Loop-mediated isothermal amplification test development, implementation and evaluation for yaws eradication

LAMP 4 YAWS

Version and date: 1.1 / 18/08/2020

STUDY SPONSOR: London School of Hygiene & Tropical Medicine

SPONSOR ADDRESS: Keppel St, London, WC1E 7HT, United Kingdom

CHIEF INVESTIGATOR: Dr Michael Marks

INTERNATIONAL STUDY COORDINATOR: Rebecca Handley

FUNDERS: European and Developing Countries Clinical Trials Partnership (EDCTP)

STUDY COORDINATION CENTRE: London School of Hygiene & Tropical Medicine

MAIN CONTACTS:

Chief Investigator: Dr Michael Marks, Michael.marks@lshtm.ac.uk; Keppel St, London, WC1E 7HT, UK, 0207 927 2457

Study Coordination: Rebecca Handley, Rebecca.Handley1@lshtm.ac.uk, Keppel St, London, WC1E 7HT, UK, 0207 927 2866. For general queries, supply of trial documentation, and collection of data, please contact: Rebecca Handley.

Sponsor: London School of Hygiene & Tropical Medicine is the main research sponsor for this study. For further information regarding the sponsorship conditions, please contact the Research Governance and Integrity Office: London School of Hygiene & Tropical Medicine, Keppel Street, London, WC1E 7HT, UK; Tel: +44 207 927 2626,

This protocol describes the LAMP4YAWs study and provides information about procedures for entering participants. Every care was taken in its drafting, but corrections or amendments may be necessary. These will be circulated to investigators in the study. Problems relating to this trial should be referred, in the first instance, to the study coordination centre. This trial will adhere to the principles outlined in the International Conference on Harmonisation Good Clinical Practice (ICH GCP) guidelines, protocol and all applicable local regulations.

Table of Contents

1 STUDY OVERVIEW AND PROTOCOL SUMMARY

1.1 Study title:

Loop-mediated isothermal amplification test development, implementation and evaluation for yaws eradication

1.2 Acronym:

LAMP4Yaws

1.3 Study design:

This study will take place in three sub-Saharan African countries known to be endemic for yaws. We aim to screen approximately 60,000 participants, of all ages. Screened participants will undergo primary clinical examination for yaws-like lesions and will be classed as either suspected yaws or negative for active yaws. A proportion of the yaws free participants (≈10%) and all those with suspected yaws (≈6000 people) will be invited to answer a questionnaire relating to household demographics and possible risk factors for yaws. If resources allow they will also be asked to provide dried blood spots for future serological analysis of yaws and other co-endemic neglected tropical diseases. Rapid point of care (POC) serological tests will then be used to confirm the clinical yaws diagnosis in the field, to allow for recruitment into the diagnostic evaluation study. Those with suspected yaws lesions will be tested with the SD Bioline syphilis test and those who test positive will be confirmed with the Chembio Dual Path Platform Syphilis Screen and Confirm POC test (herein DPP). As some cases of yaws occur in individuals who have not yet sero-converted, in order to improve the *Treponema pallidum pertenue* (TPE) prevalence estimate accuracy, we will collect lesion samples from all individuals with a dual seropositive DPP (treponemal and non-treponemal) and a subsample of the participants (1/10) negative for the SD Bioline or the DPP (either treponemal antibodies, non-treponemal antibiodies or both). Two swabs, collected simultaneously from the same lesion, will be used for the diagnostic evaluation of the *T. pallidum* and *Haemophilus ducreyi* Loop-mediated isothermal amplification (TPHD LAMP) assay. Up to three further swabs may be collected from the same or additional lesions of the participant and from individuals with non-seroreactive tests but yaws-like lesions. We will also collect swabs of asymptomatic skin and of fomites of the house from some participants with and without yaws. These additional swabs will allow us to investigate other pathogenic causes of lesions using specific nucleic acid amplification tests and next generation sequencing. We will also be able to perform supplementary laboratory analysis on swab collection and DNA extraction methodologies, investigate the role fomites may have in the transmission of TPE and *H. ducreyi* and further understand the role of *H. ducreyi* as a cause of skin ulcers. No more than five lesion

swabs will be collected from any one individual. Further to this, we will enrol people from yaws endemic communities, healthcare workers and key stakeholders into sub-studies examining knowledge, attitudes and practices in relation to yaws, skin disease, and their diagnosis.

1.4 Study Sites:

Field studies and laboratory testing will take place in three countries in West Africa: Cameroon, Cote d'Ivoire and Ghana. These countries have been selected as they have currently the highest reported incidence of yaws in Africa. Within each country, national reporting data will be used to select three districts that are highly endemic for yaws, to allow for more rapid attainment of the required number of participants with active yaws for the LAMP diagnostic evaluation.

1.5 Study participants and planned sample size:

Patients of all ages in endemic districts will be recruited into the study. In total, we plan to perform an initial clinical screen on approximately 60,000 participants of whom we expect 6,000 will present with yaws-like lesions. Of these we expect around 600 individuals will have a dual positive DPP test and will therefore be eligible for the diagnostic evaluation.

1.6 Planned Study period:

36 months

1.7 Research question/Aim:

LAMP4Yaws is a multidisciplinary study aiming to determine whether the *T. pallidum* and *H. ducreyi* Loop-mediated isothermal amplification (TPHD LAMP) test is suitable, feasible and cost-effective for use in national yaws eradication programmes and/or routine yaws surveillance. The main aim is supported by additional, linked social science and laboratory analysis that we hope will allow us to learn more about risk factors associated with disease, local transmission dynamics, other skin conditions and build in country capacity to run efficient yaws eradication campaigns.

1.8 Primary objectives:

1) Perform active case searching and collect household and community level data to identify risk factors associated with yaws transmission and disease.
2) Assess the accuracy, acceptability and feasibility of the TPHD-LAMP assay for *T. pallidum* and *H. ducreyi* diagnosis under programmatic conditions in yaws endemic areas in Ghana, Cote d'Ivoire and Cameroon to determine its suitability as a diagnostic in yaws eradication programmes.
3) Testing the *Treponema pallidum* macrolide resistance (TPMR)-LAMP assay in a field setting.

4) Establish other infectious causes of non-yaws ulcers using specific PCR tests and next generation sequencing techniques, including novel devices such as employing the Minion for nanopore sequencing.
5) Collect data on community, patient and healthcare worker knowledge, attitudes and practices in relation to yaws.
6) Determine the acceptability and feasibility and cost effectiveness of the TPHD-LAMP assay.

1.9 Secondary objectives:

1) Build within-country capacity for future national yaws eradication campaigns.
2) Refine and evaluate the current TPHD-LAMP assay to enable accurate detection of macrolide resistance in a field setting.
3) If macrolide resistance is detected we will use whole genome sequencing to explore the evolution and transmission routes of resistant strains.
4) Develop an external quality assurance scheme for molecular diagnosis of yaws across endemic countries.
5) Collect an archive of swabs from people and fomites, which can be used to evaluate alternative methodologies for swab collection, DNA extraction, molecular testing, and investigations of bacterial transmission.
6) Collect an archive of dried blood spots from participants for future serological analysis to detect other neglected tropical diseases and/or previous exposure to Treponemes.
7) Establish which other skin conditions are commonly co-endemic in Ghana, Cote d'Ivoire and Cameroon.

1.10 Collaborating institutions:

Institute	Principal investigator	Contact details	Primary Role
Albert-Ludwigs-Universität Freiburg, Germany	Nadine Borst	Nadine.Borst@Hahn-Schickard.de	Refinement of LAMP assay
Centre Pasteur du Cameroun, Cameroon	Tania Crucitti	crucitti@pasteur-yaounde.org	Field evaluation and capacity building
Fundación Privada de Lucha contra el sida (FLS), Spain	Oriol Mitjà	oriolmitja@hotmail.com	Field study coordination

Georg-August-Universität Goettingen Stiftung Oeffentlichen Rechts, Germany	Sascha Knauf	sascha.knauf@uni-goettingen.de	External quality assurance scheme
Institut Pasteur de Cote D'Ivoire, Cote d'Ivoire	Solange Ngazoa	ngazoa_solange@yahoo.fr	Field Evaluation
London School of Hygiene and Tropical Medicine	Michael Marks	Michael.marks@lshtm.ac.uk	Lead institute: project management Field Evaluation Health economic and qualitative evaluation
MAST Diagnostics	Mohammed Bakheit	bakheit@mast-diagnostica.de	Provision and refinement of LAMP assay
University of Ghana, Noguchi Memorial Institute for Medical Research (NMIMR), Ghana	Kwasi Kennedy Addo	kaddo@noguchi.ug.edu.gh	Field Evaluation

1.11 Funding:

This study has been funded by an EDCTP grant (RIA2018D-2495)

1.12 Role of study funder:

The funder has not played any role in the study design.

2 BACKGROUND AND RATIONALE

2.1 Background

2.1.1 Yaws

Yaws is a chronic infectious disease of the skin, bone and cartilage caused by the spirochete bacterium *Treponema pallidum* subsp. *pertenue* (TPE)[1]. TPE is closely related to the syphilis-causing bacterium *Treponema pallidum* subsp. *pallidum* [2] but unlike syphilis, yaws primarily affects children and is generally not sexually transmitted [3–5]. It is one of the World Health Organization (WHO) neglected tropical diseases (NTDs) and under the WHO's new roadmap is targeted for eradication by 2030 [6]. Yaws presents initially as a bacteria-rich papilloma, which if left untreated, can ulcerate. In some cases, the disease progresses to secondary yaws and it is at this stage that it can cause further skin manifestations as well as inflammation of bones and joints. In rare cases tertiary yaws develops; this is often characterised by destructive and disfiguring lesions [3]. Untreated patients are susceptible to developing a latent infection, which can relapse, commonly within five years of the initial infection. During the latent stage of the disease there are no clinical manifestations [3]. It is thought that for every clinical case of yaws there are six latent cases [7], which has serious implications for eradication programs.

2.1.2 Historical and contemporary eradication strategies

Yaws was once reported to be endemic in more than 80 countries, but global prevalence was reduced by over by 95% as the result of a 1950's to 60's WHO/UNICEF eradication campaign involving mass drug administration (MDA) of single dose parenteral penicillin[4,8] . Most likely, a lack of resources, reduced political will and premature discontinuation of efforts led to a resurgence of yaws and the disease [9,10]. A single dose of oral azithromycin has since been shown to be highly effective at treating yaws[11–14]. This resulted in a revised WHO yaws eradication strategy, which consists of azithromycin MDA at the district or sub-district level, followed by active-case finding and treatment of remaining cases [15]. Human yaws is currently known to be endemic in 14 countries, with the majority of cases reported in West Africa, South East Asia and the Pacific [16,17]. Prevalence estimates are likely to be an underestimate due to a lack of systematic mapping data and under-reporting of the disease [1,17]. To meet eradication targets it is essential that all endemic areas are accurately mapped to allow for targeted interventions. However, this is dependent on the availability of an accurate diagnostic method, that can be deployed in all suspected yaws-endemic districts in a standardised manner.

2.1.3 Diagnostics

Accurately diagnosing yaws will be key to the success of the global eradication campaign. Traditionally, serological tests have been the mainstay of yaws diagnosis, consisting of detection of treponemal and non-treponemal antibodies. Anti-treponemal antibodies are long-lived so are useful for indicating exposure but cannot distinguish between current and past infections, nor can they distinguish between infection with any of the *T. pallidum* subsp. *pallidum* (syphilis), *pertenue* (yaws), *endemicum* (bejel) or *T. carateum* (pinta) [1]. Non-treponemal tests are used to predict a current infection, but lack specificity [1]. When used in conjunction non-treponemal and treponemal tests are a better indicator of a current treponemal infection, but also fail to distinguish between different species of Treponemes, and have been shown to miss early cases of yaws[1], which could be important for identifying resurgence of the disease after MDA.

Clinical diagnosis based on lesions alone is unreliable, often due to the presence of co-endemic ulcer-causing diseases. Recent studies using molecular tools have demonstrated only one third of yaws-like ulcers are positive for *T. pallidum*[18]. Studies from Papua New Guinea [18] and Solomon Islands [19] have shown *H. ducreyi,* the causative agent of chancroid, to be responsible for a further third of these clinically-indistinguishable lesions. Often neither *T. pallidum* nor *H. ducreyi* are detected in these lesions [18,19] and it is possible another infectious agent is responsible. Therefore, we will conduct further investigations to determine if other pathogens may be routinely detected that could influence the design of yaws eradication programs.

The urgent need for novel diagnostics is perhaps most essential in light of the emergence of azithromycin-resistant TPE. These strains have been shown to emerge independently, and have led to localized transmission [20]. Highly sensitive and specific nucleic acid amplification tests (NAATs) which include tests for azithromycin resistance are available [18,21]. However, these tests are often inaccessible and have limited use in the field setting. In light of this emerging macrolide resistance it is clear that more robust laboratory-based surveillance is required.

2.1.4 Significance and Scientific Rationale

Improved understanding of yaws transmission and novel yaws diagnostics are urgently needed to support the WHO eradication campaign. Current WHO strategy is based on clinical case searches combined with serological point of care (POC) assays [15]. An improved understanding of yaws epidemiology, informed by diagnostics, would help refine and improve case search approaches and contact tracing as part of eradication efforts. Given the limitations of existing diagnostics, improved diagnostic tests are needed. These tests should be designed to fulfil the WHO REASSURED criteria, i.e. allow for real-time connectivity, easy to collect specimens, be affordable, sensitive, specific, user-

friendly, rapid, equipment-free and deliverable to end users [22]. The TPHD LAMP test (Mast Diagnostica GmbH, Germany) is a POC test that is practical to use in resource-limited areas. LAMP does not require expensive equipment, such as thermocyclers required for traditional NAATs, and the reagents have been designed to be stored lyophilised for field conditions. The TPHD LAMP test can rapidly identify an infection with *T. pallidum* or *H. ducreyi* and has been shown to have high sensitivity and specificity in the laboratory [23]. It can also be adapted to include azithromycin resistance detection [24]. This test may, therefore, provide a suitable solution to the problems faced by current yaws diagnostics and if accurate and feasible to use in the field, has the potential to be used by yaws eradication programs, allowing for systematic real-time detection of *T. pallidum* and *H. ducreyi,* as well as azithromycin-resistant strains.

3 RESEARCH AIMS AND OBJECTIVES

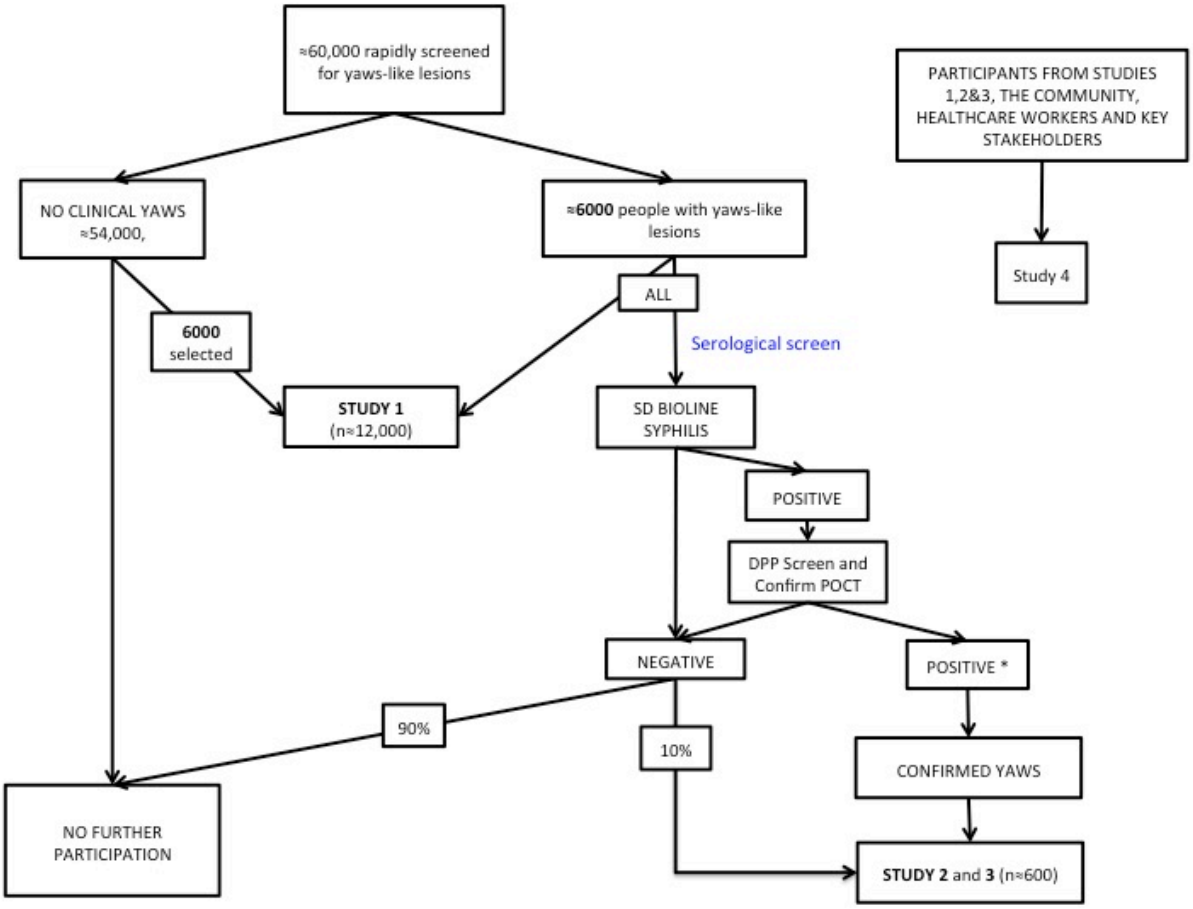
LAMP4Yaws is a multidisciplinary study aiming to determine whether the TPHD LAMP test is a suitable, feasible and cost-effective diagnostic tool for yaws eradication campaigns and surveillance. The main aim is supported by additional, linked social science and laboratory analysis that will allow us to learn more about risk factors associated with disease, local transmission dynamics, other endemic skin conditions and build in country capacity to run efficient yaws eradication campaigns. This project consists of four linked sub-studies:

1. Multi-country case searches and epidemiological analysis of risk factors for yaws and other causes of yaws-like skin lesions (SECTION 4)
2. Performance evaluation of a TPHD LAMP assay compared to qPCR for the diagnosis of yaws-like lesions and optimisation of sample collection and processing (SECTION 5)
3. Use of molecular tools and next-generation sequencing to understand the aetiology of yaws-like lesions, and optimise sequencing tools for POC use (SECTION 6)
4. A health economic and social science evaluation of healthcare worker and community knowledge-attitudes and practices in relation to yaws and related diseases, and their diagnosis (SECTION 7)

By performing these four linked studies we hope to achieve a better understanding of the epidemiology of yaws in our chosen districts including identifying key risk factors and transmission dynamics, and determining the main aetiologies of lesions. The evaluation of the TPHD LAMP assay and the social science analyses will result in data that allow us to conclude if the current assay is a useful tool for routine diagnostics and use in yaws eradication programmes. An outline of each specific sub-study is given below.

3.1 Overall Study Outline and recruitment

Figure 1: Flow chart of participant recruitment across all three-study countries. Initially we will screen 60,000 people or whom we expect 6000 will present with yaws-like lesions. These 6000 participants, along with 6000 without clinical evidence of yaws will be invited to take part in sub-study 1, the epidemiological analysis. All participants with clinically suspected yaws will be tested with a serology POC test, with positive results being confirmed by second POC test. Those with clinical and serological evidence of yaws will be invited to participate in sub-studies 2 and 3. Additionally, some participants from studies 1-3 along with healthcare workers and key stakeholders will be invited to enrol in study 4.



* A DPP test is considered positive only if sero-reactive for both treponemal and non-treponemal antibodies.

3.2 Study Sites

In each of the three countries, national reporting data will be used to select at least three districts that are highly endemic for yaws, facilitating rapid fulfilment of the sample size required number of yaws cases for the diagnostic accuracy study. The number of villages/communities selected will depend on prevalence of the disease. This exercise will be conducted with the yaws programme national coordinators in the three endemic countries.

3.3 Consent and information for participants

In each site we will conduct active case finding activities in collaboration with Ministry of Health departments and National Yaws Programmes. For sub-studies 1-3, all residents in selected communities will be eligible to participate in the study. Prior to any study procedures, written informed consent will be obtained from participants or, from parents, guardians, or legal

representatives if the participant is under the age of consent. Following national recommendations informed consent will be conducted in English or French, with the assistance of a community representative translating if necessary. If a person is illiterate the study information and consent procedure will be clearly explained to them by a member of the study team or a local translator. If a translator is used they will be required to witness the informed consent procedure. Children and minors over ten years of age and under the age of consent, will also be required to give verbal assent for enrolment. If consent is not given for future research, it will be indicated under the appropriate section in the consent form. If any participants wishes to withdraw from the study at any point post-enrolment this will be indicated to study coordinator and data managers for complete elimination of that participants information. Consent forms, approved by all relevant ethical review boards, will be available in English and French, as appropriate. Individuals at each site will be consented for participation into each sub-study based on the relevant inclusion/exclusion criteria (see sections: 4.2; 5.2; 6.2; 7.2).

4 SUB-STUDY 1: MULTI-COUNTRY CASE SEARCHES AND EPIDEMIOLOGICAL ANALYSIS OF RISK FACTORS FOR YAWS

4.1 Objectives

1) Determine the prevalence of active and latent yaws in each setting using combined clinical, serological and molecular tools
2) Identify risk factors associated with active and latent yaws
3) Collect a representative dried blood spot sample library for future serological testing

We will undertake integrated case searches for individuals with yaws-like lesions. We will collect baseline clinical, demographic and risk factor information from participants. Individuals with clinical evidence of yaws will be tested with point-of-care serological tests (SD Bioline and Chembio DPP) in line with standard WHO recommendations, and will have lesion samples collected which will form the sample set required for sub-studies 2 & 3. We may record the presence of other skin conditions seen.

4.2 Inclusion and exclusion criteria

Inclusion Criteria

- Resident in selected community
- Willingness to provide informed consent to participate in the study

Exclusion Criteria

- Refusal of village chief (for village inclusion), or refusal of individual and/or guardian (for individual inclusion)
- Not willing or able to give informed consent for the study
- Permanent disability that prevents or impedes study participation and/or comprehension

4.3 Sample Size Considerations

We aim to screen approximately 60,000 individuals, 20,000 in each country. Of these we estimate 6000 individuals will present with yaws like lesions and 600 will have active yaws (i.e. present with yaw-like lesions and confirmed serologically). The remaining participants will be classed as either negative for yaws due to the absence of clinical signs or negative serology or suspected latent yaws if sero-reactive. We will aim to enrol all 6000 participants with yaws like lesions and a further 6000 without clinical yaws into this sub study. This will give us >80% power to detect risk factors associated with yaws with an odds ratio of 3. If resources allow, we will collect dried blood spots of half of the people enrolled into sub-study 1 giving us roughly 6000 dried blood spots samples. This would allow us to detect 5% seroprevalence with 0.5% precision, and a seroprevalence of 50% with a precision of 1.3%, which is deemed more than sufficient for a convenience sample [25].

4.4 Data Collection

In conjunction with Ministry of Health and local collaborators we will undertake case searches for individuals with yaws and yaws-like lesions in selected communities. All study staff involved in the field surveys will be extensively trained in the clinical, diagnostic, and public health aspects of yaws. The initial screening will involve a quick examination to determine if people have yaws lesions present. All people with lesions and around 6000 people without any clinical evidence will then be invited into sub study 1. Those enrolled will provide demographic data and risk factor data (Appendix A and B). Those with yaws will undergo a more thorough skin examination to look for evidence of clinically active yaws and this will be recorded into a case report form (CRF) (see Appendix B) and may note the presence of other skin conditions. Yaws-like lesions will be assessed using WHO recommended procedure [26] (Appendix C), with a simplified system of WHO yaws pictorial guide[27] used to define each yaws-like lesion. These will be marked on a diagram and lesions will be photographed for quality control purposes (Appendix B). Each participant will be assigned a unique study identification number and all data will be recorded and analysed with this unique identification number. Study data will be electronically entered into a Good Clinical Practice (GCP)-compliant, offline, trial-specific mobile application [Research Electronic Data Capture (REDCap), Nashville, TN, USA] or Open Data Kit (ODK).

4.5 Sample collection and storage

As per WHO recommendations, people with clinically suspected yaws lesions will be screened using a *T. pallidum* POC test, and those that test positive will be confirmed with the DPP Screen and Confirm POC test detecting both treponemal and non-treponemal antibodies. In individuals with a positive DPP assay, we will collect lesion swabs for molecular testing according to WHO procedure [26] (Appendix C). As some cases of yaws occur in individuals who have not yet sero-converted we will also collect swabs from 1/10 people with suspected yaws but non-reactive serological tests. Two swab samples will be simultaneously obtained from each suspected yaws lesion (i.e. rolling both swabs over the lesion at the same time); one to conduct the TPHD-LAMP assay at the district level hospital, the other for PCR at the national reference laboratory for the detection of *T. pallidum* and *H. ducreyi* and monitoring resistance to antibiotics. A maximum of three other swabs may be taken, from the same or different lesions from people with or without yaws, and further swabs may be collected from asymptomatic skin or from fomites in the house, for additional infection detection analysis. Swab samples will be collected dry, or in suitable transport medium, and all tubes will be labelled with the participant's unique identification numbers and collection date. Half of those enrolled into sub-study 1 will be asked to provide a dried blood spot sample. This will give us an archive of around 6000 dried blood spots with which we can perform serological testing in the future. For this we will collect capillary finger blood onto a filter

paper designed specifically for blood collection following published procedures (see Appendix D). These will be air dried for at least 12 hours prior to being stored with desiccant sachets at ambient temperature.

4.6 Laboratory testing

The dried blood spots will be used to conduct multiplex serological assays on the Luminex platform to look for antibodies to Treponemes and other infectious diseases. By measuring levels of anti-Treponemal antibodies in children we will be able to estimate the number of latent yaws cases within the community. We will choose a panel of infectious diseases that we know, or suspect, to be endemic in our populations and measure antibody responses specific to these pathogens in order to indicate exposure and seroprevalence at the individual, community and district level [28].

4.7 Follow-up

All samples for the primary analysis are collected at the first visit and therefore we do not anticipate loss to follow-up or non-compliance to be an issue. All individuals with a clinically suspected yaws lesion or ulcer, regardless of screening-assay result, will be offered treatment with azithromycin or referred to the relevant services for treatment in line with national guidelines. If other skin conditions are seen, participants will also be referred to the relevant services. As per WHO guidance, we will review patients with suspected yaws lesions at week four, when unhealed lesions will be swabbed and treatment with benzathine benzylpenicillin offered. If azithromycin resistance is detected through the laboratory analysis at any point during the study, we will work with yaws eradication programmes to implement interventions for study participants and communities.

4.8 Data Analysis

Quantitative data analysis will be performed with R, STATA and other software packages.
Initially we will perform univariable logistic regression analyses on each potential risk factor. If any factors are found to be associated with yaws we will include these in multivariate logistic regression models with age and gender included *a priori* in order to calculate odds ratio for any factors associated with active yaws. We will also calculate the 95% confidence interval for each odds ratio. The multivariate analysis will allow us to determine the strength of associations between active yaws and each factor, or combinations of factors. All statistical analysis will be performed to the 95% significance level. For the Luminex assay, we will use appropriate statistical tools and published literature to determine cut-off values for each antibody and calculate seroprevalence with 95% confidence intervals.

5 SUB-STUDY 2: PERFORMANCE EVALUATION OF A TPHD LAMP ASSAY COMPARED TO QPCR FOR THE DIAGNOSIS OF YAWS-LIKE LESIONS

5.1 Objectives

1) Evaluation of a TPHD LAMP assay performed in local hospitals compared to qPCR/PCR results obtained at the national reference laboratory in each country.
2) Testing the *Treponema pallidum* macrolide resistance (TPMR)-LAMP assay in a field setting
3) Development of an external quality assurance scheme for molecular diagnosis of yaws across endemic countries
4) Evaluating alternative methodologies for swab collection, DNA extraction and molecular testing

5.2 Inclusion and exclusion criteria

This study will consist exclusively of a subset of the individuals enrolled into sub-study 1 (case-finding). Participants will be consented for inclusion into sub-studies 2&3 at the same time as consent and enrolment into sub-study 1.

5.3 Sample size

We will collect samples for the TPHD LAMP evaluation from the 600 individuals with clinical and serological evidence of yaws, and an additional 10% of participants with clinical yaws but negative serology (sub-study 1). Based on the assumption that 35% of seropositive cases will also be positive by qPCR, this will result in approximately 210 qPCR positive *T. pallidum* cases and a similar number of *H. ducreyi* cases [18,19,29]. This sample size will allow us to confirm that the TPHD-LAMP has both a sensitivity and specificity of at least 85% compared to the reference qPCR assays for both targets. This will also provide us with about 200 samples with unknown lesion aetiology to evaluate using next-generation sequencing as part of sub-Study 3.

5.4 Sample collection and Storage

Samples will consist of lesion samples collected in sub-study 1 to be tested using both the TPHD LAMP assay (performed at the district/regional level) and qPCR/PCR (performed at each country's National Reference Laboratory). We will also test all *Treponema pallidum* positive samples for azithromycin resistance genes using the multiplex TPMR-LAMP assay and the gold standard resistance detection techniques performed at reference laboratories. Samples from the study will also be used to

undertake objectives 3-5; including development of an external quality assurance (EQA) scheme for yaws molecular diagnostics and evaluating optimal sample collection and processing approaches. These samples will also be used for sub-study 3.

5.5 Laboratory Procedures

For the diagnostic evaluation, samples will be transferred to both district and reference laboratories for testing under routine programmatic conditions. For reference PCR testing at the reference laboratory, DNA will be extracted from lesion samples. In the first instance, we will run PCRs targeting *T. pallidum* and *H. ducreyi.* A negative (no-template) control, positive controls for *T. pallidum* and *H. ducreyi* DNA and an extraction control will be included in each PCR run. In samples where *T. pallidum* is detected further PCRs targeting macrolide resistant point mutations A2058G and A2059G in the 23s RNA genes and to distinguish between different species of Treponemes will be run.

TPHD-LAMP and TPMR-LAMP will be performed at district laboratory level. DNA will be extracted using a commercial extraction kit deemed suitable for use in the field, the same kit will be used in all district laboratories. The TPHD-LAMP assay will be run in accordance with the manufacture-provided instructions. SOPs will be written by members of the LAMP4yaws consortium and provided to each of the laboratories. Incubation will be performed using the field compatible Tubescanner platform, which reads fluorescence signals for up to 4 different dyes. Final test results in terms of “positive”, “negative” or “invalid/retest” will be directly displayed on the screen using test methods developed by Mast (Mast Diagnostica GmbH, Germany).

All results, including raw and cleaned data from both reference and local laboratories will be electronically uploaded and deposited in the secure, encrypted central study database at LSHTM. Staff at district laboratories performing the TPHD-LAMP will be blinded to results from the national reference laboratories, and staff performing the PCR/qPCR at the national reference laboratories will be blinded to the TPHD LAMP results.

A subset of the additionally collected swabs taken from suspected yaws patients, the fomite swabs and some aliquots of DNA extracts will be shipped to the laboratories of the LAMP4YAWS consortium (see 1.11) for use in sub-study 3 and to run additional investigations into optimal sample collection and processing methods. These will include testing fomite swabs for the presence of *T. pallidum* or *H. ducreyi* and preforming additional viability PCRs to determine if living bacteria can be detected on fomites in order to highlight if fomites are a key route of transmission. We will also use the TPHD-LAMP assay and reference qPCR to investigate if diagnostic accuracy can be enhanced by taking swabs

from multiple lesions of an infected patient. A selection of additional swabs and/or DNA extracts may be sent to various collaborating laboratories to enable the development of an external quality assurance (EQA) scheme.

5.6 Quality assurance for laboratory procedures at study sites

The TPHD LAMP will be used at local hospital facilities close to the enrolled communities. Extensive training for this will take place prior to participant recruitment and we will conduct both internal and EQA activities throughout the study. Some aliquots of extracted DNA or additional lesion swabs will be exported to the European consortium laboratories and rechecked using both the TPHD-LAMP and gold standard (qPCR/PCR) for each pathogen. We will purposively select a higher proportion of samples for External Quality Assurance in the first six months of the laboratory testing to allow early detection of significant implementation problems. In case of significant differences between the reported results and the international reference laboratory results, an on-site evaluation will be initiated to identify the cause and solve the problem. All participating laboratories will work according to the GCLP guidelines, including internal quality assurance activities.

5.7 Data Analysis

For the evaluation of the TPHD-LAMP assay compared to qPCR for the diagnosis of yaws, we will calculate the sensitivity and specificity of the *T. pallidum* and *H. ducreyi* targets individually compared to the respective reference qPCR assays. We may perform additional sub-analyses to assess if the performance of the TPHD-LAMP assay is related to bacterial load as measured by qPCR. The positive predictive value and negative predictive value will also be determined against the qPCR results.

6 SUB-STUDY 3: USE OF MOLECULAR TOOLS AND NEXT-GENERATION SEQUENCING TO UNDERSTAND THE AETIOLOGY OF YAWS-LIKE LESIONS, AND OPTIMISE SEQUENCING TOOLS FOR POINT OF CARE USE.

6.1 Objectives

1) Use nucleic acid amplification tests to investigate the presence of *Mycobacterium ulcerans*, Leishmania or *Corynebacterium diphtheria* DNA in TPE and *H. ducreyi* negative swabs to determine if these pathogens are a significant cause of yaws-like lesions
2) Perform metataxonomic microbial analysis to determine other bacterial causes of yaws-like lesions when targeted pathogens are not detected through NAATs
3) Explore the evolution and transmission routes of resistant strains using whole genome phylogenetic analyses if macrolide resistant TPE are isolated
4) Optimise the Oxford Nanopore Minion sequencing for detection of TPE, *H. ducreyi,* and macrolide resistance strains of both pathogens, in the field
5) Perform investigations into the role of *H. ducreyi* as a significant cause of yaws-like lesions by measuring the presence of *H. ducreyi* on the skin of asymptomatic patients of all ages, using culture and molecular methods and genotyping any *H. ducreyi* detected from symptomatic or asymptomatic cases.
6) Detail the baseline resistance profiles of *H. ducreyi* using drug susceptibility assays

6.2 Inclusion and exclusion criteria

As with sub-study 2, this study will consist exclusively of a subset of the individuals enrolled into sub-study 1 (Case-finding). Participants will be consented for inclusion into sub-studies 2&3 at the same time as consent and enrolment into sub-study 1.

6.3 Sample size

There is no formal sample size for this sub-study.

6.4 Sample collection and storage

As with sub-study 2, this study will utilise the samples collected from participants in sub-study 1. These will be stored in country and a sub-set will be sent to the laboratories of the consortium in Europe.

6.5 Laboratory Procedures

Tests will be performed in the laboratories having the assays, platform and equipment in place or under development for the purpose. For all NAATs and sequencing techniques, DNA will be extracted from lesion swabs using a suitable commercial kit specially designed or adapted for swab

extraction. Targeted tests for *Mycobacterium ulcerans*, Leishmania or *Corynebacterium diphtheria* will be performed using qPCR. To detect *H. ducreyi* from fomite swabs or asymptomatic skin, swabs will be inoculated on enriched culture media before being tested using qPCR. Genotyping using whole genome sequencing (WGS) or multilocus sequence typing (MLST) will be performed on the *H. ducreyi* cultures obtained. Genotyping directly from swab DNA extracts, without the need for enrichment culturing, will also be tested. These activities will be led by the Centre Pasteur du Cameroun.

For Illumina and Minion WGS sequencing, qPCR will be used to select samples with a higher bacterial presence. DNA fragmentation and library preparation will be performed using a suitable commercial kit for the sequencing platform. We will then sequence using manufacture guidelines or methodology optimised within house. A range of open source software will be used to highlight low quality reads, clean data and complete assembly of genomes using established reference strains.

For metataxonomic sequencing, the variable region 4 (V4) of the 16S ribosomal RNA gene will be targeted. A blank control (microbial DNA-free water) and a mock control sample will be included into the analysis. Amplicon triplets will be pooled and purified using AMPure XP beads (Beckman Coulter). Sequencing will be done using Illumina technology (MiSeq 2x250 bp paired-end sequencing), following published guidelines [30].

6.6 Data Analysis

Whole genome sequencing: We will compare any sequences *T. pallidum* or *H. ducreyi* to reference strains to highlight single nucleotide polymorphisms, or any other structural differences. We will create phylogenetic trees to compare relatedness. We will use the same technology to explore how antibiotic resistance may have spread around the participating communities and if it is caused by the two common resistance mutations or novel mutations. Data management will fall under the responsibility of the lead investigator of the laboratory conducting each test and will be shared with other members of the consortium if requested.

7 SUB-STUDY 4: A HEALTH ECONOMIC AND SOCIAL SCIENCE EVALUATION OF HEALTHCARE WORKER AND COMMUNITY KNOWLEDGE-ATTITUDES AND PRACTICES IN RELATION TO YAWS AND RELATED DISEASES, AND THEIR DIAGNOSIS.

7.1 Objectives

1) Collect data on community, patient and healthcare worker knowledge, attitudes and practices in relation to yaws and identify specific needs among the community including challenges in accessing prevention, care, and treatment services
2) Determine the acceptability and feasibility of TPHD-LAMP assay routine use
3) Determine cost-effectiveness of the TPHD-LAMP assay

7.2 Inclusion and exclusion criteria for objectives 1 and 2

Inclusion criteria:

- Willingness to provide informed consent and assent to participation in the study
- Male or female and over the age of consent
- Male or female >12 and under the age of consent if accompanied by a parent or guardian
- Community members should be permanent residents in the selected communities

Exclusion

- Not able to give informed consent
- Under the age of consent with no parent/guardian available to accompany
- Not a permanent resident in the community

7.3 Data collection

Attitudes and practices in relation to yaws and identifying community needs: In each community, we will invite a sample of key groups including community health workers, opinion leaders and parents and guardians of children to participate in focus group discussions and semi-structured interviews about yaws and related skin diseases. As this is a qualitative evaluation, we have not performed a formal sample size calculation but will aim to achieve data saturation. For the community, members will be selected with an equal portion of males and females and a range of ages to ensure a representative sample. All focus group discussion and interviews will be conducted in English or French with local translators available if another language is preferred. All interviews and focus groups will be recorded, transcribed and translated if necessary. After being transcribed, recordings will be deleted. Common themes identified from the focus group discussions will be used to develop a questionnaire relating to their experiences and perceptions of: the cause of the disease and its transmission; its

diagnosis; healthcare seeking attitudes and factors (barriers/facilitators) influencing this. This questionnaire will be circulated to other communities and key stakeholders enabling us to obtain quantitative data to validate the hypotheses generated from the focus group discussions.

Acceptability and feasibility: To assess the acceptability and feasibility of the TPHD-LAMP assay to healthcare workers and laboratory staff, we will conduct semi-structured interviews and/or use standardised questionnaires to gather their overall impressions of performing traditional molecular tests and the TPHD- LAMP assay. Again focus group discussion and interviews will be conducted in English or French with local translators available if another language is preferred. All interviews and focus groups will be recorded, transcribed and translated if necessary. After being transcribed, recordings will be deleted. For the feasibility aspect of the study, we will collect a range of information such as: determining the amount of training required to perform the test, for both field workers and laboratory staff, practicality of running the LAMP test in terms of laboratory space, reagent storage, electricity, sample transport and storage and other factors such as the time taken between sample collection and result and how easy it is to get the result back to the patient.

With patients and other key stakeholders, the test acceptability, will be explored via focus group discussions and in-depth interviews, with a focus on local knowledge and perceptions: of yaws and other skin conditions; diagnostics and available treatments, perceptions of healthcare workers' decisions in relation to yaws; and barriers to acceptability of the new test.

Cost-effectiveness: For the health-economic analysis we will use an "ingredients approach to costing", gathering unit costs for each category of costing (training, personnel, capital costs, consumables, results storage and reporting, EQA, etc.). The costs of the LAMP assay will be compared with running the gold standard qPCR at national reference laboratories. The measure of effectiveness will be the number of additional yaws cases diagnosed and the number of additional yaws cases appropriately managed. If the macrolide resistance test can be successfully incorporated, then these costs will also be included, including the cost-savings of avoiding inappropriate antibiotic treatment.

7.4 Data Analysis

1) This will involve a mixed methods analysis. We will perform thematic analyses to identify common themes arising from focus group discussions and in-depth interviews. The quantitative questionnaires will be analysed using standard statistical analyses, including Chi

squared tests and logistic regression to analyse relationships between reported knowledge, attitudes and practices, and demographic variables such as gender, age and location.

2) For the feasibility and acceptability analysis we will also perform thematic analysis to generate themes of acceptance of the TPHD-LAMP assay amongst healthcare workers, laboratory staff and patients.
3) Cost-effectiveness: This analysis will involve using a decision tree model to weigh up the costs attributed to either test. The model will include the sensitivity and specificity of both tests, costs of person time required to perform the test and other variables that will affect if the TPHD-LAMP assay is suitable for routine diagnostics and surveillance.

8 SAFETY CONSIDERATION AND REPORTING

8.1 Potential risks

Risks associated with participation in this study are minimal. Swabs are non-invasive and are routinely used programmatically. Finger prick blood samples may cause slight discomfort and bruising; however, these too are routinely used programmatically. Loss of privacy is a potential risk associated with participation in any research project. In this study, it is necessary to confirm cure at four weeks post-treatment, as per WHO guidelines, so it is essential personal details are recorded at the time of recruitment. Potential for loss of confidentiality will be minimized by use of coded study numbers on data collection forms and laboratory samples, and all data will be stored in locked filing cabinets and on encrypted, password-protected computer files. As with the use of any antibiotic, there is a risk that resistance may evolve in either TPE, *H. ducreyi* or in a host's commensal microbiota. There have also been reports of microbial gut community disturbances with azithromycin administration which could have long term implications [31,32]. However, the data suggest that azithromycin antimicrobial resistance is not long-lived [33] .

8.1.1 Mitigation for COVID-19

COVID-19 transmission is occurring globally. As participation in this study entails interactions with staff from the Ministry of Health and the research team as well as interactions with other members of the community there might be an increased risk of COVID-19 transmission to staff and/or study participants. In order to mitigate these risks we will at all times follow guidance published by WHO on restarting of NTD related activities in the context of COVID-19 (https://www.who.int/publications/i/item/WHO-2019-nCoV-neglected-tropical-diseases-2020). These considerations include but are not limited to:

1) We will appoint a COVID-19 focal person in each study site to ensure appropriate SOPS are available and followed, that PPE is available, and liaise with other relevant stakeholders such as Ministries of Health

2) We will provide dedicated training to all staff on COVID-19 transmission, infection control and use of PPE.

3) Risk-benefit assessments will be carried out for each individual study activity using the framework laid out by WHO. Research activities will only proceed where the benefit is felt to outweigh the risk.

4) Before each activity participants and staff will be screened symptoms suggestive of COVID-19 and study staff will use PPE and other appropriate infection prevention and control measures at all times.

5) The majority of study activities including screening, enrolment, sample collection and treatment will be conducted outside (as is routine for community NTD screening activities) and will be conducted

using physical distancing. In-depth interviews and focus groups will be conducted using appropriate physical distancing between study staff and participants.

These steps will be reviewed in each country in line with national guidance and any further guidance provided by WHO.

8.2 Known potential benefits

All individuals with a skin ulcer will be referred to relevant health services for treatment with azithromycin, regardless of screening-assay result, as this is an effective treatment for both *T. pallidum* and *H. ducreyi*. Due to the activity of azithromycin against a broad range of bacterial pathogens there is likely to be collateral benefits to taking the prescribed dose of azithromycin for yaws, as has been shown previously [34]. By conducting this study we hope to improve in-country capacity for running field and laboratory studies. If the LAMP tests are shown to be an effective tool each country will have the capacity to use the assay in future studies and eradication campaigns.

8.3 Adverse events

As this is an observational study the likelihood of encountering adverse events is low.

8.3.1 Definitions

I. Adverse event (AE): Any untoward medical occurrence in a patient or study participant, which occurs between enrolment and follow up (four weeks post enrolment).

II. Serious adverse event (SAE): Any untoward medical occurrence that:

- results in death;
- is life-threatening;
- requires inpatient hospitalization or prolongation of existing hospitalization;
- results in persistent or significant disability/incapacity;
- consists of a congenital anomaly or birth defect.

Serious adverse events are only likely to occur in participants with a severe allergy to penicillin; anyone requiring penicillin treatment will have their history checked. SAEs are rarely associated with azithromycin [13,35].

8.3.2 Reporting Procedures

Any questions concerning adverse event reporting will be directed to the Chief Investigator in the first instance. An SAE form will be completed and submitted to the Study Coordination Centre with as much detail as available at that time. If awaiting further details, a follow up SAE report should be submitted promptly upon receipt of any outstanding information.

8.4 Follow-up

No formal study follow-up is planned for participants after the completion of study activities, however, as mentioned in section 5.6, participants will be re-visited four weeks after initial treatment and offered benzathine benzylpenicillin if treatment failure is thought to have occurred.

8.5 Data quality assurance and monitoring

Study staff will be trained on the study protocol prior to the start of study activities at each site. For laboratory testing, this will include centralised and localised training of the TPHD-LAMP technique for laboratory staff working in the local laboratories. Staff at reference laboratories will be trained. Data collection forms will be reviewed by the Study Coordinators and Principal Investigators (PIs) for completeness and accuracy and tested prior to the pilot study. As well as being trained about how to diagnose and assess yaws, field staff and healthcare workers will also be taught about the epidemiology of yaws, treatment options and the importance of good hygiene for combating the disease. All field workers will be trained to perform and interpret serological POCTs, collect lesion swabs and blood spots and other relevant components of the protocol. Refresher training of field and laboratory staff followed by a 2-week pilot period will be conducted before beginning the study at each site, which will allow the investigators and study personnel to identify and resolve potential logistical and technological problems prior to beginning data collection. Standardized protocols and SOPs will be followed for quality control/quality assurance of clinical evaluations, biological sample procurement and preparation, and all laboratory procedures. As well as regular internal audits and monitoring of quality indicators, a central quality management team consisting of the international study coordinator and other team members involved in field coordination, will help to ensure that all laboratory procedures are performed according to SOPs at all sites and with high quality standards. PIs and study coordinators from the different countries will maintain regular contact to ensure consistency in protocol implementation at each site, and will meet regularly with study staff at their site/s to ensure consistency in the collection of data. PIs and study coordinators will participate in regular study group meetings to assess progress of the study, address any difficulties and provide feedback to members of the study group.

8.6 Records

All participants will be identified throughout the study by a unique identifying number (UID) that will be assigned at recruitment using uniquely numbered and barcoded consent forms. This number will only be traceable to identifiable data by the study coordinator. All data will be backed up regularly to an off-site secure facility. Electronic data will be stored on password-protected and encrypted media. Access to the records will be limited to study staff. The investigators and study staff will allow all requested monitoring visits, audits or reviews by relevant ethical review boards.

8.7 Data capture

Clinical data will be collected using REDCAP and/or ODK. Data connections with the encrypted server can be made over mobile connections and data are transferred automatically to the server whenever an internet access point is available. This allows continuous backup of data and quality monitoring in real time.

8.8 Immediate and long-term use of the data

Data collected in this study will be compiled, analysed, and made available to collaborating partners and to ministries of health in participating countries in an anonymised way. Research data will be prepared for publication in scientific open-access peer-reviewed journals. Anonymised data may be made available to research students in participating countries and at participating institutions for educational use. The investigators will comply with international standards and guidelines regarding open access to research data. Upon completion of the study all study documents including CRFs and laboratory notes will be stored at study sites for at least ten years. Electronic data records will be stored indefinitely.

9 DISSEMINATION OF RESULTS AND PUBLICATION POLICY

A high degree of public engagement is an integral part of the research plan for this study, and forms the basis of sub-study 4. Stakeholders include research participants, clinicians, policy-makers, and the general public. In the first year of the study meetings will be held with community leaders and with the public at each site to inform local communities about the aims of the study and the methods to be used. Seminars will be organised at participating health facilities to inform medical and nursing staff about the study before it begins.

10 DURATION OF THE STUDY

This study has been funded for 36 months. The duration of participant recruitment is expected to take no more than 12 months. Laboratory testing will coincide with the fieldwork at each site. We anticipate data analysis and dissemination to take one year. The total study is expected to take 36 months.

ACTIVITY	Year 1				Year 2				Year 3			
	1	2	3	4	1	2	3	4	1	2	3	4
Final Version of Study Protocol												
Data and Material Transfer Agreements established												
Appoint of Key Project Staff												
Project Steering Group Meetings												
Training needs assessment												
Training												
Monitoring Visits												
Production of TPHD LAMP assay for field study												
LAMP SOP development												
Assessment of Reference Labs diagnostic capacity												
Laboratory procurement												
Sub study 1-3 enrolment												
Sample Testing at District and National Laboratories												
Laboratory monitoring												
Sub-study 4 - Patient and Stakeholder Interviews on Diagnostic Testing												
Sub-study 4 - Health-Economic Data Collection and Analysis												
Lab analyses for sub-studies 1-3												

Sub study 1-3 Data Analysis												
Community Dissemination Meetings												
Final Study Findings Publication												

11 ANTICIPATED POTENTIAL PROBLEMS

Table 5. Potential study problems and proposed solutions

Potential problem	Proposed solution
Delayed start of study activities due to delays in ethics approvals, procurement, other logistic impediments or due to the Covid-19 outbreak	1) Work with relevant personnel/agencies, local and national authorities as needed to minimize impact on project timelines 2) If necessary, discuss with EDCTP re: adjustment of deliverables timeline 3) Streamline field collection by increasing number of fieldworkers
Insufficient sample size (for cases and/or controls), or slower than expected enrolment	1) Work with local community leaders, health facility staff and other relevant personnel to identify and address possible causes 2) Increase number of clinical sites (within the same geographical/population area)
Adverse events and/or protocol violations	1) Report to appropriate ethical committee and EOC 2) Prevention as appropriate through protocol amendment and/or personnel training
Loss of or damage of specimens during storage or transport	1) Minimize risk with use of good-quality equipment, SOPs and training at sites, and use of well-established international courier

12 ETHICAL CONSIDERATIONS AND PARTICIPANT CONFIDENTIALITY

Ethics approval will be sought from the London School of Hygiene & Tropical Medicine ethics review committee and from national/institutional review committees in all participating countries (Ghana Health Services Ethics Committee, Cote d'Ivoire National Health Ethics Committee, Cameroon National Health Ethics Committee). In addition, we will obtain relevant data-transfer and material-transfer agreements to cover the transfer of samples and study data between countries. All data will be coded through the use of unique study IDs and held on a secure server. We may provide participants with compensation or travel expenses according to each country's ethical committee requirements.

13 SPONSOR

London School of Hygiene & Tropical Medicine (LSHTM) will act as the main sponsor for this study. Delegated responsibilities will be assigned locally.

14 FUNDING

This study has been funded by an EDCTP grant, number RIA2018D-2495

15 AUDITS AND INSPECTIONS

The study may be subject audit by the London School of Hygiene & Tropical Medicine under their remit as sponsor, the Study Coordination Centre and other regulatory bodies to ensure adherence to Good Clinical Practice.

REFERENCES:

1. Mitjà O, Asiedu K, Mabey D, 2013. Yaws. *Lancet*. *381*(9868):763-773. doi:10.1016/S0140-6736(12)62130-8.
2. Čejková D et al., 2012. Whole Genome Sequences of Three Treponema pallidum ssp. pertenue Strains: Yaws and Syphilis Treponemes Differ in Less than 0.2% of the Genome Sequence. Lukehart S, ed. *PLoS Negl Trop Dis*. *6*(1):e1471. doi:10.1371/journal.pntd.0001471.
3. Marks M, Lebari D, Solomon AW, Higgins SP, 2015. Yaws. *Int J STD AIDS*. *26*(10):696-703. doi:10.1177/0956462414549036.
4. Asiedu K, Fitzpatrick C, Jannin J, 2014. Eradication of Yaws: Historical Efforts and Achieving WHO's 2020 Target. *PLoS Negl Trop Dis*. *8*(9). doi:10.1371/journal.pntd.0003016.
5. Kazadi WM, Asiedu KB, Agana N, Mitjà O, 2014. Epidemiology of yaws: an update. *Clin Epidemiol*. *6*:119-128. doi:10.2147/CLEP.S44553.
6. WHO2020 *Ending the Neglect to Attain the Sustainable Development Goals. A Road Map for Neglected Tropical Diseases 2021-2030*.; 2020. http://apps.who.int/bookorders. Accessed April 17, 2020.
7. Marks M, Katz S, Chi KH, Vahi V, Sun Y, Mabey DC, Solomon AW, Chen CY, Pillay A, 2015. Failure of PCR to detect treponema pallidum ssp. pertenue DNA in blood in latent yaws. *PLoS Negl Trop Dis*. *9*(6). doi:10.1371/journal.pntd.0003905.
8. Yaws G, Programme E *Summary Report of a Consultation on the Eradication of Yaws Morges, Switzerland*. http://www.who.int/yaws/en. Accessed April 20, 2020.
9. Marks M, Solomon AW, Mabey DC, 2014. Endemic treponemal diseases. *Trans R Soc Trop Med Hyg*. *108*(10):601-607. doi:10.1093/trstmh/tru128.
10. WHO WHO | Yaws: A forgotten disease. https://www.who.int/neglected_diseases/diseases/yaws/en/. Accessed January 24, 2020.
11. Mitjà O, Hays R, Ipai A, Penias M, Paru R, Fagaho D, De Lazzari E, Bassat Q, 2012. Single-dose azithromycin versus benzathine benzylpenicillin for treatment of yaws in children in Papua New Guinea: An open-label, non-inferiority, randomised trial. *Lancet*. *379*(9813):342-347. doi:10.1016/S0140-6736(11)61624-3.
12. Kwakye-Maclean C, Agana N, Gyapong J, Nortey P, Adu-Sarkodie Y, Aryee E, Asiedu K, Ballard R, Binka F, 2017. A Single Dose Oral Azithromycin versus Intramuscular Benzathine Penicillin for the Treatment of Yaws-A Randomized Non Inferiority Trial in Ghana. *PLoS Negl Trop Dis*. *11*(1). doi:10.1371/journal.pntd.0005154.
13. Mitjà O et al., 2015. Mass Treatment with Single-Dose Azithromycin for Yaws. *N Engl J Med*. *372*(8):703-710. doi:10.1056/NEJMoa1408586.
14. Marks M et al., 2015. Impact of Community Mass Treatment with Azithromycin for Trachoma Elimination on the Prevalence of Yaws. *PLoS Negl Trop Dis*. *9*(8):e0003988. doi:10.1371/journal.pntd.0003988.

15. World Health Organization, 2012. Eradication of Yaws- the Morges Strategy. *Wkly Epidemiol Rec*. *87*:189-200. doi:10.1016/S0140-6736(11)61624-3.
16. GHO | By category | Status of endemicity for yaws - Data by country https://apps.who.int/gho/data/node.main.NTDYAWSEND. Accessed April 22, 2020.
17. Mitjà O et al., 2015. Global epidemiology of yaws: A systematic review. *Lancet Glob Heal*. *3*(6):e324-e331. doi:10.1016/S2214-109X(15)00011-X.
18. Mitjà O et al., 2014. Haemophilus ducreyi as a cause of skin ulcers in children from a yaws-endemic area of Papua New Guinea: A prospective cohort study. *Lancet Glob Heal*. *2*(4):e235-e241. doi:10.1016/S2214-109X(14)70019-1.
19. Marks M, Chi KH, Vahi V, Pillay A, Sokana O, Pavluck A, Mabey DC, Chen CY, Solomon AW, 2014. Haemophilus ducreyi associated with skin ulcers among children, Solomon Islands. *Emerg Infect Dis*. *20*(10):1705-1707. doi:10.3201/eid2010.140573.
20. Mitjà O et al., 2018. Re-emergence of yaws after single mass azithromycin treatment followed by targeted treatment: a longitudinal study. *Lancet*. *391*(10130):1599-1607. doi:10.1016/S0140-6736(18)30204-6.
21. Lukehart SA et al., 2004. Macrolide resistance in Treponema pallidum in the United States and Ireland. *N Engl J Med*. *351*(2). doi:10.1056/NEJMoa040216.
22. Land KJ, Boeras DI, Chen XS, Ramsay AR, Peeling RW, 2019. REASSURED diagnostics to inform disease control strategies, strengthen health systems and improve patient outcomes. *Nat Microbiol*. *4*(1):46-54. doi:10.1038/s41564-018-0295-3.
23. Becherer L, Bakheit M, Frischmann S, Stinco S, Borst N, Zengerle R, Von Stetten F, 2018. Simplified Real-Time Multiplex Detection of Loop-Mediated Isothermal Amplification Using Novel Mediator Displacement Probes with Universal Reporters. *Anal Chem*. *90*(7):4741-4748. doi:10.1021/acs.analchem.7b05371.
24. Becherer L et al., 2020. Multiplex Mediator Displacement Loop-Mediated Isothermal Amplification for Detection of *Treponema pallidum* and *Haemophilus ducreyi*. *Emerg Infect Dis*. *26*(2):282-288. doi:10.3201/eid2602.190505.
25. Solomon AW et al., 2012. A diagnostics platform for the integrated mapping, monitoring, and surveillance of neglected tropical diseases: Rationale and target product profiles. *PLoS Negl Trop Dis*. *6*(7). doi:10.1371/journal.pntd.0001746.
26. WHO *ERADICATION OF YAWS A Guide for Programme Managers*.
27. WHO2014 Yaws: Recognition Booklet for Communities. https://apps.who.int/iris/bitstream/handle/10665/75360/9789241504096_eng.pdf;jsessionid=D9C13FCC6882898076399A9A5033AD8C?sequence=1. Published 2014. Accessed June 9, 2020.
28. Lammie PJ, Moss DM, Brook Goodhew E, Hamlin K, Krolewiecki A, West SK, Priest JW, 2012. Development of a new platform for neglected tropical disease surveillance. *Int J Parasitol*.

42(9):797-800. doi:10.1016/j.ijpara.2012.07.002.

29. Marks M et al. Diagnostics for Yaws Eradication: Insights From Direct Next-Generation Sequencing of Cutaneous Strains of Treponema pallidum. *Clin Infect Dis*. *818*:66. doi:10.1093/cid/cix892.
30. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD, 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. *Appl Environ Microbiol*. *79*(17):5112-5120. doi:10.1128/AEM.01043-13.
31. Parker EPK, Praharaj I, John J, Kaliappan SP, Kampmann B, Kang G, Grassly NC, 2017. Changes in the intestinal microbiota following the administration of azithromycin in a randomised placebo-controlled trial among infants in south India. *Sci Rep*. *7*(1):1-9. doi:10.1038/s41598-017-06862-0.
32. Wei S, Steen Mortensen M, Stokholm J, Brejnrod D, Thorsen J, Rasmussen MA, Trivedi U, Bisgaard H, Sørensen J, 2018. Short-and long-term impacts of azithromycin treatment on the gut microbiota in children: A double-blind, randomized, placebo-controlled trial-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). *EBioMedicine*. *38*:265-272. doi:10.1016/j.ebiom.2018.11.035.
33. Evans JR, Solomon AW, Kumar R, Perez Á, Singh BP, Srivastava RM, Harding-Esch E, 2019. Antibiotics for trachoma. *Cochrane Database Syst Rev*. *2019*(9). doi:10.1002/14651858.CD001860.pub4.
34. Keenan JD et al., 2018. Azithromycin to Reduce Childhood Mortality in Sub-Saharan Africa. *N Engl J Med*. *378*(17):1583-1592. doi:10.1056/NEJMoa1715474.
35. Marks M, Sokana O, Nachamkin E, Puiahi E, Kilua G, Pillay A, Bottomley C, Solomon AW, Mabey DC, 2016. Prevalence of Active and Latent Yaws in the Solomon Islands 18 Months after Azithromycin Mass Drug Administration for Trachoma. *PLoS Negl Trop Dis*. *10*(8):e0004927. doi:10.1371/journal.pntd.0004927.