 -73.0)a	70 (34.8 - 98.3)a
TOTAL	43	10	53		
ZN MICROSCOPY for AFB					
POSITIVE	10	0	10		
NEGATIVE	24	5	29	29 (15.1 - 47.5)	100 (47.8 - 100)
TOTAL	34	5	39		

*****Fishers exact test.

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4.5 Determination of the positivity rate of samples stored on FTA cards for different durations

To determine the ability of the FTA cards to store DNA for longer periods and stabilize it as indicated by the manufacturers and established by many studies, the sample cards were stored for different periods and DNA extracted after proteinase K treatment. The number of samples collected depended on the number of patients recruited per month whiles samples were kept in a plastic Ziploc bag and stored at room temperature in the laboratory until it was extracted.

Positivity ratio was defined as the proportion of samples among tested specimens that yielded positive results for every month converted into percentages with 95% confidence interval.

Table 7 shows that samples stored for 6 months had positivity rate of 27% (95% confidence interval of 44- 54%) whiles that of 5 months had a rate of 58.8% (95% confidence interval of 35% -83%). For 4 months, 3 months, 2 months, and 1 month storage periods, the positivity rates were 55.5%, 44.4%, 100% and 50% respectively. The highest positivity rate was found in samples run after two months with a ratio of 100% (95% CI 100%).

This shows that the FTA cards could preserve *M. ulcerans* DNA stored on it, however there was no obvious link between storage duration and positivity ratio.

7: Proportion of positive samples per storage time.

Storage time in months	FTA PCR			
	Positive	Negative	Total	% Positivity (95% CI)
One	½	1/2	2	50 (90.0 -100.0)
Two	5/5	0/5	5	100

Table

				(100)
Three	4/9	5/9	9	44.4 (11.3-77.6)
Four	5/9	4/9	9	55.5 (22.4 – 88.6)
Five	10/17	7/17	17	58.8 (34.9 – 82.6)
Six	3/11	8/11	11	27 (0.44 – 54.0)

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4.6. Determination of agreement between FTA- DRB- PCR and CLS- DRB- PCR per lesion category and sample type.

The inter-assay agreement rates defined as the agreement between FTA- DRB -PCR and the standard CLS- DRB- PCR was determined. Swab specimens from ulcerative lesions and FNA specimen from pre-ulcerative lesions stored on FTA cards and in CLS were subjected to dry reagent based PCR at the Kumasi Center for Collaborative Research in Tropical Medicine.

The agreement rates between both methods were determined as the percentage of concordant results per type of specimen and per category of lesions and were expressed as overall agreement rates.

Overall agreement rate between FTA-PCR and CLS-PCR was 50% with a p value of 0.58 for swab samples and 67.7%, p value of 0.22 for FNA samples as shown in Table 8.

The agreement rate per category of lesions show category I being highest for FNAs with 72.7% compared with that of swabs which was 41.7%. Category II lesions for Swabs had an agreement rate of 57.1% whiles that of FNA was 55.6%. Category III lesions for swab samples

had an agreement rate of 66.7% between FTA-PCR and CLS-PCR. There were no Category III lesions for FNA samples. There was no significant difference between the agreements based on sample types and the lesion categories.

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Table 8: Inter-assay agreement rates between FTA DRB PCR and standard CLSDRB-PCR (overall agreement rates and agreement rates per type of specimen).

Specimen, Lesion Category	Concordant results of FTA PCR and CLS PCR: Positive	Negative	Total	No. of Tested Specimens	Inter-assay agreement rate(%)	p value*
SWAB						
CAT. I	4	1	5	12	41.7	0.58
CAT. II	4	0	4	7	57.1	1.0
CAT. III	1	1	2	3	66.7	1.0
TOTAL	9	2	11	22	50	1.0
FNA						
CAT. I	13	3	16	22	72.7	0.28
CAT. II	3	2	5	9	55.6	1.0
TOTAL	16	5	21	31	67.7	0.22

*** p- value <0.05 was considered significant, Fishers exact test.**

CHAPTER 5

DISCUSSION

5.1 Patient characteristics

In this study, it was observed that though there were more women 28 (53%) than men 25(47%), there was no significant difference in the sex of the patients. The ages ranged from as low as

2 years to 87years with a median age of 14years. This suggests that the disease affects persons of all age groups and gender but children below the ages of fifteen are the most affected. These findings confirm studies conducted in the early days which were aimed at finding the correlation between the sex and ages of affected persons and the disease, which could serve as a marker for acquiring the disease (Van der Werf *et al.*, 1999; Amofah *et al.*, 2002; Johnson *et al.*, 2005a). In another study, Oluwasanmi et al found no difference in the sex of patients being affected (Oluwasanmi *et al.*, 1976) while Barker in his study identified that more women were affected as reported by Amofah *et. al*. in 2002.

The current study identified that, a greater proportion, (72%) of the lesions were found on the lower limbs than the upper limbs (23%) and other parts of the body(6%) resulting from exposure of the limbs to the environment without proper protection with clothes. Farming was the major occupation of most patients reporting to the clinic and this could explain their environmental exposure. Similar observations were made in a study conducted by Van der Werf et al in 1999. It is noted that children are mostly found around water bodies where this environmental organism had been isolated and cultured. Their activities around the water bodies including domestic chores like washing and bathing as well as playing along river banks predispose them to the disease. Because most of the patients were from deprived communities where access to safe and clean water is scarce, they rely on surrounding water bodies for their daily source and an affordable recreational site. In addition, studies elsewhere suggested farming as well as small scale mining popularly known as "galamsey" are risk factors for getting infected, and these are what most of the patients engage in as their source of livelihood (Van der Werf *et al.*, 1999). The study also identified that 34 (64%) of the 53 suspected patients sampled, presented with category I lesions which is defined by the WHO as lesions with diameters less than 5cm whiles 16 (30%) of the 53, presented with category II lesions. Only 3 of the patients representing 6% of the study population presented with category III

lesions, even though 51% of the lesions presented were ulcers. The World Health organization prioritized its early detection and treatment program through outreaches to endemic regions of Buruli ulcer (WHO, 1998; WHO, 2014) to reduce the delay in the reporting of the disease leading to category III lesions. These lesions are defined as those with diameters greater than 10cm or patients reporting with multiple lesions form of the disease and these are the largest which usually results in contractures. The nature of the disease is such that it presents as a painless nodule or a boil which most patients would want to treat with local herbs. Moreover, access to health facilities in most endemic communities is difficult hence the delay, and by the time it is reported, the lesion is extensive leaving most patients with various forms of deformities which is similar to observations made in other studies(Asiedu and Etuaful, 1998; WHO, 2012). In effect, education through the outreaches would help improve on disease reporting.

5.2 The detection limit of PCR for *M. ulceran*ssamples transported on FTA cards

In this experiment, an optimization process was set up to determine the detection limit of PCR for the FTA card. Two objectives were to be achieved with the first one testing the possibility of DNA being amplified from the card without any washing steps based on the design of the cards which contains chemicals that remove inhibitors and also lyse the cells making the nucleic acid readily available for analysis (Whatman, 2009). Results however indicated that all the spotted sections that were amplified showed no band indicating a negative result, their inhibition controls were all negative, a strong indication that they were inhibited. This therefore suggests that though the preserving agents in the filter paper remove inhibitors from the samples spotted on it, they are themselves inhibitory to most reactions therefore they require some washing steps to remove them. Similar observations were made in other studies (Miles and Saul, 2011)

Results from the second objective had shown that, in absolute numbers, 1 bacterium could be detected when a concentration of 10 bacteria /100µl is spotted on the card. This one organism has about 249 copies of the insertion sequence *IS2404* in its genome hence when amplified by the dry reagent based PCR increases the chances of a positive result. This was achieved by using the in-house extraction and purification method due to the hard cell wall of the mycobacterium; it was therefore subjected to proteinase K treatment which is known to increase the yield of DNA before purification as was stated by Johanson et al. in their studies to determine the best way to extract quality DNA from FTA cards (Johanson *et al.*, 2009).

By including a negative control in the test samples, the sensitivity and specificity of the card was tested through its ability to detect the absence of mycobacterium in the TE buffer spotted on it. This was a promising alternative to the current transport medium, the CLS especially for the endemic communities during health workers' outreaches for suspected forms of the disease that need confirmation. Thus a patient's sample containing only one organism could be detected and given a true diagnostic result. The optimization experiment was done because this is the first time this technology is being tested in the study of Buruli ulcer.

5.3 Comparison between the PCR of samples kept in three transport media

The sensitivity of the FTA cards as a transport material was compared to the standard cell lyses solution for the dry reagent based PCR and RNA protect for the qPCR in this study. The test proved to be an easy and safe mode of transport for diagnostic samples, but a lower sensitivity of 58% compared to the gold standard, CLS-DRB PCR which is reported to have a sensitivity of 98% (Phillips *et al.*, 2005). This was in contrast to higher sensitivities recorded in some other experiments involving *Mycobacterium leprae* (Aye *et al.*, 2011). The specificity of FTA DRB PCR (70%) was however higher than qPCR whose specificity was 67% resulting from 4 of 6

patients identified as showing a true negative result compared to 7 of 10 patients for FTA DRB PCR. qPCR had a sensitivity of 94% compared to the standard

CLS based DRB PCR which is known to have a specificity of 100% (Phillips *et al.*, 2009a). Though it recorded a lower sensitivity, it was evident from this study that the FTA cards can store DNA which can be recovered from it however at lower yields; a similar observation was made in a study by Stangegaard *et al*., in 2011, where repeated extraction was done to give higher yields. Successful molecular diagnosis requires the availability of genomic material of an appropriate quality and integrity as well as concentration of the sample being examined (Ahmed *et al.*, 2011; Saieg *et al.*, 2012).The design of the card, as described was such that, when the samples were applied to the card the cells were lysed and the DNA became tightly associated with the card. This association therefore required that the sample is analyzed while it is still bound to the paper meanwhile this does not take into consideration the reaction volume or other PCR conditions as a result of the presence of the card in the mastermix as was observed in some studies by (Johanson *et al.*, 2009; Miles and Saul, 2011). The requirement for this study was to elute the DNA from the card in order to get the maximum volume of the reaction mix for the PCR, however initial patient samples that were run using the in-house protocol yielded less positive results which confirmed that much of the DNA was entangled in the matrix of the card and could not be eluted but this improved after the introduction of the vortexing step in which higher yields were achieved. This suggests that, a more vigorous method of extraction needs to be employed in order to elute enough DNA for the diagnostic test especially with the tough cell wall of the mycobacterium involved.

In comparison to the patient samples, sensitivity for the optimization experiment using the FTA cards spiked with known concentrations was as high as the gold standard due to the high positivity rate. This could be attributed to the introduction of other forms of inhibitors and the presence of other bacteria in the case of secondary infections during patients' sample collection.

It was suggested that, purifying the organism or lysing the cells before spotting on the cards would improve sensitivity because the *M. ulcerans* used for the optimization had undergone some form of purification before storage while samples from the field were spotted directly. A similar observation was made in a study in which trypanosome DNA stored on FTA cards was detected with a higher sensitivity when whole blood was lysed before application onto the card (Ahmed *et al.*, 2011). It is evident that though many applications have been described for the card, its use under field conditions has not been explored and this may be reduced compared to the standard conditions as observed in some studies (Borisenko *et al.*, 2008; Heim *et al.*, 2012).

Of all the three PCR methods, q PCR had the highest sensitivity of 94% above the standard DRB PCR. This would imply the use of qPCR for routine diagnosis or as a replacement for the DRB PCR; however this method is expensive to run hence it can only be useful for research purposes. Moreover, it requires highly skilled personnel and may not be available in all reference labs used for routine diagnosis for the *M. ulcerans* disease. However transporting samples to the laboratory using the cards was much easier and storage comparatively simpler. In the midst of frequent power outages as was common at the time of the study, it came handy since it could be stored at room temperature confirming observations made in similar studies (Al-Kzayer *et al.*, 2012)

5.4 Comparison of sensitivity of FTA PCR with smear microscopy for Acid Fast Bacilli as the standard

In this study the sensitivity of the FTA card was lower than the standard CLS DRB PCR, its usefulness was therefore evaluated by determining its sensitivity with smear microscopy which is the most available method. Results show that the overall sensitivity of FTA DRB (80%) was higher when compared with ZN microscopy. Of 29 study participants examined, 15(51.7%) were negative for ZN microscopy but were positive for FTA DRB PCR whiles 2 of 10 participants were positive for ZN but negative for FTA DRB PCR accounting for the

20% deficit in the sensitivity of the FTA PCR. When expanded further into sample types, the sensitivity of FNA samples (100%) was higher than the Swab samples (33%). However the specificities of PCR for both swab and FNA samples were low recording 48%, 53% and 42% respectively. A possible explanation for the high sensitivity of FNA samples could be as a result of the sampling technique; in the ulcerative lesions, it is difficult to find the most productive sampling site, more so, the swabbing of the undermined edges leads to the collection of more cells, and other microorganisms which inhibit the PCR. A similar observation was made by (Eddyani *et al.*, 2009) . Comparatively, FNA samples produce small sample of cells hence limits the possibility of collecting extra materials that would inhibit the PCR. This confirms studies that have shown that FNA samples produce small sample of cells from suspected abnormal tissues and they have a relatively high diagnostic accuracy (Layfield, 2007; Phillips *et al.*, 2009b). In another study it was observed that nonulcerative lesions had a higher positivity rate (Eddyani *et al.*, 2009) which confirms findings in this study.

Comparing both FTA PCR and microscopy to the standard CLS PCR, FTA PCR had a higher sensitivity of 58% than the microscopy that had a sensitivity of 29%. The latter had a very high specificity of 100% in contrast to FTA PCR which had a specificity of 70%. This suggests that, though the FTA has a lower sensitivity compared to the standard, it could be considered for transport of *M. ulcerans* samples in very remote areas where access to reference laboratories is limited and it can be used in addition to the microscopy as a first line diagnostic procedure in these areas due to its specificity. As the first line of treatment since the introduction of antibiotic treatment, WHO recommends laboratory diagnosis as an important step in the management of the disease (WHO, 2012). The current accepted techniques for laboratory confirmation of Buruli ulcer disease include the histopathology, isolation by culture, smear for acid fast bacilli and the *IS2404* insertion sequence detection by PCR (Herbinger et al., 2009). The non-availability of histopathology methods in most endemic areas and the slow growing

M. ulcerans in culture makes them not useful for routine diagnosis. This leaves PCR for *IS2404* and smear microscopy for AFB the commonly used diagnostic procedures. Even though PCR has the highest sensitivity and specificity, it is very expensive to run and requires special skills and equipment which is not available in most endemic areas (Portaels and WHO, 2014). Smear microscopy for acid fast bacilli therefore becomes the available method for the point of care diagnosis of Buruli ulcer disease for the ease and cost effectiveness as demonstrated in studies by Yeboah-manu et al. (2011). It however has the lowest sensitivity compared to the PCR. Meanwhile, this can be improved using two slides and good microscopy skills (Frimpong et al., 2015).

5.5 Determination of the sensitivity of samples stored on FTA cards for different durations

The study sought to establish that the FTA cards could store and stabilize the *M. ulcerans* DNA for a long period of time and still produce results as expected. In doing this the samples were stored in Ziploc bags and kept at room temperature for as long as 6months. Results have proven that the cards could store and protect M. ulcerans DNA as stated by the manufacturers and confirmed by various studies (GE Healthcare, 2011; Saieg et al., 2012). It was observed that the positivity rates were not very high, a situation that can be attributed to the localized trapping of the genomic material in the matrix of the card which is worsened by long term storage as observed by (Ahmed et al., 2011). In every month, there was a proportion of the samples collected that were PCR positive, with those stored up to two months giving off 100% positivity. This indicates the cards were able to stabilize the DNA stored on it. Another drawback about the FTA cards observed is the low yield of DNA after long term storage. This is in line with observations made in a study by Mullen et. al.(2009) in which samples were stored for up to 8 years and DNA was extracted. Their results provided evidence of ample PCR products however in limited quantities (Mullen et al., 2009). Flinders technology and associates® card (FTA cards) were designed to store genomic DNA at room temperature for

many years, as long as 17 years and be successfully amplified (GE Healthcare, 2011). Other studies have also confirmed the ability of the FTA cards to store up samples for periods of 4 years as found in the study of M. leprae (Aye et al., 2011), 18months, and 2 years (Saieg et al., 2012; Santos et al., 2012). Moreover some studies retrieved quality genomic material in good quantities better than their standards, even with RNA after 6 months of storage (Saieg et al., 2012; Santos et al., 2012). This feature of the cards would enable it to be used as a tool for archiving M. ulcerans samples for future use and making use of little space. Buruli ulcer is known to be endemic in rural communities where access to basic healthcare is limited. Usually samples have to be transported over long distances that might take a long time to arrive in the reference laboratories for diagnosis (Frimpong et al., 2015). Sometimes the transport of the samples as they are in liquid media can pose a hazard to the one transporting the sample. It usually would be safer to have samples transported as dry samples and easy to carry for space and stability of the genomic material. The FTA card as containing chemicals that preserve the nucleic acid and prevent the growth of other contaminating organisms could best be used in the transport of these *M. ulcerans* samples within these conditions.

5.6 Determination of Interassay agreement between FTA- DRB- PCR and CLS- DRB- PCR per lesion categories and sample type.

In the current study, interassay agreement was determined between the FTA DRB PCR method and the standard Cell lysing solution dry reagent based PCR. Results were stratified into specimen type and category of lesions and determined as the concordant results of both methods. It has been observed that the agreement between FNA samples, 68% was insignificantly higher than that of swab samples of 50% which could be due to sampling techniques. Generally there was a lower agreement between the FTA cards and the CLS as transport media for collection of samples for *IS2404* sequence amplification. This disagreement could be due to the use of different swab samples and aspirates though taken

from the same lesion and from adjacent sites for the FNAs or same circling of the undermined lesion. It is possible that one set of specimen does not contain the bacilli or fewer than detectable bacilli since aspirating in the first place is expected to suction the organism into the syringe. After repeated suction, it is possible for the number of bacilli to reduce or otherwise the site for subsequent suctions does not have the *M. ulcerans.* Likewise for the swabs, the first swab always has the highest bacterial load whiles the others have fewer and possibly none at all. Duplicate tests for the FTA PCR was observed to have improved positivity in most samples run since the card provided slots for that. It is therefore better to run tests in duplicates though they might give variable results as stated by (Siegmund et al., 2007).

Based on lesion categories, the category I lesions of FNA samples had the highest agreement of 72.7% but category II had a lower agreement rate of 55.6% comparatively, there was an increment in agreement from category I to category II in the swabs. The difference in the category I lesions between the two samples could be due to lesion size. It was normal for preulcerative lesions to belong to category I where organisms might be within reach to the needle , but with category I ulcers, it might be difficult to go beyond a section of the undermined edge due to fear of traumatizing the lesion. Meanwhile the bacteria could be found beyond these boundaries since they continue to move further into tissues. As the lesion extends, the level of necrosis leading to undermined lesions increases and makes swabbing easier and possible to pick bacteria onto the swabs; whereas in the pre ulcers, where the aspirates are taken from a central point, the possibility of missing the bacteria which would have been distributed at the periphery is higher hence the observed values in agreement.

Agreement between two diagnostic tests seeks to determine the ability of one (usually a new test being introduced) to give same results as the other (the standard). With good agreement rates, the new one can easily replace the current one if the need be. In view of the low

agreement, the FTA PCR cannot replace the CLS PCR, however it could be used in times when the CLS is not available for transporting samples to the lab, care should then be taken in the sampling and it should be done in duplicates to increase the chances of getting good results.

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CHAPTER 6

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

In absolute numbers, 1 bacterium could be detected from the FTA card. Hence the card can be used for the collection of diagnostic samples. In the current study, the optimization experiment identified that chemicals on the card themselves are inhibitors to samples hence samples stored need to be purified before analyzing. It was observed that the disease affected persons of all sexes, but children below the ages of 15 were the most affected. Lesions were found mostly on the limbs which was linked to activities of the patients since most of them indicated farming as their primary occupation. The number of category I lesions were more than category III lesions which is considered the late form of the and usually leads to complication compared to earlier studies due to the early detection programs by WHO. Education was therefore identified as a key to improving disease reporting.

Of the three PCR methods, qPCR had the highest sensitivity followed by the DRB PCR. FTA PCR had the lowest sensitivity of 58%. The FTA cards can be used for transport but will require a more vigorous method of extraction to elute enough DNA stored on it for amplification and analysis. Samples stored directly were noted to introduce inhibitors onto the card. This can however be avoided when samples are purified before storage onto the card. In the era of frequent power outages the card came in as a good storage medium since it does not require any cold storage but room temperature.

Though it had a lower sensitivity when compared with the gold standard, the FTA PCR would be a better choice over the smear for microscopy since it had a higher sensitivity of
80% when compared with it. However both can be used for sample storage and transport in areas where access to the reference laboratories is limited. Sampling technique too was found to affect the sensitivity of results.

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The cards can be used to store samples over long periods (up to one year) of time as demonstrated by the results of the study. Hence for archiving purposes the cards can be used and it also helps in reducing storage space. It will also serve as a safer means of transporting samples as the card dries up upon spotting.

The interassay agreement rates from the study indicated a low agreement between the FTA cards and the CLS hence the FTA technique cannot be used to replace the current available transport medium for Buruli ulcer samples but can be used in the absence of the CLS.

6.2 Recommendations

Based on results and observations from the study, it is recommended that:

i. A better way of eluting *M. ulcerans* DNA from the cards in appreciable quantities for use in PCR should be developed ii. Attention should be paid to the sampling techniques in order to increase the bacterial load on the card; the first samples taken from the lesions; both swabs and FNA be spotted on the card before samples for other tests are taken if there is any.

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REFERENCE

Affolabi D., Sanoussi N., Vandelannoote K., Odoun M., Faihun F., Sopoh G., Anagonou S., Portaels F. and Eddyani M. (2012) Effects of decontamination, DNA extraction, and amplification procedures on the molecular diagnosis of Mycobacterium ulcerans disease (Buruli ulcer). *J Clin Microbiol* 50(4), 1195-1198.

Agbavor B., Awuah A.A. and Amani M. (2012) Buruli Ulcer; Presentation of Cases, Diagnostic Methods and Treatment, a Study at the Agogo Hospital. BSc, Kwame Nkrumah University of Science and Technology.

Agbenorku P., Donwi I.K., Kuadzi P. and Saunderson P. (2012) Buruli ulcer: treatment challenges at three centres in ghana. *Journal of tropical medicine* 37(19), 15-.

Ahmed H.A., MacLeod E.T., Hide G., Welburn S.C. and Picozzi K. (2011) The best practice for preparation of samples from FTA(R)cards for diagnosis of blood borne infections using African trypanosomes as a model system. *Parasit Vectors* 4(6), 8.

Al-Kzayer L.F., Sakashita K., Matsuda K., Al-Hadad S.A., Al-Jadiry M.F., Abed W.M., Abdulkadhim J.M., Al-Shujairi T.A., Hasan J.G., Al-Abdullah H.M., Al-Ani M.H., Saber P.A., Inoshita T., Kamata M. and Koike K. (2012) Genetic evaluation of childhood acute lymphoblastic leukemia in Iraq using FTA cards. *Pediatr Blood Cancer* 59(3), 461-467.

Amofah G., Asamoah S. and Afram-Gyening C. (1998) Effectiveness of Excision of PreUlcerative Buruli Lesions in Field Situations in a Rural District in Ghana. *Tropical Doctor* 28(2), 81-83.

Amofah G., Bonsu F., Tetteh C., Okrah J., Asamoa K., Asiedu K. and Addy J. (2002) Buruli ulcer in Ghana: results of a national case search. *Emerging infectious diseases* 8(2), 167-170.

Asiedu K. and Etuaful S. (1998) Socioeconomic implications of Buruli ulcer in Ghana: a three-year review. *Am J Trop Med Hyg* 59(6), 1015-1022.

Aye K.S., Matsuoka M., Kai M., Kyaw K., Win A.A., Shwe M.M., Thein M., Htoo M.M. and Htoon M.T. (2011) FTA card utility for PCR detection of Mycobacterium leprae. *Jpn J Infect Dis* 64(3), 246-248.

Boleira M., Lupi O., Lehman L., Asiedu K.B. and Kiszewski A.E. (2010) Buruli ulcer. *Anais brasileiros de dermatologia* 85(3), 281-301.

Borisenko A.V., Lim B.K., Ivanova N.V., Hanner R.H. and Hebert P.D. (2008) DNA barcoding in surveys of small mammal communities: a field study in Suriname. *Molecular Ecology Resources* 8(3), 471-479.

Borman A.M., Fraser M., Linton C.J., Palmer M.D. and Johnson E.M. (2010) An improved protocol for the preparation of total genomic DNA from isolates of yeast and mould using Whatman FTA filter papers. *Mycopathologia* 169(6), 445-449.

Braae U.C., Johansen M.V., Ngowi H.A., Rasmussen T.B., Nielsen J. and Uttenthal A. (2013) Detection of African Swine Fever Virus DNA in Blood Samples Stored on FTA Cards from Asymptomatic Pigs in Mbeya Region, Tanzania. *Transbound Emerg Dis* 62(1), 87-90.

Bratschi M.W., Bolz M., Grize L., Kerber S., Minyem J.C., Boock A.U., Yeboah-Manu D., Ruf M.-T. and Pluschke G. (2014) Primary cultivation: factors affecting contamination and Mycobacterium ulcerans growth after long turnover time of clinical specimens. *BMC infectious diseases* 14(1), 1.

Chauty A., Ardant M.-F., Adeye A., Euverte H., Guédénon A., Johnson C., Aubry J., Nuermberger E. and Grosset J. (2007) Promising clinical efficacy of streptomycinrifampin combination for treatment of Buruli ulcer (Mycobacterium ulcerans disease). *Antimicrobial agents and chemotherapy* 51(11), 4029-4035.

Clancey J. (1964) Mycobacterial skin ulcers in Uganda: description of a new mycobacterium (Mycobacterium buruli). *The Journal of pathology and bacteriology* 88(1), 175-187.

de Bie R.P., Schmeink C.E., Bakkers J.M., Snijders P.J., Quint W.G., Massuger L.F., Bekkers R.L. and Melchers W.J. (2011) The indicating FTA elute cartridge a solid sample carrier to detect high-risk HPV and high-grade cervical lesions. *J Mol Diagn* 13(4), 371-376.

Debacker M., Aguiar J., Steunou C., Zinsou C., Meyers W. and Portaels F. (2005) Buruli ulcer recurrence, Benin. *Emerging infectious diseases* 11(4), 584-589.

Debacker M., Portaels F., Aguiar J., Steunou C., Zinsou C., Meyers W. and Dramaix M. (2006) Risk Factors for Buruli Ulcer, Benin. *Emerging infectious diseases* 12(9), 1325-1331.

Durnez L., Stragier P., Roebben K., Ablordey A., Leirs H. and Portaels F. (2009) A comparison of DNA extraction procedures for the detection of Mycobacterium ulcerans, the causative agent of Buruli ulcer, in clinical and environmental specimens. *Journal of microbiological methods* 76(2), 152-158.

Eddyani M., Debacker M., Martin A., Aguiar J., Johnson C.R., Uwizeye C., Fissette K. and Portaels F. (2008) Primary Culture of Mycobacterium ulcerans from Human Tissue Specimens after Storage in Semisolid Transport Medium. *J Clin Microbiol* 46(1), 6972.

Eddyani M., Fraga A.G., Schmitt F., Uwizeye C., Fissette K., Johnson C., Aguiar J., Sopoh G., Barogui Y., Meyers W.M., Pedrosa J. and Portaels F. (2009) Fine-needle aspiration, an efficient sampling technique for bacteriological diagnosis of nonulcerative Buruli ulcer. *J Clin Microbiol* 47(6), 1700-1704.

Eddyani M., Ofori-Adjei D., Teugels G., De Weirdt D., Boakye D., Meyers W.M. and Portaels F. (2004) Potential role for fish in transmission of Mycobacterium ulcerans disease

(Buruli ulcer): an environmental study. *Applied and Environmental Microbiology* 70(9), 5679-5681.

Etuaful S., Carbonnelle B., Grosset J., Lucas S., Horsfield C., Phillips R., Evans M., OforiAdjei D., Klustse E. and Owusu-Boateng J. (2005) Efficacy of the combination rifampin-streptomycin in preventing growth of Mycobacterium ulcerans in early lesions of Buruli ulcer in humans. *Antimicrobial agents and chemotherapy* 49(8), 3182-3186.

Frimpong M., Sarpong-Duah M., Beissner M., Sarfo F., Loglo A., Tannor E., Awuah N., Frempong M., Adjei O., Wansbrough-Jones M., Bretzel G. and Phillips R. (2015) Microscopy for Acid Fast Bacilli: A Useful but Neglected Tool in Routine Laboratory Diagnosis of Buruli Ulcer. *Journal of Tropical Diseases* 3(158), 2-5.

GE Healthcare W. (2010) Reliable extraction of DNA from Whatman™ FTA™ cards. *www.gelifesciences.com/whatman* Application note 28-9822-22 AA.

GE Healthcare W. (2011) FTA* cards. *www.gelifesciences.com/whatman* Data file 28-984354 AA.

Geraets D.T., van Baars R., Alonso I., Ordi J., Torne A., Melchers W.J., Meijer C.J. and Quint W.G. (2013) Clinical evaluation of high-risk HPV detection on self-samples using the indicating FTA-elute solid-carrier cartridge. *J Clin Virol* 57(2), 125-129.

Gonzalez P., Cortes B., Quint W., Kreimer A.R., Porras C., Rodriguez A.C., Jimenez S., Herrero R., Struijk L., Hildesheim A. and Melchers W. (2012) Evaluation of the FTA carrier device for human papillomavirus testing in developing countries. *J Clin Microbiol* 50(12), 3870-3876.

Guarner J., Bartlett J., Whitney E.A.S., Raghunathan P.L., Stienstra Y., Asamoa K., Etuaful S., Klutse E., Quarshie E. and van der Werf T.S. (2003) Histopathologic features of Mycobacterium ulcerans infection. *Emerging infectious diseases* 9(6), 651.

Heim B.C., Ivy J.A. and Latch E.K. (2012) A suite of microsatellite markers optimized for amplification of DNA from Addax (Addax nasomaculatus) blood preserved on FTA cards. *Zoo Biol* 31(1), 98-106.

Herbinger K.H., Adjei O., Awua-Boateng N.Y., Nienhuis W.A., Kunaa L., Siegmund V., Nitschke J., Thompson W., Klutse E., Agbenorku P., Schipf A., Reu S., Racz P., Fleischer B., Beissner M., Fleischmann E., Helfrich K., van der Werf T.S., Loscher T. and Bretzel G. (2009) Comparative study of the sensitivity of different diagnostic methods for the laboratory diagnosis of Buruli ulcer disease. *Clin Infect Dis* 48(8), 1055-1064.

Herbinger K.H., Beissner M., Huber K., Awua-Boateng N.Y., Nitschke J., Thompson W., Klutse E., Agbenorku P., Assiobo A., Piten E., Wiedemann F., Fleischmann E., Helfrich K., Adjei O., Loscher T. and Bretzel G. (2010) Efficiency of fine-needle aspiration

compared with other sampling techniques for laboratory diagnosis of Buruli ulcer disease. *J Clin Microbiol* 48(10), 3732-3734.

Johanson H.C., Hyland V., Wicking C. and Sturm R.A. (2009) DNA elution from buccal cells stored on Whatman FTA Classic Cards using a modified methanol fixation method. *Biotechniques* 46(4), 309-311.

Johnson P., Azuolas J., Lavender C.J., Wishart E., Stinear T.P., Hayman J.A., Brown L., Jenkin G.A. and Fyfe J. (2007a) Mycobacterium ulcerans in mosquitoes captured during outbreak of Buruli ulcer, southeastern Australia. *Emerg Infect Dis* 13(11), 1653-1660.

Johnson P.D., Hayman J.A., Quek T.Y., Fyfe J.A., Jenkin G.A., Buntine J.A., Athan E., Birrell M., Graham J. and Lavender C.J. (2007b) Consensus recommendations for the diagnosis, treatment and control of Mycobacterium ulcerans infection (Bairnsdale or Buruli ulcer) in Victoria, Australia. *Medical Journal of Australia* 186(2), 64.

Johnson P.D., Stinear T., Pamela L., Pluschke G., Merritt R.W., Portaels F., Huygen K., Hayman J.A. and Asiedu K. (2005a) Buruli ulcer (M. ulcerans infection): new insights, new hope for disease control. *PLoS medicine* 2(4), e108.

Johnson P.D.R., Timothy Stinear, Pamela L. C. Small, Gerd Plushke, Richard W. Merritt, Francoise Portaels, Kris Huygen, JohnA. Hayman and Asiedu K. (2005b) Buruli Ulcer (M. ulcerans Infection): New Insights, New Hope for Disease Control. *PLoS Med* 2(4), 0282-0286.

Kibadi K., Mputu-Yamba J., Mokassa B., Panda M. and Muyembe-Tamfum J. (2009) [Relapse after surgical treatment of mycobacterium ulcerans infection (buruli ulcer): study of risk factors in 84 patients in the Democratic Republic of the Congo]. *Medecine tropicale: revue du Corps de sante colonial* 69(5), 471-474.

Layfield L.J. (2007) Fine_needle aspiration in the diagnosis of head and neck lesions: A review and discussion of problems in differential diagnosis. *Diagnostic cytopathology* 35(12), 798-805.

Linhares D.C., Rovira A. and Torremorell M. (2012) Evaluation of Flinders Technology Associates cards for collection and transport of samples for detection of Porcine reproductive and respiratory syndrome virus by reverse transcription polymerase chain reaction. *Journal of Veterinary Diagnostic Investigation* 24(2), 328-332.

Merritt R.W., Walker E.D., Small P.L., Wallace J.R., Johnson P.D., Benbow M.E. and Boakye D.A. (2010) Ecology and transmission of Buruli ulcer disease: a systematic review. *PLoS Negl Trop Dis* 4(12), e911.

Miles M. and Saul D. (2011) Improved elution of DNA from Whatman FTA® cards using prepGEM®/forensicGEM® Storage Card extraction kits.

Moscoso H., Thayer S.G., Hofacre C.L. and Kleven S.H. (2004) Inactivation, storage, and PCR detection of mycoplasma on FTA® filter paper. *Avian Dis* 48(4), 841-850.

NFSTC (2005) DNA Analyst Training; FTA Technology. *http://www.nfstc.org/pdi/Subject03/pdi_s03_m04_02_d.htm*.

Oluwasanmi J.O., Solanke T.F., Olurin E.O., Itayemi S.O., Alabi G.O. and Lucas A.O. (1976) Mycobacterium Ulcerans (Buruli) Skin Ulceration in Nigeria. *Am J Trop Med Hyg* 25(1), 122-128.

Palomino J.C. and Portaels F. (1998) Effects of Decontamination Methods and Culture Conditions on Viability of Mycobacterium ulcerans in the BACTEC System. *J Clin Microbiol* 36(2), 402-408.

Phillips R., Horsfield C., Kuijper S., Lartey A., Tetteh I., Etuaful S., Nyamekye B., Awuah P., Nyarko K. and Osei-Sarpong F. (2005) Sensitivity of PCR targeting the IS2404 insertion sequence of Mycobacterium ulcerans in an assay using punch biopsy specimens for diagnosis of Buruli ulcer. *J Clin Microbiol* 43(8), 3650-3656.

Phillips R., Sarfo F.S., Guenin-Macé L., Decalf J., Wansbrough-Jones M., Albert M.L. and Demangel C. (2009a) Immunosuppressive signature of cutaneous Mycobacterium ulcerans infection in the peripheral blood of patients with buruli ulcer disease. *Journal of Infectious Diseases* 200(11), 1675-1684.

Phillips R.O., Sarfo F.S., Osei-Sarpong F., Boateng A., Tetteh I., Lartey A., Adentwe E., Opare W., Asiedu K.B. and Wansbrough-Jones M. (2009b) Sensitivity of PCR targeting Mycobacterium ulcerans by use of fine-needle aspirates for diagnosis of Buruli ulcer. *J Clin Microbiol* 47(4), 924-926.

Portaels F., Agular J., Fissette K., Fonteyne P.A., De Beenhouwer H., de Rijk P., Guédénon A., Lemans R., Steunou C., Zinsou C., Dumonceau J.M. and Meyers W.M. (1997) Direct detection and identification of Mycobacterium ulcerans in clinical specimens by PCR and oligonucleotide-specific capture plate hybridization. *J Clin Microbiol* 35(5), 1097-1100.

Portaels F. and WHO (2014) *Laboratory diagnosis of buruli ulcer, A manual for health care providers*. Switzlander: World Health Organization.

Portaels F.o., Johnson P., Meyers W.M. and Initiative G.B.U. (2001) Buruli ulcer: diagnosis of Mycobacterium ulcerans disease: a manual for health care providers.

Pouillot R., Matias G., Wondje C.M., Portaels F., Valin N., Ngos F., Njikap A., Marsollier L., Fontanet A. and Eyangoh S. (2007) Risk Factors for Buruli Ulcer: A Case Control Study in Cameroon. *PLoS Negl Trop Dis* 1(3), e101.

Raghunathan P.L., Whitney E.A., Asamoa K., Stienstra Y., Taylor T.H., Amofah G.K., Ofori-Adjei D., Dobos K., Guarner J. and Martin S. (2005) Risk factors for Buruli ulcer disease (Mycobacterium ulcerans infection): results from a case-control study in Ghana. *Clinical Infectious Diseases* 40(10), 1445-1453.

Rajendram D., Ayenza R., Holder F., Moran B., Long T. and Shah H. (2006) Long-term storage and safe retrieval of DNA from microorganisms for molecular analysis using FTA matrix cards. *Journal of microbiological methods* 67(3), 582-592.

Roder B., Fruhwirth K., Vogl C., Wagner M. and Rossmanith P. (2010) Impact of long-term storage on stability of standard DNA for nucleic acid-based methods. *J Clin Microbiol* 48(11), 4260-4262.

Rondini S., Mensah-Quainoo E., Troll H., Bodmer T. and Pluschke G. (2003) Development and Application of Real-Time PCR Assay for Quantification of Mycobacterium ulcerans DNA. *J Clin Microbiol* 41(9), 4231-4237.

Saieg M.A., Geddie W.R., Boerner S.L., Liu N., Tsao M., Zhang T., Kamel-Reid S. and da Cunha Santos G. (2012) The use of FTA cards for preserving unfixed cytological material for high-throughput molecular analysis. *Cancer Cytopathol* 120(3), 206-214.

Santos C.R., Franciscatto L.G., Barcellos R.B., Almeida S.E. and Rossetti M.L. (2012) Use of FTA elute card impregnated with cervicovaginal sample directly into the amplification reaction increases the detection of human papillomavirus DNA. *Braz J Microbiol* 43(1), 389-392.

Sarfo F.S., Converse P.J., Almeida D.V., Zhang J., Robinson C., Wansbrough-Jones M. and Grosset J.H. (2013) Microbiological, histological, immunological, and toxin response to antibiotic treatment in the mouse model of Mycobacterium ulcerans disease. *PLoS Negl Trop Dis* 7(3), e2101.

Sarfo F.S., Le Chevalier F., Aka N., Phillips R.O., Amoako Y., Boneca I.G., Lenormand P., Dosso M., Wansbrough-Jones M., Veyron-Churlet R., Guenin-Mace L. and Demangel C. (2011) Mycolactone diffuses into the peripheral blood of Buruli ulcer patients-implications for diagnosis and disease monitoring. *PLoS Negl Trop Dis* 5(7), e1237.

Siegmund, Ruth Thompson, Ohene Adjei, Jo¨rg Nitschke, Felicitas van Vloten, Paul Racz, William Thompson, Bernhard Fleischer, Erasmus Klutse, Karl Heinz Herbinger, Thomas Loescher and Bretze G. (2007) Dry Reagent–Based Polymerase Chain Reaction Compared with Other Laboratory Methods Available for the Diagnosis of Buruli Ulcer Disease. *Clinical Infectious Diseases* 4568-75.

Sopoh G.E., Johnson R.C., Anagonou S.Y., Barogui Y.T., Dossou A.D., Houézo J.G., Phanzu D.M., Tente B.H., Meyers W.M. and Portaels F. (2011) Buruli ulcer prevalence and altitude, Benin. *Emerging infectious diseases* 17(1), 153.

Stangegaard M., Ferrero-Miliani L., Børsting C., Frank-Hansen R., Hansen A.J. and Morling N. (2011) Repeated extraction of DNA from FTA cards. *Forensic Science International: Genetics Supplement Series* 3(1), e345-e346.

Stienstra Y., van der Werf T.S., Guarner J., Raghunathan P.L., Spotts Whitney E.A., van der Graaf W.T.A., Asamoa K., Tappero J.W., Ashford D.A. and King C.H. (2003) Analysis of an IS2404-Based Nested PCR for Diagnosis of Buruli Ulcer Disease in Regions of Ghana Where the Disease Is Endemic. *J Clin Microbiol* 41(2), 794-797.

Stinear T., Ross B.C., Davies J.K., Marino L., Robins-Browne R.M., Oppedisano F., Sievers A. and Johnson P.D. (1999) Identification and characterization of IS2404 and IS2606: two distinct repeated sequences for detection of Mycobacterium ulcerans by PCR. *J Clin Microbiol* 37(4), 1018-1023.

Subrungruang I., Mungthin M., Chavalitshewinkoon-Petmitr P., Rangsin R., Naaglor T. and Leelayoova S. (2004) Evaluation of DNA extraction and PCR methods for detection of Enterocytozoon bienuesi in stool specimens. *J Clin Microbiol* 42(8), 3490-3494.

Sudhakaran R., Mekata T., Kono T., Supamattaya K., Linh N.T., Suzuki Y., Sakai M. and Itami T. (2009) A simple non-enzymatic method for the preparation of white spot syndrome virus (WSSV) DNA from the haemolymph of Marsupenaeus japonicus using FTA matrix cards. *J Fish Dis* 32(7), 611-617.

Van der Werf T.S., Van der Graaf W.T., Tappero J.W. and Asiedu K. (1999) Mycobacterium ulcerans infection. *THE LANCET* 354(9183), 1013-1018.

Vincent Q.B., Ardant M.-F., Adeye A., Goundote A., Saint-André J.-P., Cottin J., Kempf M., Agossadou D., Johnson C. and Abel L. (2014) Clinical epidemiology of laboratoryconfirmed Buruli ulcer in Benin: a cohort study. *The lancet global health* 2(7), e422e430.

Vitha S. and Yoder D.W. (2005) High Throughput Processing of DNA Samples on FTA Paper for PCR Analysis.

Whatman G.H. (2009) DNA Extraction from FTA® Cards Using the GenSolve DNA Recovery Kit. *http://www.whatman.com* 370-2007-032 15Dec074.

WHO (1998) Buruli Ulcer; The Yamoussoukro Declaration on Buruli ulcer. *http://www.who.int/entity/buruli/en/*.

WHO (2012) *Treatment of Mycobacterium Ulcerans Disease (Buruli Ulcer) Guidance for Health Workers*. Geneva: WHO.

WHO (2013) Drug treatment and new treatment modalities, pp. Publication: WHO.

WHO (2014) Buruli ulcer Objective and strategy for control and research. *http://www.who.int/entity/mediacentre/factsheets/en/*Fact sheet N°199 (Updated May 2015).

Yeboah-Manu D., Asante-Poku A., Asan-Ampah K., Ampadu E.D. and Pluschke G. (2011a) Combining PCR with microscopy to reduce costs of laboratory diagnosis of Buruli ulcer. *Am J Trop Med Hyg* 85(5), 900-904.

Yeboah-Manu D., Danso E., Ampah K., Asante-Poku A., Nakobu Z. and Pluschke G. (2011b) Isolation of Mycobacterium ulcerans from swab and fine-needle-aspiration specimens. *J Clin Microbiol* 49(5), 1997-1999.

Yeboah-Manu D., Kpeli G.S., Ruf M.-T., Asan-Ampah K., Quenin-Fosu K., Owusu-Mireku E., Paintsil A., Lamptey I., Anku B. and Kwakye-Maclean C. (2013) Secondary bacterial infections of buruli ulcer lesions before and after chemotherapy with streptomycin and rifampicin. *PLoS Negl Trop Dis* 7(5), e2191.

Yeboah-Manu D.K. (2006a) Bacteriological and immunological studies towards effective control of *Mycobacterium ulcerans* disease (Buruli ulcer). PhD Universität Basel.

Yeboah-Manu D.K. (2006b) Bacteriological and immunological studies towards effective control of" Mycobacterium ulcerans" disease (Buruli ulcer), University of Basel.

Yee E.Y., Zainuddin Z.Z., Ismail A., Yap C.K. and Tan S.G. (2013) Identification of hybrids of painted and milky storks using FTA card-collected blood, molecular markers, and morphologies. *Biochem Genet* 51(9-10), 789-799.

APPENDIX

APPENDIX I

Buruli Ulcer treatment forms (BU01)

Buruli ulcer clinical and treatment form – new case **BU 01.N**

Health facility: _____ Date of clinical diagnosis or admission (dd/mm/yy): ___/___/___

Name of health worker treating patient : _____ Date of complete healing (dd/mm/yy): ___/___/___

Name of patient: _____ ID#: _____ Age (yrs): _____ Sex: ☐ Male ☐ Female

Address (village or town): _____ District: _____

Province/Region/State: _____ Country: _____ Weight (kg): _____ Profession: _____

CLINICAL HISTORY AT DIAGNOSIS

Duration of illness before seeking care (weeks): _____

Use of traditional treatment: ☐ Yes ☐ No

Limitation of movement at any joint: ☐ Yes ☐ No

Previous treatment with streptomycin: ☐ Yes (duration in days: ____) ☐ No

REFERRED BY: ☐ Village health worker ☐ Self-referral ☐ Former patient ☐ Family member ☐ Schoolteacher ☐ Health worker ☐ Other (specify): _____

CLINICAL FORMS

☐ Nodule (N) ☐ Plaque (Q) ☐ Oedema (E) ☐ Ulcer (U) ☐ Osteomyelitis (O) ☐ Papule (P)

CATEGORIES ☐ **Category I**: A single lesion ≤ 5 cm in diameter ☐ **Category II**: A single lesion 5–15 cm in diameter ☐ **Category III**: A single lesion > 15 cm in diameter, multiple lesions, lesions at critical sites, osteomyelitis

LOCATION OF LESION(S) ☐ Upper limb (UL) ☐ Lower limb (LL) ☐ Abdomen (AB) ☐ Back (BK) ☐ Buttocks and perineum (BP) ☐ Thorax (TH) ☐ Head and neck (HN)

CRITICAL SITES ☐ Eye ☐ Breast ☐ Genitalia

LABORATORY CONFIRMATION

Specimen(s) collected: ☐ **Yes** ☐ **No** Date first specimen(s) taken: __/__/__

Specimen(s) type(s): ☐ Swab ☐ Fine needle aspiration (FNA) ☐ Biopsy

Results

☐ ZN :	☐ *Positive*	☐ *Negative*	☐ *Positive*	☐ *Negative*
☐ PCR :	☐ *Positive*	☐ *Negative*	☐ *Positive*	☐ *Negative*
☐ Histo :	☐ *Positive*	☐ *Negative*	☐ *Positive*	☐ *Negative*

TREATMENT TYPE (Tick all applicable) ☐ Dressings ☐ Antibiotics ☐ Surgery (date: ____/____/____) ☐ POD (prevention of disability)

DOSAGES **Rifampicin:** _____ **(mg)** **Streptomycin:** _____ **(g)** **Other (name):** _____: _____ **(mg)**

Cross out each day (X) after administering the antibiotics; if antibiotics are not taken, indicate with the symbol Ø

Day / Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Total Doses

TREATMENT OUTCOME

☐ 1a: Antibiotic treatment completed ☐ 2a: Healed without surgery ☐ 3a: Healed without limitation of movement at any joint ☐ 4: Referred for further treatment

☐ 1b: Antibiotic treatment not completed ☐ 2b: Healed with surgery ☐ 3b: Healed with limitation of movement at any joint ☐ 5: Lost to follow up ☐ Died

APPENDIX II

REAGENTS AND SOLUTIONS

Reagents carbol fuchsin decolourising (20% H_2SO_4) solution 70% ethanol

Methylene blue

Phenol

KNUST

Materials and instruments

Slides

Pencil

Storage and preparation of reagents

1) Stock alcoholic fuchsin

Fuchsin (basic)	3g
Ethanol (95%)	100ml

The basic fuchsin is dissolved in 100 ml ethanol.

2) 5% Phenol solution

Phenol melted	5m
Distilled water	95m

To liquefy pure phenol crystals, loosen the cap of the phenol reagent bottle, place it into a hand warm water bath. Measure it with a warm pipette to avoid re-crystallization. Mouth pipetting is prohibited. Add the melted phenol slowly to the distilled water while stirring.

3) Ziehl's solution (working carbol fuchsin solution)

Stock alcoholic fuchsin	10ml
5% Phenol solution	90ml

Mix the stock alcoholic fuchsin with 5% phenol while stirring.

Filter the solution before use to remove fuchsin crystals or particles.

4) 20% Sulphuric acid solution

Sulphuric acid (conc H_2SO_4)	20ml
Distilled water	80ml

Add the sulphuric acid slowly to the distilled water using a safety pipette. Never add water to sulphuric acid!

5) 0.3% methylene blue solution

Methylene blue	0.3g
Distilled water	100ml

Dissolve the methylene blue in the distilled water.

Filter the solution before use.

DNA EXTRACTION

Reagents contained in the Genomic DNA Purification Kit

Cell Lysis Solution (CLS)

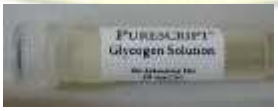
DNA Hydration Solution (DNA Hyd)

Protein Precipitation Solution (PPS)

Reagents not contained in the Genomic DNA Purification Kit

Ethanol

Glycogene 20 mg/ml



Isopropanol

Lysozyme 10 mg/ml

Proteinase K 20mg/ml

Lyophilization of Primers

Reagents

Purified destilled water (Carl Roth, Karlsruhe, Germany)

Primer MU5 primer stock
agc gac ccc agt gga ttg gt
(TibMolbiol, Berlin, Germany)

Primer MU6 primer stock cgg
tga tca agc gtt cac ga
(TibMolbiol, Berlin, Germany)

Gel Electrophoresis

Reagents
10 X TBE Buffer

100 bp DNA ladder

Agarose

Agarose gelelectorophoresis chamber

Materials and instruments

DNase-free Pipette tips 10 µl, 20 µl

Gloves nitrile

Loading Dye

Reaction tube rack

Waterresistent pens, pens

Pipettes
0,1-10 µl, 2-20 µl

Microwave oven

Plastic Erlenmeyer flask

KNUST