UK PUBLIC HEALTH RAPID SUPPORT TEAM RESEARCH

Field Validation Study of an ELISA assay to identify Lassa virus specific antibody responses in oral fluids

Case-control assessment of a novel immunoassay comparing antibody responses from oral fluids and blood samples in Lassa Fever survivors and unexposed controls in Sierra Leone

Study protocol

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Contents

1 Summary 3
2 Introduction 4
2.1 Host & transmission 5
2.2 Sero-prevalence & incidence of infection 5
2.3 Assay Development 6
2.4 Study Sites 6
2.5 Timeline 6
3 Aims and objectives 7
4 Methods 8
4.1 Recruitment 8
4.2 Study participants 8
4.4 Eligibility & consent 9
4.5 Sample collection 9
4.6 Data collection 10
4.7 Sample size 10
4.8 Data analysis 11
4.9 Dissemination of results 11
5 Human subjects' protection 12
5.1 Risks to participants 12
5.2 Intellectual property 13
5.3 Data handling 13
5.4 Storage of study participants' samples 13
6 References 14
7 Appendices 15
7.1 Consent/assent form 15
7.2 Questionnaires and control eligibility check list 15
7.3 Patient information leaflet 15

1 Summary

This proposal describes a collaborative study between the UK Public Health Rapid Support Team (UK-PHRST), London School of Hygiene and Tropical Medicine (LSHTM), and Kenema Government Hospital (KGH), Sierra Leone to develop and validate a novel enzyme-linked immunosorbent assay (ELISA) to investigate antibody responses to Lassa virus (LASV) infection in oral fluid samples.

The study will compare oral fluid and blood samples from Lassa Fever (LF) survivors and unexposed controls in order to validate oral fluid samples as a means of establishing anti-LASV antibody prevalence in endemic settings.

There is limited recent evidence on the prevalence of LASV infection and the longevity of immune responses to LASV within endemic communities. A robust antibody assay suitable for use with non-invasive oral fluid sampling could greatly improve ability to accurately estimate prevalence of infection, describe the spectrum of disease, and facilitate longitudinal incidence, immunity and transmission studies through improved acceptance of testing.

A prototype ELISA for use with oral fluid has been created in the UK by experts in this form of testing. A small number of stored convalescent human samples (n=6) from Sierra Leone consented for research purposes will be obtained through official channels to finalise the prototype development in the UK. Validation of the assay, including determination of sensitivity and specificity, will be done in Sierra Leone by national laboratory staff supported by UK-PHRST laboratory scientists, using oral fluids and blood samples collected from individuals confirmed to have had LF by the Lassa Fever Unit of KGH, Kenema, Sierra Leone. Samples from individuals without LF diagnosis or known LASV exposure will be collected as controls.

A validated non-invasive, community-acceptable, and logistically simpler tool for measuring LASV sero-prevalence and sero-conversion in communities in endemic countries will greatly facilitate the understanding of LASV epidemiology and support the development and trialling of LASV vaccines and other control measures.

2 Introduction

Lassa Fever (LF) is a viral haemorrhagic illness endemic in parts of West Africa including Sierra Leone, Liberia, Guinea, Mali and Nigeria where the illness was first identified.[1] In endemic settings, it is believed that the majority of those infected with Lassa virus (LASV) experience sub-clinical infection or mild disease with only around 20% infections resulting in hospitalisation.[2] Overall mortality from infection is thought to be ~1% but in hospitalised cases, case fatality rates of up to 50 to 70% have been reported,[3] often linked to late presentation. LF outbreaks can cause significant public health emergencies, often triggered by patients presenting in health facilities that are unaccustomed to receiving haemorrhagic fever cases, with the accompanying risks of health worker and nosocomial infection, as well as the burden of investigating and managing large numbers of suspect cases.

During clinical LASV infection, early symptoms may include fever, headache, and muscle aches, mimicking symptoms of diseases such as malaria and typhoid fever which are common in LASV endemic areas. As clinical infection progresses, patients may go into shock, develop multi-organ failure, and sometimes bleed from the mouth, nose, eyes, or rectum.[4] In addition, there is evidence that LF survivors suffer long-term sequelae such as hearing loss, depression, joint pain and hair loss.[3] There are currently no vaccines to prevent LF nor approved drugs to treat, and although accepted wisdom is that early clinical use of the nucleoside drug ribavirin can reduce mortality in clinical cases, there is little scientific evidence to support this.

It is estimated that up to 300,000 LASV infections occur annually resulting in 5,000 deaths,[2] but these data are based largely on the results of a single study carried out in the 1980s.[5] Similarly, there is little evidence for the 20-80% proportions cited for sub-clinical and clinical disease. The need for more robust estimates based on up-to-date data has been recently emphasised by WHO in its Research & Development Blueprint which identified LASV as one of eight priority diseases for urgent attention.[6] More precise estimates of these parameters, and deeper understanding of the exposure and immune status of populations in the endemic countries, will also be critical to developing, targeting, and evaluating the efficacy of LASV vaccine candidates and other control measures.

LASV is an arenavirus composed of an enveloped, segmented negative single-stranded RNA and classified as a Category A pathogen requiring high biosafety handling.[7] Several methods are used to diagnose LF cases including reverse transcriptase polymerase chain reaction (RT-PCT), a recombinant LASV (ReLASV) ELISA, as well as antigen detection tests and a lateral flow immunoassay rapid diagnostic test, all of which require blood samples.[8] Virus isolation by cell culture is the gold standard for LASV diagnostic purposes, but cannot be performed in normal diagnostic facilities due to the high-level biosafety laboratory facilities required for live virus work.

2.1 Host & transmission

The natural host for LASV is the common African soft-furred rat, *Mastomys natalensis*, in which infection is thought to be lifelong and harmless. Recently there have been reports of other host rodents and investigations into this are continuing.[9] Viral transmission occurs - via aerosol or contact - from infected rats or their excretions to humans, and subsequent LASV infection in humans can be lethal. The virus can also be transmitted between humans via blood and tissue fluids, particularly in hospitals with weak infection prevention control measures, and among contacts exposed to body fluids through caring practices, resulting in high mortality rates.[3, 10]

2.2 Sero-prevalence & incidence of infection

At present, sero-prevalence and persistence of LASV antibodies and incidence of infection (measured by sero-conversion) can only be studied through surveys using blood-based sampling. Significant bio-security, community and psycho-social issues accompany the collection of blood samples in affected countries. Indeed, the drawing of blood can be one of the most contentious issues in a clinical trial process potentially arousing political, cultural and social antipathy,[11-14] as well as requiring considerable logistics to correctly and safely collect and store specimens.

"Oral fluid" is found in the gingival crevice (between teeth and gums) and contains considerably higher levels of IgG than saliva.[15] Oral fluid sampling has major advantages over blood collection: it is non-invasive, more acceptable to subjects of all ages (due to absence of pain and low or no perceived risk of contamination), and easier to collect without the need for medically-trained personnel. It is safer for collectors, removing the risk of needle-stick injury and other collection and storage-related exposures.[16]

The availability of a non-blood-based, non-invasive alternative method to detect LASV antibodies, and thus past infection, would allow more comprehensive sampling due to its higher acceptability by populations. This would greatly facilitate the community-based research needed to gather critical information on the full burden of LASV infection and disease and on levels of immunity, while also potentially providing a non-invasive tool to track responses to immunisation when trials are underway.

Oral fluid has been used in surveillance activities to detect antibodies to viral infections such as mumps, measles, rubella, and is routinely used for the diagnosis of HIV and Hepatitis A and C.[15, 17] During the 2013-16 West Africa Ebola outbreak, a reverse IgG-capture ELISA for oral fluid samples was developed by the Public Health England (PHE) team involved in this proposal, and used to investigate the sero-prevalence of Ebola virus infection in affected households and communities, with high sensitivity (97.4%, 95% CI, 92.5%-99.5%) and specificity (99.7%, 95% CI, 98.4%-99.7%).[18]

2.3 Assay Development

Laboratory scientists from Public Health England and Imperial College are in the process of developing a prototype LASV immunoassay for use with oral fluid incorporating single or combined LASV antigens (glycoprotein 1 (GP1), GP2 or recombinant matrix protein (rMP)). To complete the development stage and provide a reliable assay for validation, a small number of anonymous convalescent human samples will be requested from the KGH LF unit under a Material Transfer Agreement. Once the assay kits are complete, all subsequent validation work will be done in Sierra Leone in order to support increased research capacity in country.

2.4 Study Sites

Kenema District is one of the 'hotspots' of Lassa Fever transmission in Sierra Leone and the KGH LF Unit is the national centre for diagnosis, treatment and research. Together with the Irrua Specialist Teaching Hospital in Nigeria, KGH is one of only two facilities globally where LASV testing and LF patient admission is continuous, and there is a programme of survivor monitoring and follow-up, which will facilitate the recruitment of the convalescent LF survivors needed for this validation work.

The KGH laboratory, medical and outreach teams at KGH have extensive experience of diagnosing treating and collecting data on LF, supported over many years by partners including Tulane University, New Orleans, USA. KGH carries out clinical and diagnostic research including an ongoing 5-year study of sequelae and immunity in survivors of viral haemorrhagic fevers (referred to hereafter as the Survivors Study). This validation study will be embedded in the Survivors Study for efficiency and to ensure a coordinated approach to survivors. The study presented here shares a principal investigator (Dr Grant) with the Survivors Study and the study teams will collaborate on all activities.

2.5 Timeline

Activity	2018	2019											
	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
• Oral Fluid Assay Development (UK)													
• LSHTM/Sierra Leone Ethical approval for sample collection													
• Team training and Community Sensitisation (Sierra Leone)													
• LF survivor single time point sample collection (Sierra Leone)													
• Unexposed single time point sample collection (Sierra Leone)													
• ELISA assays (Sierra Leone)													
• Study and Data analysis													
• Research paper / report draft													

3 Aims and objectives

The aim of this study is:

- To develop and examine the validity of a new assay to measure evidence of LASV infection for use with oral fluids by comparing naturally acquired antibody responses to LASV in oral fluids and blood serum samples from LF survivors and unexposed controls.

The specific objectives are:

- To complete the development of a novel ELISA to investigate naturally acquired antibody responses LASV infection in non-invasive oral fluid samples
- To validate this tool and determine its sensitivity and specificity by measurement of LASV-specific antibodies in oral fluids and blood samples from LF survivors and unexposed controls
- To evaluate the feasibility of oral fluid as an alternative sampling tool using these findings
- In LF survivors, to correlate LASV-specific antibody responses with time since clinical infection, original clinical characteristics and potential re-exposure events.

4 Methods

4.1 Recruitment

Inclusion Criteria

For LF survivor participants

- History of LF as documented by a positive anti-LASV Ag or IgM ELISA in the KGH diagnostic laboratory
- Age ≥6 years
- No fever at recruitment (Temperature below 37.5^{o}C)
- Willingness to provide informed consent (or assent, if applicable)
- Willingness to undergo phlebotomy for blood samples and provide oral fluid samples

For unexposed control participants

- No history of LF clinical illness and/or LASV positive test,
- No history of residence, work or visiting in LF endemic zones
- No history of contact with a LF case
- Age ≥6 years
- No fever at recruitment (Temperature below 37.5^{o}C)
- Willingness to provide informed consent (or assent, if applicable)
- Willingness to undergo phlebotomy for blood samples and provide oral fluid samples

4.2 Study participants

Up to 70 LF survivors admitted to the KGH Lassa Ward for treatment of confirmed LF and discharged up to 15 years prior to the start of this study will be recruited. The study team will work with LF programme outreach staff at KGH to identify LF survivors and implement the study protocol.

Unexposed controls will be recruited from among individuals understood to have no exposure to the *Mastomys natalensis* rodent or LF cases. After agreement with the relevant authorities, control participants will be sought from among medical and/or nursing students in Freetown (an area considered outside the LASV endemic zone) as a relatively easily-accessed cohort of individuals for whom taking part in a research study may be of interest. The study brings no inherent benefit to participants, however those involved in study for health care/public health careers may benefit from taking part in a well-conducted research study. We will also collaborate with college authorities to offer lectures on the conduct of research studies and on LF as one of Sierra Leone's priority emerging

disease issues. A questionnaire including past travel/residence/contact information will be used to exclude candidates with possible exposure (Appendix 2: Control eligibility check).

4.4 Eligibility & consent

After holding meetings with key community leaders and Lassa survivor communities about the aims of the study, individual survivors will be approached for recruitment. The purpose and objectives of the study will be explained verbally, and a patient information leaflet provided for those able to read (Appendix 3: Patient information leaflet). Study investigators will explain the role of the participant in the study and discuss potential risks of participation. Informed consent will be in English. If English is not the patient's native language or they are unable to read, the patient will be informed about the study and related risks in his or her native language by a collaborating investigator fluent in that language who will read the consent form to the potential participant in the presence of a witness.

Potential participants will have the opportunity to raise any concerns, after which, written informed consent will be sought from all adult participants and from adult guardians of child participants (Appendix 1: Consent form). For survivors, consent will include approval to access clinical records of their LF admission and to information previously given to the Survivors Study. All participants will be asked to consent to storage of their oral fluid and blood samples for possible future research; samples from participants who do not agree to storage will be destroyed after the validation study.

Following provision of informed consent, clinical, demographic and risk factor information for the participant will be collected from medical records completed during admission and from the Survivor Study database. Participants will be asked to respond to a short questionnaire specific to the validation study focussing on possible antibody-boosting exposures (Appendix 2: Questionnaires). Data will be entered into a password-protected electronic database.

4.5 Sample collection

A 5ml blood sample will be collected and stored in a cool box for daily transfer to the laboratory. Serum will be processed then stored at -20°C prior to testing antibody responses.

Oral fluid will be collected by the participant rubbing a small sponge swab firmly on the gums for 90 seconds, then placing it in a stoppered plastic tube and in a sealable plastic bag and stored in a cool box, then transferred for storage at -20°C at the end of the day prior to testing antibody responses. Experience suggests that oral fluid collection is acceptable to participants of all ages.[18]

Oral fluid and blood samples will be tested to detect anti-LASV antibodies using the new ELISA tool. Further comparative tests may be conducted with assays currently in use in KGH.

4.6 Data collection

Data will be collected using a study-specific questionnaire (Appendix 2), participants' medical records during admission to KGH Lassa Fever Unit, and data held in the Survivors Study database. Variables for survivors will include demographics, symptoms and clinical information at the time of admission, dates of LF onset and discharge, laboratory test results at discharge, and information on any known exposure to LASV or LF patients since recovery, in order to explore potential immunity-boosting events.

Unexposed participants will be asked a series of questions regarding exposure to LASV hosts and/or cases to determine if they fulfil the inclusion criteria, and if not excluded based on responses, will be asked to respond to a brief demographic questionnaire.

Both groups will be asked to give feedback on using the oral fluid swab, including perceptions of the method as a follow-up and research tool.

4.7 Sample size

We will recruit at least 70 LF survivors and 70 unexposed controls. This will allow for an estimation of specificity of at least 61.2% at 95% confidence interval (CI) with10% relative precision if true sensitivity is 85%, and higher precision if true sensitivity is greater (Table 1).

Table 1: Sample size estimation for a range of sensitivity levels

Sensitivity point estimate (%)	Relative precision (%)			
	5	10	20	50
80	385	97	25	4
85	272	68	17	3
90	171	43	11	2
95	81	21	6	1

4.8 Data analysis

Analyses will:

- Determine the sensitivity and specificity of the novel assay to detect naturally acquired LASV-specific antibodies in LF survivors and unexposed controls in oral fluids compared to blood samples
- Describe antibody levels among study participants as a function of time since clinical infection and in relation to original clinical characteristics and potential re-exposure events.
- Investigate characteristics of any apparently unexposed controls found to have positive antibodies

4.9 Dissemination of results

The results of the study will be disseminated through reports, academic papers, and presentations. We will ensure that negative results are also reported. All publications will be open-access. We will also share the results directly with the LASV team at KGH, government of Sierra Leone and other organizations working on LASV. A report will be shared with the Sierra Leone Ethics and Scientific Review Committee.

Survivor participants will not be informed of their individual results as knowledge carries no individual benefit to health or wellbeing. Unexposed controls will be informed if they are found to have positive antibodies and the meaning of this will be explained.

5 Human subjects' protection

5.1 Risks to participants

The study protocol will be submitted to the Sierra Leone Ethics and Scientific Review Committee and at LSHTM for approval. No implementation will begin until approval is received. Written informed consent will be sought as described in section 2.3 above.

There is a risk that participants may not be properly informed about the study. Risks of drawing blood from participants are typically minor, with a bruise at the puncture site being the most common adverse event and a very small risk of infection. There is risk that participants may experience duress during the recruitment and interview process or in providing samples for analysis and a risk of loss of privacy. There are no risks in providing oral fluids.

We will take appropriate measures to minimize these risks to participants by providing clear training, guidance and supervision to members of the study team. We will be sensitive to any breach of confidentiality due to the stigmatizing nature of LASV.[19] Study data will be managed according to a clear operational protocol, with only authorised research team members having access to de-identified data. We will also minimise risk to participants by engaging staff who have already built a rapport with LF survivors.

There will be no costs to the participant for participating in this research study. All costs related to the study, including phlebotomy and laboratory testing, will be paid for by the study.

Recruitment and participation will require a maximum time commitment of 1 hour from each participant, financial compensation of 100,000 Leones (approximately $11) will be provided for inconvenience, transport and loss of work time. This amount is consistent with the amount paid per follow-up visit by the Survivors Study.

Child survivors will be recruited into the study after the informed consent from their parent or guardian. In accordance with Sierra Leonean age cut-offs for child participation in research, children older than 12 years will also be asked to provide assent to participate. Pregnant women will be eligible to participate in either study cohort.

Participants will be informed that participation is voluntary and that they are free to discontinue their participation at any time with no adverse consequence. As the primary aim of this work is to validate an assay, individuals who do not wish to provide any oral fluid and/or blood samples will not be recruited. Participation in, or discontinuation from the study will not affect the participant's ability to receive medical care provided by the Sierra Leone health service. The study will not offer or provide any medical care, other than to respond to an emergency unexpected reaction to the sampling procedure.

5.2 Intellectual property

Participants will be informed that the researchers and study team will not benefit financially from the development and testing of this assay. No intellectual property rights (IPR) will be sought for the new assay, and IPR linked to the conjugate component of the assay will be waived for production of the assay by state laboratories in endemic countries. Sample donors will be asked to freely donate a single blood and oral fluid sample and to relinquish all rights, title and interest to these samples.

5.3 Data handling

Maintaining participant confidentiality is an essential part of medical research. Staff will receive training on ensuring patient confidentiality, and all data collected on study participants will be secured (locked if paper or password-protected/encrypted if electronic). All participants will be assigned a unique identifying number. An electronic file as well as a paper log book will be kept by the study coordinator linking patient names with the unique identifying number. All electronic master lists will be kept on password-protected computers in locked offices or in the restricted access laboratory in the Lassa unit. Paper log books will be kept in a locked cabinet in locked offices. All electronic copies of medical records and laboratory documents will be kept on password-protected computers in locked offices with restricted access.

An anonymised version of the raw database(s) stripped of all identifying information such as participant name, address, etc. will be created for analysis. Only authorised members of the LSHTM and Lassa Fever Unit data team will have access to the raw data including the personal identifiers, and only for the purpose of entering the information into an electronic database, checking for errors, and producing the anonymised version of the database for analysis at LSHTM. The electronic database will be password-protected and stored on a secure server owned by the London School of Hygiene & Tropical Medicine.

De-identified data will be shared with KGH investigators. No individual data or samples will be sold to or shared with third parties beyond the collaboration.

5.4 Storage of study participants' samples

Study participants will be asked to consent to allowing any residual samples to be stored for future testing and given the option to refuse. Participants who change their mind regarding storage of their samples may contact a study investigator at any time to request that their samples be destroyed. Future use of specimens by other investigators and collaborators will be limited to their receipt as anonymized samples.

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7 Appendices

7.1 Consent/assent form

7.2 Questionnaires and control eligibility check list

7.3 Patient information leaflet