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EDITORIAL

Introduction to the First Issue of East Africa Science: Search, Discover, Develop

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On behalf of the East African Health Commission (EAHRC), we are delighted to share the inaugural issue of East Africa Science (EASci) with our readers. We would like to take this opportunity to acknowledge all of the contributions from authors, reviewers, and the editorial team that have made this first issue possible.

EASci is a no-fee, open access, peer-reviewed journal published by the EAHRC of the East African Community (EAC, www.eac.int). The journal will publish papers on a wide range of topics relevant to basic science, technology, and innovation in health. The aims are to contribute to knowledge dissemination, continuing professional development of researchers in the region, and play a part in the mentoring of young, up-and-coming scientists. EASci is the sister journal of the East African Health Research Journal (EAHRJ), which is dedicated to articles informing health policy and practice.

EASci will not only promote research in the basic sciences but also the application of science, technology and innovation (STI) as well as information and communication technology (ICT) in health. This will include clinical trials on investigational pharmaceutical products, devices, and diagnostics, as well as featuring the application of health technologies and solutions, among other related matters. Issues of the journal will include peer-reviewed articles, original articles, reviews, short communications, surveys, commentaries, opinions, book reviews, supplementary issues, essays, and reports related to the advancement in health and medical sciences.

EASci is open access with no article processing charges. The journal will initially be published once annually but will eventually build up to 2 issues per year. EASci will be available online at www.eahealth.org and in hard copy. Hard copies will be distributed to all relevant stakeholders, such as government institutions, research institutions, academic institutions, and relevant civil society organisations.

EASci, among other things, aims to:

- Present East African innovations in the health sector
- Provide solutions through research, discovery, and development to priority issues that are relevant to the East African region
- Be a catalyst for innovation and the use of science and technology, including digital health technologies and solutions
- Guide the development of products in health that harness the advancement of science and technology
- Be a forum for sharing innovations and knowledge related to health
- Provide an avenue for closing the ‘knowledge gap’ in research and development in health that exists between developing and developed countries
- Enable scholarly recognition of professionals and institutions
- Support the career development of professionals
- Provide a forum for researchers from the EAC to become more visible globally
- Provide direction in setting up health research priorities in the region
- Contribute to economic and social development through research, innovation, and development in health
We are publishing the inaugural issue of the journal in conjunction with the 7th East African Health and Scientific Conference (EAHSC), taking place in Dar es Salaam, the United Republic of Tanzania, from 27 to 29 March 2019. The main theme of the conference is ‘Technology for Health Systems Transformation and Attainment of the UN Sustainable Development Goals’. The conference participants will have the opportunity to discuss the 5 subthemes:

1. Technologies Supporting Data for Health System Decision-making
2. Technologies for Disease Surveillance, Disease Outbreak Detection and Response, and Cross-border Mobility and Disease tracking
3. Innovative Technologies and Solutions for Application in, and Improvement of, Health-Care Service Delivery and Health Outcomes
4. Costing and Financing Health: The Role of Digital Health, International Remittances, Universal Health Care, and EAC Status on the UN SDGs
5. Health Knowledge Management Through Digital Technologies and Solutions in East Africa: Health Research, Training, and Care

As digital health technologies are increasingly being used in health care to improve health services and delivery, the EAHRC management thought that it was imperative to propose to the 14th EAC Sectoral Council of Ministers of Health that the main theme of the 7th EAHSC to be in line with digital health technology. The Sectoral Council of Ministers of Health, having granted their approval, will allow for the consideration of recommendations and strategies on how to harness the potential of digital health technologies to transform the health sector in the East African region. The EAHRC will also launch the Digital Regional East Africa Community Health (Digital REACH) Initiative during the 7th EAHSC. The Digital REACH Initiative is a new, groundbreaking initiative within the EAC that will implement ICT across all dimensions of the health sector in East Africa. This has the potential to transform health outcomes across the region for millions of people.

The Digital REACH Initiative will be implemented through its 10-year strategic plan (2019-2028). The strategic plan presents a common regional vision and strategic approach for regional collaboration in health. It also serves as a platform for development partner and private sector input to support coordination and shared investment. The plan allows the EAC to pursue the funding necessary to scale-up operations.

The EAC is the first African Union Regional Economic Community to create an ambitious and coordinated approach to digital health, which is prioritised for investment by all presidents of its partner states. The successful implementation of the Digital REACH Initiative will fully support the EACs integration of the agenda, “One people, One Health System”.

In addition to this first issue of EASci, the EAHRC will also launch 2 supplementary issues, EASci volume 1 supplement 1, 2019, and EAHRJ volume 1 supplement 1, 2019. These 2 supplementary issues will publish the abstracts submitted for presentation at the 7th EAHSC.

Competing Interests: None declared.


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ABSTRACT

*Mycobacterium tuberculosis* has caused tuberculosis (TB) in humans for at least 3 millennia, but the disease has evaded eradication efforts by all human civilisations despite promising technological advancements. The World Health Organization (WHO) has set a target of ending the TB epidemic by 2035. Going by the current rate of progress, it is estimated that it will take another 160 years to realise the WHO End TB Strategy’s target. Accelerating the eradication of TB will require effective tools for diagnosis, vaccines and medicines to treat the disease, and efficient implementation thereof. This presents a great opportunity for innovators in East Africa and the world over to chip in and develop the best technologies to end TB. With funding from the European and Developing Countries Clinical Trials Partnership (EDCTP), partnerships between the UK-based University of St Andrews and research institutions in East and Southern Africa have led to the development of the first ever test – the molecular bacterial load assay (MBLA) – that measures the number of TB bacteria in a patient and reveals if this number is declining as a patient progresses on treatment. Assay results are available within 4 hours. Real-time knowledge of patient mycobacterial burden and the effectiveness of prescribed medications are crucial for timely clinical decisions on patient management.

INTRODUCTION

*Mycobacterium tuberculosis* has caused tuberculosis (TB) in humans for at least 3 millennia, but the disease has evaded eradication efforts by all human civilisations despite promising technological advancements. The disease is transmitted via the inhalation of aerosols laden with TB bacilli, expelled when infected individuals cough. One-third of the world’s population is latently infected with TB, reflecting one of the largest reservoirs of disease through asymptomatic carriers, from which an unfortunate 5% of cases progress to active disease.\(^1\) Close to 10 million new cases and over 1 million TB-related deaths were reported in 2017.\(^2\) Although TB is a global disease, the burden varies greatly between high- and low-income countries. Asia and Africa, which host the most low- and middle-income countries (LMICs), account for the highest burden of TB.\(^2\) According to the World Health Organization (WHO), 30 countries are categorised as high-TB-burden countries (HBCs) and account for 87% of the global TB burden. Seventeen (56%) of the HBCs are in Africa, of which 8 (47%) are in eastern and southern Africa.\(^3\) Countries from the eastern–southern African HBC block (ES)\(^4\) are recognised as having the highest burden of multidrug-resistant TB.\(^1\)-\(^3\) The need for these countries to invest in developing effective tools to solve the TB problem cannot be overemphasised.

The WHO has set a target of ending the TB epidemic by 2035.\(^4\) Going by the current rate of progress, it is estimated that it will take another 160 years to realise the WHO End TB Strategy’s target.\(^5\) Accelerating the eradication of TB will require effective tools for diagnosis, vaccine and medicines to treat the disease, and efficient implementation thereof. This presents a great opportunity for innovators in ES and the world over to chip in and develop the best technologies to end TB. With funding from the European and Developing Countries Clinical Trials Partnership (EDCTP), partnerships between the UK-based University of St Andrews and research institutions in ES have led to the development of the first ever test – the molecular bacterial load assay (MBLA) – that measures the number of TB bacteria in a patient and reveals if this number is declining as a patient progresses on treatment.\(^6\) Assay results are available within 4 hours. Real-time knowledge of patient mycobacterial burden and the effectiveness of prescribed medications are crucial for timely clinical decisions on patient management.
edge of patient mycobacterial burden and the effectiveness of prescribed medications are crucial for timely clinical decisions on patient management.

This is a great improvement over the tools currently used for TB diagnosis and treatment monitoring. The gold standard test – *M. tuberculosis* culture – takes weeks or months to deliver results, which is too slow to inform patient management. Furthermore, culture is acutely sensitive to endogenous and exogenous contaminants, such as non-TB bacteria and fungi, which grow rapidly and cause false-positive time to culture positivity (TTP) results.

Consequently, a result from culture requires 3 extra confirmatory tests: (1) acid-fast microscopy to confirm acid-fast bacilli, (2) blood agar culture to rule out contamination, and (3) an antigen test, MPT64, to confirm the presence of *M. tuberculosis*. These extra tests consume more person-hours and increase the total cost associated with culturing. Smear microscopy, which is widely available and less expensive, is less sensitive and cannot distinguish between live and dead bacteria. This limits the utility of smear microscopy when it comes to low-burden TB patients, such as children and people living with HIV, and makes it a poor tool for monitoring treatment responses. MBLA, therefore, is a technological solution to these challenges drawing on recent advances in molecular biology.

**THE MBLA TEST**

The MBLA test is specific to TB, and it has a faster turnaround time (TAT) than culture, taking only 24 hours for results to be received at the clinic. It is a reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) assay that uses 16S-rRNA as a marker to detect and quantify the load of viable *M. tuberculosis* bacteria in a patient’s sputum sample. The process starts with RNA isolation from *M. tuberculosis* bacteria, followed by RT-qPCR to quantify the RNA, then conversion of the RT-qPCR output (cycle threshold [CT] values) into bacterial load values (estimated colony forming units per millilitre [eCFU/ml]) using a standard curve (Figure 1).

Unlike DNA, which can persist long after cells have died, RNA degrades following cell death, implying that the RNA measured by MBLA is from viable cells. The principle is that for patients responding to treatment, the number of viable *M. tuberculosis* bacilli remaining following treatment should be fewer than before treatment, and this number should continue declining until there is zero (undetectable) mycobacterial load. Changes in mycobacterial load can be detected as early as the third day of treatment follow-up. This means that drug resistance or nonadherence to treatment can be detected earlier and appropriate measures can be taken. The EDCTP-funded Pan-African Biomarker Expansion (PANBIOME) study – conducted in Malawi, Mozambique, and Tanzania – demonstrated that patients, on average, clear 1 log_{10} eCFU/ml of *M. tuberculosis* bacteria per week of treatment (Figure 2).

**TAKING THE MBLA INTO POLICY AND PRACTICE**

Many tools are invented but are never implemented in practice or are too expensive to be accessed by the majority. The other challenge with new technologies is their frequent incompatibility with existing infrastructure or environmental conditions in the target areas of implementation. Taking MBLA to policy and practice refers to all processes – including technical optimisation and validation, assessment of appropriateness, acceptability and cost-effectiveness – that are required for the tool to be adopted for the routine clinical management of TB. After a stakeholders conference, the WHO identified MBLA as having the potential to replace smear microscopy and culture for monitoring TB treatment responses.

Importantly, the development of MBLA has taken a co-development model with stakeholders to ensure that the capacities needed to sustain the test at sites of implementation are developed in advance.

The first trial that demonstrated MBLA as a culture-free biomarker test for rapidly measuring patient mycobacterial load was conducted at South Africa’s Stellenbosch University. This study also demonstrated that the reduction in mycobacterial load measured by MBLA was consistent with the increase in TTP measured by automated TB liquid culture, Mycobacterium Growth Indicator Tube (MGIT). The difference between the 2 technologies is that MBLA is quantitative, contamination-free, and produces results within hours, compared with 5 to 42 days for MGIT. A second study, conducted at Tanzania’s National Institute for Medical Research (NIMR)–Mbeya Medical Research Centre, revealed superior performance of MBLA over solid TB culture in quantifying mycobacterial load and the assay results TAT.

Funded by the EDCTP, the first multi-site evaluation of the test was conducted at the University of Malawi College of Medicine, Instituto Nacional de Saude, Kilimanjaro Clinical Research Institute, and NIMR–Mbeya Medical Research Centre in Malawi, Mozambique, and Tanzania, respectively. The study revealed that MBLA results are reproducible in different laboratory settings and that it is superior to existing tools for the long-term follow-up of TB patients and monitoring treatment response (Figure 2). Unlike culture, wherein reported contamination rates were as high as 30% in some settings, MBLA was found to be unsusceptible to contamination and inhibition.

Further trials are ongoing in Uganda, Tanzania, Vietnam, Thailand, and the UK. The Pan African Consortium for the Evaluation of Antituberculosis Antibiotics (PanACEA) has adopted MBLA as a primary test in their clinical trials at over 10 sites in East and Southern Africa, as well as Gabon in Central Africa.

With support from the Global Challenges Research Fund (GCRF) and the Scottish Funding Council (SCF), the first-ever MBLA stakeholders conference was held at the University of St Andrews from 11 to 13 June 2018. Directors of national TB control programmes, laboratory scientists, academics, and industrialists representing 16 countries and WHO participated.
in the conference (Figure 3). Seventeen delegates participated in the 2-day MBLA training session, among whom 41% were from East and Southern Africa (Figure 4). WHO was represented by the Laboratories, Diagnostics, and Drug Resistance Unit of the Global TB Programme. The conference discussed ways by which the MBLA test can be translated into routine clinical practice and accessed by the TB patients who need it the most. The conference underscored what the development of TB diagnostics should focus on – tests that identify true TB infections, monitoring the effectiveness of treatment, and determining cure. In addition to the current evidence, the conference unanimously recommended a large-scale, multisite, multinational, near–routine-practice evaluation of MBLA to conclusively answer technical and operational questions required by WHO to approve the test for routine practice. This work will answer questions about MBLA test sensitivity and specificity, sample type and frequency of testing, reproducibility, appropriateness, acceptability, and cost-effectiveness in relation to the current standard of care tests for TB diagnosis and treatment monitoring.

While commonly applied in viral disease management, the molecular measurement of pathogen load (the number of microbes causing the disease) is new in bacteriology. As with other bacteria, the measurement of TB bacterial load has depended on the laboratory cultivation of sputum (and other patient samples) in culture media to count \(M.\) \textit{tuberculosis} colonies or determine TTP as an index of mycobacterial load. Unfortunately, \(M.\) \textit{tuberculosis} takes weeks to grow, and this is too long for results to inform initial patient management decisions.\(^8\) Discovered 136 years ago, and despite its shortcomings of low sensitivity and inability to distinguish between dead and viable bacteria, semiquantitative smear microscopy remains the most widely used test for TB diagnosis and treatment monitoring.\(^9\) Microscopy is often unreliable for samples from children and HIV-positive patients who often have a low TB bacterial load. Additionally, because of its low sensitivity, smear microscopy is often negative a few weeks into treatment when patients still have a significant mycobacterial load.\(^14\)
FIGURE 2. Treatment Response Curves of Tuberculosis Patients Followed for 12 Weeks of Treatment

Mycobacterial load was measured at baseline before the initiation of antituberculosis therapy and weekly during treatment. Each curve represents a patient. The red curve demonstrates the biphasic decline in mycobacterial load as patients responded to treatment. Note the steeper slope (fast rate of sputum clearance) in the first 2 weeks of treatment, and then a plateauing curve (slow rate of sputum clearance) in the later weeks of treatment.

FIGURE 3. The Molecular Bacterial Load Assay (MBLA) Stakeholders Conference Delegates
Rapid Test for Measuring Individual TB Bacterial Load

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THE TB CONTROL COMMUNITY SHOULD ACCELERATE IMPLEMENTATION

Like slow-growing *M. tuberculosis* colonies, the uptake of TB diagnostic and therapeutic innovation has been disappointingly slow. After the discovery of *M. tuberculosis* bacilli using a rudimentary microscope in 1880, it took 127 years to adopt the use of light-emitting diode (LED) technologies for microscopy and for the first molecular diagnostic tool – the line probe assay – to be adopted. The Xpert MTB/RIF, a rapid test that provides TB diagnostic results within 2 hours, was adopted in the 128th year. In contrast, within 10 years of describing HIV disease, viral load measurement for monitoring the response to antiretroviral therapy was adopted in clinical practice. After 136 years, the microscope remains the only WHO-approved tool for the routine monitoring of TB treatment, and there is still no effective rapid test to measure mycobacterial load and monitor treatment responses in routine practice. The MBLA has been developed to fill this gap and ensure the early identification and appropriate management of patients responding poorly to treatment. With MBLA, clinicians can undertake early investigations of adherence and drug resistance when the patient’s bacterial load does not decline following the initiation of therapy.

CONCLUSION

Through partnerships with local and international technology developers, East Africa will leverage its capacity for biomedical innovation. In some respects, there is no need to reinvent the wheel when it comes to technologies that are already available. Frugal innovation, using existing knowledge and technology is the way to go; this way, existing technologies can be customised and repackaged for similar functions or repurposed to serve different functions of interest at affordable costs. It is important to note, however, that innovation and entrepreneurship do not happen in a vacuum. Without a conducive environment and financial resources, innovative ideas can only go so far. Governments in East Africa and Africa at large should take it upon themselves to create business incubation centres to nurture ideas from young entrepreneurs and policy frameworks to protect intellectual property rights, assuring innovators and their funders of good returns on their investments.

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Rapid Test for Measuring Individual TB Bacterial Load

Research Fund, the Pan-African Consortium for the Evaluation of Antituberculosis Antibiotics (PanACEA), and University of St Andrews School of Medicine made the MBLA stakeholders conference possible. Thanks to the conference organising team, trainers, the School of Biology teaching laboratory team, the School of Medicine research support office, and the delegates who made the conference a success. We also acknowledge the research institutions in East and Southern Africa who have significantly contributed to the development of the MBLA test.

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ABSTRACT

The ubiquity of mobile phones offers an opportunity for a paradigm change in health-care delivery, which may offer solutions to some of the challenges faced by the health sector in Uganda. The Medical Concierge Group (TMCG) is a digital health company, headquartered in Uganda, which leverages on mobile phone-based platforms – such as short messaging service (SMS), voice calling – and social media to deliver health services. Just over two-thirds (68%) of users of TMCG’s services are males between 18 and 30 years of age. SMS reminders have improved the honouring of health facility appointments among HIV-positive clients, from 60% to 90%; retention rates at supported health facilities have improved from 45% to 89%. Furthermore, information dissemination has been achieved via mobile SMS, wherein subscribers can access health content on diverse topics – such as HIV/AIDS prevention and family planning – by sending messages to a pre-defined short code to a phone line. Over 900 beneficiaries have accessed health content via SMS subscriptions. Social media platforms, including Facebook and Twitter, are used for health information dissemination and have enabled a wider reach to over 13 million beneficiaries accessing health information on TMCG’s Facebook page alone. Tailoring mobile phone-based health content to meet the target beneficiaries’ needs is critical for TMCG’s impact and uptake. With rising rates of phone ownership and Internet connectivity in Uganda, mobile phones offer an affordable and proven adoptable avenue to overcome the chronic challenges faced by the health sector.

INTRODUCTION

To date, mobile health (mHealth) does not have a standard definition. For the purposes of this article, we adopted the World Health Organization’s definition, which refers to mHealth as a component of electronic health that deals with the provision of health services and information via mobile technologies.1 Uganda’s phone ownership rate increased from 10% in 2002 to 65% in 2014.2 This has driven different sectors within the country – including the health sector – to leverage the ubiquity of mobile phones to improve services.3 Mobile technologies have great potential to overcome challenges in health-care delivery, as they provide cost-effective means of accessing quality health-care services.4 Such technologies provide an avenue for addressing some of the challenges that are of particular concern for health systems in developing countries, including low health worker-to-patient ratios, accessibility and transport to health facilities, poor health information systems, long waiting times at health facilities, and the poor or non-existent patient follow-up systems.5 This article discusses health-care delivery through mobile technologies, as implemented by The Medical Concierge Group (TMCG), a digital health company in Uganda.

mHealth in Uganda

The integration of digital technologies in health-care delivery in Uganda has gained momentum in past decade, coinciding with increased access to the Internet across the country.6 Mobile phones provide an ideal means of targeted health information dissemination because of their portability and ubiquity.7,8 Short messaging service (SMS) functionality is used, for example, in aspects of disease surveillance as well as patient notifications – to remind people about health facility appointment dates or to take their medications – all as means of improving public health outcomes.9,10

Interventions relying on mHealth have also been implemented to improve health worker performance; for example, during Malaria Consortium’s programme implementation in the northern and eastern parts of Uganda, text messaging was used to increase awareness and improve coverage of intermittent preventive treatment for malaria among health workers.11 Additionally, mobile phones have been used to facilitate remote consultations among health workers.12 Mobile phone approaches have also been used for the timely delivery of medical laboratory test results. A
study done in southwestern Uganda revealed a high level of acceptability of text messaging as a mode of delivery for laboratory investigation results among a cohort of HIV-positive clients. Mobile technologies have also been used for data and information collection, for example, by the Uganda Ministry of Health for tuberculosis and malaria monitoring.

In Uganda, mHealth programmes have employed a variety of strategies and technologies, including SMS – which accounts for the majority of mobile phone-based health services – as well as personal computers, personal digital assistant-based applications. Web-based applications and voice calls – for example, with interactive voice recognition (IVR) or even call-in services – have also gained momentum.

**METHODS**

TMCG was chosen for this case study, which was conducted from October 2016 to October 2017 because it has operated a functional mHealth platform for the past 7 years and, therefore, provides an appropriate example of the landscape of mHealth implementation in Uganda. Focus group discussions with key personnel at TMCG were scheduled: these included 1 company director, 1 health-care worker, and the senior software engineer at the company. Data were also collected via a researcher-administered, open-ended questionnaire that covered details about the mobile phone platforms deployed and how they are used, the target beneficiaries, and the kinds of health services they facilitate.

Demographic information about the end users of the mHealth services – including age, gender, and location – was collected from the databases of the different mHealth platforms assessed. Additionally, we analysed anonymised user data, including HIV status information, categories of health enquiries made, and health services utilised. These data are routinely collected on an optional basis and documented within the different service provision platforms. The data, when exported from the different digital health platforms, were made available in the form of Excel (Microsoft Corp., Redmond, WA, USA) spreadsheets for analysis within Microsoft Excel 2018.

**RESULTS**

**The Medical Conierge Group**

TMCG is a digital health company, headquartered in Kampala, Uganda, which has been operational since 2012. TMCG operates a call centre that provides free access to health professionals 24 hours a day, 7 days a week, for anyone seeking health consultations or information.

**Company Mission**

The mission of the company is “to design and deploy scalable models for unlimited access to holistic, affordable, and quality health-care products and services guided by innovation and user engagement”. The company has achieved this through deploying a combination of concepts in health-care delivery via mobile technologies, including voice calling, SMS, social media platforms (Facebook and Twitter, for example), and messaging services (like WhatsApp). Focusing on live interactions to provide a unique personal experience of individualized responses in every interaction is the main objective of the company’s model of mHealth delivery.

**Voice Calling Services**

The voice calling platform allows for immediate and real-time interactions between health workers and end users. Data are collected using an open source application called Asterisk. The voice calling platform is used to handle health-related enquiries, coordinate care for chronically ill patients, facilitate treatment adherence, assist in the retention of patients in care, follow-up antenatal care clients, and contribute to pharmacovigilance.

The majority voice platform users are males (60%) between 18 and 35 years of age (80%). Calls are received from different parts of the country, but the majority (67.2%) come from the central region.

Interactions cut across different health domains, with those related to HIV/AIDS accounting for 30.6%. Most HIV/AIDS-related enquiries deal with myths and misconceptions about HIV transmission and prevention. Enquiries about sexual and reproductive health account for 10.6% of interactions, and these cover topics such as family planning and menstrual hygiene. Maternal, newborn, and child health concerns account for 12.7% of interactions, and gastrointestinal complaints account for 9.2%.

**Messaging Services**

TMCG’s messaging service involves both *pushes* and *pulls*. With *push* interactions, users consent to receiving mobile messages with health information. For *pull* messaging services, individuals subscribe to mHealth content by sending a keyword to a short code. For example, to receive messages about family planning, a user sends the keyword “FP” to 8884. HIV/AIDS prevention, with 282 subscriptions, is the most highly subscribed topic, followed by family planning (270 subscriptions); adolescent health (201 subscriptions); and maternal, newborn, and child health (153 subscriptions).

For the *push* system, the SMS platform was integrated into RapidPro, an open source messaging software application that is used for health campaigns, wherein content – including reminders – can be sent to beneficiaries. Monthly reminders were sent to 10,371 clients on antiretroviral treatment, and this improved the appointment uptake rate from 60% to 90% which consequently improved client retention at supported health facilities.

Additionally, health content is disseminated via mobile messages that cover different thematic areas, including family planning; maternal, newborn, and child health; and HIV/AIDS awareness and prevention, among others. The mobile messages are available in different local languages. In addition to being used for health information dissemination, the SMS platform can be used for individualised case management based on client enquiries, allowing personalised interactions between health workers and end users.
Social Media Platforms
TMCG uses its social media accounts on Facebook (https://www.facebook.com/TMCGLtd/) and Twitter (https://twitter.com/TMCGLtd) to reach a much larger audience for information dissemination. Health content to be shared on the pages undergoes a development process that starts with, for example, a topic chosen according to a date specified by the international health calendar or any major health event in the country. After the topic is chosen, data and research are compiled to inform content curation, after which the content is organised, reviewed, and revised before it is posted, shared, or otherwise disseminated. Metrics reflecting the impact of the

A beneficiary who calls into the call centre [1] is answered by the doctor who addresses the caller’s health inquiry [2], for example, by referring the client to a health service or offering health advice. The doctor documents a summary of the call into the electronic records system for archiving in the database [3].
DISCUSSION

The 24 million mobile subscribers\textsuperscript{18} and 13 million Internet users in Uganda represent a huge opportunity for overcoming barriers hindering universal health-care accessibility in the country. Social media, in particular, can reach far more people in a shorter period of time, and at a lower cost, than the traditional methods of cascading health information through physical gatherings.\textsuperscript{19} According to TMCG data, mHealth strategies have shown to be effective at reaching male clients, whose poor health-seeking behaviour has been shown to pose challenges that hinder preventive health programming and interventions.\textsuperscript{20} Among other factors, men are often discouraged from seeking health-care services because of long waiting hours at health facilities, fear of projecting weakness instead of the perceived expectation of "manliness", and the low levels of privacy at many health facilities.\textsuperscript{20} Despite these barriers and the well-established pattern of the relatively low uptake of health-care services by men relative to women, during the study period, 68% of TMCG's voice calling service users were males, and 32% were females.

Mobile phone features, such as SMS, offer opportunities for improving patient outcomes. For example, daily SMS reminders for asthmatic patients have been shown to increase adherence to treatment over 3 months of follow-up.\textsuperscript{21} In rural Uganda, SMS reminders for patients on antiretroviral therapy led to improved adherence.\textsuperscript{22} The TMCG data corroborate these study findings, as users of TMCG mHealth services exhibited improved hospital appointment uptake and retentions in HIV care programmes (Figure 3).

Important considerations for SMS platforms and content include contextualisation, comprehensibility, and accessibility tailored to the target audience.\textsuperscript{23} Multi-language support is, therefore, a key feature of TMCG's messaging platforms. Additionally, continuous content review, feedback, and editing are necessary for content to remain relevant and useful. This is exemplified by TMCG's social media content development process flow.

The limitations of mHealth interventions in low-income countries – especially in remote, rural areas – may include accessibility to mobile technology and the Internet. TMCG's mHealth clients are disproportionately distributed in urban areas, particularly in Kampala and Wakiso.

CONCLUSION

Mobile phone-based health innovations provide an affordable and flexible way to overcome the chronic challenges to health-care delivery in Uganda, including the low doctor-to-patient ratio, long waiting times at health facility, and poor or non-existent patient follow-up systems. Such mHealth services allow for large numbers of beneficiaries to be served at minimal expenditure of time and resources relative to traditional models of health-care delivery, which require physical interactions in most circumstances. Mobile technologies are particularly useful for preventive interventions, such as behavioural change communication for HIV/AIDS prevention.
Mobile Health (mHealth) in Uganda

The TMCG further reaffirms the effectiveness of SMS for improving patient outcomes, especially for chronically ill patients, for example, in terms of improved adherence to hospital appointments among HIV-positive clients. The demographic reach of TMCG’s mobile phone-based platforms – notably of particular interest to males and clients of both genders between 18 and 30 years old – provides insight to the plausibility of pilot mHealth interventions targeting men and young people. Effective communication via mobile phone-based channels requires targeted messaging that is tailored to the needs of the audience, easy to understand, and context-specific for the beneficiaries, even if it means translating messages and content to local languages. Finally, given the potentially sensitive nature of health data, ensuring data security and patient confidentiality is critical if the growth of such platforms is to be realised and sustainable.

Acknowledgements: We would like to acknowledge the staff of TMCG, who took the time to take part in the interviews, as well as the graphic designer, Michael Katerega, who helped in the designing of the illustrations used in the article. Funding for the study was from TMCG.

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FIGURE 3. The Retention Rates of HIV-positive Clients Under Care at Facilities Implementing Mobile Health (mHealth) Interventions

![Graph showing retention rates of HIV-positive clients under care at facilities implementing mobile health (mHealth) interventions.](image-url)


Peer Reviewed

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Prevalence and Distribution of Multidrug-Resistant Mutations in Mycobacterium tuberculosis in Tanzania

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ABSTRACT

Background: Molecular identification of mutations resulting in multidrug-resistant tuberculosis (MDR-TB) is an important approach for improving understanding of MDR-TB epidemiology and planning for appropriate interventions. We aimed to estimate the prevalence and distribution of mutations causing MDR-TB as well as determine the gene distribution among patients previously treated for TB.

Methods: This was a cross-sectional, hospital-based study conducted from April 2017 to October 2018 at Kibong’oto Infectious Diseases Hospital (KIDH). KIDH is the national MDR-TB referral hospital. Participants were patients presumptively diagnosed with MDR-TB and referred to KIDH from district and regional hospitals across Tanzania. Sputum samples were collected and analysed using the Xpert MTB/RIF assay, direct sputum smear fluorescence microscopy, culture on Lowenstein-Jensen medium, and line probe assay using the GenoType MTBDRplus VER 2.0 system. Demographic information and mutation frequencies were reported as counts and percentages and analysed using descriptive statistics.

Results: A total of 208 (69.3%) participants had \( rpoB \) gene mutations conferring resistance to only rifampicin; 92 (30.7%) had \( rpoB \), \( katG \), and \( inhA \) mutations conferring resistance to rifampicin and isoniazid; 78 (26%) had \( rpoB \) and \( katG \) mutations conferring resistance to rifampicin and isoniazid; and 14 (4.7%) had \( rpoB \) and \( inhA \) mutations conferring resistance to rifampicin and isoniazid.

Conclusion: The mutation prevalences identified in this study indicate the most frequent mutations were the S531L mutation of the \( rpoB \) gene, the S315T mutation of the \( katG \) gene, and the S315T mutation in the promoter region of the \( inhA \) gene. To control the emergence and spread of MDR-TB, drug sensitivity testing must be carried for GeneXpert-confirmed TB patients prior to initiating second-line anti-TB regimens.

INTRODUCTION

Tuberculosis (TB) continues to be a disease of public health importance worldwide. The emergence of multidrug-resistant TB (MDR-TB) is a serious challenge to TB control. MDR-TB, defined as resistance to both isoniazid and rifampicin, is a growing public health problem in resource-poor regions where adequate diagnosis and treatment are often unavailable. The World Health Organization (WHO) report of 2018 estimates that there were 558,000 new cases of rifampicin-resistant TB in 2017, 82% of which were MDR-TB. MDR-TB is estimated to affect 3.5% of patients diagnosed with TB for the first time and 20.5% of those previously treated for TB. WHO estimates that about 5% of all TB patients progress to MDR-TB, which had a peak mortality rate of more than 40% in 2013. Tanzania is among the 30 countries most affected by TB and MDR-TB, with an MDR-TB prevalence of 1.1% in 2010.

Resistance to anti-TB agents by Mycobacterium tuberculosis is caused by mutations in genes or the promoter regions of genes involved in drug activation or encoding drug targets. Molecular mechanisms for resistance to rifampicin are known to involve chromosomal mutations in the RNA polymerase \( \beta \)-subunit gene (\( rpoB \)), which encodes the \( \beta \) subunit of the M. tuberculosis RNA polymerase chain. Isoniazid is one of the most effective anti-TB drugs. The majority of the mutations responsible for high-level M. tuberculosis isoniazid resistance are found in the catalase peroxidase gene (\( katG \)). Isoniazid resistance also commonly results from mutations in the...
Multidrug-Resistant M. tuberculosis Mutations in Tanzania

The promoter region of the enoyl acyl carrier protein reductase gene (inhA)\textsuperscript{15}; these mutations increase inhA expression and confer low-level resistance to isoniazid.\textsuperscript{16} The lower susceptibility to isoniazid is associated with mutations in the structural region of inhA, which lower affinity to drug-NAD adducts.\textsuperscript{16}

The molecular detection of M. tuberculosis mutations is important for the understanding of TB epidemiology, as it can help predict transmission rates and identify dominant strains, strains with enhanced capacity to spread, and strains associated with outbreaks\textsuperscript{17} and severe disease.\textsuperscript{18,19} Effective genotypic monitoring of the emergence of drug-resistant strains of M. tuberculosis is pivotal to TB control, more so than the detection of drug resistance by phenotype, which suffers from protracted identification of resistant strains. There is accumulating evidence correlating gene mutations with phenotypic resistance; however, the relevant data are sparse and inconsistent, particularly in sub-Saharan Africa where the disease burden is highest.\textsuperscript{18}

This study aimed to estimate the prevalence and distribution of mutations causing MDR-TB as well as determine the gene distribution among patients previously treated for TB who presented at Kibong’oto Infectious Disease Hospital (KIDH) in Sanya Juu, Tanzania.
Multidrug-Resistant M. tuberculosis Mutations in Tanzania

METHODS

Design and Settings
This was a hospital-based cross-sectional study conducted from April 2017 to October 2018 at KIDH, a national referral hospital for patients from all parts of Tanzania. KIDH is currently the country’s largest referral hospital for MDR-TB management, with a dedicated 40-bed capacity in addition to separate facilities for treating drug-susceptible TB.

Study Participants and Inclusion Criteria
We enrolled referred inpatients aged 18 years and older. A structured questionnaire was administered by interviewers to collect sociodemographic information in addition to that obtained from the hospital files. We excluded admitted patients on treatment for diseases other than TB and those coming from outside of Tanzania.

Sample and Data Collection
Sputum samples were collected from each participant in a sterile Falcon tube (BD Biosciences, Bedford, MA, USA). The Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) for molecular detection of MDR-TB was used to test all sputum samples.20 Direct sputum smear fluorescence microscopy was used to test for acid-fast bacilli (AFB) for all enrolled patients. All AFB-positive sputum samples were cultured on the Lowenstein-Jensen (LJ) medium (HiMedia Laboratories GmbH, Einhausen, Germany). Recovered M. tuberculosis colonies from LJ medium culture-positive sputum samples were used to perform a line probe assay (LiPA) employing the GeneType MTBDRplus VER 2.0 system (Hain Lifescience GmbH, Nehren, Germany) according to the manufacturer’s instructions (Figure 1).

LiPA
M. tuberculosis DNA was extracted from recovered M. tuberculosis colonies using the GenoLyse DNA extraction kit (Hain Lifescience GmbH) followed by a polymerase chain reaction sequence to amplify the rpoB gene encoding the β-subunit of RNA polymerase, the katG gene encoding for catalase peroxidase, and the promoter region of the inhA gene encoding for NADH enoyl ACP reductase, for the detection of genomnic mutations associated with MDR-TB. The primers and polymerase included in the MTBDRplus Ver 2.0 assay kits were used to amplify the genes, and the H37Rv quality control M. tuberculosis strain was used as the positive control. The M. tuberculosis mutations in the rpoB gene associated with rifampin resistance, and those in the katG and inhA genes associated with isoniazid resistance, were interpreted and determined by the band patterns on the LiPA strips after reverse hybridisation of the gene amplificates.

Statistical Analysis
Data were analysed using IBM SPSS Statistics version 20 (IBM Corp., Armonk, NY, USA). Data were summarised using frequency distributions and charts for categorical data and descriptive statistics (mean, median, standard deviation, and interquartile range) for numerical data. Chi-square tests were applied to assess patient sociodemographic and clinical characteristics associated with drug resistance, and Fisher’s exact was used to calculate P values when comparing small frequencies (less than 5). The mutations rates in the rpoB, katG, and inhA genes, in relation to MDR-TB–negative and MDR-TB–positive status, were also estimated.

Ethical Considerations
Ethical approval was obtained from the Kilimanjaro Christian Medical University College Research and Ethics Committee (Certificate number 2039, April 2017). Permission from KIDH management was obtained from the medical officer in charge. All participants consented to participate in the study voluntarily after the study was explained to them. The patients’ confidentiality and privacy were strictly observed.

RESULTS
A total of 428 presumptive sputum specimens were collected from newly referred patients to KIDH. Of these, 100 tested negative for TB using Xpert MTB/RIF, and these patients were
excluded from the study and treated as per routine hospital guidelines. Sputum smear fluorescence microscopy was carried out on 328 samples, which were then cultured on LJ medium. Twenty of the samples were LJ medium culture-negative, and the patients who submitted these samples were excluded and treated as per routine hospital guidelines. DNA extraction – using GenoLyse kits according to manufacturer’s instructions – was performed on 308 specimens that showed growth in AFB culture. The LiPA was performed for 308 specimens, and 8 specimens returned invalid LiPA results; the patients who submitted these 8 specimens were excluded from the analysis and treated as per standard-of-care guidelines.

Out of the 300 patients recruited, 191 (63.7%) were male, and 109 (36.3%) were female. The mean patient age was 37.5 ± 10 years. Patients between of 20 and 40 years old were most affected by MDR-TB (Table 1). There were 208 (69.3%) isolates that had mutations conferring resistance to only rifampicin (rifampicin-monoresistant) and 92 (30.7%) isolates that had mutations conferring resistance to both rifampicin and isoniazid (multidrug-resistant [MDR] isolates) (Figure 2). The S531L mutation was observed in 182 (60.7%) isolates. The frequency of the S531L mutation in the rpoB gene was significantly higher among rifampicin-monoresistant isolates than among MDR isolates (P < .01). Mutations in the rpoB gene region encompassing codons 513 to 519 were significantly more common among non–MDR-TB isolates than among MDR-TB isolates (P < .01) (Table 3).

**Rifampicin Resistance–Associated Mutations**

Rifampicin resistance–associated mutations involving the *rpoB* gene were the most frequently encountered mutations in this study, appearing in 208 (69.3%) isolates (Table 3). The S531L (TCG→TTG) *rpoB* mutation was observed in 182 (60.7%) isolates. The frequency of the S531L mutation in the *rpoB* gene was significantly higher among rifampicin-monoresistant isolates than among MDR isolates (P < .01). Mutations in the *rpoB* gene region encompassing codons 513 to 519 were significantly more common among non–MDR-TB isolates than among MDR-TB isolates (P < .01) (Table 3).

**Isoniazid Resistance–Associated Mutations**

The *katG* 315ACC mutation was the most common isoniazid–rifampicin multidrug resistance–associated mutation identified in this study. The S315T1 *katG* gene mutation – with a codon change of AGC→ACC – occurred in 78 (84.8%) of 92 MDR isolates detected. Mutations at the promoter region of the *inhA* gene were also detected. Overall, the 315ACC mutation was found in 14 (15.2%) of the 92 MDR isolates; 7 with the MUT1 S315T variant (TCG→TGG), 1 with the WT 315 variant, and 6 with the WT1 C15T variant (GGC→ACC) (Table 3).

**DISCUSSION**

The present study highlights the prevalence and distribution of MDR *M. tuberculosis* mutations in Tanzania. The majority patients were between 20 and 40 years old, with male patients predominating. The frequency of MDR-TB in this age group has substantial socioeconomic implications, as young adult males are an important component of the economically productive population. A high MDR-TB prevalence among young adults has an acute impact on the national economy. TB control strategies need to set specific targets for all age groups, but for this group in particular. Our findings are comparable...
with observations from previous studies in India, South Africa, and Zimbabwe, which revealed higher rates of MDR-TB among youths and young adults.²⁷⁻²⁹

We found a higher prevalence of MDR-TB among men in our study. Although the explanations regarding differences in immunity between men and women are incomplete, it is generally accepted that infectious diseases rarely affect males and females equally.³⁰ Females exhibit a more robust immune response to antigenic challenges, such as infection and vaccination, than males.³¹ This is mediated largely by sex hormones, the role of which – in TB – is supported by the fact that the male disadvantage does not arise until puberty.³² Sex hormones have diverse effects on many immune cell types, including B cells, T cells, neutrophils, dendritic cells, macrophages, and natural killer cells.³³ Other reasons for these gender differences may be related behavioural and exposure differences – including regarding social roles and risk behaviours, such as alcohol and tobacco consumption – between the sexes, which make males more likely to acquire TB.³⁰,³³⁻³⁵ In this regard, our findings are contradictory to what was previously reported in Pakistan and Afghanistan, where TB has been reported to be more prevalent among women than men³⁶; however, our results are comparable with other studies done in other parts of the world.³⁰,³⁷⁻⁴² It has been reported that nearly twice as many men as women have been diagnosed with TB globally.⁴³,⁴⁴ These findings are relevant for planning different TB control strategies and programmes, especially in low-resource settings.

We found a high prevalence of rifampicin monoresistance determined by *rpoB* gene mutations. Drug resistance is multifactorial and – in the presence of HIV infection, for example – higher rates anti-TB drug resistance could be attributable to HIV-associated malabsorption, mismanagement of TB cases, adherence challenges and antiretroviral and anti-TB drug interaction.⁴⁵⁻⁴⁹ Rifampicin is the most vital drug for TB treatment; therefore, resistance to rifampicin

#### TABLE 2. Prevalence and Distribution of Gene Mutations in Mycobacterium tuberculosis by Patient Characteristics (N=300)

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Non–Multidrug-Resistant (n=208)</th>
<th>Multidrug-Resistant (n=92)</th>
<th>Multidrug-Resistant (n=78)</th>
<th>Multidrug-Resistant (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>rpoB</em> (Mono-resistance)</td>
<td><em>katG and rpoB</em> (n=78)</td>
<td><em>rpoB and inhA</em> (n=14)</td>
<td></td>
</tr>
<tr>
<td>MUT1, MUT2B, (S315T1), (H526D) n=5 (%)⁶</td>
<td>MUT3 (S531L) n=182 (%)⁶</td>
<td>WT3 (S13-517) n=9 (%)⁶</td>
<td>WT4 (S19-519) n=7 (%)⁶</td>
<td>MUT1 n=78 (%)⁶</td>
</tr>
<tr>
<td>MUT1 n=78 (%)⁶</td>
<td>WT7, WT8 n=5 (%)⁶</td>
<td>MUT1 n=7 (%)⁶</td>
<td>WT, WT1 n=7 (%)⁶</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-30</td>
<td>2 (40%) 51 (28.02%) 1 (11.2%) 2 (28.57%) 1 (20%)</td>
<td>24 (30.7%) 2 (28.57%) 2 (28.57%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>31-40</td>
<td>1 (20%) 58 (31.9%) 4 (44.4%) 2 (28.57%) 3 (60%)</td>
<td>28 (35.9%) 3 (42.86%) 2 (28.57%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>41-50</td>
<td>2 (40%) 53 (29.1%) 0 (0%) 3 (42.86%) 1 (20%)</td>
<td>18 (23.1%) 2 (28.57%) 3 (42.86%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>0 (0%) 20 (0.11%) 4 (44.4%) 0 (0%) 0 (0%)</td>
<td>8 (10.3%) 0 (0%) 0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>3 (60%) 58 (31.9%) 3 (33.3%) 3 (42.86) 1 (20%)</td>
<td>35 (44.9%) 2 (28.7%) 4 (57.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2 (40%) 124 (68.1%) 6 (66.7%) 4 (57.14%) 4 (80%)</td>
<td>43 (55.1%) 5 (71.3%) 3 (42.9%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

⁶For each entry in the respective columns, the denominators are 5, 182, 9, 7, 78, 7, and 7, respectively, which are counts for every mutation reported in a column across participant age groups and genders.
### TABLE 3. Prevalence and Distribution of Mutations, Amino Acid Changes, and Nucleotide Change in *Mycobacterium tuberculosis* Isolates (N=300)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Band Missing (WT#)/ Mutation Present (MUT#)</th>
<th>Mutation or Codons Involved</th>
<th>Amino Acid Change</th>
<th>Nucleotide Change</th>
<th>Non- MDR-TB n=208</th>
<th>MDR-TB n=92</th>
<th>Fisher’s Exact $\chi^2$</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>rpoB</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUT1</td>
<td>S315T1</td>
<td>Ser→Thr</td>
<td>AGC→ACC</td>
<td>2</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUT2B</td>
<td>H526D</td>
<td>His→Asp</td>
<td>CAC→GAC</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUT3</td>
<td>S531L</td>
<td>Ser→Leu</td>
<td>TCG→TTG</td>
<td>182</td>
<td>0</td>
<td>191.48</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>WT3</td>
<td>517-519</td>
<td>Asn→Lys</td>
<td>AAC→AAA</td>
<td>9</td>
<td>0</td>
<td>5.967</td>
<td>.02</td>
<td></td>
</tr>
<tr>
<td>WT4</td>
<td>513-517</td>
<td>Lys→Phe</td>
<td>AAA→TTC</td>
<td>7</td>
<td>0</td>
<td>208.232</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>WT7</td>
<td>526-529</td>
<td>His→Arg</td>
<td>CAC→CGC</td>
<td>3</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT8</td>
<td>530-533</td>
<td>Leu533→Pro</td>
<td>CTG→CCG</td>
<td>2</td>
<td>0</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>katG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUT1</td>
<td>S315T1</td>
<td>Ser→Thr</td>
<td>AGC→ACC</td>
<td>0</td>
<td>78</td>
<td>2229.57</td>
<td>&lt;.01</td>
<td></td>
</tr>
<tr>
<td>inhA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MUT1</td>
<td>S315T</td>
<td>Ser→Thr</td>
<td>TCG→TGG</td>
<td>0</td>
<td>7</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>315</td>
<td>0</td>
<td>1</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT1</td>
<td>C15T</td>
<td>Cys→Thr</td>
<td>GGC→ACC</td>
<td>0</td>
<td>6</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
has enormous implications for TB control programmes. Our findings are comparable with results from a study done in South Africa, which reported a rise in rifampicin monoresistance.\(^{45,50}\) A study conducted to evaluate anti-TB drug resistance surveillance in 19 countries reported the presence of rifampicin monoresistance in all of the countries.\(^{51}\) Rifampicin resistance has often been considered as a surrogate marker of MDR-TB, because it is highly correlated with concomitant isoniazid resistance.\(^{52,53}\) In this regard, all patients with rifampicin monoresistance ought to be treated as MDR-TB patients. Our findings, however, differed from study findings from Iran and Nigeria, which failed to detect rifampicin monoresistance.\(^{25,54}\)

We found a high prevalence of mutations in codon 315, with predominance of the ACC nucleotide sequence in the \(katG\) gene, which resulted in resistance to both rifampicin and isoniazid. This indicates that the amino acid at position 315 of \(katG\) is prone to mutation. These results correspond to what other researchers have reported, and it may be attributable to lifestyle factors, delays or difficulties in accessing health facilities, and patient immunocompromise or noncompliance to treatment.\(^{55,56}\) Mutations of the \(katG\) gene more strongly influence the development MDR-TB than mutations of the \(inhA\) gene.\(^{57}\)

We also observed the presence of \(rpoB\) and \(inhA\) gene mutations at codon 315 (S315T) and 15 (C15T) that resulted in resistance to both rifampicin and isoniazid. Our findings are comparable with those of other studies, whereby it has been reported that mutations at the \(katG\)-315, \(rpoB\)-531, and \(inhA\)-15 positions are associated with high rates of isoniazid-resistant TB.\(^{11,27}\) Furthermore, other studies have reported that mutations in codon 315 and the promoter region of the \(inhA\) gene are the most common and are associated with isoniazid resistance.\(^{14,35}\) These observations suggested that repeated administration of the same anti-TB drugs increases the risk of resistance, including multidrug resistance.

**CONCLUSION**

The most frequently detected mutations in our study were the S315L \(rpoB\) mutation, the S315T \(katG\) mutation, and the S315T mutation in the promoter region of the \(inhA\) gene. MDR-TB control strategies require an understanding of the evolution of these mutations. Further studies to evaluate these mutations in detail would increase our understanding of the epidemiology and transmission dynamics of drug-resistant \(M.\) tuberculosis in Tanzania to inform the planning, design, and implementation of innovative TB control strategies.

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**REFERENCES**


Peer Reviewed

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ABSTRACT

Background: Globally, over 325 and 170 million people are infected with hepatitis B virus (HBV) and hepatitis C virus (HCV), respectively. If untreated, these infections can progress to cirrhosis or hepatocellular carcinoma. The primary aim of this study was to determine the prevalence, genetic diversity, and factors associated with HBV and HCV among couples attending antenatal care in rural Rwanda.

Methods: This was a cross-sectional survey of HBV and HCV seroprevalence. Study participants were administered a brief structured questionnaire to obtain information on sociodemographic and behavioural risk factors for HBV and HCV. Participant blood samples were screened for hepatitis B surface antigen (HBsAg) and anti-HCV antibodies (anti-HCV) using rapid diagnostic kits; confirmatory testing was done by enzyme immunoassay and nucleic acid tests. HBV genotypes were determined using nested polymerase chain reaction; HCV genotypes were determined by reverse transcriptase PCR followed by hybridisation with sequence-specific oligonucleotides. Statistical associations between risk factors and infection status were determined using Chi-square tests and bivariate logistic regression.

Results: In total, 220 individuals participated in the study. This includes 110 pregnant women and 110 male partners who were attending antenatal care at Gitare and Cyanika health centres. Two participants (0.9%) had serological evidence of HBV infection, and 4 participants (1.8%) were infected with HCV. HBV genotype A accounted for all HBV infections; HCV genotype 4 accounted for all HCV infections. None of the assessed factors were associated with HBV infection while occupation type and scarification were significantly associated with HCV infection ($P$ values were .03 and <.01 respectively). All cases of infection were discordant with their respective partners.

Conclusion: Prevalence rates of HBsAg and anti-HCV are low in couples attending antenatal clinics in rural Rwanda. Consideration should be given to interventions aimed at reducing the risk of transmission in discordant couples and infants of infected mothers.

INTRODUCTION

Globally, hepatitis B and C viral infections account for a considerable proportion of morbidity and mortality from liver disease. In 2015, an estimated 257 million individuals worldwide had chronic hepatitis B virus (HBV) infection, leading to approximately 887,000 deaths.\(^1\) During the same period around 71 million people were chronically infected with hepatitis C virus (HCV); each year approximately 1.7 million people are newly infected with HCV and 400,000 HCV-related deaths occur.\(^1\)

Prevalence rates of HBV and HCV vary widely between world regions and countries.\(^2\) Hepatitis B surface antigen (HBsAg), a marker of active infection, has a prevalence rate >8% in several African countries, including Nigeria, Namibia, Gabon, Cameroon and Burkina Faso. HBsAg prevalence is intermediate (2%-8%) in Kenya, Zambia, Ivory Coast, Liberia, Sierra Leone, Senegal and Rwanda, and low (<2%) in North Africa.\(^3,4\) Survey data indicate that HBsAg prevalence in Rwanda is 1.9% to 3.2% among blood donors and 3.7% among pregnant women attending antenatal clinics.\(^5\) Based on studies of small patient groups and data from neighbouring countries, in Rwanda, the prevalence of anti-HCV antibodies (anti-HCV) is estimated to be 3.1% in the general population and 4.7% among people living with HIV.\(^6,8\)
Globally, in 2015, there was an estimated 1.75 million new HCV infections. The availability of effective antiviral treatment has revolutionised treatment prospects, although access to treatment remains a significant challenge for most developed countries and remains out of reach for developing nations. However, the incorporation of hepatitis B vaccination into the expanded immunisation programmes has led to substantial decreases in chronic hepatitis B infection rates in children.

In addition to variations in prevalence, HBV and HCV display genotypic diversity between world regions and countries. HBV has 10 well-described genotypes (A to H), and 2 genotypes that are tentatively defined according to their genome divergences (I and J). People infected with HBV genotypes A or C are at a higher risk of progressing to chronic hepatitis than those exposed to genotypes D and B; nevertheless, all genotypes can lead to acute or chronic infections.

In Africa, genotype A predominates in Southern and Eastern Africa, genotype D predominates in northern Africa, and genotype E predominates in the vast region from Senegal to Namibia and eastward to the Central African Republic. In Rwanda, HBV genotypes A, B, C, D and E have been reported. HCV has 6 well-described genotypes (1 to 6) and 5 genotypes that are tentatively defined (7 to 11). Genotype 1 accounts for about 46% of all HCV infections, genotype 3 accounts for about 30% of HCV infections, and genotypes 2, 4, 5, 6 collectively account for about 24% of HCV infections. Recent studies have correlated the different HCV genotypes with varying levels of risk of disease progression and varying responses to antiviral therapy.

In Rwanda, HCV genotypes 1 and 4 predominate.

To reduce the burden of HBV-associated liver disease, in 2002 Rwanda introduced universal administration of the HBV vaccine in neonates; thus, most youth born from 2002 onwards are protected from HBV infection. However, perinatal transmission, which accounts for the majority of HBV transmission worldwide, continues to occur in Rwanda. In adults, sexual transmission and risky behaviour, including injection drug use, tattooing, body piercing, and scarification without using sterilised equipment are also routes of HBV transmission. Routes of ongoing HCV transmission in Rwanda remain unknown, but prevalent cases are thought to have resulted from unsafe injection and blood transfusion practices in earlier eras.

In 2012, Rwanda initiated a viral hepatitis B and C control programme. The programme was intensified in 2015 with the introduction of more efficacious treatments for HCV. For Rwandans chronically infected with HBV, tenofovir and entecavir are available at all public health facilities offering HIV services. These drugs have been shown to suppress HBV viral replication and prevent disease progression and subsequent mortality. For treatment of HCV infection, ribavirin, ledipasvir, daclatasvir and Harvoni (ledipasvir + sofosbuvir) are being used. The programme includes primary prevention activities to reduce infection by increasing awareness of HBV and HCV in the general and high-risk populations. Secondary and tertiary prevention activities focus on early detection of HBV and HCV, timely treatment to prevent progression to liver disease, and targeted HBV vaccination of high-risk populations.

National guidelines recommend screening and treatment of hepatitis infections in pregnant women and their partners. There are 2 separate rapid diagnostic tests routinely implemented: 1 designed to detect HBsAg for HBV screening and another to detect anti-HCV antibody for HCV screening. However limited implementation of these guidelines has undermined the efficiency of universal neonatal HBV immunisation, which is normally given at 6 weeks of age, but is given within 24 hours of birth in combination with hepatitis B immunoglobulin when the mother is recognised to be HBV positive.

We conducted the current study to provide data on prevalence rates, genotypes and risk factors for HBV and HCV infection in couples attending antenatal clinics in rural Rwanda. These data may inform the country’s viral hepatitis disease control programme, aimed at reducing perinatal and sexual transmission.

**METHODS**

**Study Design and Setting**

We conducted a cross-sectional study from August 2017 to February 2018 to assess the prevalence and genetic diversity of HBV and HCV among couples attending antenatal clinics in rural Rwanda. The study was carried out at 2 randomly chosen health centres, Gitare and Cyanika, located in Burera District, Northern Rwanda; these health centres serve a population of 57,805 people and are adjacent to borders of Ugandan and Democratic Republic of Congo border.

**Sample Size**

We determined the minimum sample size using Fisher’s formula for cross-sectional studies, assuming an HBsAg prevalence rate of 5% and an anti-HCV prevalence rate of 4%. The minimum sample size was estimated by Fisher’s formula expressed as samples size (SS) = \( \frac{Z^2 \times P(100-P)}{\epsilon^2} \), where, \( Z \) is the value (1.96 for 95% confidence level [CI]), \( P \) represents, and \( \epsilon \) is the minimal tolerable error at 95% CI, expressed as a decimal (0.05). This formula yielded a minimum sample size of 73. To increase precision, an anticipated design effect of 3 was applied to yield a final sample of 220.

**Data Collection and Diagnostic Procedures**

Participants were recruited as couples during routine antenatal clinic visits. Participants were consecutively recruited from antenatal clinics; only those who met the enrolment criteria were invited to participate in the study.

Couples were recruited at health centres. Inviting men to accompany women to antenatal clinic is considered an important strategy for reducing maternal morbidity and mor-
tality by enabling couples to sufficiently prepare for birth and avoid care-seeking delays for obstetric emergencies. Presently, Rwanda records a distinctly high number of attending partners – 87% at the first visit – resulting from previous campaigns done by community health workers. We excluded couples who had a prior history of HBV vaccination as well as couples in which at least 1 partner was undergoing treatment for HBV or HCV infection.

After obtaining written informed consent, study participants were administered a structured one-on-one interview to obtain information on sociodemographic factors, medical history and risk factors for HBV and HCV. A 5 ml venous blood sample was collected for HBV and HCV screening by rapid diagnostic test (RDT), confirmatory testing and genotype analysis. RDT results were communicated back to the study participants within 45 minutes of blood collection; confirmed results were communicated to participants. Participants with a confirmed positive test result for HBV or HCV were counselled and referred to hospital for clinical management. The unused portions of the samples were transported to Kigali to retest the same samples using advanced diagnostic tests (enzyme-linked immunosorbent assay for HBV and polymerase chain reaction [PCR] for HCV), as recommended by national guidelines. All positives samples were also positive upon retesting, and we invited those who were confirmed positive to provide another 5 ml of blood for genotype testing (as the manufacturers’ instructions recommended whole fresh blood samples). The genotypes results were available after

<table>
<thead>
<tr>
<th>Variables</th>
<th>HBsAg Detected n (%)</th>
<th>P Value</th>
<th>Anti-HCV Detected n (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-30</td>
<td>129</td>
<td>1 (0.8)</td>
<td>.88</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>31-40</td>
<td>72</td>
<td>1 (1.4)</td>
<td>.75</td>
<td>3 (4.2)</td>
</tr>
<tr>
<td>41-50</td>
<td>16</td>
<td>0 (0.0)</td>
<td>.07</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>&gt;51</td>
<td>3</td>
<td>0 (0.0)</td>
<td>.71</td>
<td>0 (0.0)</td>
</tr>
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<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>110</td>
<td>1 (0.9)</td>
<td>.75</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>Female</td>
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<td>1 (0.9)</td>
<td></td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>Education level</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>48</td>
<td>1 (2.0)</td>
<td>.74</td>
<td>1 (2.0)</td>
</tr>
<tr>
<td>Primary</td>
<td>120</td>
<td>1 (0.8)</td>
<td></td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>Secondary</td>
<td>41</td>
<td>0 (0.0)</td>
<td>.71</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Tertiary</td>
<td>11</td>
<td>0 (0.0)</td>
<td>.71</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>Occupation</td>
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<td></td>
</tr>
<tr>
<td>Not employed</td>
<td>132</td>
<td>2 (1.5)</td>
<td>.71</td>
<td>1 (0.8)</td>
</tr>
<tr>
<td>Private sector</td>
<td>13</td>
<td>0 (0.0)</td>
<td></td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Public sector</td>
<td>8</td>
<td>0 (0.0)</td>
<td>.71</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>Self-employed</td>
<td>67</td>
<td>0 (0.0)</td>
<td></td>
<td>1 (1.5)</td>
</tr>
</tbody>
</table>

TABLE 1. Distribution of Hepatitis B Surface Antigen and Anti-HCV Seroprevalence Rates Across Sociodemographic Characteristics
Prevalence and Diversity of Hepatitis B and C in Rwanda

Prevalence and Diversity of Hepatitis B and C in Rwanda

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one month and were communicated to the respective participants.

RDT screening for HBsAg was done using Cypress Diagnosis anti-HBsAg Dipstick, Langdorp, Belgium. RDT screening for hepatitis C virus IgG (anti-HCV) was done using SD BIOLINE HCV One Step Hepatitis C Virus Test (Standard Diagnostics Inc., Korea). RDT results were confirmed by enzyme immunassay (EIA) using the Murex HBsAg kit (version 3; Murex Biotech, Dartford, Kent, United Kingdom). Confirmed HBV-positive samples were genotyped using hybridisation with sequence-specific oligonucleotides. The PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide and examined under UV light using the Bio-Rad Gel Doc 2000 System (Bio-Rad Laboratories, Segrate, Milan, Italy). For HCV positive samples, viral load and genotype were determined by the reverse transcriptase-PCR (RT-PCR) followed by hybridisation with sequence-specific oligonucleotides. HCV RNA was extracted by using the QIAamp DSP virus kit in combination with the QIAvac 24 Plus vacuum system (Qiagen GmbH, Hilden, Germany). Probes were bound to a nitrocellulose strip by a poly (T) tail. All laboratory assays were carried out according to the manufacturer’s instructions.

Data Processing and Statistical Analysis

Study data were double entered into Microsoft Excel, cleaned, and validated. The data was exported into IBM SPSS for Windows version 20.0 (IBM Corp, Armonk, NY, USA) for analysis. Descriptive statistics were used to summarise the sociodemographic and behavioural characteristics of study participants. Seroprevalence of HBV and HCV was expressed as a percentage of the entire study population. Chi-square tests and bivariate logistic regression were used to assess associations between dependent and independent variables. Odds ratios were estimated at a 95% confidence interval; statistical significance was set at a P value <0.05.

Ethical Approval

Ethical clearance for the study was obtained from the Institutional Review Board of the College of Medicine and Health Sciences of the University of Rwanda (Reference number 311/CMHS/2017). Documentation of ethical clearance was presented to research sites administration before starting data collection.

RESULTS

A total of 220 participants were recruited into the study, of whom 110 were male (50%) and 110 female (50%). The mean age of female and male study participants was 30.26 and 30.24 years, respectively (data not shown). The level of education among participants was generally low: 53.6% had only completed primary education (Table 1). Eighteen per cent of men and 42% of women were unemployed (data not shown).

Two participants (0.9%) including 1 female from Gitare and 1 male from Cyanika had a confirmed HBV infection.

### TABLE 2. Distribution of HCV Infection Across Behavioural and Medical History Factors

<table>
<thead>
<tr>
<th>Variables</th>
<th>Participants</th>
<th>Anti-HCV Detected n (%)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Knowledge of HCV infection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>30</td>
<td>1 (3.3)</td>
<td>.50</td>
</tr>
<tr>
<td>No</td>
<td>190</td>
<td>3 (1.5)</td>
<td></td>
</tr>
<tr>
<td>Intravenous user</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>209</td>
<td>4 (1.9)</td>
<td></td>
</tr>
<tr>
<td>Multiple sex partners</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>40</td>
<td>2 (5.0)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>180</td>
<td>2 (1.1)</td>
<td>.09</td>
</tr>
<tr>
<td>History of scarification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>21</td>
<td>2 (9.5)</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>No</td>
<td>199</td>
<td>2 (1.0)</td>
<td></td>
</tr>
<tr>
<td>History of body piercing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>216</td>
<td>4 (1.9)</td>
<td>.84</td>
</tr>
<tr>
<td>History of tattooing</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>3</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>217</td>
<td>4 (1.8)</td>
<td>.81</td>
</tr>
<tr>
<td>Ever had blood transfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>209</td>
<td>4 (1.9)</td>
<td>.64</td>
</tr>
<tr>
<td>Ever had hospital admission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>80</td>
<td>1 (1.3)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>140</td>
<td>3 (2.1)</td>
<td>.68</td>
</tr>
</tbody>
</table>

Continued

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Prevalence and Diversity of Hepatitis B and C in Rwanda

TABLE 2. Continued

<table>
<thead>
<tr>
<th>Variables</th>
<th>Participants</th>
<th>Anti-HCV Detected</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>Ever had tooth extracted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>27</td>
<td>1 (3.7)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>193</td>
<td>3 (1.6)</td>
<td>.43</td>
</tr>
<tr>
<td>Ever had surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>17</td>
<td>1 (5.9)</td>
<td>.19</td>
</tr>
<tr>
<td>No</td>
<td>203</td>
<td>3 (1.5)</td>
<td></td>
</tr>
<tr>
<td>Ever catheterised</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>19</td>
<td>1 (5.3)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>201</td>
<td>3 (1.5)</td>
<td>.24</td>
</tr>
<tr>
<td>Ever consumed alcohol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>83</td>
<td>3 (3.6)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>137</td>
<td>1 (0.7)</td>
<td>.12</td>
</tr>
</tbody>
</table>

Four participants (1.8%), including 2 females from Cyanika health centre and 2 males (1 from Gitare and another from Cyanika health centre) had confirmed HCV infection. All HBV and HCV infections were discordant to their respective partners. Two HBsAg positives cases were due to HBV genotype A; all 4 cases of HCV were due to genotypes 4.

In univariate analysis, HCV prevalence varied significantly by occupational group. HCV infection occurred in 12.5% of participants employed in the public sector and 7.7% of participants employed in the private sector, compared to <2% of participants who were self-employed or unemployed (Table 1). HCV infection was significantly associated with scarification (Table 2).

DISCUSSION

To our knowledge, ours is among the first studies to assess the prevalence and genetic diversity of HBV and HCV among couples attending antenatal care in rural Rwanda. We observed an HBsAg prevalence rate of 0.9% and an anti-HCV prevalence rate of 1.8% among study participants. This corresponds to the World Health Organization’s definition of low HBV endemicity. Genotypic analysis of viral types revealed that genotype A represented all confirmed HBV infections, while genotype 4 represented all confirmed HCV infections. No demographic or behavioural factors were associated with HBV status. However, HCV infection was associated with occupational group and history of scarification.

We compared results of the present study with similar studies, finding that others report substantially higher prevalence rates of HBsAg among non-pregnant adults. Studies from Rwanda report that the prevalence of HBsAg was 2.9% in health care workers and 2.5% in commercial sex workers. Surveys of HIV-infected adults from urban Uganda and Western Kenya report HBsAg prevalence rates of greater than 4%. These differences may be explained by the low-risk profile of our study’s rural participants, compared to that of population groups known to be at elevated risk of HBV transmission. However, rural status and pregnancy have not consistently been associated with a low risk of HBV. Although in Uganda, polygamous pregnant women had an HBsAg prevalence rate of 0.9%, other studies of pregnant women from rural African settings report HBsAg prevalence rates ranging from 3.8% to 12.7%. A study from Kenya reported an HBsAg prevalence rate of 2.1% in adult residents of a rural area.

Reports of HCV prevalence in Rwanda vary markedly. Our finding of an HCV prevalence rate of 1.8% is substantially lower than other surveys conducted in Rwanda, which report HCV prevalence rates of 3.1% in the general population, 4.7% in HIV-infected adults and 9.6% in patients attending Rwanda Military Hospital. However our findings are consistent with the HCV prevalence rates of 1.3% and 1.4% reported in Rwandan health care workers and commercial sex workers, respectively. We conjecture that these variations in HCV infection rates within Rwanda reflect the tendency of HCV to cluster within population subgroups and geographic areas.

We found that all individuals infected with HBV or HCV, were discordant to their respective partners, a finding which has been reported elsewhere. In China, 11.4% of rural couples who planned to conceive were affected by HBV infection and most of these infections were discordant. Discordance in HBV and HCV infection status may expose seronegative partners to infection, and may be an area where targeted vaccination could be employed. However, numerous large prospective cohort studies did not show an increased risk for HCV transmission among heterosexual discordant couples (married or stable partners), even after 10 or more years of observation.

In our study, all HBV infections were due to HBV genotype A. This is consistent with surveys conducted in Rwandan adults and blood donors, which found the majority of HBV infections in Rwanda were due to genotype A. In the East African sub-region, HBV infections have been attributed to genotypes A, B, C, D, E and H, but HBV genotypes A and D predominate.

In our study, HCV genotype 4 was the only one identified in confirmed HCV infections, which is consistent with a report on hepatitis care referral patients from Rwanda, in whom 96.7% HCV infections were due to HCV genotype 4. A recent phylogenetic analysis of HCV infection in Rwandan blood donors demonstrated the predominance of genotype 4 subtypes 4k, 4q, and 4r, with no geographical difference in their distribution.
We assessed whether sociodemographic and behavioural factors were associated with occurrence of HBV and HCV infection in our cohort. We found that none of the factors evaluated predicted HBsAg seropositivity, but that occupation type was associated with risk of HCV infection, which had the highest frequency in participants employed in the public and private sectors. Our finding that HCV infection was associated with occupation type but not with other factors partly accords with what has previously been reported. Studies from Africa have found that increased risk of HCV infection is associated with X,Y, sharing personal belongings8 and occupation types that involve exposure related to blood.13 Our study did not examine occupational activities, so we were unable to assess if the association between HCV infection and occupation type in our cohort was due to occupational exposures to blood or other job-related factors.

Limitations
This study had several limitations. The small sample size and the low occurrence of HBV and HCV infection in study participants may have reduced the statistical power to detect associations between purported risk factors and infection. Our HBV and HCV genotype analyses were based on a few cases, and, therefore, may not have accurately reflected the general population's genotypic diversity. Finally, study inclusion criteria required that both the male and female partners be enrolled in the study. This may limit the generalisability of study findings to individuals who are in a stable sexual partnership.

CONCLUSION
Our study revealed low prevalence rates of HBV and HCV in couples attending antenatal clinics in rural Rwanda. Consistent with other data from Rwanda and the sub-region, HBV genotype A and HCV genotype 4, accounted for all confirmed infections. All individuals infected with HBV or HCV were discordant with their respective partners. We recommend that larger studies of HBV and HCV prevalence be conducted in childbearing couples to obtain precise estimates of the prevalence, genetic diversity and risk factors associated with these infections. Such studies will inform target preventive measures, especially those aimed at reducing risk among children born to infected mothers.

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Prevalence of Wuchereria bancrofti Infection in Mosquitoes from Pangani District, Northeastern Tanzania

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INTRODUCTION

Wuchereria bancrofti is a filarial nematode that has a thread-like appearance in its adult stage. The female nematodes are about 10 cm long and 0.2 mm wide, while the males are only about 4 cm long. The adults reside and mate in the lymphatic system, where they can produce up to 50,000 microfilaria per day. The microfilariae are 250 to 300 µm long, 8 µm wide, and they circulate in the peripheral blood. They can live in the host as microfilaria for up to 12 months. Adult worms take 6 to 12 months to develop from the larval stage and can live between 4 and 6 years. The parasites are transmitted to humans when infected mosquito vectors deposit infective larvae onto the human skin. The larvae penetrate the skin, migrate to the lymphatic vessels, and develop into male and female adult worms over a period of months. Microfilaria ingested by a vector during a blood meal will develop to infective larvae in about 10 to 14 days. These migrate to the mosquito's proboscis and may then be transmitted to a new human host during a subsequent blood meal. Mosquitoes thus play an essential role in maintaining the lifecycle of W. bancrofti and disseminating the infection.

A blood smear is a simple and accurate diagnostic tool, provided the blood sample is taken during the day when the juveniles are in the peripheral circulation. A polymerase chain reaction (PCR) test can be performed to detect a minute fraction – as little as 1 pg of filarial DNA. Some infected people do not have microfilaria in their blood. As a result, tests aimed to detect antigens from adult worms are used. Ultrasonography can also be used to detect the movements and noises caused by the movement of adult worms.

Wuchereria bancrofti causes lymphatic filariasis, which is a disfiguring and disabling disease that is associated with severe suffering and socioeconomic burden in endemic communities. Current estimates suggest that more than 1 billion people living in endemic areas, who are at risk of the infection, and that more than one-third of these at-risk individuals are in sub-Saharan Africa. In Tanzania, about 34 million people are at risk, while 6 million people are already affected by lymphatic
Wuchereria bancrofti in Mosquitoes in Northeastern Tanzania

filariasis. Lymphatic filariasis is widespread in Tanzania; particularly high endemicity is seen along the coast of the Indian Ocean and in areas adjacent to the Great Lakes. In the Tanga Region of Tanzania, recent reports after mass drug administration (MDA) estimate circulating filarial antigen (CFA) and microfilaraemia rates of 15.5% and 3.5%, respectively, which are down by 75.5% and 89.6% – and 2.3% for CFA in school children – from baseline. Ongoing vector control measures against W. bancrofti in Tanzania consist of indoor residual spraying, long-lasting insecticidal nets, larval source management, mosquito repellents and coils, and house modifications.

It has previously been shown in Tanzania that MDA treatment regimen drastically reduce W. bancrofti microfilarial load. Other studies have revealed a decrease in the transmission of lymphatic filariasis associated with a decline in anopheline mosquitoes. Although a decline in anopheline mosquitoes has been documented in Tanga, information on vector burden and vector infection rate with W. bancrofti is still lacking. Therefore, this study assessed vector burden and vector infection rate with W. bancrofti.

METHODS

Study Setting
This study was carried out in 5 rural villages of Pangani District, which has an area of 1,830 km², making it the smallest district in Tanga Region. It is located in the southern part of Tanga, extending from 5°15.5’ to 6° S and from 38°35’ to 39° E. It is bordered by Handeni District to the west, the Indian Ocean to the east, Pwani Region to the south, and Muheza District to the North. Altitude ranges from 0 to 186 m above sea level. The Pangani District is administratively divided into 13 wards and 23 villages.

Study Design
This was an 8-month cross-sectional study, which involved the trapping of mosquitoes for laboratory examination of W. bancrofti. The 8 months were divided into 2 rounds, and 5 villages were randomly selected. Houses for mosquito collection were randomly selected from each village. The mosquitoes were sampled using standard Centers for Disease Control light traps with incandescent light bulbs (Model 512, John W. Hock Company, Gainesville, FL, USA).

Traps were hung beside beds occupied by at least 1 person sleeping in unimpregnated bed nets. Briefly, the shield of each trap was left to touch the side of the net with 150 cm clearance above the floor. The light traps were set between 20:00 hours and 06:00 hours and retrieved in the morning at 06:00 hours.

Mosquito Storage and Identification
The mosquitoes collected at each village were held separately and transported to the National Institute for Medical Research’s Tanga Centre for identification based on morphological identification keys. Female mosquitoes were organised into pools of 20, stored in cryogenic vials with silica gel, and transported to Sokoine University of Agriculture in Morogoro for screening of W. bancrofti.

DNA Extraction from Mosquitoes
DNA from the pools of 20 mosquitoes was extracted using a modified version of the Qiagen DNeasy kit protocol (Qiagen, Hilden, Germany). Briefly, mosquitoes were crushed in phosphate buffered saline, lysed, and then proteins were precipitated out using ethanol. The supernatant was passed through a silica column, followed by washing of the bound DNA. Afterwards, the silica was dried and DNA eluted into RNase-free Eppendorf tubes. DNA was stored at -20 °C until PCR were done.

Detection of W. bancrofti Using PCR
PCR assays to detect W. bancrofti were performed using NV1 and NV2 primers. The target sequence for these primers is the Ssp I repeat, a gene present at ~500 copies per haploid genome. Amplification with these primers yields an 188 bp fragment. Each 20 µl PCR reaction contained 1× Qiagen Taq buffer; 50 Mm MgCl₂, 50 mM each of dATP, dCTP, dGTP, and dTTP; 10 pmol/ µl of NV1 and NV2 primer; 1.25 U HotStar Taq DNA polymerase; and 2 µl genomic DNA. PCR reactions were run on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Jurong, Singapore), and reaction conditions consisted of a single step of 95°C for 10 minutes, followed by 94°C for 30s econds, 54°C for 45 seconds, and 72°C for 45 seconds. The final step was a 10-minute extension at 72°C. PCR products were size fractionated on 1.5% agarose gel stained with GelRed (Biotium, Hayward, CA, USA). Agarose gels were run at 100 V for 40 minutes and visualised under ultraviolet light using a gel documentation system (EZ Gel Imager, Bio-Rad Laboratories, Hercules, CA, USA). A positive control mosquito pool, known to be infected with W. bancrofti – a kind donation from the National Institute for Medical Research, Amani Tanga Centre – was used, along with negative controls, which were run concurrently with the samples to ensure that the PCR amplification was not contaminated. This helped prevent false positive results and ensure that all the reagents were working properly.

Determination of Estimated Rate of W. Bancrofti Infection in Mosquito Vectors
The calculation of vector infection rates from pool screening was addressed via an application of the binomial distribution. A maximum likelihood estimation algorithm was used to estimate the maximum likelihood of W. bancrofti infection at the 95% confidence level in mosquitoes, whereby total pools screened, the number of positive pools, and pool sizes was entered into PoolScreen 2 software to obtain infection rate. PoolScreen 2 software was obtained from the Depart-
ment of Biostatistics and Division of Geographic Medicine, University of Alabama at Birmingham, USA. The programme relies on the fact that the PCR assay is sensitive enough to detect a single infected insect in a pool containing large numbers of uninfected insects.

**Ethical Considerations**

Ethical approval for this study was obtained from the Medical Research Coordination Committee (MRCC), based at the National Institute for Medical Research, Dar es Salaam, Tanzania (Ref: NIMR/HQ/R.8a/Vol. IX/1834). Permission to conduct study was also obtained from regional, district, and respective village authorities. Moreover, written informed consent was sought from the heads of the households where mosquito collection was carried out.

**RESULTS**

**Lymphatic Filariasis Vector Abundance**

A total of 951 female mosquitoes were collected: 174 from Bweni, 301 from Madanga, 180 from Meka, 137 from Msaraza, and 159 from Pangani West. Among the 951 collected mosquitoes, by far the majority (99.36%) were *Cx. quinquefasciatus*, followed by *Anopheles gambiae sensu lato* (0.32%), and *Culex cinereus* and *Culex pipiens* (0.32%) (Table 1).

**Presence of *W. bancrofti* in Mosquitoes**

All *Cx. quinquefasciatus* mosquitoes collected in Pangani District were screened for *W. bancrofti* infection. From 47 mosquito pools screened for *W. bancrofti*, 24 (51%) pools tested positive and 23 (48%) tested negative. Positive pools produced a PCR product of approximately 188 bp, an expected size after *Ssp I* amplification using NV1 and NV2 primers. Figure 2 shows an example of the agarose gel after performing PCR for the detection of *W. bancrofti* in mosquito pools.

**Estimated Rate of Infection of *W. bancrofti* in Mosquito Vectors**

A total of 951 female mosquitoes were screened for infection with *W. bancrofti* using Poolscreen 2 software, which uses maximum likelihood at the 95% confidence level based on likelihood rates for determining the infection rates. Msaraza village had the highest estimated rate of infection, at 3.34%, and Bweni village had the lowest estimated rate of infection, at 2.9% (Table 2).

**DISCUSSION**

Monitoring infection rates among humans and vectors is an essential component of any lymphatic filariasis control programme. Such monitoring informs decision making, for example, deciding when to stop MDA and to certify the elimination of the disease. Monitoring transmission or infection in vectors is ideal, as mosquitoes may offer a real-time estimate of transmission, even though the manifestation of microfilaremia may be marginally quicker in humans. Low-level microfilaremia may also not be easy to detect in human populations.

The results obtained from the present study indicate that *Cx. quinquefasciatus* was the most abundant vector species caught during the study. These observations concur with a study carried out in Dar es Salaam, which reported that out of 12,096 vector mosquitoes caught using light traps, the great majority (99.0%) were *Cx. quinquefasciatus*, followed by a few *Anopheles gambiae* (0.9%) and *Anopheles funestus* (0.1%).

The higher abundance of *C. quinquefasciatus* in the present study might be because mosquitoes were collected during the dry season, during which the overall mosquito population is normally relatively low. The observed mosquito abundance has important implications on the transmission of both malaria and lymphatic filariasis, but the low anopheline mosquito abundance observed in the present study has greater implications on malaria transmission.

*Wuchereria bancrofti* infection in mosquitoes was found in all 5 villages, with an overall infection rate of 3.3%. Derua et al. reported that the overall rate of *W. bancrofti* infection among 3 sibling species – *An. gambiae*, *Anopheles merus*, and *Anopheles arabiensis* – in their study area in northern Tanzania, was 3.6%, which is similar our calculated rate. It should be noted that these infection rates are based on all vector-borne stages of the parasite, since the PCR testing method used cannot distinguish between the different larval stages. There is a need to determine the presence of the infective stages of *W. bancrofti* to estimate the risk of lymphatic filariasis trans-
mission by these mosquitoes.\textsuperscript{23} The detection of infection in mosquito vectors is an indication that there may be infected humans in the area, and a high rate of \emph{W. bancrofti} in the vectors might reflect a high prevalence of microfilaraemia in the human population. A previous study reported the overall prevalence of 24.5\% for \emph{W. bancrofti} microfilaria among people over the age of 1 year.\textsuperscript{24} In a similar study, the prevalence of \emph{W. bancrofti}-specific circulating antigen was 53.3\%.\textsuperscript{24}

While annual MDA remains the standard intervention for interrupting the transmission of lymphatic filariasis, vector control to reduce the number of potential mosquito vectors is increasingly recognised as a complementary strategy in some contexts.\textsuperscript{25} A combination of more than 1 vector control method would probably enhance the impact on vector populations and lymphatic filariasis transmission reduction, particularly if the methods address different stages of the mosquito lifecycle or if they have different modes of action. To further explore the findings and implications of this study, we recommend that further research – with much larger sample sizes and encompassing parasites from different geo-climatic regions – be conducted to enhance our understanding of \emph{W. bancrofti} vector infection status. Additionally, further research comparing the prevalence of \emph{W. bancrofti} in the human population with that among mosquito vectors in the study area and other endemic areas is of paramount importance, to draw clear conclusions regarding \emph{W. bancrofti} infection prevalence in Tanzania.

**CONCLUSION**

A high \emph{W. bancrofti} vector infection rate of 3.3\% was found in the present study, indicating a high likelihood of human infection in the area. Most mosquitoes collected were \emph{Cx. quinquefasciatus}, which calls for integrated mosquito control interventions to lower the risk of \emph{W. bancrofti} transmission from mosquitoes to humans. Additional research is needed to gain an in-depth understanding of the \emph{W. bancrofti} larval stages in mosquitoes, their drug sensitivity and susceptibility profiles, and fecundity. Such information would inform treatment strategies and decision making related to, for example, how long to run MDA programmes and the optimal size of the human population treatment unit.

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**REFERENCES**


**TABLE 2. Infection Rates of Mosquitoes with \textit{Wuchereria bancrofti}, as Determined by Polymerase Chain Reaction Pool Screening**

<table>
<thead>
<tr>
<th>Village</th>
<th>Mosquitoes n</th>
<th>Screened Pools n</th>
<th>Positive Pools n</th>
<th>Estimated Rate of Infection (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bweni</td>
<td>174</td>
<td>11</td>
<td>5</td>
<td>2.9 (8.9–7.04)</td>
</tr>
<tr>
<td>Madanga</td>
<td>301</td>
<td>15</td>
<td>7</td>
<td>3.09 (1.15–6.54)</td>
</tr>
<tr>
<td>Meka</td>
<td>180</td>
<td>7</td>
<td>4</td>
<td>4.15 (1.04–1.08)</td>
</tr>
<tr>
<td>Msaraza</td>
<td>137</td>
<td>6</td>
<td>4</td>
<td>5.34 (1.3–1.42)</td>
</tr>
<tr>
<td>Pangani West</td>
<td>159</td>
<td>8</td>
<td>4</td>
<td>3.4 (8.6–8.79)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>951</strong></td>
<td><strong>47</strong></td>
<td><strong>24</strong></td>
<td><strong>3.3 (2.13–5.37)</strong></td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; ERI, estimated rate of infection.


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Competing Interests: None declared.

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ABSTRACT

Background: Sumilarv 0.5G (Sumitomo Chemical Co., Ltd., Tokyo, Japan) is a granular insecticide developed for the control of mosquito and fly aquatic stages. The active ingredient is pyriproxyfen (4-phenoxyphenyl (RS)-2-(2 – pyridyloxy) propyl ether), a juvenile hormone analogue that acts as an insect growth regulator. Sumilarv 0.5G functions by inhibition of adult emergence from pupae. In this study, the Tropical Pesticides Research Institute in Tanzania carried out laboratory, semifield, and full-field evaluation on a new candidate of pupicide, Sumilarv 0.5G. The present study, therefore, sought to test the bioefficacy of Sumilarv 0.5G in laboratory, semifield, and full-field conditions in Mabogini, northern Tanzania.

Methods: Standard World Health Organization laboratory bioefficacy evaluations of Sumilarv 0.5G and untreated microcosms were prepared and monitored for inhibition of the larvae introduced to the habitats, while field plots were monitored for 5 weeks after the introduction of Sumilarv 0.5G using manufacturer-recommended doses.

Results: Sumilarv 0.5G biolarvicide was highly efficacious in its pupicidal effect, with an adult emergence inhibition rate of up to 90% in all conditions. In both laboratory and semifield experiments, the emergence inhibition was dose-dependent, with the lowest adult emergence being recorded in association with the highest Sumilarv 0.5G dose of 0.03 ppm of active ingredient. Under field conditions, the application rate recommended by the manufacturer – 5 mg ai per m\(^2\) – reduced the adult emergence rate by 90% to 96% for up to 5 weeks.

Conclusion: We demonstrated the long-lasting biological activity of Sumilarv 0.5G under field conditions. Notably, the field efficacy was attained using the recommended dose of 5 mg per m\(^2\), thus making it economical to apply this product, which is capable of inhibiting mosquito productivity in natural habitats for longer periods than achieved by existing products, the efficacy of which is usually about 1 week.

INTRODUCTION

In Africa, the main malaria vectors are members of Anopheles gambiae sibling species complex and the Anopheles funestus complex.\(^1\,\!^2\) The An. gambiae complex consists of An. gambiae sensu stricto (s.s.), Anopheles arabiensis, Anopheles merus, Anopheles melus, Anopheles bambwae, Anopheles colluzzii, and Anopheles ahmaricus.\(^1\,\!^2\) The An. funestus complex vectors are An. funestus s.s., Anopheles leesoni, Anopheles rivulorum, and Anopheles vaneedem.\(^1\,\!^3\,\!^8\) The main frontline malaria vector control tools are long-lasting insecticidal nets (LLINs) and indoor residual spraying (IRS).\(^7\) Insecticide resistance has emerged as an urgent threat to these tools\(^10\,\!^12\) that requires alternative or complementary solutions. Larval source management (LSM) is of paramount importance in the fight against malaria, through vector control, to complement LLINs and IRS. Larval control is a new avenue which, if effectively implemented, can have an impact on malaria epidemiology.\(^11\,\!^14\) LSM has been shown to be more effective when combined with other tools that target adult vectors.\(^14\,\!^15\) Insecticide resistance among disease vectors is achieved via a variety of mechanisms at different vector developmental stages,\(^16\) but the main advantage of LSM is that it targets the immobile immature stages of mosquito vectors, thus controlling both outdoor and indoor resting and biting vectors.\(^14\,\!^17\)

Commercially available chemical and microbial larvicides are highly effective for short-lasting control of the aquatic stages of the main malaria vectors.\(^11\,\!^14\,\!^18\,\!^20\) The major challenge of existing larvicides is their short duration of activity in environmental conditions,
which means that they require weekly reapplication. Labour and larvicide supply are the major costs associated with large-scale vector management, which aims to reduce costs by maximising the reapplication intervals. Also, the toxicity of larvicides to untargeted aquatic insects limits the practicality of regular larvicide programmes.

Sumilarv 0.5G (Sumitomo Chemical Co., Ltd., Tokyo, Japan) is a granular insecticide that was developed to control the pupal stages of mosquitoes and flies. The active ingredient is pyriproxyfen (4-phenoxyphenyl (RS)-2-(2-pyridyloxy) propyl ether), a juvenile hormone analogue that acts as an insect growth regulator. Pyriproxyfen generally inhibits the adult emergence of target insects species.

However, pyriproxyfen causes delayed effects on reproduction among female adult mosquitoes exposed to sublethal doses at the larval or adult stage. Sumilarv 0.5G has exceptional residual activity of up to more than 1 month for the control of mosquito species in their natural breeding sites. Furthermore, pyriproxyfen has been evaluated as a safe insecticide for application in drinking water, with limited impact on untargeted aquatic insects and the environment. Nevertheless, Sumilarv 0.5G has never been evaluated for the control of An. arabiensis, the major malaria vector in Tanzania.

This study aimed to evaluate the efficacy of Sumilarv 0.5G as a pupicide against An. gambiae s.s. in laboratory and semifield conditions, and against An. arabiensis under field conditions.

**METHODS**

**Study design**

This was laboratory and field experimental study.

**Sumilarv 0.5G Formulation**

Sumilarv 0.5G was applied only to mosquito breeding habitats that did not drain into natural water bodies. According to the manufacturer's instructions, the amount of Sumilarv 0.5G applied was determined by the volume of water in the respective habitats (width×length×depth), based on a target concentration of 0.01 to 0.05 ppm of active ingredient (ai) or (0.2-1.0 oz/100 ft$^3$). The targeted habitats were temporary or permanent water holding sites amenable to treatment: ornamental ponds, fountains, cesspools, abandoned swimming pools, gutters, construction site depressions, septic tanks, flooded basements, gutters, animal waste lagoons, livestock runoff lagoons, sewers, sewage effluent, tire tracks, waste water impoundments associated with organic pollutants and industrial run off, waste water and settling ponds, and vegetation-choked phosphate pits. Other potentially treatable sites include natural and artificial water holding containers: hollow trees and tree holes, potted plants, bird baths, tire dumps, landfills, rain barrels, flooded roof tops, flower pots, buckets, salvage yards, abandoned vehicles, vehicle impounds, and junkyards. The targeted mosquito species for Sumilarv 0.5G are Aedes aegypti, Aedes albopictus, Aedes vexans, anophelines, Culex pipiens, Culex quinquefasciatus, Culex tarsalis, and Culex restuans.
Mosquito Rearing
Mosquitoes originated from a colony of *An. gambiae* s.s., established in Kisumu, Kenya, in 1992, and were reared at the Tropical Pesticides Research Institute TPRI. The laboratory larval rearing of protocol is described elsewhere. In the insectary, larvae were fed TetraMin (Spectrum Brands Pet, Blacksburg, VA, USA) fish food at rate of 0.003 g/larva. Third instar larvae were used for trials, as recommended by the World Health Organization protocol. The photo phase in the insectary was 12 light:12 darkness, with a temperature of 27°C ± 2°C and a relative humidity of 78% ± 2%.

Dose–Response Bioassays
Experiments were done in the laboratory and in semifield conditions – experiments conducted in controlled conditions outside the laboratory but with restricted interaction with nature. Before the dose–response experiments, a range-finding test was implemented by exposing test larvae to a wide range of test concentrations and a control. This was used to find the activity range of the insecticide for tested species.

A range of concentrations between 10 ppm ai and 1.0 × 10⁻³ ppm ai were tested. After determining the emergence inhibition of the larvae in a broader range, concentrations causing emergence inhibition of between 10% and 95% were chosen and used in dose–response bioassays. Fifteen serial dilutions were made, and the best 11 doses causing emergence inhibition for use in laboratory and semifield trials were selected. A stock solution was prepared by grinding the granular formulation into a fine powder, following the procedure described Sihuincha et al. Using a pestle and mortar, 5 g of SumiLarv 0.5 G (25 mg ai) was ground and added to 500 ml of unchlorinated tap water. This produced a stock solution of 10,000 ppm SumiLarv 0.5G (50 ppm ai). The top of the vial was covered with aluminium foil, and the solution was left to agitate for 1 hour on a shaker. The mixture was left overnight to allow the active ingredient to be released into the solution. The next morning, the mixture was again agitated on a shaker for 30 minutes to prepare a homogenous mixture, as some of the inert ingredients of the formulation – potentially still containing some active ingredient – had settled overnight. Serial dilutions were made immediately after shaking in unchlorinated tap water to produce the test concentrations.

The laboratory-reared colony of *An. gambiae* s.s. was evaluated against different concentrations of Sumilarv 0.5G. Each test concentration and a control were replicated 6 times, and 200 ml of each test solution was set up in 300 ml glass bowls. The test was repeated 3 times for each concentration. Separate batches of 25 insectary-reared third instar larvae of test species were introduced into each test concentration and the control.

Larvae were fed with TetraMin (Spectrum Brands) fish food only when the experiments were monitored for more than 24 hours. Bowls were covered with netting to prevent any emerging adults from escaping. The pupae were monitored until emergence or death. The number of dead larvae, pupae, and emerging adults were recorded until the end of the experiment, when all pupae had emerged or died. Live
pupae from each bowl were transferred into a separate bowl containing 20 ml of water from the habitats. These bowls were covered with netting for monitoring adult emergence. Separate pipettes were used to collect pupae from treated and control bowls to avoid cross-contamination.

**Semifield Studies**

Semifield trials were carried out in an open field with artificial microcosms. Six artificial microcosms were made up using small washing basins (diameter, 21.5 cm; depth, 10 cm) filled with 1 kg of soil and 1,500 ml of water to resemble a natural larval habitat. Microcosms were paired between treatments and controls at 3 m intervals. Monitoring of the microcosms was conducted daily until the first pupa was observed, then monitoring was conducted twice a day. All pupae were collected with some water from each microcosm. Batches of 25 insectary-reared third instar larvae were introduced into each microcosm.

**Field Trials**

Two pairs of rice plots were selected for the field trials, with larval abundance evaluated before treatments (Figure 1). Two were control plots, and the other 2 were treatment plots. The plots measured 70 m × 20 m. Larvae were sampled 3 times per week (Monday, Wednesday, and Friday) for 5 weeks. All collected control and treatment pupae were kept in labelled paper bowls and monitored for adult emergence. The effect of Sumilarv 0.5G was measured as adult emergence inhibition in all treated plots, while for the control, emergence inhibition was considered as natural mortality.

**Parameters Measured**

The percent inhibition of adult emergence (%IE) was calculated following the World Health Organization guideline, using the following formula:

\[ IE(\%) = 100 - \left( \frac{T \times 100}{C} \right) \]

Where,

- \( T \) = percentage survival or emergence in treated batches
- \( C \) = percentage survival or emergence in control batches

**Data Analysis**

Data were analysed using IBM SPSS Statistics version 25 (IBM Corp., Armonk, NY, USA). Descriptive statistics were deployed for data analysis to obtain the confidence intervals and mean differences. Excel (Microsoft Corp., Redmond, WA, USA) spreadsheets were used to calculate percentage inhibition of larvae. The comparison between treatment and control was done using paired samples t-tests. Probit analysis was used to calculate the LC50 and LC95. \( P \) values less than .05 were considered statistically significant.
Ethical Considerations

Approval for this study was granted by the Tanzania Pesticides registrar’s office (experimental permit number 2310, issued in 2015).

RESULTS

Laboratory Trials

A series of doses were evaluated, and those with emergence inhibition effects were considered for laboratory trials. Selected trial doses ranged from $10^{-6}$ to 0.03 ppm ai. Percentage inhibition was dose-dependent (Figure 2). The dose of 0.03 ppm ai caused 100% emergence inhibition. The percentage inhibition among treatment doses was statistically significant (df=11, $F=242.9$, $P<.001$).

Semifield Trials

Similar results to those observed in laboratory studies were found in the semifield experiments. Emergency inhibition was dose-dependent, and the highest inhibition levels resulted from the highest doses (Figure 3). Inhibition percentage was statistically different between the groups, with more adult emergence observed at lower doses (df=11, $F=367.86$, $P<.001$).

Field Trials

In the first week of sampling after the plots were treated, the emergence rate for pupae differed significantly between treated and control plots, with more adults emerging from the pupae sampled in the control than the treated plot arms (df=4, $t=74.1$, $P<.001$). The same was observed in weeks 2 (df=4, $t=70.5$, $P<.001$), 3 (df=4, $t=70.5$, $P<.001$), 4 (df=4, $t=69.7$, $P<.001$), and 5 (df=4, $t=81.1$, $P<.001$), as shown in Figure 4.

DISCUSSION

Our findings shown that wild An. arabiensis and laboratory An. gambiae s.s. populations had similar responses to Sumilarv 0.5G under different environmental conditions. Similar findings were presented by a study conducted in Kenya by Mbare et al. Sumilarv 0.5G inhibited over 90% of the total adult emergence over a period of 5 weeks in irrigated rice fields at an application rate of 5 mg ai per m$^2$. This level of inhibition is consistent with observations of a study targeting the control of An. gambiae sensu lato (s.l.) in Kenya, where a concentration of 0.003 ppm pyriproxyfen was enough to completely inhibit emergence for up to 1 month. In the present study, however, weekly emergence rates remained low for up to 5 weeks, even at the lower doses. Weekly emergence inhibition was frequently higher than 90%, the threshold recommended by the World Health Organization Pesticide Evaluation Scheme for the successful control of immature mosquito stages. Application rates in field conditions were increased up to several times the minimum dose instructed by the manufacturer to obtain sufficient control under field conditions. The findings were consistent with those observed in previous studies. The higher dose of 5 mg ai per m$^2$ in field conditions inhibited over 80% of adult emergence for 5 weeks.
Further field tests to establish the optimum dose for operational control in a variety of different habitats are necessary. However, based on the results presented in this study, it is likely that the optimum dose lies between the doses tested here. This concurs with the maximum dose recommended by the manufacturer (0.05 ppm ai) for field operational control of anopheline mosquito species. Kawada et al. previously found emergence inhibition for An. gambiae to be 4 times less than the level found in the present study. The same dose for An. gambiae, however, was found to be in the range recommended for culicine and Aedes species. The observed differences in these studies were attributed to factors such as differences in container types and pyriproxyfen formulations. The study by Kawada et al. used an emulsifiable concentrate (5%) formulation, while the present study used a granular formulation. Moreover, in the present study, the plastic bowls used during the bioassays were found to have retained high larvicidal amounts leading to a higher residual effect relative to the aluminium bowls used by Kawada et al. Plastic materials are known to retain substantial amounts of active ingredient and to release it slowly, which could explain the higher emergence inhibition found in this study at all doses used. Our experiments have shown that Sumilarv 0.5G is effective at low active ingredient concentrations. The required concentration of pyriproxyfen is substantially lower than that of other microbial agents used as larvicides.

A previous study on culicine mosquitoes also demonstrated that pyriproxyfen operates effectively at very low concentrations. The efficacy of lower concentrations will lower the stress caused by growth regulators is known to affect the adult sex ratio and reduce blood feeding ability. The observed residual impact on An. arabiensis emergence rates was similar to what was found in previous studies on other mosquito species.

In previous studies under field conditions, Sumilarv 0.5G at 0.02 ppm ai and 0.05 ppm ai effected complete emergence inhibition for 6 weeks for Anopheles quadrimaculatus, Culex nigripalpus, Aedes taeniorhynchus, Ae. albopictus, and Ae. aegypti. This granular formulation is released and produces its effects relatively slowly compared to other biolarvicides, exhibiting extended residual effects, particularly when applied to mosquito breeding containers. It was very effective against Aedes larvae even when the habitats where flushed with untreated water. In Sri Lanka, a single dose of 0.1 mg/l was shown to be sufficient for six months against anopheline malaria vectors in pots and small pits, meaning that 2 applications per annum were sufficient. In Peru, it was observed that 0.003 g ai pyriproxyfen/m² was sufficient to extend emergence inhibition for 5 months in water tanks housing Ae. aegypti. Overall, it can be concluded from previous and our own study that the efficacy and residual activity of different pyriproxyfen-containing products depends on the formulation, dose, habitat types, and vector species.

We did not observe that the efficacy of Sumilarv 0.5G is reduced in turbid water, as reported by Mbare et al. Some of the turbidity observed in that study might have been due to algae and bacteria growing in the established habitats. It is possible that the debris absorbed some of the active ingredients of the Sumilarv 0.5G, reducing its efficacy. Debris in aquatic habitats is an important parameter that is often associated with the abundance, development, and survival of An. gambiae s.l. larvae. In the recent past, anopheline larvae have been found to exploit aquatic habitats with varying degrees of water turbidity and pH, from sunlit and ephemeral to permanent, large water bodies in both urban and rural areas. Debris and other decaying materials provide mosquitoes with food particles that enhance their aquatic survival, thus increasing adult emergence from turbid water bodies. This condition in natural habitats needs to be considered and monitored in field operations for the effective control of aquatic stages. At 5 mg ai per m², reproduction by female mosquitoes declined by over 90% as a consequence of the sublethal effect of Sumilarv 0.5G on emergence for 5 weeks. Similar effects of pyriproxyfen have been shown for Anopheles, Aedes and Culex species in both laboratory and field conditions. Another effect is to suppress the viability of eggs, thus reducing emergence rates and subsequently reducing intervention costs. The outcome of stress caused by growth regulators is known to affect the adult sex ratio and reduce blood feeding ability. The same phenomenon is observed among adult mosquitoes exposed to pyriproxyfen. Insect growth regulators have also been found to suppress ovarian and egg development.

Caution should be taken when considering the continuous use of pyriproxyfen, as resistance might develop, as has occurred with other insecticides. Examining the population of insects surviving after exposure for tolerance against pyriproxyfen must be built into malaria vector control strategies.

**CONCLUSION**

We have shown that An. arabiensis and An. gambiae s.s. are highly susceptible to Sumilarv 0.5G at low doses. This product would, therefore, be useful for targeting productive natural habitats of malaria vectors and help control wild mosquito populations. Such reduction in population size can happen within a relatively short period, as this study has shown that Sumilarv 0.5G significantly inhibits adult emergence and egg viability. We recommend further studies to better understand and standardize re-treatment intervals in both dry and rainy seasons.

**Acknowledgements:** We wish to thank the technicians at the Tropical Pesticides Research Institute, field officers, and the lower Moshi community members who participated in data generation and collection. This study was funded by the company that manufactures Sumilarv 0.5G, Sumitomo Chemical Co., Ltd., of Tokyo, Japan. The funder had no role in data collection, data analysis, results interpretation, or the conclusions derived from this study.
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Sumilarv 0.5G as an Anopheles Insecticide in Northern Tanzania
This study was funded by the Sumitomo Chemical Co., Ltd., (Diptera: Culicidae) in urban areas.  


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Determination and Quantification of Gallic Acid in Raw Propolis by High-performance Liquid Chromatography–Diode Array Detector in Burundi

Ramadhan Nyandwi, Aysel S. Kilic, Meltem Celik, Hasan H. Oruc

ABSTRACT

Background: Honey, pollen, and propolis are among the products that bees process and derive from plants and flowers. Propolis is a resinous material that bees gather from the buds and bark of some trees and small plants. Propolis from temperate climates mainly contains phenolic compounds, in contrast with propolis from tropical climates, which mainly contains terpenes. This study aimed to determine, characterise, and quantify the phenolic content of raw propolis from Burundi.

Methods: In this study, a total of 6 samples were collected from the provinces of Rumonge, Cibitoke, and Ruyigi in Burundi. Fifteen phenolic compounds (caffeic acid, ferulic acid, epigallocatechin gallate, isoferulic acid, cinnamic acid, caffeic acid phenethyl ester, gallic acid, apigenin, chrysin, galangin, quercetin, kaempherol, rutin trihydrate, naringenin, and pinocembrin) were used as high-performance liquid chromatography (HPLC) standards for qualitative and quantitative analyses of the propolis samples.

Results: Among the 15 phenolic compounds checked, only 1 – gallic acid – was detected at a measurable level using an HPLC-diode array detector system.

Conclusion: In addition to terpenes, propolis found in sub-Saharan Africa may contain phenolic compounds. Further advanced investigation of sub-Saharan African propolis is required for more detailed characterisation.

INTRODUCTION

Honeybees process and produce various substances from plants and flowers, such as honey, pollen, and propolis. The products are components of foods and cosmetics, and they have applications in both traditional and medical practice.

Propolis is a resinous material collected by honeybees from buds and cracks in the bark of certain plants, typically from poplar, beech, horse chestnut, birch and conifer trees. Bees mix this substance with beeswax and bee enzymes (β-glycosidase) that they secrete during propolis collection. Propolis contains mainly resin and plant balsam, beeswaxes, essential oils, as well as organic and mineral compounds. Honeybees use propolis to plug holes, straighten inner walls, and soften the walls of their hives for protection against external invaders and hive temperature regulation. Propolis is useful for fending off potential fungal and microbial infections.

In veterinary medicine, propolis is mostly used for its antimicrobial properties. It is also used to enhance livestock growth and productivity. For example, propolis has been reported to increase weight gain among chickens, subsequently improving meat quality. Propolis also increases egg laying rates among laying hens. Furthermore, propolis has positive effects on livestock feed consumption. It can also be used in veterinary medicine to treat canine fungal otitis and dermal mycosis infections as well as bovine dermatophytosis.

Propolis is also an ingredient of cosmetic creams and lotions. It can be used in shampoos and hair lotions, acne treatments, shaving products, deodorants, antiperspirants, as well as antidiarrheal and sebum-stabilising agents. In addition to its pharmaceutical uses, propolis may be used as a food preservative, owing to its natural antioxidant and antibacterial effects.

Despite its beneficial properties, propolis also has adverse effects, particularly when used by or administered to sensitive individuals and in high doses. In humans, it can cause allergic and other adverse reactions, including contact dermatitis or oral mucositis.
Data about bees and bee products are available from almost every known period of human history. Propolis has been used by the Egyptians, Greeks, Persians, Arabs, and Romans since ancient times. Moreover, most religious books have spoken about bees and honey. Propolis has been used in traditional and modern medicine as an alternative and supportive treatment for its antibacterial, antifungal, anti-inflammatory, antiviral, antioxidant, gastroprotective, neuroprotective, anti-ulcerative, antispasmodic, chelator, immunomodulatory, and sedative properties. It has also been used to treat and prevent acne, allergies, liver disease, diarrhoea, diabetes, pain, ageing, malaria, and heart disease.

More than 300 active compounds have been identified in propolis, with the composition varying by season, plant source, bee species, and altitude. The most important components with pharmacological activity in propolis are flavonoids, like flavones, flavanols, flavanone, and various phenolic and aromatic substances. Flavonoids are not toxic when consumed in fruits and vegetables; they are potentially useful compounds for the human body and protect plants against microbial infection and insect infestation.

Many countries have investigated the chemical composition of their indigenous propolis and identified its biopharmaceutical effects. For instance, propolis has been extensively studied in Europe and South America (especially in Brazil), and there are ongoing detailed studies in China, Japan, Taiwan, and Turkey, among other countries. However, studies on African propolis are few and limited to North African countries, such as Morocco, Algeria, and Egypt.

African vegetation is suitable for beekeeping and, therefore, suitable for propolis production. However, there is a lack of data related to African propolis, and in some countries and regions, including Burundi, there is no available information about the chemical content of the local propolis. Nevertheless, research on propolis in sub-Saharan Africa has shown that, in general, African propolis – like the propolis found in other tropical regions – is rich in terpenoids, including α-amyrine, β-amyrin, α-amyryl acetates, β-amyryl acetates, n-alkanes, n-alkenes, methyl n-alkanoates, and long-chain wax esters, among other compounds.

The price of propolis varies according to its source and quality. For example, Chinese propolis is offered for sale at about €25 to €50 euros per kg, while the Brazilian propolis is offered at higher prices (€100 to €150 euros per kg). For this reason, propolis production can be beneficial for a nation’s economy in general and particularly profitable for beekeepers. However, for this to be the case, the propolis produced must be of good quality and have the desired pharmacological effects. It is, therefore, necessary to carry out chemical analyses of total phenolic content to assess propolis quality for consumer use. This study aimed to identify, quantify, and characterise the presence of phenolic compounds in Burundian propolis.

### METHODS

#### Sample Collection

A total of 6 propolis samples were randomly collected from 3 provinces in Burundi by placing at least 4 propolis traps in each province. First, in Rumonge Province, samples were collected from the Rumonge (2 samples) and Buyengero (2 samples) communes. Second, in Cibitoke Province, 1 sample was collected from Murwi. Finally, in Ruyigi Province, 1 sample was collected from Kinyinya. Propolis samples were collected using plastic propolis traps (42 × 49.5 cm) (Civan Incorporation, Bursa, Turkey). Propolis traps were placed on the hive in place of the inner cover for a month during the spring season. Propolis samples were immediately stored in a refrigerator before any analysis.

#### Instruments, Chemical Reagents, and Standards

All reagents used for this study – including methanol, acetonitrile, ethanol, and formic acid – were high-performance liquid chromatography (HPLC)–quality grade. A PURELAB flex 4 (ELGA LabWater, Buckinghamshire, UK) system was used to purify water. For quantitative analyses, we used HPLC coupled with diode array detector (DAD) (Shimadzu Corporation, LC-20 AD/SPD-M20A, Tokyo, Japan) analysis. A vacuum drier system (Jouan Inc., RC 10-10, Winchester, VA, USA) was used to concentrate propolis extracts.

The standards of phenolic compounds used in the analyses were:

1. Caffeic acid (3,4-dihydroxy cinnamic acid, trans-3,4-dihydroxy cinnamic acid; molecular weight: 180.16, Chemical Abstracts Service [CAS] number: 331-39-5; Sigma-Aldrich, C0625, ≥98%)
2. Epigallocatechin gallate ((−)-cis-2-(3,4,5-tri hydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol 3-gallate, (−)-cis-3,3 EG, 4-, 5,5 fla, 7-hexahydroxy-flavane-3-gallate, EGCG, Teavigo; molecular weight: 458.37, CAS number: 989-51-5; Santa Cruz, sc-200802, ≥98%)
3. Trans-isonerulic acid (trans-3-(3-hydroxy-4-methoxyphenyl) acrylic acid, trans-3-hydroxy-4-methoxy cinnamic acid; molecular weight: 194.1, CAS number: 25522-33-2; Fluka, 05407, ≥98%)
4. Ferulic acid (3-hydroxy-4-methoxy cinnamic acid, caffeic acid 3-methyl ether, coniferic acid; molecular weight: 194.18, CAS number: 537-98-4; Fluka, 52229, 99%)
5. Trans-cinnamic acid (trans-3-phenylacrylic acid, trans-cinnamate, trans-3-phenylacrylate; molecular weight: 148.16, CAS number: 140-10-3, Sigma-Aldrich, C80857, ≥99%)
6. Caffeic acid phenetyl ester (CAPE) (2-phenylethyl caffeate; molecular weight: 284.31, CAS number: 104594-70-9; Sigma-Aldrich, C8221, ≥97%)

7. Gallic acid (3,4,5-trihydroxybenzoic acid; molecular weight: 170.12, CAS number: 149-91-7, Sigma-Aldrich, G7384, ≥97.5%)

8. Apigenin (4 in, 5,7-trihydroxyflavone, narigenin chalcone; molecular weight: 270.24, CAS number: 520-36-5, Fluka, 42251, ≥97%)

9. Chrysin (5,7-dihydroxyflavone; molecular weight: 254.24, CAS number: 480-40-0; Sigma-Aldrich, C80105, 97%)

10. Galangin (3,5,7-trihydroxyflavone; molecular weight: 270.24, CAS number: 548-83-4, Sigma-Aldrich, 282200, 95%)

11. Quercetin hydrate (3,3',4',5,7-pentahydroxyflavone; molecular weight: 302.24, CAS number: 849061-97-8; Sigma-Aldrich, 337951, ≥95%)

12. Kaempherol (3,4,35,5,7-tetrahydroxyflavone; molecular weight: 286.24, CAS number: 520-18-3, Fluka, 96353, ≥99%)

13. Rutin trihydrate (quercetin-3-rutinoside trihydrate, vitamin P trihydrate; molecular weight: 664.56, CAS number: 250249-75-3; Fluka, 78095, ≥95%)

14. Naringenin (4',5,7-trihydroxyflavanone; molecular weight: 272.25, CAS number: 67604-48-2; Fluka, 52186, ≥95%)

15. Pinocembrin (5,5-dihydroxyflavanone, dihydrochrysin, galangin flavanone; molecular weight: 256.25, CAS number: 480-39-7; Fluka, P5239, ≥95%)

Sample Extraction and Analysis

Propolis extraction was done at a dilution rate of 1:9, mainly using the extraction method described by Oruç et al. Raw propolis samples were frozen at −20°C for 30 minutes, then ground into powder using a De’Longhi KG49 electric coffee grinder (De’Longhi, Treviso, Italy). Powdered propolis samples were thoroughly mixed, and 2 g of each sample was weighed for extraction. Then, 18 ml of 70% ethanol was added to obtain a propolis-ethanol extract (PEE). This mixture was first stirred with an orbital shaker (SL-350, Nüve, Ankara, Turkey) for 1 hour, followed by ultrasonication in an ultrasonic bath (Sonorex RK100, Bandelin, Berlin, Germany) for 15 minutes. This process was carried out twice. The mixture was then filtered, first using grade 1 rectangular filter paper and then grade 1 Whatman filter paper (125 mm). The obtained PEE was preliminarily collected in weighed empty tubes and then concentrated using a vacuum drier. After drying the PEE, the tubes were weighed again, and the dry weights were calculated. The amount of dry PEE per tube was recorded, and the tubes were stored at −20°C until the HPLC-DAD analysis stage. The dry PEE was solved with methanol (1:10, w:v) and filtered through a polyvinyl difluoride syringe filter (Millipore Millex-HV, 0.45 μm), and 5 μl injected into the HPLC-DAD system.

We mainly used the methods described by Pellati et al. for HPLC-DAD analysis. The PEE samples were prepared in tubes and filtered again through 0.45 μm polyvinyl difluoride (Millipore Millex-HV, 0.45 μm), and 5 μl of the obtained solu-
### TABLE 2. Characteristics of Collected Samples

<table>
<thead>
<tr>
<th>Location</th>
<th>Color</th>
<th>Sticky Consistency</th>
<th>Aromatic Scent</th>
<th>Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>Black</td>
<td>Brown</td>
<td>Strong</td>
<td>Weak</td>
</tr>
<tr>
<td>Rumonge 1</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>56</td>
</tr>
<tr>
<td>Rumonge 2</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>87</td>
</tr>
<tr>
<td>Buyengero 1</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>64</td>
</tr>
<tr>
<td>Buyengero 2</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>98</td>
</tr>
<tr>
<td>Murwi</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>86</td>
</tr>
<tr>
<td>Kinyinya</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>128</td>
</tr>
</tbody>
</table>

### TABLE 3. Burundian Propolis Component Concentrations

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration, µg/g, According to Geographic Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rumonge 1</td>
</tr>
<tr>
<td>GAL</td>
<td>33</td>
</tr>
<tr>
<td>EGKG</td>
<td>-</td>
</tr>
<tr>
<td>KA</td>
<td>-</td>
</tr>
<tr>
<td>FR</td>
<td>-</td>
</tr>
<tr>
<td>IFR</td>
<td>-</td>
</tr>
<tr>
<td>QE</td>
<td>-</td>
</tr>
<tr>
<td>SA</td>
<td>-</td>
</tr>
<tr>
<td>NR</td>
<td>-</td>
</tr>
<tr>
<td>AP</td>
<td>-</td>
</tr>
<tr>
<td>KF</td>
<td>-</td>
</tr>
<tr>
<td>CR</td>
<td>-</td>
</tr>
<tr>
<td>PN</td>
<td>-</td>
</tr>
<tr>
<td>GL</td>
<td>-</td>
</tr>
<tr>
<td>CAPE</td>
<td>-</td>
</tr>
<tr>
<td>RT</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviation: GAL, gallic acid; EGKG, epigallocatechin gallate; KA, caffeic acid; FR, trans-ferulic acid; IFR, isoferulic acid; QE, quercetin; SA, cinnamic acid; NR, naringenin; AP, apigenin; KF, kaempherol; CR, chrysin; PN, pinocembrin; GL, galangin; CAPE, caffeic acid phenethyl ester; RT, rutin trihydrate
tion was injected into the HPLC-DAD system automatically. The method is gradient where mobile phase A (deionised water plus 0.1% formic acid, V/V) and mobile phase B (HPLC grade acetonitrile plus 0.1% formic acid, V/V) were used as solvent carriers (Table 1). The mobile phase’s total flow rate was 1 ml/min between 80 and 90 bar of pressure on the pump with a total injection volume of 5 μl at temperatures between 25°C and 33°C. During the analyses, the column temperature was kept at 30°C. Detection of the propolis content was carried out at a wavelength of 270 nm, and the compounds were obtained by identification and comparison of retention time values and peaks of samples and standards.

The propolis extraction and HPLC-DAD analysis procedures were carried out at Uludag University’s Department of Pharmacology and Toxicology within the Faculty of Veterinary Medicine, in Bursa, Turkey. The phenolic compound concentrations in the dry PEEs were calculated in μg/g.

RESULTS AND DISCUSSION

Analysed propolis samples were sticky, red, black, and brown with a slight aromatic smell (Table 2). There were also differences between the participating communes in terms of the propolis sample volumes collected, and, overall, the volume of propolis samples collected was small (Table 3). During collection, propolis was generally found in the edge sections of the traps used for harvesting, with the middle sections being empty or slightly waxy. Propolis is typically described as having a fibrous, matte, sticky, and bright appearance, with colours ranging from red, yellow, and green to dark brown depending on the plant source and region; our propolis samples matched these characteristic descriptions. Many researchers have also described the aromatic scent of propolis.²³,²⁵,²⁶

Among all of the samples collected, only gallic acid was determined at a measurable level. The mean gallic acid concentration was 216.2 μg/g (Table 3), which is low compared to propolis found in temperate regions. In this study, according to the method used, chromatogram peaks were weak, small, and low in number and mainly appeared from 1 minute up to 15 minutes. This finding is in contrast to observations reported by Oruç et al, who – using the same methods as we did – investigated propolis samples from the temperate climate of Turkey and found that peaks were consistent, high, and numerous; their chromatogram peaks appeared between 1 minute and 70 minutes.

Gallic acid is a phenolic compound with antibacterial²⁸ and antitumour³⁸ activity. The constituent phenolic compounds found in Burundian propolis are quantitatively and qualitatively different from the propolis components found in temperate climates. Previous studies conducted in sub-Saharan Africa have revealed propolis from this region to contain a high terpenoid content.²⁹,³⁰ This has been confirmed by researchers, such as Zhang et al, who investigated propolis samples from Uganda, Tanzania, Malawi, Zambia, South Africa, Cameroon, Nigeria, and The Gambia. They concluded that African propolis was rich in diterpenoids and triterpenoids. Papakroni et al found, for the first time, triterpenes, such as lupen non, β-amirin and lupeol, diprenyl-flavonoid lonicarpol, and 6,8-diprenyl-eriodictyol in propolis samples from the Democratic Republic of Congo. Rushdi et al found α-amyrin, β-amyrin, α-amyrin acetates, β-amyrin acetates, n-alkanes, n-alkenones, methyl n-alkanoates, and long-chain wax esters in propolis samples from Ethiopia.

CONCLUSION

The absence of phenolic compounds other than gallic acid in Burundian propolis samples from is in concordance with studies that have reported terpenoids as the main constituents of propolis found in tropical areas, including sub-Sahara Africa. Despite the limitations of our study – including the small number of samples and narrow geographical distribution of propolis – the detection of gallic acid is important in terms of propolis quality and consumption and can be looked at as a starting point for designing new methods for characterising and quantifying the phenolic content of propolis from sub-Saharan Africa.

Acknowledgements: The project was supported by The Commission of Scientific Research Project of Uludag University (Project No: HDP (V)-2017-25). We are grateful Uludag University for providing laboratory and logistical support. We also acknowledge the support of Prof. Songül Sonal, Prof. Murat Cengiz, and Meltem Cayci.

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Quantification of Gallic Acid in Raw Propolis from Burundi


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Peer Reviewed

Competing Interests: None declared.

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ABSTRACT

Background: Zika virus infection during pregnancy has been recently associated with congenital microcephaly and other severe neural tube defects. However, the magnitude of confirmed cases and the scope of these anomalies have not been extensively documented. This review focuses on the magnitude of laboratory-confirmed congenital Zika virus cases among probable cases and describing the patterns of congenital anomalies allegedly caused by the Zika virus, information which will inform further research in this area.

Methods: We conducted a literature search for English-language articles about congenital Zika virus infection using online electronic databases (PubMed/MEDLINE, POPLINE, Embase, Google Scholar, and Web of Knowledge). The search terms used were, “zika”, “pregnancy”, [year], “microcephaly”, “infants”, “children”, “neonates”, “foetuses”, “neural tube defect”, and “CNS manifestations” in different combinations. All articles reporting cases or case series between January 2015 and December 2016 were included. Data were entered into a Microsoft Excel database and analysed to obtain proportions of the confirmed cases and patterns of anomalies.

Results: A total of 24 articles (11 case series, 9 case reports, and 4 others) were found to be eligible and included in this review. These articles reported 919 cases, with or without microcephaly, presumed to have congenital Zika virus infection. Of these cases, 884 (96.2%) had microcephaly. Of the 884 cases of microcephaly, 783 (88.6%) were tested for Zika virus infection, and 216 (27.6%; 95% confidence interval, 24.5% to 30.8%) were confirmed to be Zika virus-positive. In addition to microcephaly, other common abnormalities reported – out of 442 cases investigated – were calcifications of brain tissue (n=240, 54.3%), ventriculomegaly (n=93, 20.8%), cerebellar hypoplasia (n=52, 11.7%), and ocular manifestations (n=46, 10.4%).

Conclusion: Based on the available literature, Zika virus infection during pregnancy might lead to a wide array of outcomes other than microcephaly. There is a need for more epidemiological studies in Zika-endemic areas, particularly in Africa, to ascertain the role of Zika virus in causing congenital neurological defects.

INTRODUCTION

Zika virus (ZIKV) is an arthropod-borne virus transmitted mainly by mosquitoes of the genus Aedes. ZIKV was discovered in East Africa in 1947 from rhesus monkeys in Uganda’s Zika forest, and human cases were confirmed a few years later in Tanzania and the Central African Republic. In recent years, the public health impact of ZIKV has been underscored by its association with Guillain-Barré syndrome and central nervous system congenital abnormalities. Between 2007 and 2016, several ZIKV outbreaks were reported in 52 countries and territories, including 40 countries that reported autochthonous transmission. By January 2016, locally transmitted cases from Puerto Rico and 19 countries or territories in the Americas had been reported to the Pan Africa Health Organization. A total of 51,473 suspected cases of ZIKV had been reported in Brazil alone by March 2016. In sub-Saharan Africa, outbreaks have been reported in Gabon, and recently, sporadic cases were reported in Angola.

ZIKV is transmitted by mosquitoes, primarily of the genus Aedes. Aedes species, including Aedes aegypti, Aedes africanus, Aedes hensilli, and Aedes albopictus have been linked with ZIKV transmission. For example, A. hensilli mosquitoes were implicated in the 2007 Yap State outbreak in Micronesia. In Africa, the predominant Aedes species vector has not been definitively identified, although viral isolation studies suggest that A. albopictus was likely the vector connected to the 2007 ZIKV outbreak in Gabon. ZIKV infection is known to result from intrauterine or intrapartum transmission from a
viraemic mother to her foetus or newborn (both resulting in congenital infection), sexual transmission, blood transfusions, and laboratory exposure. Although ZIKV RNA has been detected in breastmilk, transmission through breast-feeding has not been documented. ZIKV infection is asymptomatic in about 80% of cases, when symptoms are present, they are usually mild and self-limiting. Commonly reported symptoms include rash, fever, arthralgia, myalgia, fatigue, headache, and conjunctivitis. These symptoms usually resolve within 2 weeks. Several clinical sequelae have been associated with ZIKV infection. ZIKV-associated congenital abnormalities have been observed in many countries outside of Africa. Following increasing reports on its association with congenital microcephaly in the Americas and Europe, the World Health Organization (WHO) declared ZIKV a public health emergency in early 2016.

The evaluation of clinical symptoms alone is unreliable in the diagnosis of ZIKV infection because of clinical overlap with other arboviruses. The diagnosis relies on laboratory testing: molecular amplification with real-time polymerase chain reaction (RT-PCR) assays on serum samples remains the most specific diagnostic approach and is the preferred testing method for ZIKV during the acute phase (<7 days from symptom onset). Serologic testing for ZIKV is another option, although it is limited by potential cross-reactions with other flaviviruses, particularly dengue virus.

There is no specific treatment or vaccine available for ZIKV infection. Management is based on supportive care, including rest, fluids, antipyretics, and analgesics. Other general measures focus on the prevention of mosquito bites include personal protection by wearing long pants, long sleeves, light-coloured clothing, and using insect repellents and bed nets. Pregnant women residing in countries that are not ZIKV endemic are advised against travel to affected countries. Testing should be offered to all pregnant women who have travelled to areas with ongoing Zika virus transmission.

Previous and ongoing studies have investigated the apparent causal link between ZIKV infection and congenital anomalies and sequelae, with the majority of reported cases falling under the “probable” category because of a lack of laboratory evidence to support the hypothesised causality. This obfuscates the magnitude of the problem. There are limited data to help quantify confirmed cases and the associated pattern of anomalies. It is important to generate, analyse, and compile such data because most anomalies allegedly caused by congenital ZIKV infection can also be caused by other pathogens, such as rubella virus, cytomegalovirus, Toxoplasma gondii, parvovirus B19, Treponema pallidum, and herpes simplex virus type 2, for example. This review aimed at documenting the magnitude of the reported laboratory-confirmed congenital ZIKV cases and the patterns of congenital anomalies potentially caused by congenital ZIKV infection. This information may be useful for ascertaining the knowledge gap to be filled by future research, especially as there are still more questions than answers regarding congenital ZIKV infection.

**METHODS**

**Search Strategy**

We conducted a literature search for English-language articles about ZIKV infection published between 2015 and 2016. During this period, WHO and different organisations, such as the US Centers for Disease Control and Prevention (CDC) laid down clear guidelines for ZIKV diagnosis. The search terms used were, “zika”, “pregnancy”, [year], “microcephaly”, “infants”, “children”, “neonates”, “foetuses”, “neural tube defect”, and “CNS manifestations” in different combinations. After each search, the links displayed in the abstracts were followed to yield more results. The bibliographies of the retrieved articles were carefully reviewed to yield more articles.

**Information Sources**

Information was searched from the online databases PubMed/MEDLINE, POPLINE, Google Scholar, Embase, and Web of Knowledge between 1 July and 31 December 2016. Only studies published in scientific journals were included. We used a checklist to extract the relevant information from each included article, and no authors were contacted for additional information.

**Eligibility Criteria and Study Selection**

As this review aimed to quantify laboratory-confirmed cases and delineate the pattern of anomalies, we included case reports, case series, and cross-sectional studies with descriptions of cases published in English between 2015 and 2016. All search results reporting on cases were included in this review. Articles were screened for the completeness of data; articles reporting ZIKV infection in the general population, news and epidemiological alerts, review articles, and articles with evidence of data repetition were excluded. All studies were assessed for methodological quality using a checklist.

A total of 2,134 articles and abstracts were obtained; 1,761 were excluded because of duplication, leaving 373 abstracts and articles to be analysed for eligibility, of which 320 were excluded. Finally, 53 full articles were critically reviewed. Only 24 articles (11 case series, 9 case reports, 2 cross-sectional studies, 1 case–control study, and 1 cohort study) were found to be eligible and included in this review (Figure 1).

**Data Collection Process**

Two independent reviewers performed data extraction using a checklist with the headings Author, Study Period, Year of Publication, Study Area/Country, Sample Size, Study Design, Clinical Presentation, Imaging Results, Cases Tested, Diagnostic Technique, and Mother Symptoms, where applicable.
All studies described cases, and laboratory confirmation was done using recommended techniques, such as RT-PCR and IgM immunoassays.19,20

Summary Measures
Unweighted analysis was carried out, whereby the outcomes of individual studies were counted to quantify the proportions of cases with microcephaly and laboratory-confirmed cases, as well as describe patterns of congenital anomalies. We calculated 95% confidence intervals using 2-sample proportion tests.

Ethical Approval
Ethical approval to conduct and publish this review was obtained from the Joint Catholic University of Health and Allied Sciences/Bugando Medical Centre (CUHAS/BMC) Ethical Review Committee (CREC/043/2014/2015/2016).

RESULTS
Study Characteristics
Of the 24 articles included in the final analysis, 20 (83.3%) were case series or case reports. Most of the data were collected between February 2015 and June 2016. The majority (n=21, 87.5%) of the studies were conducted in South America (Table). Seventeen (70.8%) of the articles reported on laboratory results associated with ZIKV infection. Five articles reported PCR as the sole laboratory technique, while 13 articles reported the use of IgM immunoassays along with PCR assays. All articles reported on the presence or absence of microcephaly. Nineteen (79.2%) of the studies investigated other causes of congenital anomalies, such as rubella, T. gondii, cytomegalovirus, parvovirus B19, T. pallidum, herpes simplex type 2, and HIV. Various combinations of ultrasound, computed tomography, magnetic resonance imaging, and plain x-rays were employed to establish imaging findings. Seventeen (68%) articles reported on maternal clinical signs and symptoms during pregnancy.

Synthesis of Results
A total of 919 cases with central nervous system manifestations with or without laboratory confirmation of ZIKV infection were reported in the 24 articles. The majority of these studies were from Brazil (Table). There were 884 (96.2%) cases of microcephaly. Maternal clinical data were available for 309 mothers whose babies had microcephaly. Among these 309 women, the common signs and symptoms were rash (n=189, 61.8%), arthralgia or myalgia (n=65, 21%), and fever (n=62, 20.1%). The details of the analysed articles are summarised in the Table.

Diagnosis of ZIKV Infection
Out of 884 cases of microcephaly, 783 (88.6%) were tested for ZIKV infection. Of the 783 tested samples, 216 (27.6%; 95% CI, 24.5% to 30.8%) were confirmed to be positive for ZIKV infection. Of 35 cases without microcephaly, 11 (31.4%) tested positive for ZIKV infection. Of the 227 confirmed cases, most (n=153, 67.1%) were confirmed by IgM enzyme immunoassay (EIA). The commonest sample type used for laboratory confirmation of ZIKV infection was blood (n=761 of 783,
97.2%), followed by cerebrospinal fluid in 86 (11%) of 783 cases. Both amniotic fluid and tissue samples were used in 14 cases, urine was used in 8 cases, and amniotic fluid only was used in 8 cases. Four cases relied on only tissue samples for diagnosis. Histopathological findings using haematoxylin and eosin staining and immunohistochemistry were investigated for 6 cases, all of which had calcifications, with macrophages and perivascular infiltrates observed in 1 case.

**Imaging Findings**

Of the 883 cases with microcephaly, imaging results were available for 442 (50.1%). The commonest finding was calcifications (n=240, 54.3%), followed by ventriculomegaly (n=92, 20.8%), cerebellar hypoplasia (n=52, 11.7%), and ocular manifestations – including chorioretinal scarring, macular pigmentation, fovea and reflex loss, microphthalmia, intraocular calcifications, cataracts, and microphthalmia (n=46, 10.4%). Other findings included cortical hypogyrination (n=23, 5.2%), arthrogryposis (n=15, 3.4%), lower limb manifestations (n=6, 1.4%), cerebral atrophy (n=2, 0.5%), and hydrocephalus (n=1, 0.25%). The details of the findings are summarised in Figure 2. Adverse pregnancy outcomes observed were miscarriages (n=7), intrauterine growth retardation (n=4), hydrops foetalis (n=1), and stillbirths (n=2).

**DISCUSSION**

This report summarises the magnitude of confirmed congenital ZIKV infection and the scope of anomalies that might be caused by ZIKV. An important finding is the magnitude of the confirmed cases, which reflects progress in investigating the link of causality. Only a quarter of “probable cases” were laboratory confirmed as cases of ZIKV infection. The small proportion of confirmed cases could be due to the unknown course of the disease, the time taken for viral markers to disappear after congenital infections, and the sensitivity of the available assays. There is a need for a coordinated effort to develop inexpensive, specific, and sensitive tests to diagnose ZIKV infection. To date, only a few assays have been approved and recommended by WHO. Another important observation is that three-quarters of ZIKV studies in this review ruled out other causes of congenital anomalies – such as rubella virus, T. gondii, cytomegalovirus, parvovirus B19, T. pallidum, herpes simplex type 2, and HIV – which is important for providing more robust evidence for the association between ZIKV and congenital anomalies. It is crucial to screen for other causes of congenital anomalies during the diagnosis of potential ZIKV cases, particularly in developing countries where these infections are most prevalent.

The majority of the articles in this review included well-studied cases highlighting the widely varying patterns of congenital ZIKV presentation. As has been done for the
TABLE. Summary of the Articles Included in the Review

<table>
<thead>
<tr>
<th>Study Number</th>
<th>Study Design*</th>
<th>Study Location</th>
<th>Sample Size</th>
<th>CNS Manifestations N</th>
<th>Microcephaly n</th>
<th>Lab results n</th>
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<tr>
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<td>2</td>
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<td>ND</td>
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</tr>
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<td>19</td>
<td>19</td>
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<tr>
<td>23</td>
<td>Case series</td>
<td>Brazil</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>24</td>
<td>Case series</td>
<td>Brazil</td>
<td>13</td>
<td>13</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td><strong>994</strong></td>
<td><strong>919</strong></td>
<td><strong>884</strong></td>
<td></td>
</tr>
</tbody>
</table>

*The diagnosis included 3 tissues samples
Abbreviation: ND, not determined
congenital rubella syndrome, there is a need to establish a pathognomonic features list and case definitions for congenital ZIKV infection.

Regarding laboratory diagnosis of ZIKV infection, half (n=12) of the included articles confirmed ZIKV infection using ELISAs and PCR assays. The diagnosis of ZIKV infection – similarly to other closely related viruses, such as the chikungunya, dengue, and yellow fever viruses – has become challenging due to cross-reactivity, which hinders the specificity of the available diagnostic techniques. Sensitive and specific assays to confirm ZIKV infection are high demand at this time of increasing anxiety related to ZIKV and its associated sequelae in both developed and developing countries. Given that most congenital infections are associated with similar maternal clinical presentations during pregnancy and given the high incidence of congenital anomalies with unidentified causes being reported in developing countries, reliable and affordable diagnostic techniques for the screening these infections at the point of care are paramount. This review highlighted the common clinical manifestations of congenital ZIKV infections. Cerebral calcifications were the most common imaging finding, followed by ventriculomegaly, cerebellar hypoplasia, and ocular anomalies. This suggests that congenital ZIKV infection causes a wide array of congenital anomalies, aside from microcephaly, which calls for thorough clinical examinations and investigations of all neonates with congenital anomalies. Even though ZIKV is known to be neurotropic, manifestations involving other organ systems are possible, and this must be remembered when considering ZIKV’s role in causing congenital anomalies, particularly in endemic developing countries where the majority of congenital anomalies have not identified causes. Furthermore, apart from the congenital abnormalities mentioned herein, ZIKV might also cause a diverse range of adverse pregnancy outcomes, such as miscarriages, stillbirths, and intrauterine growth retardation.

In general, this review provides an overview of the current situation regarding confirmed congenital ZIKV infection along with the scope of outcomes, which may be useful for future research and for designing control strategies. Nevertheless, we must mention several drawbacks. For example, some of the articles did not clearly indicate how many microcephalic newborns underwent imaging studies. This might lead to an inaccurate understanding of the patterns of imaging findings. Similarly, most of the articles did not report maternal clinical features during pregnancy, information that could lead clinicians to have a high index of suspicion for ZIKV infection when appropriate. Another limitation was that some studies did not rule out other causes of congenital anomalies.

CONCLUSION
Congenital ZIKV infections might be associated with a wider spectrum of anomalies aside from microcephaly. This necessitates thorough clinical examination and investigation whenever a congenital infection is suspected. Diagnostic assays to confirm ZIKV infection are urgently needed, especially in areas where ZIKV is endemic. There is a need to develop a standardised format for reporting results regarding congenital ZIKV infections to allow pooling of data for public use. Additionally, we recommend further research on ZIKV strains in relation to the pattern of congenital anomalies.

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