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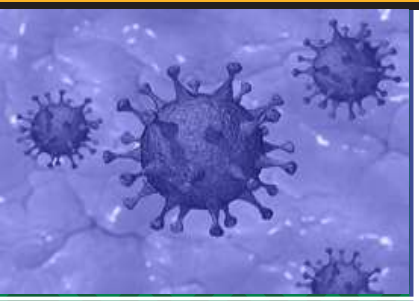

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EAHRC
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REVIEW ARTICLE

COVID-19 Awakening: Preparedness, Readiness and Response during the pandemic

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ABSTRACT

At the end of December 2019, the China public health authorities were informed on cases of unusual pneumonia detected in Wuhan City, mainland China. The causative agent was later identified to be a novel coronavirus (2019-nCoV) currently referred to as the severe acute respiratory syndrome coronavirus 2 (SARS CoV-2). The World Health Organization declared the disease a public health emergency of international concern on 30th January 2020 and named it Coronavirus disease 2019 (COVID-19) on 11th February 2020. By 20th April 2020, over 2.4 million cases of COVID-19 had been reported from 210 countries and territories worldwide. The rate at which COVID-19 spreads and the fact that asymptomatic individuals can transmit the disease has risen concerns on how to control the pandemic in resource-constrained countries. We conducted a review of literatures and summarized the key lessons to inform policy and decision makers on strategies necessary for an effective response during the pandemic. We recommend comprehensive implementation of the proposed approaches that encompass robust containment strategies and flexible transition to scaling up mitigation strategies.

Key words: Preparedness and Response, SARS CoV-2, COVID–19, Pandemic, Epidemic, Outbreak.

INTRODUCTION

Pandemics have appeared to be increasing in frequency in the past few decades. Since the 2002-2003 severe acute respiratory syndrome coronavirus (SARS-CoV) epidemic that started in Guangdong province southern China, spreading across 26 countries and affecting over 8000 people, the world has experience at least 10 major outbreaks including the 2014-2016 Ebola outbreak in West Africa that claimed over 11,000 lives [1-3]. On 31st December 2019, the World Health Organization (WHO) China Country Office was notified of unusual cases of acute respiratory syndrome in Wuhan City, Hubei province, China. On 7th January the causative agent was identified to be a novel coronavirus (2019-nCoV), currently referred to as the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[4]. The World Health Organization (WHO) declared the disease a public health emergency of international concern on 30th January 2020 and named it Coronavirus disease 2019 (COVID-19) on 11th February 2020. By 20th April 2020, the COVID-19 pandemic had spread across 210 countries and territories, affecting over 2.4 million people and causing more than 160,000 deaths worldwide[5]. Its estimated global case fatality rate is 3.4%, but higher rates of up to 6.16% and 9.26% have been reported in Spain and Italy respectively[6,7]. At least 600 confirmed COVID-19 cases and over 20 related deaths have already been reported from 6 member States of The East African Community (EAC) as seen in ***Figure 1*** and ***Figure 2***[5] as of 19th April 2020. It has been demonstrated that the type of outbreaks and response measures taken against them can determine the level of social and economic disruption, political stresses and tensions, violence and even changes in individual behaviors[8].

A comprehensive outbreak response is a complex multifaceted systematic plan that has to be harmoniously coordinated. We have observed concerns on how to apply knowledge about infection prevention and the control of the pandemic especially in resource-constrained countries[9]. In order to address this concern, we reviewed relevant articles of interest after conducting an online literature search through PubMed, Google Scholar and the World Wide Web using the keywords: ''2019-nCoV'', ''SARS CoV-2'', ''COVID-19 Preparedness and Response"; ''Pandemic/outbreak/epidemic containment and Mitigation''; ''Screening'', ''Sensitivity'', ''Specificity'', and ''Predictive values'' in different combinations.

In this article, we provide an overview to policy and decision makers; enlightening the understanding on the spectrum of activities necessary for effective response against the COVID-19 outbreak by using two different models of approach. We also provide suggestions on different screening approaches that may be applied with respect to the dynamics of an outbreak. This will help in providing guidance on how to respond to both the current and future outbreaks.

FIGURE 1. The cumulative frequency of confirmed COVID-19 cases in EAC member states over time

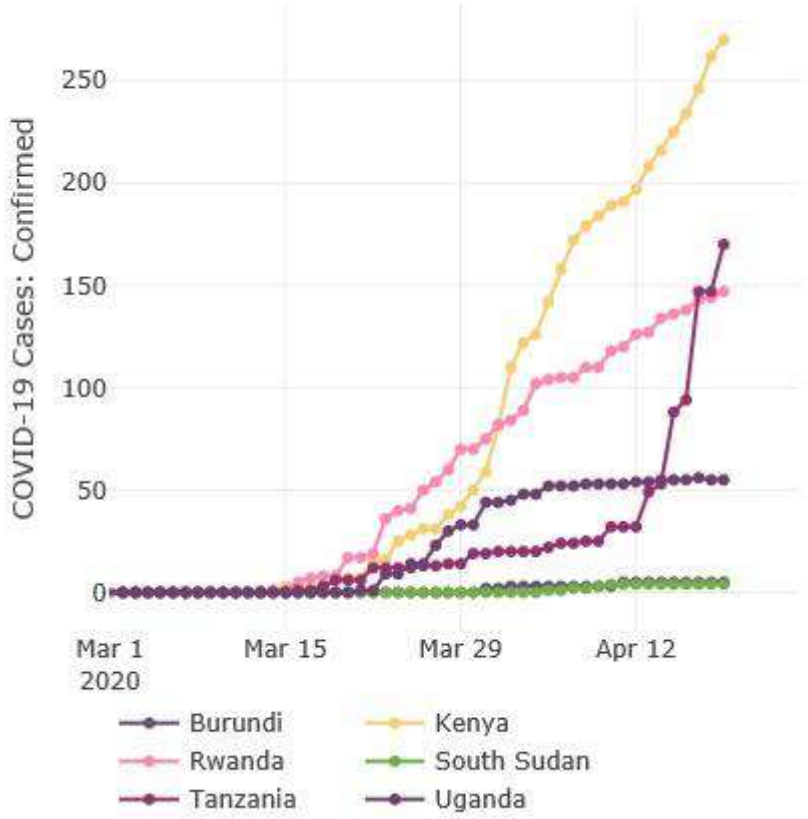

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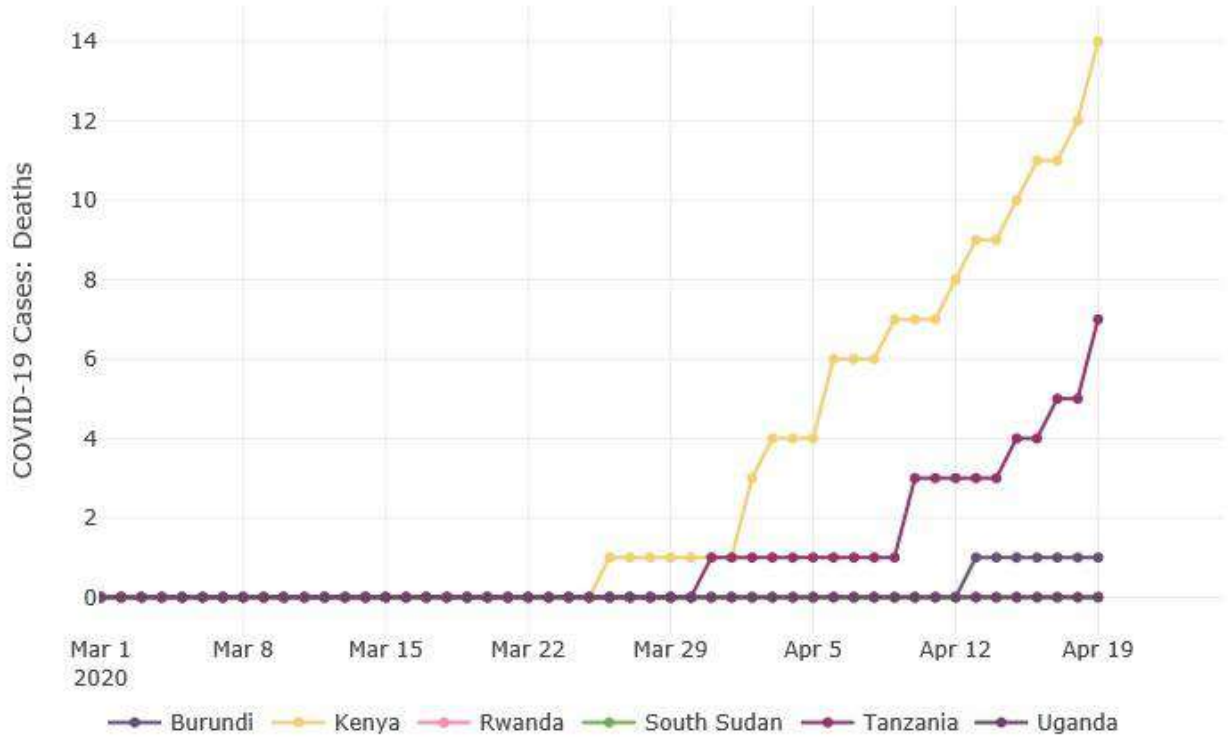
FIGURE 2. The cumulative frequency of COVID-19 related deaths in EAC member states overt time



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UNDERSTANDING THE RESPONSE APPROACH TO AN OUTBREAK

Effective preparedness against health threats is essential for a robust and sustained response during an outbreak. Such kind of preparedness will require adequate investment to strengthen the existing health care systems[10]. Controlling the spread of highly transmissible diseases like COVID-19 require swift mobilization of personnel and resources in order to rapidly implement effective interventions capable of preventing the devastating impacts that are likely to result from a pandemic. Key public health interventions should be rapidly implemented, operationalizing multi-sectoral coordination and collaboration to ensure the welfare of the society and health care workers. This includes improved access to health care facilities, access to safe water for proper hand hygiene, and access to Personal Protective Equipment (PPE) for the health care workers. Governments should enact and enforce policies that will ensure availability and affordability of key products such as masks, hand sanitizers and PPE which are essential in halting the spread of the virus. Physical distancing measures should be harmoniously enforced with public health education in order to enhance compliance and avoid resistance since social and public gatherings are common practices in African communities. Media outlets and all leaders (political, government, traditional, religious etc.) play a key role in educating the public on how to react during the outbreak due to their inherent ability to influence and reach a large number of public. They must therefore be well informed and educated in order to provide correct evidence-based information and directives. Addressing a pandemic or epidemic call for joint actions towards the interest of saving lives by practicing proven effective preventive, containment and response

measures, leaving aside any political, religious, traditional differences and beliefs.

Preparedness plans based on lessons learned from past encounters ought to align with scenarios that include periods of either, no reported cases, sporadic cases or clustered cases and community transmission. Prior to the outbreak when no case has been reported, screening and triage protocols at country's points of entry and all levels of the health care system should be installed in addition to designating isolation areas for suspected, probable or confirmed cases. It is important to identify and address the drivers that are likely to facilitate the spread of the disease and to map vulnerable communities and locations such as high-risk elderly populations and high-density towns or cities. With reported sporadic cases, containment measures aiming at reducing the risk of amplification of the disease must be instituted rapidly. In order to meet the minimum required surge capacity, it is necessary to repurpose or build new structures and community facilities i.e. temporary field hospitals, when clustered cases are identified with evidence of community spread indicating amplification of the outbreak.

For simplicity, we categorize the response spectrum into three phases which are not distinct but rather a continuous sequence of activities; these phases include the 'containment phase', 'transition phase' and the 'mitigation phase'. ***Figure 3*** and ***Figure 4*** summarize the proposed models of two different response approaches that can be applied during outbreaks. Depending on the feasibility and time period of the initial response, these phases/approaches can be implemented either sequentially or concomitantly.

FIGURE 3. Sequential response approach during an outbreak

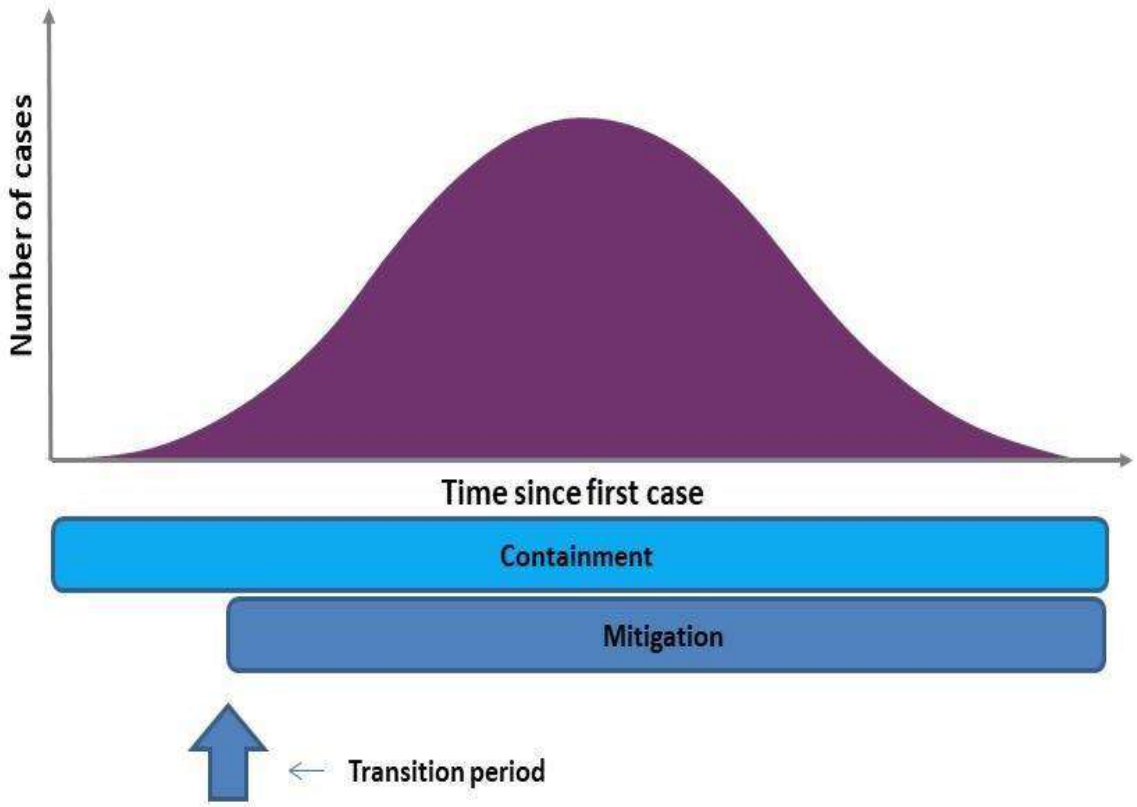
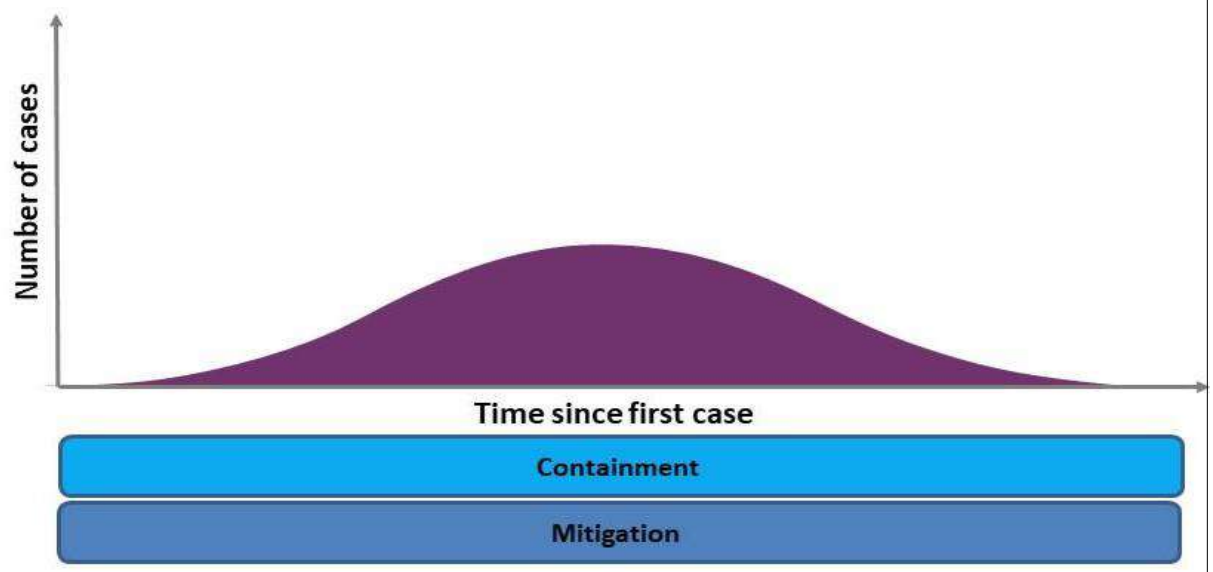


FIGURE 4. Concomitant response approach during an outbreak



The 'containment phase'

Containment strategies are applied at the early stages (and spans to the end of an epidemic) of an outbreak aiming at confining the virus at source and avoiding spillage into the community. These strategies have to be implemented as soon as the index case is detected as seen in ***Figure 3*** and ***Figure 4***. It will involve case/contact tracing together with border control in order to regulate the movement of people from regions with reported cases; thus, curtailing the number of imported cases. It is crucial to allocate areas for isolation and quarantines so as to limit the circulation of the diseased in the community or within health facilities while ensuring that treatment and care is provided to the patients. Of note is that, isolation and quarantine is required not only for confirmed cases but also for persons who have been exposed to either a known or a suspected case (contacts). The recommended length of the quarantine period for COVID-19 is at least 14 days, corresponding to the incubation period of 2-14 days[11]. Test results (where necessary) and the progress of the diseases are also determinants of the length of the isolation period. During this phase, Research & Development (R&D) activities aimed at gaining insight on the nature of the disease outbreak and hastening the availability of diagnostics, vaccines and therapeutics are to be strongly supported with adequate resources[3]. Aggressive implementation of containment strategies will halt the spread of the virus at an early stage of the outbreak reducing the necessity of up-scaling the mitigation strategies.

The 'transition phase'

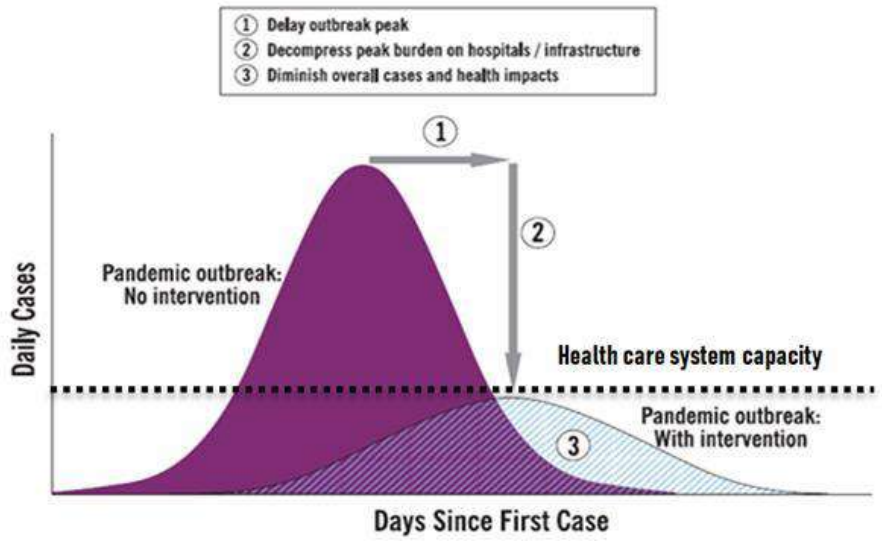
The transition phase as seen in ***Figure 3*** set-in when there is failure to contain the disease, resulting into spillage of the virus, threatening or causing sustained transmission at community level. A given threshold of successful contact tracing is essential to control early spread of the virus in the community. This threshold value can be analyzed by utilizing different statistical models that use the basic reproduction number (R_0) which is the expected number of secondary cases generated by one primary case in a susceptible population. A stochastic SARS-CoV-2 transmission model developed by Joel et al[12] demonstrated that at least 70% and 90% of successful contact tracing is required to control the outbreak with a R_0 of 2.5 and 3.5 respectively. Reports of community spread should alarm and

prompt execution of mitigation strategies in addition to ongoing containment efforts. Deciding to implement the mitigation strategies might be extremely difficult for policy and decision makers especially in low-income countries as they may appear to damage the already weak economic status. Bold and well-targeted measures when implemented early and rapidly will save the economy from severe destruction and difficult as it may seem to be, the decisions have to be made quickly. However, they have to be evidence informed decisions and not panic driven decisions.

The 'mitigation phase'

As it is seen in ***Figure 3*** and ***Figure 4***, mitigation strategies can be applied during the amplification phase or right after detection of the index case. The goal is to reduce the disease incidence, morbidity and mortality as well as minimizing the social, political and economic impact of an epidemic[3]. Mitigation also serves to delay the outbreak peak thus providing time for rapid re-deployment of personnel and reallocating resources to avoid overstretching of the health care system capacity by the prolongation of the epidemic period as demonstrated in ***Figure 5*** [13,14]. The disease control strategies initially employed in the containment phase should be ongoing throughout this phase and may target particular cohorts with a high incidence rate that have a high likelihood of further spreading the disease into the rest of the community or to highly vulnerable cohorts[13]. These highly vulnerable cohorts (individuals at highest risk for severe disease and mortality) include people aged more than 60 years and those with chronic underlying conditions such as hypertension, diabetes, cardiovascular disease, chronic respiratory disease and cancer. Physical distancing measures in order to reduce human to human transmission are of essence. Studies have shown that voluntary isolation at home is a feasible strategy as compared to drastic physical distancing measures that should be reserved for severe outbreaks[15]. Additional measures to be undertaken will depend on the dynamics of the outbreak; theses may include non-pharmacological interventions such as hand hygiene, respiratory hygiene, face mask ordinances, or strict physical distancing measures. Pharmacological interventions will aim at either treatment or prevention of the disease. These include specific treatments, prophylaxis or vaccinations when available. Provided that pharmacological interventions are of value, non-pharmacological interventions are the mainstay due to lag in development of tests, therapeutics and vaccines.

FIGURE 5. Goals of Community Mitigation



Adapted from: CDC. Interim pre-pandemic planning guidance: community strategy for pandemic influenza mitigation in the United States—early, targeted, layered use of non-pharmaceutical interventions. https://stacks.cdc.gov/view/cdc/11425.

The value of early up-scaling of mitigation strategies

Several studies have assessed the effectiveness of implementing multiple interventions in reducing the rate of disease transmission during outbreaks. Reduction of the effective reproduction number from approximately 3.0 to to 1.0 in less than one month period following implementation of cordons sanitaire, traffic restriction and home quarantine in controlling the COVID-19 outbreak in Wuhan, China has been demonstrated[16]. Assessment of the intensity of the 1918-1919 influenza pandemic showed that cities in which multiple interventions were implemented at an early phase had peak death rates of approximately 50% less as compared to their counterparts[17]. In addition to the layered and early implementation of public health interventions, sustained interventions are strongly associated with a lowered and delayed peak of mortality[18].

A common finding in these studies suggest that early implementation of multiple sustained interventions has a promising outcome on responding to epidemics. These findings provide evidence to the gentleness of the curve in (***Figure 4)*** that corresponds with the concomitant approach in responding to an epidemic. Although less feasible as compared to the sequential approach (***Figure 3***), the concomitant approach has to be advocated whenever failure to contain the diseases is anticipated.

Screening during different phases of the outbreak

The real-time reverse transcriptase–polymerase chain reaction (rRT-PCR) test used for qualitative detection of nucleic acid of SARS-CoV-2 from nasopharyngeal swabs has typically been used to confirm the clinical diagnosis of COVID-19[19]. However, rapid diagnostic tests (RTDs) are of particularly interest due to the speed in obtaining test results and less reliance on laboratory-based services especially in countries with reduced laboratory diagnostic capacity. Nonetheless, based on current evidence, the WHO does not recommend the use of COVID-19 antigen-based RDTs for clinical decision-making due to lack of validation of the rapid test kits with an expected low test sensitivity ranging between 34% and 80%[20].

Since tests with high sensitivity are required at the screening phase, in the absence of highly sensitive tests, parallel testing involving the use of two or more tests applied to a patient at the same time can be employed in order to increase test sensitivity[21,22]. High levels of test sensitivity are essential since missing cases can have devastating consequences. Caution should also be taken in declaring cured cases in the face of tests with poor specificity which is the ability of a test to correctly classify an individual without a diseased. This can be achieved by applying serial testing in order to improve the test specificity[22].

Testing in containment phase

Target screening approach which involves testing persons who meet the given standard case definition is economic and may be preferred. We presume that at the beginning of the outbreak the prevalence of the diseases in the community is low, resulting into a subsequent low Positive Predictive Value (PPV) of a given screening test which is the probability that subjects with a positive screening test truly has the disease of interest[14]. Applying mandatory mass screening in a population with low disease prevalence will result into large numbers of false positives which will be a waste of time and resources especially in resource constrained areas. Such resource would rather be reallocated to prioritize core preparedness activities for the mitigation phases including improving diagnostic and treatment capabilities, repurposing facilities to meet the surge capacity, training and ensuring availability of PPE for health care providers.

Testing in mitigation phase

Mandatory mass screening approach will most likely be productive during the amplification phase of the pandemic. We presume that the prevalence of the disease in the community at this phase is high; thus, a given screening test will yield a high PPV with subsequent lesser number of false positives[23]. Mass community testing is important since, mildly symptomatic and asymptomatic cases will be identified, isolated and treated; hence limiting the disease transmission.

RECOMMENDATIONS

In summary, the following action points are recommended:

- Countries should seize COVID-19 pandemic as an opportunity to invest adequately in sustainably strengthening their health systems' capacities for preparedness and response against public health threats.
- Countries and the region should establish and continuously update national and regional teams of experts ready for rapid deployment during outbreaks.
- Responsible national and regional authorities should provide resources for rapid activation of Research & Development activities to address the Pandemic.
- Continuous education on personal and environmental interventions such as personal hygiene and large-scale disinfection efforts taken during the outbreak should be maintained as routine practices even after the pandemic to avoid a secondary peak and subsequent outbreaks.
- Countries must rapidly develop and institute policies facilitating implementation of containment strategies to stop early progression of the pandemic in order to avoid social and economic consequences.
- Countries must implement mitigation measures early (at the earliest signal of sustained community spread), in order to prevent prolonged lockdown, save lives and reduce related economic hardship.

CONCLUSION

COVID-19 pandemic has already affected millions of people worldwide. Due to the alarming trends of the disease spread particularly in EAC partner states, decision makers should work together to scale up containment and mitigation strategies in order to slow the spread of the disease across the region. Quarantine, lock down and travel restrictions must be enforced at the earliest signals of community spread while maintaining personal measures like proper hand hygiene and avoidance of crowds and congregations. Leaders must adhere to WHO guidelines, disseminate evidence-based messages and seek any clarifications and guidance from their national or regional experts. COVID-19 respects neither borders nor political, religious or traditional beliefs. Leaders must avoid issuing wishful messages based on their own opinion or beliefs as many lives may be at stake if conflicting messages are issued. Let us be educated by current knowledge and follow scientific preventive, containment, and mitigation measures. Current interventions must be sustained not only to avoid a secondary peak of the outbreak, but also to allow the culture of hygiene that may prevent future threats. The current COVID-19 pandemic is an opportunity to revive hygienic culture, strengthen health systems and invest in sustaining the capacities, ready to respond to both the current and future outbreaks.

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REVIEW ARTICLE

Tracking characterization of Mycobacterium strains in Tanzania and some sub-Saharan African Countries: An overview on genotyping studies, implication and trends in advancing technology

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ABSTRACT

Tuberculosis (TB) is a devastating chronic debilitating infection that imposes a considerable negative impact on human health. The disease also affects the quality of livestock, wildlife and their products. *Mycobacterium tuberculosis* (*M. tuberculosis*) is the causative agent for human TB, while *Mycobacterium bovis* (*M. bovis*) predominantly causes TB in animals, though is also zoonotic. In Tanzania and other developing countries timely diagnosis of disease is hampered by poor access to appropriate capacity and technology to characterize the pathogen. This review explores the diverse methods available for the diagnosis and characterization of Mycobacterial species and strains isolated from both humans and animals in sub Saharan Africa. The review will identify knowledge gaps and highlight direction for future investigation of the interface TB risk, which could lead to a better regional control strategy. A systematic search of PubMed, Google and Google Scholar retrieved 716 published articles on TB and methods used with the aim of tracking the advancement in technology and to reflect where we are and what can best be done to improve the strategy for best control of the disease. The search terms included but not limited to *"(Whole Genome Sequencing AND sub-Saharan Africa);* (*Diverse TB strains + Human +Animals) AND Tanzania + Africa + Sub-Saharan Africa;* (*Diverse TB strains + human+ animals) OR Tanzania + Africa + Sub-Saharan Africa)"*. The review illustrates an advancement of technology from 1950s to 2000s with only 17.7% studies having been done using DNA-based methods and 81.3% being studies that used conventional methods. Most of the molecular studies cluster in the mid- to late 2000s which could be due to lack of expertise, slow adoption of technology or the high cost of running these valuable molecular tests. This overview on genotyping studies and trend in molecular studies highlights the need for more investment in this region of the world. An increased use of molecular methods will help in increasing the chances of detecting new TB strains in circulation and identifying potential risks for cross-transmission at humans-livestock-wildlife interface. A strengthened ability to detect and characterizes disease will better support country and regional control strategies.

Key words: Mycobacterium strains, Trends, Genotyping studies, Advancing technology, Tanzania.

INTRODUCTION

Tuberculosis (TB) is a devastating chronic debilitating infection which negatively impacts on the health and wellbeing of humans, and livestock and wildlife and their products. With potential for cross-transmission of the disease amongst species, an inter-sectoral participatory approach that involve multiple disciplines (One Health approach) could be relevant as a control and disease management strategy, particularly in developing countries. Whilst *Mycobacterium Tuberculosis* is predominantly the

causative agent for human TB, *Mycobacterium bovis* predominantly causes TB in animals, though is also zoonotic. Zoonotic TB risk factors include consumption of animal products, particularly raw milk and uncooked meat. Transmission of bovine tuberculosis (bTB) by *M. bovis* from cattle to humans is well known but little is known of transmission of bTB between animals in Africa and risk of transmission to from humans. Many societies in Africa share shelters with animals, particularly cattle and goats. It is therefore important to explore the disease dynamics in these interactions. Understanding the habitual practices, and being able to identify the prevalent TB strains in circulation in sub-Saharan Africa are important to design suitable interventions to improve health in both humans and animals. In this review, we explore the diversity of Mycobacterial species and strains isolated from both humans and animals in sub-Saharan Africa in order to identify knowledge gaps to drive further research and support an evidence based regional control strategy.

TB occurs worldwide with about 10 million cases and 1.6 million deaths in 2017 (WHO). TB is amongst the top causes of deaths in women aged 15 to 44 years[1] and is even more prevalent in males. According to the World Health Organization (WHO) reports the largest number of new TB cases occurred in Asia, (62% of new cases globally in the last 2 years) and then sub-Saharan African (25% of all new cases in 2017)[2]. The global impact of TB is exacerbated by co-infection with HIV[8] and drug resistance[3-10]. Extensive drug resistant TB (XDR-TB) has been widely reported in many TB endemic countries[8,11-17]. Tanzania is among the African countries with highest burden of TB in the world; having approximately 295 TB cases per 100,000 adults. In addition, Tanzania has a national HIV prevalence of 5%, which increases risk of TB infection by over 30 times[1].

Existing economic and social obstacles challenge global TB elimination[5]. Treatment and prevention strategies are hampered by socio-economic problems and limited political will. Such strategies rely on better diagnostics to increase case detection[5], and to reduce the risks of exposure. The implementation of disease control policies, including case management, require adequate and consistent funding[5]. Part of the strategy should bring in the concept of 'One Health' with medical, veterinary and other stakeholders such as environmentalists which could be a key control strategy[18].

Animal tuberculosis, zoonotic and epizoonotic implications

Bovine TB (bTB) in humans is a well-documented zoonosis and is thought to be acquired mainly from cattle through the consumption of infected meat or milk, or via the respiratory route. However, humans can also be a source of infection to livestock, for example there are reports of the isolation of *M. tuberculosis* in goats and sheep in Ethiopia[19] and even a wild elephants in South Africa[20]. The epizoonotic and zoonotic nature of TB would benefit from a One Health approach to control. For example, the use of genotyping and whole genome sequencing methods, can help to identify sources and transmission routes within and across species and geographic regions[21], which may identify points for intervention. These advances in technology, however, need committed and coordinated efforts from both within and outside the health sector[22]. In addition, it will require willingness from governments in endemic areas to invest in TB disease control programs[18] and not entirely depend on donor-funded programs.

There is insufficient information on the distribution, epidemiology patterns and zoonotic potential of bTB in all environments, but perhaps most pertinently in traditional rural environments[23]. Putting in place diagnostic programs focusing on isolation, culture and pattern discrimination of mycobacteria from human and animal TB cases is important for pinpointing the mechanisms and routes of disease transmission, spread and zoonotic impact. Undiagnosed cases contribute towards the force of infection and disease recurrence[24]. This is particularly so in resource-poor countries (mostly disease endemic African countries) where rare resource sharing could be critically important. In such resource sharing fashion, mycobacterial culture as well as isolation facilities could be done at reduced cost, consequently, enhancing effective and efficient diagnosis[23]. However, diagnosis is only the first step towards control and unfortunately, the test and slaughter strategy for TB positive animals as a control measure is rarely used in African countries, for a variety of reasons including cultural backlash and the lack of funds for adequate compensation. Thus, without change, developing countries (and particularly African countries) will remain focal points for a number of infections in both humans and animals, adversely affecting their economy. The eradication of TB will not be possible without good cooperation and collaboration between medical and veterinary personnel for effective and cost-efficient investigation, prevention, control and management of the disease[23]. This is well supported by the East African Community which has so far conducted a series of curriculum validation meetings to advocate regional pandemic preparedness with a 'One Health' approach. This is an important step because, although it targets pandemics other than TB, it builds the capacity of practitioners for the development of a common approach for the control of zoonotic infections. A missing link previously identified by Mbugi et al[25].

The epidemiology of tuberculosis has been extensively studied for decades, and considerable technological advancements have increased our ability to explore the

disease and its causative agent. In more recent times pathogens such as *M. tuberculosis* and other bacteria have been characterized using their nucleic acids (DNA and RNA) and proteins to study factors such as lineage or strain diversity, drug resistance as well as other physico-chemical properties important for pathogen survival in the host. These methods have provided a particularly suitable tool to investigate the epidemiology of bacterial diseases, including sub-Saharan Africa[26]. Several molecular tools, including polymerase chain reaction (PCR), restriction fragment length polymorphisms (RFLP) analysis, spacer oligonucleotide typing (spoligotyping), mycobacterium interspaced repetitive units variable number tandem repeats (MIRU-VNTR) and sequencing have gradually been adopted in many countries to study the epidemiology of tuberculosis. The advantage of these molecular methods in tuberculosis studies rests on their ability to not only discriminate between isolates and identify strains but also to distinguish antibiotic sensitive from resistant strains in *M. tuberculosis* complex infection. These methods have been used successfully to identify chains of transmission and to trace the origins of infection (or pathogen)[27,28].

Although various genotyping tools have been instrumental in advancing our understanding of TB dynamics, it is becoming clear that whole genome sequencing is superior to conventional genotyping for *M. tuberculosis* studies, particularly to provide a better understanding of *M. tuberculosis* genome evolution over time in its natural host context. Thus evaluation of the trend of advancement in using these new and modern technologies over time is necessary to get a clear picture on stage of our diagnostic capacity. Diagnostic approaches range from conventional genotyping to sequencing, allowing for diverse species and strains of *M. tuberculosis* complex (MTBC) to be discerned. This approach has been useful in tracing the evolution of strains over time[29], the geospatial distribution of strains[30] as well as the strains of *M. tuberculosis* that seem to be independently acquiring drug resistance[31,32]. Proper diagnosis is important, since complimentary studies on non-tuberculous mycobacteria (NTM) have shown the potential of diverse NTM species for causing disease in both animals and humans [33]. Despite the availability of these genotyping methods, very few studies tracing TB strains in human and animals in sub-Saharan Africa have been conducted.

Literature Search Strategy

A literature search was conducted using PubMed with search teams "Whole Genome Sequencing AND sub-Saharan Africa". To ensure that the retrieved articles are relevant, PRISM guidelines (http://www.prisma-statement.org/) were used, and the search included sufficient terms to extract maximum number of informative publications.

The search, initially included only studies done in Tanzania, then some sub-Saharan Africa. The initial search retrieved 40 published article ***(Table 1)***, three[34-36] done in Tanzania and the rest (37 articles) done in other areas. Most of these articles were from South Africa and Ethiopia with little coming from Djibouti (1), Mali (1), Nigeria (1), Equatorial Guinea (1), Malawi (1) and Uganda (1). The value of whole genome sequencing has been important in tracking evolutional changes in mycobacterial genome that gradually resulted into development of TB drug resistance.

RESULTS AND DISCUSSION

Search results and implication

A total of 716 publications from search were eligible for inclusion in this review 442 (61.7%) of which, originated from Tanzanian studies and 274 (30.3%) publications originated from other countries ***(Table 2)***. The geographic spread of these latter publications were variably and regionally distributed along South Africa, the Horn of Africa, West Africa, East and Central African countries. In the general research results, both conventional and molecular studies were unevenly distributed. The uneven distribution of these studies is a reflection of country and regional variability in adoption of modern technology in disease screening and diagnosis.

In Tanzania there were just 16 studies from 2001 to 2018 which have characterized TB in both humans and animals to genetic level ***(Table 1)***. All of those studies used genotyping methods, of which 6 focused on characterization of the infective bacteria (*M. bovis*) in cattle[37-41] and rodents [42], only nine[34,35,43-50] dealt with infection in humans while the rest were mainly studies that involved conventional methods both in humans and animals. The 16 genetic studies include those carried out at human-animal interface. One study (Katale et al)[33] explored species diversity of non-tuberculous mycobacteria isolated from humans, livestock and wildlife at their interface.

In Tanzania between 2014 and 2018, there was a gradual increase of genotyping studies resulting in 66 publications (retrieved from PubMed). Similarly, 15 publications have been published that use the rapid GeneXpert test (Cepheid, USA), a molecular test for TB detecting the presence of TB bacteria, as well as testing for rifampicin resistance ***(Table 2)***. Despite the approximately 4-fold increase in genetic based studies over the previous 4 years compared to those prior to 2014, only four (4/66, 6.1%) of the studies[33,39,48,49], have focused on the animal-human interface in tuberculosis. The rest focused on human TB alone or animal TB alone or were simply diagnostic, leaving a large gap in our understanding of the dynamics of tuberculosis at the interface.

Genotyping and phylogeographical analyses done in Tanzania have highlighted the predominance of the CAS, T, EAI, and LAM MTBC lineages in Tanzania with the most frequent Spoligotype International Types (SITs) being SIT21/CAS1-Kili, SIT59/LAM11-ZWE and SIT126/EAI5. Other circulating lineages include Haalem, Beijing, LAM, S, T, and X and Manu. Studies done in the past four years while mapping the MTBC genetic diversity in Tanzania, neighbouring East African and other several African countries[49], have described a new lineage (designated EAI3-TZA) that seems to be specific to Tanzania[48,49]. The studies collectively highlighted the absence of clear evidence for recent cross-species transmission of either *M. tuberculosis* or *M. bovis* between humans, livestock and wild animals[33,39,48,49] as well as identifying a novel *M. bovis* strain which have not been previously reported in the Serengeti ecosystem [48]. This information is important when evaluating prevailing strains in an area where humans and animals are in intense contact with potential for cross-transmission.

Those studies focusing exclusively on animal to animal transmission have also highlighted that, despite wild animals being at risk of acquiring *M. bovis* infection from livestock due to occasional interactions in sharing of pasture and water sources, no *M. bovis* was isolated from hunted wild animals. It should be noted that such studies may have been biased to low endemic areas and have not sampled large numbers of animals, nor targeted animals that were visibly unhealthy and therefore the presence of *M. bovis* cannot be ruled out and this does not rule out a call for integrated efforts by all stakeholders for effective control of spread of tuberculosis[41].

In Uganda both *M. tuberculosis* and *M. bovis* have been studied in humans and animals. Characterization of modern *M. tuberculosis* strains has indicated spoligotypes and drug susceptibility patterns of isolates from tuberculosis patients to consist of strains mainly belonging to the Uganda genotype and with a low anti-TB drug resistance rate[51]. A wide diversity of strains had previously been reported[52] with the majority of the TB cases supposedly due to reactivation rather than re-infection. Studies have also shown that despite TB epidemic genotypes being predominantly localized[53,54], strain types were not associated with drug resistance nor HIV sero-status[53]. Despite having little impact on the clinical course for individual patients, infection with multiple MTBC strains has been shown to occur in patients with a first episode of pulmonary disease, in settings with high TB incidence[55].

Characterization of *M. bovis* isolates in Uganda have indicated some spoligotype patterns that had not been previously reported[56], indicating the lack of comprehensive studies in this country. It was reported that infected carcasses, even with multiple sites of infection, are not routinely condemned as unfit for human consumption, and even if this should be done, people may illegally obtain meat from the carcasses for consumption, particularly in rural areas [56]. This situation occurs in most of the traditional African communities and societies where cattle are kept for prestige rather than as a source of wealth and food. This cultural difference between so-called developed and developing countries necessitates the need for specific and relevant approaches in control strategies[57].

TABLE 1. ***Published Tuberculosis research in humans and animals performed in Tanzania from the year 2000 to 2018*.***

Infection category	Target host	Method	Year	Author
Bovine tuberculosis	Cattle	Tuberculin test	2001	Kazwala et al [58]
M. bovis infection	humans	Culture and biochemical typing	2001	Kazwala et al [59]
Diseases of humans and their domestic mammals	Humans and domestic mammals	Species database construction	2001	Cleaveland et al [60]
M. tuberculosis and M. bovis	humans	Interview	2003a	Mfinanga et al [61]
M. tuberculosis and M. bovis	humans	Interview	2003b	Mfinanga et al [62]
Bovine tuberculosis and non-specific infections	Cattle	Tuberculin test	2003	Shirima et al [63]
M. tuberculosis, M. bovis, non-tuberculous mycobacterium and HIV	Humans	Culture and comparison of isolates	2004	Mfinanga et al [64]
Bovine tuberculosis	Wildlife	Enzyme Immunoassay	2005	Cleaveland et al [65]
M. bovis	Cattle	PCR, RFLP and spoligotyping	2006	Kazwala et al [40]
M. tuberculosis	Humans	Spoligotyping	2006	Eldholm et al [43]
Human tuberculosis	Humans	Culture	2007	Søborg et al [66]
M. tuberculosis in HIV infection	Humans	Spoligotyping	2007	Kibiki et al [46]

Human tuberculosis	Human	Microscopy, culture, drug susceptibility testing, Chest X-ray and CD4+T cells count (blood)	2008	Ngowi et al [67]
Common zoonoses	Humans	Structured questionnaire	2008	John et al [68]
Bovine tuberculosis and atypical mycobacterioses	Cattle	Tuberculin test, milk culture, RNA sequencing and PCR	2009	Durnez et al [38]
Various zoonoses	Human, animal and environment	Review	2009	Mazet et al [69]
TB infection	Humans	TB detection using rats from sputum samples	2009	Weetjens et al [70]
TB infection	Humans	TB detection using rats from sputum samples	2010	Poling et al [71]
Bovine tuberculosis	Cattle	Spoligotyping, VNTR typing, microarray analysis, deletion typing and IS*6110* RFLP typing	2011	Berg et al[#] [37]
Non-specific mycobacteria	Small mammals (rodents)	Culture of isolates and PCR	2011	Durnez et al [42]
Bovine tuberculosis	Cattle and wildlife	Deletion typing and spoligotyping	2013	Mwakapuja et al [41]
TB infection	Humans	*M. tuberculosis* DNA sequencing for resistance mutations in rpoB	2013	Mpagama et al [36]
Non-tuberculous mycobacteria	Cattle-human interface	Culture, PCR and sequencing	2014	Katale et al [33]
TB infection and drug resistance	Humans	Culture, PCR, spoligotyping	2014	Kidenya et al [34]
TB infection	Humans-animal interface	Culture, PCR, spoligotyping, MIRU-VNTR	2015	Mbugi et al [48]
Bovine tuberculosis	Human-animal interface	Culture, PCR, spoligotyping, MIRU-VNTR	2015	Katale et al [39]
Disseminated TB in HIV-infected patients	Humans	GeneXpert	2015	Gamell et al [72]
TB infection	Humans	Microarrays-based spoligotyping, MIRU-VNTR	2016	Hoza et al [44]
TB infection	Human-animal interface	Spoligotyping and molecular data mining	2016	Mbugi et al [49]
TB infection	Humans	GenoType®	2016	Hoza et al [45]
Tuberculous spondylitis	Humans	GeneXpert MTB/RIF	2016	Sikalengo et al [73]
TB infection	Humans	GeneXpert MTB/RIF	2017	Sariko et al [74]
TB infection	Humans	GeneXpert®	2017	Mnyambwa et al [75]
TB infection	Humans	GeneXpert (MTB/RIF) assay and culture on the Lowenstein Jensen (LJ) media	2017	Kidenya et al [50]
TB infection	Humans	Xpert MTB/RIF assay	2017	Mbelele et al [76]
TB infection	Humans	GeneXpert GxAlert platform	2018	Mnyambwa et al [77]
TB infection	Humans	Fluorescent smear microscopy, GeneXpert MTB/RIF and Löwenstein-Jensen (LJ) culture	2018	Beyanga et al [78]
TB/HIV Co-infection	Humans	GeneXpert® MTB/RIF assay and the MPT64 test	2018	Jørstad et al [79]
TB infection	Humans	Culture, spoligotyping and WGS	2018	Kidenya et al [47]
TB Infection	Humans	Whole genome shotgun sequencing and comparative microbial genomic analyses	2018	Mnyambwa et al [35]

**** The survey (Table 1) was based on research published in internationally reputable journals retrievable from PubMed and other electronic databases (Google and Google Scholar)***

[#] Samples were obtained from cattle in Tanzania, and laboratory work done elsewhere

Disease dynamics and risk of resurgence

Major factors potentiating TB resurgence include poverty, consequently poor health facilities, which lead to failures in the treatment systems as well as immigration[80]. In addition, a more recent risk is co-morbidity with HIV infection, which increases risk of progression to active disease and latent TB

reactivation[81-83]. There is also an alarming rise in drug resistant TB cases. In most localities, it is not known whether the drug resistant cases result from treatment failures, i.e. acquisition, or are from transmission. Genotyping and sequencing studies are ideal tools to establish the major driver of these epidemics and are crucial for design of appropriate intervention and treatment [84-89]. However, this is only possible where there is adequate funding and health care facilities. Therefore, studies using advanced tools have been sporadic and limited in scope while their implementation for routine screening has been impossible in most countries. For example only three published studies[35,36,47] investigated *M. tuberculosis* at the whole genome sequencing level in Tanzania, while another one (Katale et al, Bacterial Zoonozes Community of Practice under SACIDS, Katale, personal communication) has been done but is not yet published. However, evidence suggests regional TB epidemics in Africa, characterized by genetically distinct lineages of *M. tuberculosis*. *M. tuberculosis* in these regions may have been introduced from either Europe or Asia and has spread through pastoralism, mining and war[30].

Emergence of migrating, highly virulent strains of *M. tuberculosis,* sometimes in association with multidrug resistance, is a warning sign of a serious threat to TB control[90]. This necessitates the identification of major driving forces for the transmission dynamics within specific populations, which in turn may have significant impact on disease control and vaccine development strategies[30,91]. Such goal cannot be achieved without use of advanced molecular methods for screening, diagnosis and comparative molecular epidemiological studies. Advancement in genotyping methods for examples, can provide sufficient information that can determine the approach for planning control strategies. In African settings where there is close interaction and intense contact between humans and animals (including wild animals), the risk of possible cross-transmission of antibiotic resistant TB strains between species is high. This may be an emerging problem, as suggested by the increasing isolation of *M. tuberculosis* from animals, including pigs[92]. In such circumstances, the increase in cross-transmission would consequently mean cross-transmission of resistant TB (*M. bovis* or *M. tuberculosis*) strains, identification of which, needs advanced diagnostic tools. Improved diagnostic facilities as well as the use of isoniazid prophylactic therapy in endemic areas [5] may be the best global TB control strategy particularly in this era where the focus can as well be on better management of HIV and TB co-infections. All these require early screening and diagnosis at point of care, which can be ensued via advancement in technology.

TB strain diversity in animals

A variety of molecular methods ranging from simple techniques (Polymerase Chain Reaction, PCR) to Next Generation Sequencing, have variably been adopted to characterize TB strains in diverse clonal complexes both in humans and animals. Similar to TB strain distribution in humans, studies carried in bTB, have found a degree of regional and geographic distribution. While a clonal complex of *M. bovis* (Af2) was isolated at high frequency from cattle in Uganda, Burundi, Tanzania, and Ethiopia and was identified as an East African strain[37]**,** another clonal complex (Af1) has been found to be a predominantly West African strain[93] with high frequencies of isolation from Mali, Nigeria, Cameroon, and Chad**.** Similar to clonal complexes that have been identified and destined in different regional foci in Africa are the European *M. bovis* clone complexes Eu1[21] and Eu2[94] which, within Europe, are of high frequency in certain countries and low in others, but have also been identified in Africa, most likely imported with cattle originating from Europe. Within the clonal complexes, there are specific families and strains[95], allowing further discrimination and which will allow contact tracing, infection route identification and design of interventions. All these groupings of TB strains and their distribution country-wide and regionally, has been made possible via advancement in molecular-based mycobacterial studies.

Contribution of MOTT in TB infection

Mycobacteria other than *M. tuberculosis* (MOTT), commonly known as NTMs (non-tuberculous mycobacteria), are mostly ubiquitous environmental mycobacteria found in soil, water, dust as well as in food[96-99]. They do not usually cause infection or illness in healthy animals or humans, however many can cause life threatening illness under situations when the immune response becomes weakened, for example[100-102]. Included in this group are *Mycobacterium avium complex (MAC), Mycobacterium intracellulare, Mycobacterium kansasii, Mycobacterium marinum, Mycobacterium ulcerans* which all habitually cause opportunistic infections. People with critically compromised immune response such as those with HIV/AIDS or chronic lung diseases are very vulnerable to infection by these organisms and symptomatically the disease manifests similarly to TB infection (primarily in lungs, disseminating to other body tissues), however antibiotic susceptibility is often quite different from *M tuberculosis*[103,104]. As a result, these opportunistic infections may often be confused with TB infection. As such, these mycobacteria have also been referred to as atypical mycobacteria to differentiate them from the typical mycobacterium causing tuberculosis. Contradicting views are available on the spread of atypical mycobacteria in man. Despite atypical mycobacteria living on human skin or in the nose, there is no or little evidence of the mechanism of dissemination. Reports propose the idea of atypical

mycobacterial infections as being not spread from person to person but through direct contact with the bacteria in the environment[111] including drinking water[105]. Direct transmission from animals to humans has again been questioned and considered unimportant for human infection[106] and infected patients are not necessarily isolated as a protective measure to limit human to human transmission[103]. Regardless of their mode of transmission, the impact of MOTT should receive more attention. At present there are no reliable clinical predictors for the identification of MOTT infection that have been identified for adults and in children, or animals[107]. Furthermore, identification of MOTT infection in resource-limited countries is sorely lacking[98]. A recent study in South Africa[108] has revealed the diversity of NTM species in cattle and African buffaloes ascribed to at least eight clusters, with a possible eight different NTM species. This report is particularly important as it suggests that inclusion of NTM in investigative and control strategies incorporated in various TB control programs could be important. In so doing any chances of spread of infection both in humans and animals might, to a greater extent, be minimized. The presence of NTM species in all likelihood negatively affects the host immune response to tuberculosis and thus progression to TB disease and will possibly complicate immune based diagnostics[108].

Trends in Molecular based Mycobacterial studies

We found published work on tuberculosis in Africa ranging from 7 articles in the 1950s to 600 publications in the 2000s as retrieved from PubMed which is over 80 folds higher in the 2000s than in the 1950s (Search strategy: *(Diverse TB strains + Human +Animals) AND Tanzania + Africa + Sub-Saharan Africa;* (*Diverse TB strains + human+ animals) OR Tanzania + Africa + Sub-Saharan Africa)*. The search was also complemented by Google and Google Scholar search to widen the chance of capturing more articles. Microsoft Excel sheet was used to add up the number of articles in each category and for calculating proportions where needed to. Where specific tests were needed, we included in the search term e.g. DNA sequencing, GeneXpert accordingly. A total of 716 articles ***(Table 2)*** were obtained and manually categorized by EVM into different categories namely molecular, GeneXpert or others (all other approaches than molecular-based). The contribution of published molecular work in TB to the general research work is 17.7% of the total retrieved published work from 1950-2018. Among these, 15.1% are typical genotyping emanating publications and about 2.6% from GeneXpert ***(Table 2)***. In the Table we also note that the move from conventional to genotyping molecular methods has been increasing with time with larger proportions of molecular work clustering in the years 2000 – 2009. The trend however is not uniform which could be a reflection of donor dependence for funding such projects and thus periodicity. Too much dependence on donors may have a detrimental effect (boom and bust cycles) and since donors may not prioritize on developing country problems, instead being rather biased to donor priorities. Abuzeid[109] highlights problems with foreign aid flows from developed to developing countries. This paper further emphasizes the need for examination of such aids for potential detrimental effects, instead of providing solutions to poverty as envisaged. The study also uncovers that most of GeneXpert-based studies falls in the years 2010 onwards, an indication that the technique is relatively new in terms of implementation in Africa.

Table 2: ***Track record of published work in TB done in Tanzania and other sub-Saharan countries indicating the contribution of molecular methods approach in general research profile.***

SN	No. of Manuscripts	Year of publication	Molecular	Xpert MTB/RIF	Other areas	Tanzania	Others
1	39	2018	11	3	25	20	19
2	79	2017	21	5	53	42	37
3	50	2016	13	3	34	22	28
4	71	2015	12	2	57	25	46
5	56	2014	9	2	45	38	18
6	46	2013	5	2	39	34	12
7	53	2012	5	1	47	42	11
8	33	2011	1	1	31	24	9
9	32	2010	4	0	28	24	8
10	26	2009	6	0	20	14	12
11	22	2008	2	0	20	16	6
12	22	2007	4	0	18	19	3

13	19	2006	3	0	16	12	7
14	11	2005	1	0	10	7	4
15	6	2004	1	0	5	5	1
16	11	2003	1	0	10	7	4
17	4	2002	1	0	3	1	3
18	11	2001	0	0	11	9	2
19	9	2000	3	0	6	5	4
20	7	1999	0	0	7	3	4
21	5	1998	1	0	4	3	2
22	6	1997	0	0	6	4	2
23	10	1996	1	0	9	8	2
24	18	1995	1	0	17	12	6
25	12	1994	2	0	10	8	4
26	6	1993	0	0	6	4	2
27	3	1992	0	0	3	3	0
28	10	1991	0	0	10	4	6
29	6	1990	0	0	6	4	2
30	2	1989	0	0	2	2	0
31	1	1985	0	0	1	1	0
32	2	1984	0	0	2	2	0
33	1	1983	0	0	1	1	0
34	2	1982	0	0	2	2	0
35	1	1978	0	0	1	0	1
36	1	1977	0	0	1	1	0
37	2	1976	0	0	2	1	1
38	1	1975	0	0	1	1	0
39	1	1974	0	0	1	1	0
40	1	1973	0	0	1	1	0
41	1	1972	0	0	1	1	0
42	3	1971	0	0	3	1	2
43	1	1970	0	0	1	0	1
44	2	1968	0	0	2	2	0
45	1	1967	0	0	1	0	1
46	1	1962	0	0	1	1	0
47	1	1961	0	0	1	1	0
48	1	1960	0	0	1	0	1
49	1	1957	0	0	1	0	1
50	1	1953	0	0	1	0	1
51	3	1952	0	0	3	3	0
52	1	1951	0	0	1	1	0
53	1	1950	0	0	1	0	1
Total	**716**		**108**	**19**	**589**	**442**	**274**

CONCLUSION AND RECOMMENDATIONS

In Africa, the occurrence of zoonosis is common, in the main part from the close proximity of humans and animals. Exploring the disease dynamics at these interfaces could provide the key for developing successful interventions to improve health in both humans and animals. We now have molecular tools which can examine the transmission dynamics of Mycobacterial disease in these settings. In order to do this, a strategy that involves medical and veterinary professions as well as other stakeholders will be needed. The Southern African Centre for Infectious Disease Surveillance (SACIDS), as well as other consortia working under the One Health concept including CORDS (Coordinating Organizations for Regional Disease Surveillance) may be a good starting point for such an approach. From this review we found an increase in published work in retrievable papers of over 80-fold in late years compared to earlier years, which is an indicator of trend-wise development in research. Most of molecular studies, however, cluster in the mid- to late 2000s, with little developments since, suggesting a slow adoption of technology and a high cost of running molecular tests. This overview of genotyping studies and trend in molecular studies should therefore provide an insight at regional level for more dedicated efforts to invest in this area. This review lays the foundation for a more robust investigation of TB particularly at human-animal interface to reduce the potential risk of cross-species transmission. This can be achieved by using molecular screening methods to assess the disease dynamics at this interface for early detection to provoke deployment of best control strategies.

Limitations

Possibility of missing of some papers and reports that were not published is potential limitation that we think might have been the case for our review. However, the 716 published articles in the area we focused are believably, sufficient to reflect the trend in which technological advancement has been growing. We encourage researchers to publish their research work so that can be kept tracked. Our future plans are to further do a systematic review and meta-analysis that could cover the whole of Africa with an improved search strategy.

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REVIEW ARTICLE

Ethnopharmacology, Pharmacology and Phytochemistry of *Aristolochia bracteolata* Lam: A Review of an Antimalarial Plant

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ABSTRACT

Malaria remains one of the most common infectious diseases in the sub-Sahara African countries and other developing countries. Among the medicinal plants used in the endemic countries for the treatment of malaria is *Aristolochia bracteolata* Lam. due to its availability, accessibility, and traditional use. This study therefore reviewed the ethnomedicinal use, pharmacology, and the chemistry of *Aristolochia bracteolata*. Different electronic databases such as Medline/Pubmed, Cochrane Library, and Embase were searched to identify all published articles on *Aristolochia bracteolata* Lam. Key search words included ethnopharmacological use, pharmacological and phytochemical parameters of *A. bracteolata*. Retrieved articles were reviewed and synthesized. In addition, the reference list of retrieved articles was reviewed and articles which were not retrieved by previous search were hand searched. The review included original research articles that has investigated *Aristolochia bracteolata* Lam. of any study design. Only published original articles, any languages, any time of publish, and grey literature (Conference paper, theses both PhD. and Msc. technical report) were included. Those articles with full text not available, those without information of interest, e.g ethnopharmacology, pharmacology and phytochemistry of *A. bracteolata* were excluded. Despite having multiple use, the plant is mainly used in the treatment of malaria with a reported antiplasmodial activity. Aristolochic acids (AAs) were reported as the major and active ingredient among other components in the plant. The review revealed that *A. bracteolata* has various traditional use with promising pharmacological activity. However, information on its safety is limited.

Keywords: *Aristolochia bracteolata*, Ethnopharmacology, Pharmacology, Phytochemistry, South Sudan, Malaria, Aristolochic acids.

INTRODUCTION

Medicinal plants played a very important role in human life right from the ancient times till today. They comprise many chemical constituents with different pharmacological effects thereby regulating different biological mechanisms and treating different types of diseases[1]. They have a vital role in treating and preventing various diseases. Some of these medicinal plants have been reported for their antimalarial activities and have been the source of new lead drugs including artemisinin, quinine, etc.[2,3]. In addition to antimalarial efficacy, some of these plants have been reported to exhibit antidiuretic, anti-inflammatory, anti-analgesic, anticancer, antiviral, antibacterial and antifungal activities. The use of herbal medicine (HM) has become an alternative source of treatment over the past three decades to address the gap of

high cost, resistance to conventional drugs and as alternative drug for primary healthcare (PHC)[4]. Medicinal plants have played important roles in drug discovery through phytochemicals which can be directly used as medical remedy, structural basis for chemical synthesis or act as structural model for semi-synthetic drugs[5]. Medicinal plants are the richest bio-resource of drugs of traditional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, antimalarial drugs etc[4]. Many plants are useful to human lives as source of food, food supplement or therapeutic purpose, however, some have been reported to have mutagenic and genotoxic effect *in vivo*[6]. Plant toxicity may arise from contaminants like lead, mercury, arsenic and other that can be absorbed from the soils or from the end products of plant metabolism. Current studies have focused more on ethnomedicinal use, pharmacology and phytochemistry of medicinal plants used by humans. This is very significant in order to guarantee the safety of the consumers of plant products[7]. The plant toxicity may originate from different contaminants which may be chemical (organic pollutant, toxic metals or non-metals), biological (parasitic or microbiological) or agrochemical residues[8]. A number of bioassays are used in research to ascertain toxicity level of medicinal plants or herbal extracts which may be *in vivo* using laboratory animals[9] or *in vitro* using cell line cytotoxicity studies[10]. Identification of phytochemicals responsible either for biological activity or toxicity is important for enhancing the bioactive effect or preventing the toxic effect. In malaria endemic countries, medicinal plants are used as alternative for treatment of the different ailments including malaria, and has remained a first line source of novel drugs such as quinine, artemisinins etc. *Aristolochia bracteolata* is used for the treatment of various diseases in many countries including South Sudan but review on its safety, phytochemistry and efficacy is limited. This review synthesized information on ethnopharmacology, pharmacology and phytochemistry of *Aristolochia bracteolata* Lam.

METHODS

Different electronic databases searches were performed in Medline/Pubmed, Cochrane Library, Google scholar, proquest library and Embase to identify all published articles on *Aristolochia bracteolata* Lam. The key words included ethnopharmacological use, pharmacological and phytochemical parameters of *Aristolochia bracteolata*. In addition, the reference list of retrieved articles was reviewed and articles which were not retrieved by previous search were hand searched. The review included original research articles that has investigated *Aristolochia bracteolata* Lam. of any study design. Only published original articles, any languages, any time of publish, and grey literature (Conference paper, theses both PhD. and MSc., technical report) were included. Full text not available, those without information of interest, e.g. ethnopharmacology, pharmacology and phytochemistry of *A. bracteolata* were excluded.

RESULTS

Search results

After searching the data bases and hand searching a total of 215 articles were obtained. After reviewing articles for relevance, 73 were excluded. Since 23 full text were not available, 5 were reviewed articles, and the remaining does not have the information of interest. Therefore, 42 articles were finally included in this review.

Botany of Aristolochia bracteolata Lam.

The Plant *A. bracteolata Lam.* belongs to the family Aristolociaceae. The genus aristolochia has over 500 species, but those reported to be found in Africa includes; *A. elegans, A. chilensis, A. clematitis A. albida, A. baetica. A. embergeri, A. heppi, A. hockii, A. fontanesii, A. paucinervis, A. pistolochia, A. rigida, A. sempervirens and A. bracteolata*[11]. *Aristololochia bracteolata* is a climbing perennial plant with cordate leaves and dark–purple colour tubular flowers widely distributed in tropical Asia, Africa and South America[12]. It is commonly known as worm killer and classification details are provided in ***Table 1***.

TABLE 1. ***Classification of Aristolochia bracteolata* Lam.**

Family	**Aristolochiaceae**
Genus	Aristolochia
Species	*A. bracteolata*
Scientific name	*Aristolochia bracteolata* Lam.
Synonyms	*Aristolochia bracteata* Retz., *Aristolochia benadirana* Flori., *Aristolochia abbyssinica* Klotzch., *Aristolochia mauritiana* Pers. *Aristolochia crenata* Ehreb ex.Duch.
Common names	Wormkiller, Dikeritimelo. Morodi.
Habit	Climbing herb
Habitat	Dry areas, black cotton soil, riverbanks, bush lands, desert grassland and sandy soil.
Propagation	Seed

Ethnopharmacology of Aristolochia bracteolata Lam.

Aristolochia bracteolata Lam. was the leading antimalarial plant reported in the list of medicinal plants in South Sudan[13]. Other various plants that are used for the treatment of malaria include *Gardenia thunbergia, Cucumis dipsaceus, Tamarindus indica, Balanites aegyptiaca, and cassia nigricans*[13]. Apart from treating malaria, *A. bracteolata* is also used for treatment of various diseases and ailments in South Sudan traditional health system. These uses include dysentery, headache, fever, general body pain, snake bites, scorpion bites, high blood pressure, diabetes, diarrhea and stomach ache[13]. The whole plant has been reported to be of medicinal importance[13]. The plant *A. bracteolata* Lam. is the most commonly used as an antimalarial plant and sold in the markets as a source of income for the local inhabitants in South Sudan. The whole plant is either administered fresh or after sun dried. For the topical use, the plant paste is applied in the affected area and seeds are swallowed for the treatment of malaria and other stomach conditions. Its root is also powdered, infused in water and administered orally for the treatment of malaria, fever, headache, general body pain, stomachache, diarrhea and flu[13]. ***Table 2*** depicts ethnopharmacological uses of different parts of *A. bracteolata*.

TABLE 2. ***Ethnopharmacological uses of different parts of Aristolochia bracteolata* Lam.**

Plant part	Used	Reference
Whole Plant	Is crushed, soaked in water taken orally as gastric stimulant treatment, cancer, lungs inflammation dysentery, and snake bite	14
Whole Plant	For treatment of Malaria, convulsions, abdominal pain, scorpion stings, flu, vomiting, pneumonia, polymeorrhea and edema	15
Root	Root paste as vulnerary agent: 100g of fresh roots taken processed and ground to paste. It is mixed with 1 spoonful of turmeric powder, warmed and applied on wounds	16
Root and Leaf	Roots used for Scorpion stings and anti-inflammatory, leaves for malaria	17
The Whole Plant	For the treatment of malaria and other conditions like, fever, headache, general body pain, stomachache, diarrhea and flu.	13

Pharmacological activity of Aristolochia bracteolata Lam.

Aristolochia bracteolaae Lam. has been reported to have antibacterial, antifungal[18], anti-arthritis[19], hypotensive, hypothermia, antioxidant, anti-inflammatory, antihyperglycemic and antihyperlipidemic[20] activities. Hexane extract of *A. bracteolata* showed *in vitro* antiplasmodial activity on chloroquine sensitive *P. falciparum* MRC-2 strain with IC_{50} of 16 µg/mL.[21]. In another study, methanol extract of seed and root of *A. bracteolata* showed *in vitro* antiplasmodial activity on chloroquine resistant and pyrimethamine sensitive strain with IC_{50} less than 5 µg/mL. Likewise, petroleum ether/chloroform extract of whole plant of *A. bracteolata* showed *in vitro* antiplasmodial activity of 100% inhibition against *P. falciparum* at 50 µg/mL concentration[17,22]. This confirms local community claim that the plant has effect on malaria parasite. Antimicrobial activity[23] anti-arthritis activity[24], anti-allergic activity[19] and anti-oxidant property[25] were also exhibited by the plant. The ethyl acetate, acetone and methanol extracts of the root showed promising antibacterial activity on Gram positive and Gram negative bacteria, with ethyl acetate extract being the most effective[14]. *Aristolochia bracteolata* showed a promising hyperuricemia in a metabolic arthritis rat model[1] and showed a potent *in vitro* wound healing action through anti-inflammatory and proliferative effect on human dermal fibroblasts and keratinocytes[26].

Phytochemistry of Aristolochia bracteolata Lam.

Phytochemical screening of *Aristolochia bracteolata* Lam. showed that it contains presence of alkaloids, saponins, flavonoid, phenol and tannin[16]. Methanol extract of *A. bracteolata* subjected
to phytochemical screening has shown the presence of phenolic compounds, flavonoids, triterpenoids, alkaloids, steroids, cardiac glycosides, saponins and aristolochic acids A-D [4]. The stem and the root were reported to contain the alkaloid and aristolochic acids. The chief active principle of the drug is aristolochic acid, though aristolic and p-coumaric acids also appear to contribute to the activities of the drug. Aristolochic acid is 8-methoxy-3; 4-methylenedioxy – 10 – nitrophenanthrene – 1–carboxylic acid. It is intensely bitter and is optically inactive. It is the same as iso-aristolochic acid, aristolochia yellow, aristinic and aristolochic acids, but is different from aristolochine now identified as 1-curine. The aristolochic acids were host of phenanthrene derived metabolites in which the aristolactams also possessed the similar skeleton[27]. Both aristolochic acids (AAs) I and II are the major components of the plant in aristolochia genus. Phytochemical screening

of *A. bracteolata* using different solvents is presented in ***Figures 1*** and ***2*** and ***Table 3*** depict structures of aristolochic acid I and II respectively. However, in another study, methanolic extract of *A. bracteolata* Lam was purified and toxic compounds identified as AAs were isolated using different purification techniques. It was noted in previous studies that the whole plant (200g) was defatted to produce dark green oily residue (5.35%). High performance liquid chromatography (HPLC) data also showed that AA-II was represented in a higher calculated quantity of 49.03 g/kg compared to AA-I (12.98 g/kg) in *A. bracteolata L*. whole plant[31,32]. Although evidence of the presence of aristolochic I and II in *A. bracteolata* Lam. is reported by Achenbach and Fischer[33], Kumar[34,35] reported absence of Aristolochic II in this plant. Variation in their results may be explained by the different techniques and methods of analysis used.

FIGURE 1: ***$C_{17}H_{11}NO_7$; Relative molecular mass: 341.27***

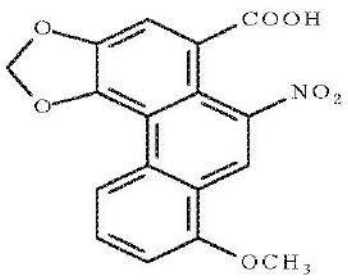

FIGURE 2. ***$C_{16}H_9NO_6$; Relative molecular mass: 311.25***

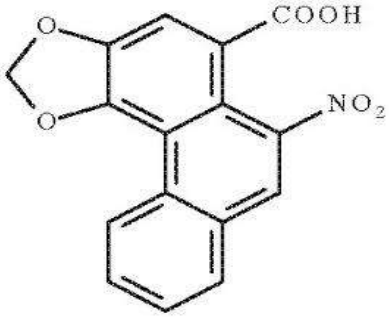

TABLE 3. ***Phytochemical screening of Aristolochia bracteolata* Lam. *using different solvents***

Plant Part Used	Extract Solvents	Phytochemicals	Reference
Whole Plant	Methanol Extract	Presence of alkaloids, triterpenoids, glycosides, steroids, tannins, phenolic compounds, flavonoids and cardio glycosides	27
Whole Plant	Methanol	phenolic compounds, flavonoids, triterpenoids, alkaloids, steroids, cardiac glycosides, saponins and aristolochi acid-A, and aristolochic acid-D	24
Leaf Part	Methanol & ethyl acetate	Presence of alkaloids, glycosides, phytosterol, saponins, tannins, phenol, carbohydrates	28
Leaf	Increasing order of polarity from petroleum ether to benzene, chloroform, acetone and alcohol extract	Presence of alkaloids, saponin, glycosides, steroids, tannins, phenolic compounds, flavonoids	29
Leaf	Methanol extract	Presence of alkaloids, saponin, steroids, tannins, terpenoids, flavonoids and glycosides	30
Leaf	Aqueous extract	Presence of alkaloids, saponins, steroids, tannins, phenol flavonoids, carbohydrates and glycosides	28

Toxicity of Aristolochia bracteolata Lam.

Most of the plant family Aristolochiaceae are said to contain aristolochic acids (AAs)[15]. Pure AAs from *A. bracteolata* plant has been reported for nephrotoxic, mutagenic and carcinogenic in the tested animals after a prolong administration. In experimental animals, high doses of aristolochic acids administered either orally or intravenously caused severe necrosis of the renal tubules[36]. However, there is limited evidence in human on the carcinogenicity of the plant. The acute oral toxicity study on *A. bracteolata* extract showed no mortality and any sign of toxicity after dosing at 2000 mg/kg[7]. In a similar study,[29,32] the ethanol extract of *A. bracteolata* administered orally at 1000, 2000, 3000, 4000, and 8000 mg/kg did not produce any sign of toxicity and mortality in rats when observed for 14 days post-administration, which could be safety-acutely.

The kidney is an important organ required by the body to perform several important functions including the maintenance of homeostasis, power of hydrogen (PH), blood pressure (BP), regulation of the extracellular environment, such as detoxification, and excretion of toxic metabolite and drugs. Kidney is also a major site of organ damage caused by drug toxicity[37]. Nephron is a basic unit structure in the kidney which functions to remove waste products, stray ions and excess water from the blood. Therefore, the kidney can be considered as a major target organ for exogenous toxicants due to nephrotoxicity[38–40].

Aristolochic acid administered orally on rats at 50 mg/kg for three days neoplastic lession on the kidneys were reported[41]. In another study, aristolochic acids administered through intraperitoneal injection on rabbits at 0.1 mg/kg for 17-21 months reported kidney tumors, ulcers, and peritoneal cavity[34].

However, it is important to recognize that safety concerns must be incorporated into a general 'risk-benefit' analysis and that toxicity of a drug does not necessarily mean that it should not be developed or approved. The aminoglycoside antibiotics, the cancer drug cisplatin and the antiviral tenofovir were some of the few mentioned examples of drugs which are proved to be nephrotoxic but efficacious in terms of treatment[42].

CONCLUSION

This review study has shown that *Aristolochia bracteolata* Lam. is used as remedy for different ailment and unlike pure aristolochic acids which is toxic, the extracts did not show any sign of toxicity from the literature. The plants have also shown a promising antiplasmodial activity which could be recommended for antimalarial study *in vivo*. It could be concluded that the plant contains different chemical constituents with aristolochic acids being the marker which is reported for a degenerative effect on the organs. This plant however has shown a promising pharmacology which could be explored in the development of future drugs development.

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EAHRC
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ORIGINAL ARTICLE

Multi-drug resistant facultative pathogenic bacteria colonizing the vagina of pregnant women with premature rupture of membrane, Tanzania

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ABSTRACT

Background: Premature rupture of membrane (PROM) contributes to approximately one-third of premature birth and 10% perinatal mortality worldwide. Here, we report the patterns of facultative pathogenic bacteria colonizing the vagina of pregnant women to guide prophylactic antibiotic treatment in the management of PROM.
Methods: This comparative cross-sectional study was conducted between August 2015 and March 2016. High vaginal swabs were collected and processed to detect the presence of facultative pathogenic bacteria. Isolate identification and antibiotic susceptibility testing was conducted using MALDI-TOF MS and VITEK-2 system, respectively. Data were analyzed using STATA version 13.
Results: A total of 175 pregnant women with PROM and 175 without PROM were investigated. The median age of the pregnant women with PROM was significantly higher than that of pregnant women without PROM: 27 [21-32] vs. 25[21-29], p=0.026. Pregnant women with PROM were significantly more likely to be colonized with facultative pathogenic bacteria 59/175 (33.7%), 95% CI; 26.7-40.7 than pregnant women without PROM; 27/175 (15.4%), 95% CI; 10.1-20.7, P<0.001. *Escherichia coli* were significantly more isolated from pregnant women with PROM than those without PROM: 36 (73.5%) vs. 13(26.5%), p<0.001. The proportion of resistance among pathogenic isolates from women with PROM to ampicillin, trimethoprim/sulfamethoxazole and cefotaxime were 100%, 66.7% and 40%, respectively.
Conclusions: The vagina of pregnant women with PROM is significantly more colonized by multi-resistant facultative pathogenic bacteria than that of pregnant women without PROM. Further studies should be done to elucidate the impact of these bacteria in relation to PROM and the pregnancy outcome.

Key words: Vaginal colonization, facultative pathogenic bacteria, pregnancy, premature rupture of membrane, multi drug resistance

INTRODUCTION

Worldwide, premature rupture of membranes (PROM) among pregnant women has been found to range from 3.3% to 10%, with 80% of them occurring at term[1,2]. PROM leads to the loss of the natural protection of the fetus hence posing a threat to bacterial infections[3]. In addition, PROM is highly associated with the increased pregnancy complications such as preterm labor, fetal demise, respiratory distress syndrome, neonatal sepsis, umbilical cord prolapse, postpartum endometritis, disseminated intravascular coagulopathy (DIC) and chorioamnionitis[1,4]. The pathogenesis of PROM has been linked to the isolation of facultative

pathogenic bacteria in the vagina[5-8], there is a strong association between pathogens colonizing the vagina and subsequent chorioamnionitis. Bacteria and protozoan parasites (*Trichomonas vaginalis*) secrete proteases and other factors that degrade the collagen and weaken the fetal membrane[9,10]. Furthermore, host inflammatory responses due to pathogenic bacteria can induce the production of prostaglandin which can lead to uterine irritability and membrane collagen degradation hence increasing the risk of PROM[8,11].

In developing countries, *Escherichia coli*, *Klebsiella pneumoniae*, Group B Streptococcus (GBS), *Staphylococcus aureus* and *Streptococcus pyogenes* have been found to be the commonest facultative pathogenic bacteria colonizing the vagina and implicated in PROM [5-7,12.] These pathogenic bacteria have also been implicated in chorioamnionitis[13,14]. Despite 12% contribution of the PROM to antenatal hospital admissions in East Africa[15] and black women being reported to have the highest risk of being colonized by the potential pathogenic bacteria[16], the spectrum of the respective bacteria colonizing the vagina of pregnant women with and without PROM in East Africa is not well understood.

A previous study[17] in Uganda among pregnant women with PROM, noted the resistance to most commonly used antibiotics such as ceftriaxone, ampicillin, trimethoprim/sulfamethoxazole and erythromycin was high with good susceptibility to expensive antibiotics such as vancomycin and meropenem. The World Health Organization (WHO) recommends the use of antibiotic prophylaxis among pregnant women with PROM[18]. Different regimen involving penicillin and erythromycin have been recommended. However, number of factors should be assessed such as population of women to be offered antenatal prophylactic antibiotics and types of antibiotics to be used. With increase antibiotic resistance among the pathogens involved in PROM local susceptibility data are crucial in establish empiric treatment protocol with emphasis on the individual tailored treatment. This comparative cross-sectional study was designed to investigate the patterns of facultative pathogenic bacteria colonizing pregnant women with and without PROM and their antimicrobial susceptibility.

METHODS

Study design, area, and population

The comparative cross-sectional study was conducted between August 2015 and March 2016 at Bugando Medical Centre (BMC), Sekou Toure Regional Hospital, Nyamagana District Hospital and Buzuruga Health Center in the city of Mwanza, Tanzania. The selected hospital are the highly populated faith base and government hospital which cover large percentage of the Mwanza population. Bugando Medical Centre is the tertiary consultant teaching hospital of Catholic University of Health and Allied Sciences with bed capacity of 900. Sekou Toure Regional Referral hospital is located in Ilemela district with bed capacity of 375 serving the all referrals from six district hospitals of Mwanza region. While Nyamagana District hospital has maternity bed capacity of 30 and Buzuruga health center has maternity ward with a bed capacity of 15. The study involved all pregnant women with gestation age of 36 weeks and above with and without PROM during the study period.

Sampling, inclusion, and exclusion criteria

Using Kirkwood formula for comparative studies[19] and assumption of the 10% effect size by the prevalence obtained in the previous study[12,] the minimum sample size obtained was 350 pregnant women (175 pregnant women with and 175 pregnant women without PROM). The pregnant women with and without PROM were recruited serially until the sample size was reached. The study excluded all pregnant women with cervical incompetency, polyhydramnios, mal-presentation, multiple pregnancies, fever, abdominal pain, foul smelling per vaginal leakage and history of antibiotic therapy in the past two weeks prior to the study to minimize the bias in relation to the colonization of the facultative bacteria. In the current study, pathogenic bacteria were defined as bacteria which are potentially capable of causing clinical infections in the genital urinary tract[20].

Sample collection and laboratory procedures:

By the use of sterile Cusco speculum, cervix was exposed, and high vaginal swab was taken using a sterile swab. The swabs were transported to the microbiology laboratory using Stuart transport media (HiMedia, India) within 2 hours of collection. All swabs were cultured on the 5% sheep blood agar (BA) and MacConkey agar (MCA) (Oxoid, UK) and aerobically incubated at 37ºC for 24-48 hours. Identification to species level was done by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Germany) on extracted cells as previous described[21,22.] Only facultative pathogenic bacteria were included for statistical analysis and subsequent antimicrobial susceptibility testing. Potential contaminants, e.g. *S. epidermidis*, *Bacillus* spp., were excluded. The current study mainly concentrated on the antibiotics that are recommended for PROM management. The tested antibiotics included: ampicillin (AMP), trimethoprim/sulfamethoxazole, (SXT), ciprofloxacin (CIP), Gentamicin (CN), ceftazidime (CAZ), ceftriaxone

(CRO) and meropenem (MRP) (Oxoid, UK). Antimicrobial susceptibility testing was done using VITEK-2 system (bioMérieux, France) and interpreted as per EUCAST (http://www.eucast.org/clinical_breakpoints/) guidelines. Using excel sheet, data were double entered, cleaned and transferred to STATA version 11 for analysis. Categorical variables such as residence, marital status, education, occupation, gravidity, history of PROM, number of antenatal care (ANC) visit, presence or absence of PROM and positive or negative bacterial growth were summarized as proportions. Continuous data (age, gestation age and parity) were summarized using median and inter quartile range. The statistical significance was set at a *p* value of less than 0.05. Two-sample test of proportions was used to compare the pattern of facultative pathogenic bacteria colonization among pregnant women with PROM and those without PROM.

Ethics approval and consent to participate

The joint CUHAS/BMC research ethics and review committee granted ethical clearance with certificate number CREC/096/2015. Permission to conduct the study was sought from all hospital administrations. All patients were requested to sign a written informed consent before recruitment was done.

RESULTS

Demographic characteristic

A total of 350 pregnant women (175 with PROM and 175 without PROM) were enrolled and analyzed. The majority of studied women resided in urban areas 286(81.7%) and had primary school education 185 (52.9%). The median age of pregnant women was 26 [21-31] years. The median age of pregnant women with PROM was significantly higher than pregnant women without PROM 27 [21-32] vs. 25 [21-29], p=0.026 ***(Table 1)***.

The median gestation age of pregnant women at the time of enrollment was 38[36-40] weeks for pregnant women with PROM and 38[37-39] for pregnant women without PROM. History of PROM in previous pregnancy was higher among pregnant women with PROM than pregnant women without PROM 20 (11.4%) vs. 6 (3.4%), p=0.004. In addition, history of preterm birth in the previous pregnancy was higher among pregnant women with PROM than those without PROM 16(72.7%) vs. 6 (27.3%), p=0.026. Regarding antenatal visits, high proportion of pregnant women with PROM had more than 4 antenatal visits compared to pregnant women without PROM 92 (52.6%) vs. 54 (30.9%), p< 0.001 ***(Table 1)***.

Bacterial colonization pattern

Of 350 women screened, 86 (24.6%) were colonized with facultative pathogenic bacteria. Pregnant women with PROM were significantly more often colonized with facultative pathogenic bacteria 59/175 (33.7%), 95% CI; 26.7-40.7 than pregnant women without PROM 27/175 (15.4%), 95% CI; 10.1-20.7, p<0.001. The most frequently isolated bacteria were *Escherichia coli* 49(39.2%) and *Pseudomonas spp*. 22(17.6%). *E. coli* isolates were significantly more from pregnant women with PROM than those without PROM, 36 (73.5%) vs. 13 (26.5%), p<0.001 ***(Table 2)***. Of 175 pregnant women with PROM, 13(7.4%) had double colonization with two different species of facultative pathogenic bacteria. All pathogenic bacteria isolated from pregnant women with PROM were resistant to ampicillin, while those isolated from pregnant women without PROM were 92.6% (25/26) resistant to ampicillin. The proportion of resistant bacteria from women with PROM to ampicillin, trimethoprim/sulfamethoxazole and cefotaxime were 57(100%), 46(66.7%) and 28(40%), respectively while for women without PROM the proportion was 25(96.2%), 21(52.5%) and 20(48.8%), respectively ***Table 3***. *E. coli* isolates from pregnant women with PROM were significantly more resistant to trimethoprim/sulphamethoxazole than *E. coli* isolates from women without PROM 82.9% (29/35) vs. 58.3% (7/12), p<0.001 ***Figure 1***. One *E. coli* isolate from pregnant woman with PROM was resistant to all antibacterial agents tested (ampicillin, ciprofloxacin, gentamycin, trimethoprim/sulphamethoxazole, ceftriaxone, ceftazidime, cefotaxime, ertapenem, meropenem).

TABLE 1. ***Socio-demographic and clinical data of 350 pregnant women studied***

Patient characteristic	Total n=350 (%)	PROM n=175 (%)	Non-PROM n=175 (%)	*p* –value
Age* years	26 [21-31]	27 [21-32]	25 [21-29]	0.026
Residence				
Urban	286 (81.7)	131 (74.86)	155 (88.6)	
Rural	64 (18.3)	44 (25.14)	20 (11.4)	0.001
Marital status				
Single	73 (20.85)	46 (26.3)	27 (15.44)	
Married	277(79.15)	129 (73.7)	148 (84.57)	0.013
Education				
Primary	185(52.85)	91 (52)	89 (50.9)	
Secondary	123(35.14)	68 (38.9)	55 (31.4)	
University	42 (12)	16 (9.14)	31 (17.7)	0.071
Gravidity				
Prime	100(28.6)	36 (20.6)	64 (36.6)	
Gravid 2	175(50.0)	86 (49.0)	89 (50.9)	
Multigravida	75 (21.4)	53 (30.3)	22 (12.6)	<0.001
GA* weeks	38 [36-40]	38 [36-40]	38 [37-39]	0.012
PROM before				
No	324(92.6)	155 (88.6)	169 (96.6)	
Yes	26 (7.4)	20 (11.4)	6 (3.4)	0.004
GA at booking*	20 [18-22]	20 [18-22]	20 [18-22]	0.236
ANC visit				
Below 4	79 (22.6)	29 (16.6)	50 (28.6)	
4	125(35.7)	54 (30.9)	71 (40.6)	
Above 4	146(41.7)	92 (52.6)	54 (30.9)	<0.001

GA is gestation age, * Are variables were median is the measure of central tendency

TABLE 2. ***Vaginal pathogenic bacteria colonizing 350 pregnant women in Mwanza***

BACTERIA	PROM N (%)	Non-PROM N (%)	p- value
E. coli (49)	36 (73.5)	13 (26.5)	<0.001
Pseudomonas spp. (22)	12 (54.6)	10 (45.5)	0.273
K. pneumoniae (17)	11 (64.7)	6 (35.3)	0.043
Enterobacter spp. (13)	8(61.5)	5(38.5)	0.119
Pathogenic GPB* (10)	9(90)	1(10)	0.002
Acinetobacter spp. (7)	1(14.3)	6(85.7)	0.0038
Other GNB* (7)	5(71.4)	2(28.6)	0.054
Total (125)	82(65.6)	40(34.4)	<0.001

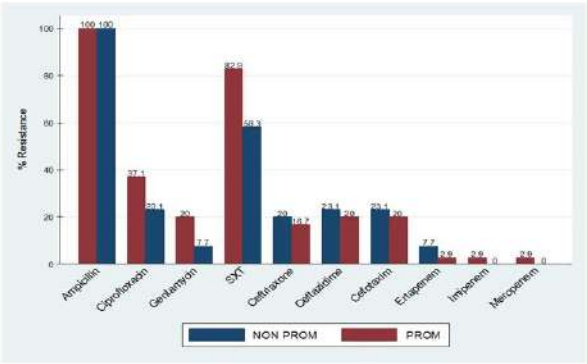
Pathogenic GPB stands for pathogenic gram-positive bacteria which include *S. aureus, S. haemolyticus, E. faecalis* and *S. saprophyticus* and other GNB stands for other gram-negative bacteria which include *Proteus* spp., *Morganella morganii* and *Escherichia hermannii.

TABLE 3. ***Antimicrobial resistance pattern among vagina pathogenic bacteria isolates***

	PROM		Non-PROM	
Antibiotic	**Tested**	**Resistant (%)**	**Tested**	**Resistant (%)**
Ampicillin	57	57 (100)	26	25 (96.2)
Ciprofloxacin	69	22 (31.9)	41	7 (17.1)
Gentamycin	69	16 (23.2)	41	8 (19.5)
SXT*	69	46 (66.7)	40	21 (52.5)
Ceftriaxone	57	14 (24.6)	22	3 (13.6)
Ceftazidime	70	17 (24.3)	40	9 (22.5)
Cefotaxim	70	28(40)	41	20 (48.8)
Ertapenem	57	1(1.8)	26	1 (4)
Imipenem	68	3(4.4)	41	0 (0.0)
Meropenem	68	2(2.9)	41	0 (0.0)

***SXT: trimethoprim/sulphamethaxazole**

FIGURE 1: ***Antibacterial resistance pattern of E. coli colonizing vagina of pregnant women with and without premature rupture of membrane***



***SXT: trimethoprim/sulphamethaxazole**

DISCUSSION

Premature rupture of the membrane (PROM) contributes to approximately one third of premature births[2,23,24], and approximately 10% of perinatal mortality[25]. A significant proportion of pregnant women with PROM in the current study had history of PROM and premature delivery in the previous pregnancy. This has also been reported elsewhere[26, 27] and could be due to the possibility of the pregnant woman genetic defect in collagen synthesis that can affect the structure and function of the fibrillar collagens[28]. The weakening of the connective tissue by the enzymatic depolarization of the collagen fibers in fetal membrane can also explain the observed findings[29,30]. Women with PROM in the current study were significantly older than those without PROM pointing to the possibility of age-dependent collagen synthesis[31]. However, there is no documentation of the influence of age and facultative pathogenic bacteria colonizing vagina during the reproductive age. The age has been found to influence normal flora before puberty and after menopause[32, 33.]

Vaginal colonization with facultative pathogenic bacteria was significantly more often observed in pregnant women with PROM than in pregnant women without PROM. This has also been reported in previous studies in Tanzania and India[12,25]. Bacteria in the vagina have been found to secrete enzymes that can either degrade the fetal membranes or increase production of prostaglandins[4,12,34-39] high concentration of prostaglandins can stimulate the uterine contractions leading to the membrane rupture. This is further supported by the fact that the presence of pathogenic bacteria has been associated with chorion thinning among PROM pregnant women[40.]

As it was also previously reported in Tanzania and India[12,41], *E. coli* and *Pseudomonas* spp. were the commonest bacteria detected. This could partly be explained by the fact that these pathogens belong to the normal flora of the gastrointestinal tract and might therefore be present in perineum with increased chance to colonize the genital tract. *Staphylococcus aureus* was the commonest gram-positive bacterial species detected among pregnant women with PROM in this study. Similar observations were made previously[12 25]. Detection of *S. aureus* colonizing women with PROM has been linked with other factors like urinary tract infections and bacterial vaginosis[25], these factors were not investigated in the current study.

Bacteria isolated from pregnant women with PROM were more resistant to ampicillin and trimethoprim/sulfamethoxazole. Similar results have been reported in other studies from pregnant women[42,43] and post-delivery women in similar settings[44]. This could partly be explained by the fact that ampicillin and trimethoprim/sulfamethoxazole are the commonest class of antibiotic in use in the study settings. Despite the fact that this study has clearly demonstrated the significant differences in patterns of bacteria colonizing pregnant women with PROM and those without PROM, neonatal outcomes which could give more evidence on the association of vagina colonization of the facultative pathogenic bacteria and clinical fetal infections especially for the pregnant women with PROM were not recorded. Furthermore, the presence of *Candida* species were not assessed, this has been recommended for future studies.

In conclusion, vagina of pregnant women with PROM was more colonized by multi-resistant facultative pathogenic bacteria than the one of pregnant women without PROM. *Escherichia coli* strains were the commonest pathogenic bacteria and were highly resistant to ampicillin and trimethoprim/sulphamethoxazole. Further studies should be done to elucidate the impact of these pathogens in relation to PROM and the pregnancy outcome. There is a need to adjust the empirical prophylaxis treatment of PROM based on the local susceptibility profile.

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EAHRC
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ORIGINAL ARTICLE

The prevalence of antibodies to Hepatitis B core antigen among blood donors in a tertiary institution in Nairobi County, Kenya

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ABSTRACT

Background: Infections linked to blood transfusion or tissue transplants prove to be a major challenge globally because of the serological window period (WP) and a latent stage exhibited by most viral infections. The objective of this study was to determine the prevalence of anti-HBc antibodies in HBsAg negative donors at Aga Khan University Hospital Nairobi.

Methods: The current project was a cross-sectional study in which 76 donor samples that tested negative for HBsAg marker were reevaluated with an anti-HBc ELIA kit (Elecsys reagent kit) following Cobas 601(Roche, Stuttgart, Germany) machine operation manual. Anti-HBc positive samples were confirmed for positivity using polymerase chain reaction (PCR). Donor demography and risk factors such as age; gender, marital status, employment status, and donor type were reviewed and documented from the archived questionnaires.

Results: In total, 76 archived samples were screened during the period of the study. The study population had an average age of 32 ± 8 years which male donors dominating at n=61 (79.2 %) and female n=16 (20.8 %). The prevalence rate of anti-HBc Total was 13 % during the period of the study. On further analysis of the 10 anti-HBc positive samples with PCR, there was no detectable HBV-DNA. The majority (63.6 %) of Anti-HBc positive cases were first-time donors and blood donors aged between 25–45 years being the majority.

Conclusions: The introduction of anti-HBc as a routine screening tool is recommended to all potential blood donors and even in other medical procedures such as dialysis. These support the need for a duo approach when screening potential donors to rule out occult infection.

Key words: Antibodies, Hepatitis B virus, anti-Hepatitis B core total, Transfusion, Blood donor, Window period

INTRODUCTION

Transfusion of blood and tissue transplants is one of the channels in which disease causing pathogens to get transmitted to the blood or tissue recipients if the donors were not screened for infectious diseases[1]. Hepatitis B virus infection is characterized by the detection of HBV-DNA, anti-HBc, anti-HBs, and HBsAg among other seromarkers, while seronegative is described by undetectability of both HBV-DNA, anti-HBc, HBsAg and anti-HBs[2]. Hepatitis B surface antigen (HBsAg) was the first marker introduced for the purpose of screening donor tissues for HBV infection since the early 1970s and is currently in use in most blood banks to date. Eventually, this sows a reduction in the number of post-transfusion HBV infection after a blood transfusion or tissue transplant[3].

Infections associated with blood transfusion or tissue transplant is still a global challenge in transfusion science and organ transplant[4,5]. Different modalities have been put in place all over the world to minimize the risk of transfusion-transmitted infections (TTIs)[6]. Some of these modalities include the use of voluntary blood donation, coming up with donor recruitment guidelines, incorporation of the quality control program in blood donor screening, and minimize blood usage by requesting clinicians[7].

A prevalence of 5.4 % anti-HBc (IgM) for instance was reported from donors with undetectable levels of HBsAg negative blood donors in a remote region of Nigeria posing a higher risk to blood recipients[8]. Immunoglobulins to hepatitis B virus core antigen (IgM) are detectable in individuals with acute hepatitis B virus (HBV) infection or with reactivation of disease in chronic carriers[9]. Total

hepatitis B core antibodies (anti-HBc IgM and IgG) immunoglobulins, on the other hand, are detected in chronic HBV infection, individuals with the previous exposure, or in the acute stage of the infection[10]. A systematic review from various countries has shown that in 2017, about 1.1 million people were newly infected with chronic HBV infection globally[11,12]. This review reported a bigger difference in the total estimates of HBV prevalence across countries; this in itself explains the difference in risk levels and mode of transmission HBV across continents. Prevalence estimated at a regional level indicated a significant burden of infection in most of the Africa countries and some countries in the Western Pacific region in particular. These reviews highlighted a continuous need for prevention strategies on HBV transmission[13,14].

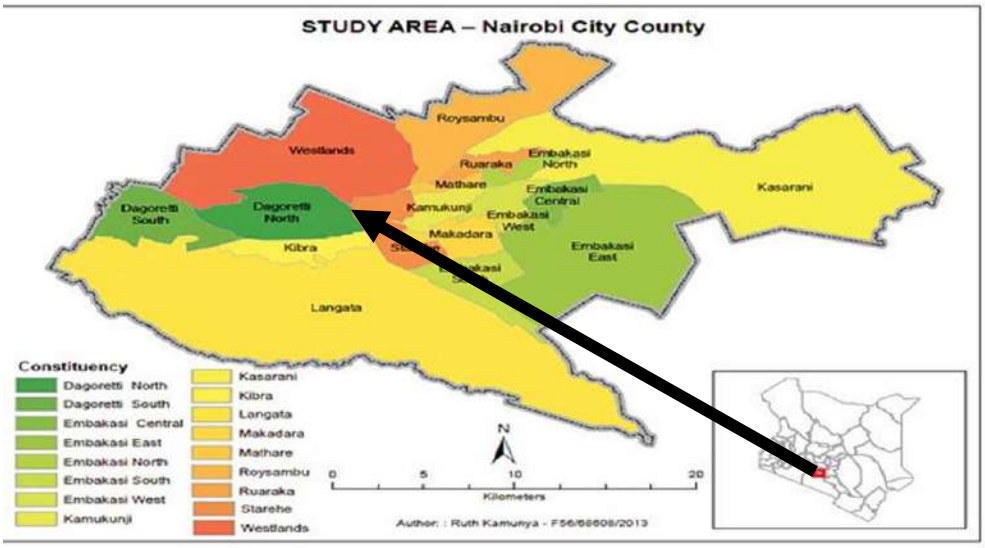
The hepatitis B virus and hepatitis C virus are the leading cause of infectious diseases that leads to higher mortality and morbidity in many communities, draining families financially and destroy economies of most countries which therefore has become a major global health burden[15]. In Sub-Saharan Africa for instance, 12.5% of recipients who receive blood transfusion are at greater risk of post-transfusion hepatitis infection[16,17]. In Kenya and other Sub Saharan Africa (SSA) countries, blood donated is screened for HIV 1 and 2, syphilis, Malaria parasite, hepatitis C virus (HCV) and HBV alone[18]. The only intervention to the prevention of transmission of HBV infection and other infectious diseases during blood transfusion is through blood screening[19]. Several scientific publications have reported the existence of HBV infection in individuals who tested negative for HBsAg but having a detectable amount of HBV virion in their hepatic cells or blood circulation[20–24]. Some of these publications have reported HBV infections as a result of transfusion of blood screened and found to be HBsAg negative[25,26]. There is strong evidence that HBsAg-negative blood donation still poses a risk of inducing post-transfusional hepatitis in recipients[27,28]. With this uncertainty of HBV screening in mind, the purpose of this study was to determine the prevalence of seropositivity of anti-HBc among healthy Kenyan blood donors and highlight its risk in blood transfusion.

METHODS

Study area

The study was carried out in Nairobi County ***(Figure 1)*** at the Aga Khan University teaching hospital (AKUHN), Parklands Sub County. Aga Khan University Hospital, Nairobi is a private not-for-profit teaching hospital that provides tertiary and secondary level healthcare services to the vast population of Nairobi County. The University Hospital has been providing high-quality care for the people of East Africa and beyond for several years now. The laboratory has a transfusion service that includes a blood bank and apheresis. In this laboratory, blood is routinely screened for malaria, syphilis, HBV, HCV, and HIV. screening of HBV is routinely done by using HBsAg assay on the Architect 1000SR. Any positive results are then confirmed on the Roche Cobas e601 (Roche, Stuttgart, Germany). Any discrepant result between the two HBsAg assays is considered indeterminate. All pints with a positive or indeterminate result for any of the TTIs screened are usually discarded.

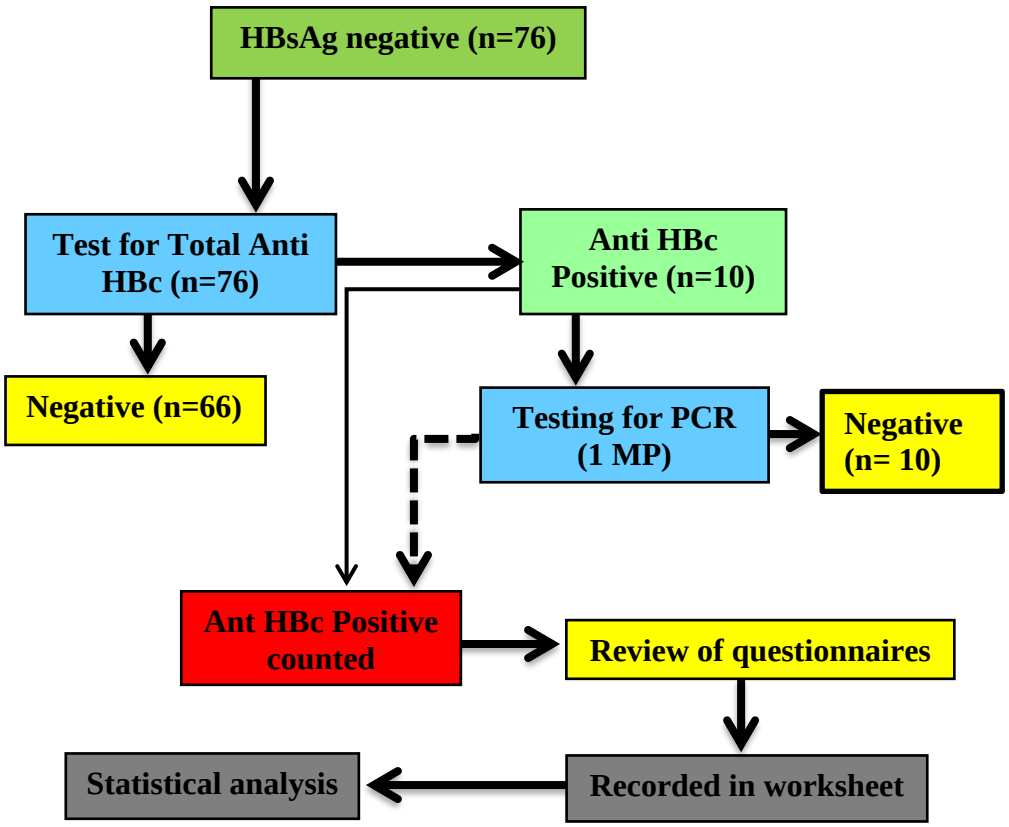
FIGURE 1. ***Study Area***



Study design

This was a laboratory-based cross-sectional study ***(Figure 2)*** design, where all serum samples screened for HBsAg during the initial blood testing of respective donors at the Aga Khan University hospital Nairobi donor unit were re-analyzed using anti-HBc Total as a marker for HBV. All samples that screened positive for Anti-HBc, plasma samples were obtained and aliquoted and were further screened by Polymerase Chain Reaction (PCR) to confirm anti-HBc positivity. Donor demography and risk factors such as age; gender, marital status, employment status, and history of blood transfusion for positive samples were reviewed by examining archived donor questionnaires stored at the donor unit.

FIGURE 2. ***Prevalence of antibodies to hepatitis B core antigen among blood donors in Nairobi City-County, Kenya***



Study population, Inclusion and Exclusion criteria

The study population included all archived samples from healthy blood donors that were initially recruited at the Aga Khan university hospital blood transfusion unit. All HBsAg negative samples collected between April 2019 to May 2019 were eligible for the study. On average, the blood bank receives 300 donors per month thus we were able to achieve the desired sample size within the specified period. All HBsAg negative blood archived samples with no signs of deterioration were included in the study. However, samples that were found to be positive for HBsAg and that showed visible deterioration were excluded from this study.

Sample Size

The minimum sample size (n= 76) was determined using the Cochran formula[29] required to allow for adequate statistical power: $n = \frac{z^2pq}{e^2}$ where z is 1.96 at 95% confidence interval; e^2 the level of desired precision (0.05); p is prevalence of occult hepatitis B infection (0.6%)[30]; q is (1-p).

Sampling procedure

Seventy-seven serum archived samples negative for HBsAg were purposively sampled for three weeks and reanalyzed for hepatitis B virus core immunoglobulins using ELIA kit (Elecsys reagent kit) following Cobas 601(Roche, Stuttgart, Germany) machine operation manual. Plasma samples for

the positive anti-HBc total were obtained from the respective donor pack, aliquoted in 1.8 ml vials and stored at -20 ^{0}C till they were analyzed. The corresponding blood questionnaires were retrieved from the blood bank storage archives. Donor demography and risk factors such as age; gender, marital status, employment status, and donor type were reviewed and documented from the archived questionnaires.

Testing for Anti-HBc

Hepatitis B core antibodies Total were analyzed using a commercial kit (Elecsys, Stuttgart, Germany) by following the manufacturer's instructions for Cobas 601.[31] At the main laboratory, the samples were allowed to attain room temperature before analysis using the immunoassay technique on Cobas 601(Roche, Stuttgart, Germany) following a written and approved SOP. Before processing, the selected samples were provided with a unique code for easier identification. The results were entered on a laboratory sheet bearing the unique code for the sample. The report was either read reactive for anti-HBc Total or non-reactive for anti-HBc Total.

Ethical consideration

Because the study was dealing with archived samples no consent was sort from the donors, but an approval was granted by the AKU research ethical Committee (REC)- Ref: 2019/REC-27 (VL).

Data analysis

The data generated was cleaned and entered into an excel worksheet then analyzed using IBM SPSS version 20 (IBM Corporation, New York, USA). Chi-square was applied to determine if there was any association between donor socio-demographic characteristics, exposure to risk factors and antiHBc positivity. Where $p < 0.05$ was of statistical significance. The prevalence of HBV was then calculated and expressed in percentage.

RESULTS

From the beginning of April 2019 to the end of May 2019, 76 we purposively selected blood donor archived samples and reevaluated them see if Total antiHBc was present. The study population had a mean age of 32 ± 8 years with the majority of the samples coming from the male donors at n=61 (79.2 %) and female n=16 (20.8 %) ***(Table 1)***. At the conclusion of the study, the prevalence rate of anti-HBc Total was 10/76 (13 %) ***(Figure 3)***. We further analyzed the 10 anti-HBc positive samples with PCR to confirm the seropositivity, there was no detectable HBV-DNA in the positive samples. We also noted that the majority (63.6 %) of Anti-HBc positive cases were mostly first-time blood donors ***(Table 2)*** and aged between 25–45 years ***(Figure 4)***.

TABLE 1. ***Socio-demographic characteristics of 76 hepatitis B surface antigen negative blood donors***

Characteristics		Frequencies (n)	Percentages (%)
Age(years)	16-24 yrs.	12	15.6
	25-45 yrs.	58	75.3
	46-55 yrs.	6	7.8
	56-65 yrs.	1	1.3
Sex	Male	61	79.2
	Female	16	20.8
Marital status	Single	35	45.5
	Married	41	53.2
	Widowed	1	1.3
Employment	Self-employed	54	70.1
	Employed	15	19.5
	Unemployed	8	10.4
Type of donor	First time donor	40	51.9
	Repeat Donor	31	40.3
	Regular Donor	6	7.8

FIGURE 3. ***Prevalence of anti-HBc Total in the study samples***

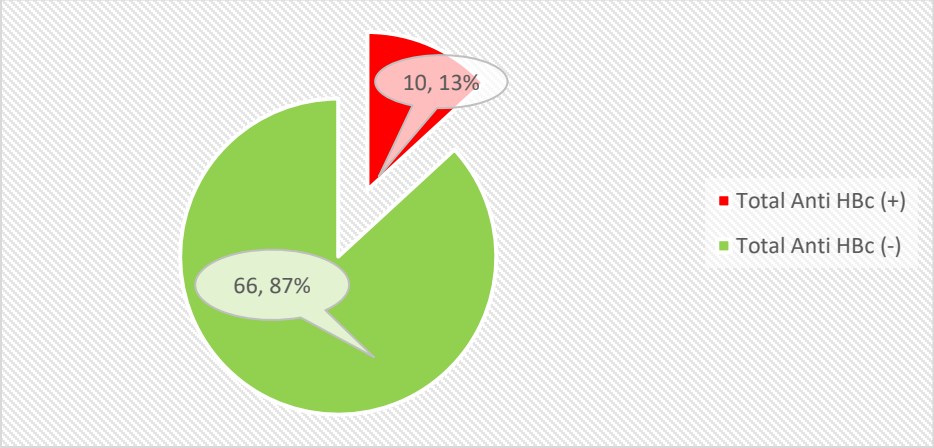


TABLE 2. Socio-demographic characteristics of 76 hepatitis B surface antigen negative blood donors

Characteristics	Exposed	Not exposed	X^2	P value
Age (years)				
16-24	2 (2.6 %)	10 (13.2%)	1.294	0.731
25-45	9 (11.8%)	49 (64.5%)		
46-55	0	6 (7.9%)		
56-65	0	1 (1.3%)		
Gender				
Male	10 (13.1%)	51 (67.1%)	1.065	0.301**
Female	1 (1.3%)	15 (19.7%)		
Marital status				
Single	6 (7.9%)	29 (38.2%)	6.920	0.031**
Married	4 (5.3%)	37 (48.7%)		
Widowed	1(1.3%)	0		
Employment				
Self-employed	9(11.8%)	45(59.2%)	0.982	0.612
Employed	1(1.3%)	14(18.4%)		
Unemployed	1(1.3%)	7(9.2%)		

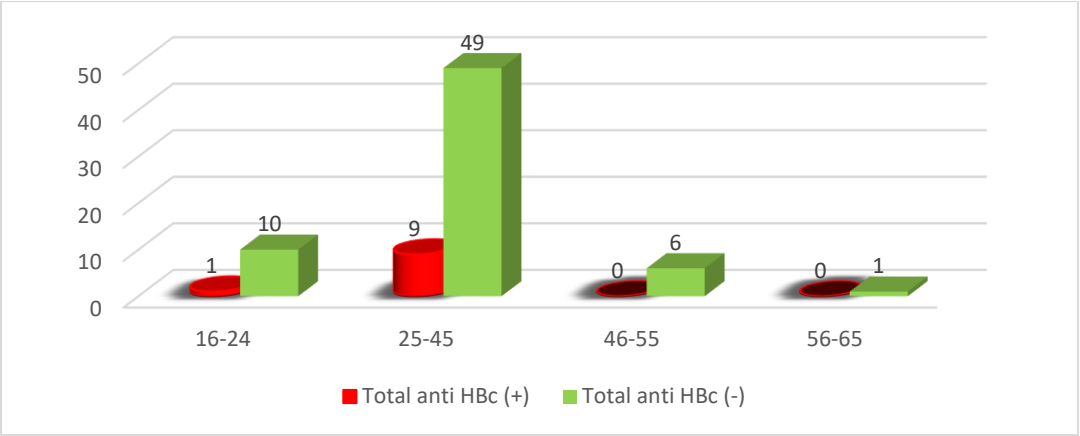
Prevalence of Anti-HBc in the Study Population

In total we analyzed 76 HBsAg negative samples on various during the study period. A total of *n*=10 samples were reactive for anti-HBc representing a 13% prevalence rate ***(Figure 3)***. The current picture indicates that apart from the study donors being negative for HBsAg, at one point they had exposure to the hepatitis B virus. These might present a donor with previous exposure or a donor who is recovering from hepatitis B infection. Such information is missed out if a donor is screened for HBsAg alone, a situation that endangers the respective blood recipient. The 87% represents individuals lacking the two serological markers (that is HBsAg and Total antiHBc), though this in itself does not rule out the existence of other HBV markers especially if the infection is in its initial stages.

Prevalence of Anti-HBc across age groups

The study also sorted to evaluate the distribution of anti-HBc marker among the blood donors. The results described in ***Figure 4*** shows that blood donors between the age of 25 to 45 had the highest reactivity to Total anti-Hbc at 11.7%. The other reactivity was seen between the age of 16-24 years at 2.6%. There were no cases reported in individuals above the age of 46 years.

Figure 4. ***Prevalence of anti-HBc Total in the study samples***



Characteristics of the Selected Archived Samples

A total of 76 archived samples at Aga Khan University Hospital in Nairobi County were selected into this study. The donor's socio-demographic characteristics are presented below.

Age of the Donors

The average age of the blood donors selected for the study samples was 32 *(SD* = 8; range 19-71) years. There were two age group peaks; of which 75.3 % of the donors were aged 25 to 45 years and 15.6 % were aged between 16 to 24 years. Other age categories included 7.8 % aged between 46 to 55 years (7.8%) and aged above 56 years (1.3%) being the list aged group ***(Table 1)***.

Gender of the Donors

In the current study, 61 (79.2%) were male donors while less than a quarter of 16 (20.8%) were female donors. Across all age groups, the majority of the participants were males. Among those aged 25-45 years' male was 44 (75.9%) while females were 14 (24.1%); those aged 16 to 24 years' male were 11 (91.7%) while females were 1 (9.3%). Among those aged 46 - 55 years (83; 69.7%) were females while (36; 30.3%) were males. For those aged 41 to 50 years 5(83.3%) were males while 1 (16.7%) were female. Those aged ≥ 55 years we had only 1 male as shown in ***Table 1***.

Occupation and Marital status of the donors

About 70.1 % of the donors were self-employment, 19.5 % were engaged either in formal or informal employment follow by 10 % donors who were students. Half of the donors (53.2%) were married, 45.5 % were single and only 1.3% widowed. Most of the donors in this study we self-employed and half of them were married ***(Table 1)***.

Risk Factors Associated with Occult Among Blood Donors

Table 2 summaries the socio-demographic characteristics of selected donors and the risk factors associated with anti-HBc positivity. Most of the donors were males 79.2 % and the female 16 % (X^2= 1.065, p = 0.301). we performed a statistical analysis to see if the was any association between the donor's socio-demographics and anti HBc seropositivity. On performing a Chi-square statistical analysis, there was no significant association between gender and anti-HBc positivity. However, this study showed that the male donors had the highest prevalence rate of 12.1 % (n=9) as compared to female donors with a prevalence rate of 1.3% (n=1).

Around 11.8 % of the anti HBc Total reactive donors were aged 25 to 45 years versus 1.3 % aged < 24 years (p = 0.731). Donors who were currently single (7.9%) were with the highest prevalence of anti-HBc Total, followed by the married donors at 5.3%. There was a significant difference between those who were single and married regarding anti-HBc positivity with a p value of 0.031. This was statistically significant, showing a strong association between marital status and anti-HBc reactivity. There was no association between donor's type (p = 0.500); age (p= 0.731), presence of a tattoo (p= 0.471); occupation (p= 0.621) and anti HBc positivity.

DISCUSSIONS

There is an increased risk of blood recipients getting infected with HBV from blood donors if blood screening is not done properly and the existence of occult hepatitis B infection in donors with HBsAg negative results. Therefore, these results show that the anti-HBc positivity was probably due to previous exposure that had resolved. Blood safety is among the major challenges encountered in blood transfusion practice today. As outlined in most blood transfusion guidelines HBsAg detection is the number one serological marker utilized for the detection of HBV in most blood transfusion centres in Kenya[32].The study also revealed that the selected samples were positive for total anti-HBc an indication that the donors had an initial exposure to HBV. However, this finding was found to be higher than the 4% described by Kisangau *et al*, in (october 2018) among the health care workers in Makueni county[33]. Similarly, it was also different to a 4.4 % prevalence rate as described in a study by Salawu *et al*, (2011) in Nigeria for anti-HBc among blood donors[34]. On a global comparison, the current study had a higher prevalence as compared to 6.3% and 8% in Saudi Arabia and Iran among potential blood donors respectively[35,36].

Nonetheless, other studies reported a higher prevalence. For instance, there was a higher prevalence rate of anti-HBc of 16.6% from Egyptian donors as described by Said *et al*, (2013).[37] Similar findings of 10.5% were reported by Lavanya *et al*, (2012) in India[38]. The distinguishing factors between this study and other studies that were done in other global regions could be attributed to varying levels of endemicity of HBV infection and differences in societal dynamics across geographical regions[39]. This lower endemicity seen in developed countries could be attributed to various factors which include; the geographical location, cultural practices regarding marriage, the availability of highly sensitive test kits, and stringent donor selection procedures, literacy levels among their population, voluntary donations, and differences in their level of civilization from the study settings[40]. The current study was carried out in a country that is still developing and facing a lot of societal issues that predispose individuals to be more vulnerable to hepatitis B infection.

In Kenya, the seroprevalence of anti-HBc is low as shown from this study in comparison to other African countries, and consequently, screening of donor blood for either anti-HBc IgM or total anti-HBc may be used in some circumstances especially if there is a historical suspicion of a donor being exposed. However, the lack of HBsAg detection as seen from the current study in itself does not guarantee blood safety because there is evidence of post-transfusion HBV infection in recipients who receive blood reactive for anti-HBc alone as reported by other researchers[41–43]. There is a compelling existence of HBV genome in blood donors reactive for only anti-HBc among the other viral markers from previous studies[44,45]. Therefore, screening for HBsAg does not rule out the possibility of HBV transmission, as the donor might be in the window phase, and detection of anti-HBc would serve as a convenient serologic marker during this period[46,47]. In Kenya, testing for HBsAg forms the initial laboratory diagnostic tool applied to confirm HBV infection in potential donors, but it does not give information on previous exposure to HBV[48]. In the current Kenya blood transfusion service (KBTS) guidelines, anti-HBc is not applied as a screening test for HBV. Therefore, as shown in this study, the safety of blood products and tissue organs should be a major priority in transfusion practice and organ transplants[49,50]. Hepatitis B surface antigen is the only diagnostic marker used in most blood transfusion centres in African countries including Kenya and it does not offer protection against HBV infection[51,17].

The current study findings have exhibited a significant association between anti-HBc positivity and some sociodemographic characteristics of the donors. There was a clear association of anti-HBc reactivity with factors such as marital status, type of donor, age and gender of the donors. From the current study people who were single were more likely to have been exposed to HBV as compared to the married or widowed an observation also reported by other authors[52]. Other studies have also reported a significant association between anti-HBc positivity with factors such as gender, age, educational level, intravenous drug use and men having sex with men activity[53,54]. From the current study, most of the positive samples were from individuals aged between 25-45 years of age. According to Alves *et al*., (2014) they showed that HBV infection was significantly associated with age between 25 and 50 years[55]. Therefore, the current research findings were in agreement with previous studies, which highlighted that middle age was associated with a higher risk of HBV exposure[56,57]. This may be because individuals in this age group are more sexually active and with a lot of risky social behaviours that expose them to HBV infection.

In this study, females were less likely to be anti-HBc positivity. A similar finding was also described by Antar *et al*., (2010) in Egypt showing that HBV infection was significantly associated with the male gender.[58] Being male was a factor for HBV exposure as reported in this study and similar finding has also been reported previously in other studies possibly due to high-risk social behaviours the makes them vulnerable to HBV infection[59]. History of using the drug of abuse and hypersexuality infection within this age bracket has been attributed to being male, and especially to those still single[60].

In this study, all the anti-HBc reactivity occurred among donors who reported not having involved themselves in risky social behaviour. This is in contradiction with other studies undertaken in southern Brazil in the years 2009 and 2010 pinpointed that tattooing, multiple sex partners, ear piercing and use of IDU increase the chance of one getting infected by HBV during adulthood[61]. Other factors that are associated with anti-HBc positivity that we either did not measure, included a history of hepatitis in the family of the donor, visiting barbershops and homosexual behaviour[62].

CONCLUSION

Following evidence of exposure to HBV infection, there is a significant chance of HBV transmission irrespective of a donor testing negative for HBsAg, and this is an important message for medical practitioners in deciding whether to transfuse blood or not. The introduction of anti-HBc as a routine screening tool is recommended to all potential blood donors and even in other medical procedures such as dialysis.

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Conflict of Interest

The authors declare no conflict of interest related to this study

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ORIGINAL ARTICLE

Antibacterial Spectrum and Susceptibility of Bacterial Pathogens Causing Diarrheal Illnesses: Cross Sectional Study of Patients Visiting Health Facility in Lake Victoria Region - Kenya

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ABSTRACT

Diarrheal illness has been studied extensively among children under five years, however little information is known among population over five years. This study determined the spectrum and antibiotic susceptibility of bacterial pathogens causing diarrhea in above five-year-old patients in a health facility along Lake Victoria region. In a cross-sectional study, 400 patients aged five years and above with diarrhea visiting Port Victoria health facility along Lake Victoria region were recruited in this study from 2013 to 2016. Stool samples were collected and cultured following standard microbiological procedures. Bacterial agents were isolated, identified and subjected to antibiotic susceptibility test. A total of 317 organisms (283 bacteria, 31 parasites and 3 others) were identified from 303 (76%) patients. Most frequently isolated pathogens being Non O1 *Vibrio cholera* 98(30.9%), *Salmonella species* 97(30.6%), *Shigella species* 47(14.8.0%) and *Aeromonas species* 38(12.0%). Non O1 *Vibrio cholerae* isolates were less sensitive to tetracycline, ampicillin and nalidixic acid. *Salmonella* species were less sensitive to ampicillin but sensitive to nalidixic acid. *Shigella* species isolates were more sensitive to chloramphenicol, cipplofloxin and nalidixic acid. Similar trend was observed for *Aeromonas* species isolates. The susceptibilities expressed were fairly good. However, some of the resistances shown pose a threat to public health, especially in unexposed populations. With the observed trends in this study, bacterial species have responded differently to antibiotics. It may be beneficial for clinicians to consider antibiotic susceptibility testing prior to management of patients to slow down resistance development.

Key words: *Diarrheal illness, Bacterial pathogens, Antibiotic susceptibility, Vibrio cholerae, Salmonella*

INTRODUCTION

Diarrheal diseases are common world-wide and among the leading causes of morbidity and mortality in developing nations. World Health Organization (WHO) report showed that diarrheal disease was the fifth most common infection accounting to mortality of 3.7% (2.2 million) in 2004[1]. In 2010, 1.4 million people, across all ages, died from diarrheal diseases, and the age-standardized mortality rate was 20.9 per 100,000, which has fallen to 49% between by 2010[2]. Diarrhea is the global second leading cause of mortality, and nearly one in five children under the age of five die as a result of dehydration, weakened immunity or malnutrition associated with diarrhea[3]. Mortality rates among children declined globally from 146 per 1,000 in 1970 to 79 per 1,000 in 2003[4]. However, the situation in Africa is strikingly different compared with other regions of the world. African region shows the smallest reductions in mortality rates and the most marked slowing down trend[5]. Diarrheal illness is the third leading cause of morbidity and mortality in children younger than 5 years of age in Africa and were responsible for 30 million cases of severe diarrhea and 330,000 deaths in 2015[6]. Diarrhea remains a major public health problem in East African, for instance, the prevalence of diarrhea among children less than five years ranged from 11-54% with an average of 27% in Kenya, Ethiopia, and Somalia from 2012 to 2017[7]. Between

2001 and 2003, diarrhea was responsible for 11.2 % hospitalized cases in Kenya. Fatality rate was 8.0% with more adults than children[8]. Diarrhea is the third most common pediatric diagnosis in rural western Kenya and particularly among adults with HIV infection[9,10,11]. Although extensive diarrheal studies have been carried out in children below the age of five years, paucity exists in individuals older than this age. Therefore, this study sought to determine bacterial agents causing diarrhea in individuals older than five years and also determine the antibiotic susceptibility test using commonly available antibiotics.

Diarrhea is the passage of three or more loose stools per day by an individual[12]. It is usually a symptom of gastrointestinal infection. Diarrhea diseases due to infection constitute a major burden of disease[12]. Systematic analysis for the Global Burden of Disease (GBD) indicated that there are various aetiologies for diarrhea in the cause list for 2010[2]. Diarrhea can be caused by bacteria, viruses or parasites[13,14,15]. Among the major diarrheal bacteria include *Vibrio species*, *Salmonella paratyphi*, *Shigella*, *Enterotoxigenic E. coli*[16,17,18]. In fact, some constitute acute infectious diarrhea[19]. Cholera remains an important cause of illness in many developing countries and has been estimated to result in more than 120,000 deaths each year[20]. The most readily recognized bacterial agents of gastroenteritis in industrialized countries are *Salmonell*a spp and *Campylobacter* Spp[13,21,22]**.** Bacillary dysentery due to either *Shigella dysenteriae* type 1 or Entoamoeba contributes to a large degree of bloody diarrhea illness based on reports from Kenya, Egypt, Ethiopia and Nicaragua[23,24,25]. The various *Shigella species* can cause diarrhea or the syndrome known as bacillary dysentery. Severe acute diarrhea is more common in travelers and older people. Travelers are exposed to drinking contaminated water and eating contaminated food prepared by other people at their destinations whereas older people have lower immunity to fight against pathogens causing diarrhea[26]. *Globally, Shigella* was the second leading cause of diarrheal mortality in 2016, accounting for 212 438 deaths among all ages and responsible for 63 713 deaths among children younger than 5 years[27].

World-wide, the etiology of bacteria causing diarrhea appears to differ depending on the geographical area. For example, a report from Spain indicated that *Campylobacter* spp. and *Salmonella* spp. were the primary bacterial pathogens, accounting for 22.2 % and 16.4% of cases of acute diarrhea in children respectively[28]. In Ecuador, *Shigella* spp. and *Campylobacter jejuni* were reported to be the main etiological causes of diarrhea[29]. Whereas in Turkey, *Salmonella* spp. (25.6%) and *C. jejuni* (18.3%) were the main causes of acute gastroenteritis in children[30]. In developing countries, Shigella, non-typhoidal Salmonella (NTS), Campylobacter, and Yersinia are the major enteropathogens causing diarrhea across all ages[31]. 22.2% diarrheal cases were caused by Aeromonas species in Pakistan and Bangladesh between 2009 and 201232. A Case-Control Study in Ifakara, Tanzania reported 47.8% children with diarrhea caused by enteropathogen of which Shigella species was the most prevalent[33].

As much as in Kenya *Vibrio* species has been isolated from stool of diarrheal patients during outbreaks[9,10,11] a prevalence of 18.8% and fatality rate of 2.5% was reported among diarrheal patients across all the ages[9]. *Salmonella* species have been isolated in studies carried out in Kenya [34,35,36,37,38,39,40,41,42,43]. However, a prevalence of 14 was reported among children of less than 12 years of age[44]. In a study carried out in Kenya on patients with sporadic bloody diarrhea, *Shigella* species was the most prevalent pathogen affecting 44% of the studied population followed by *Salmonella* species with 17%[45]. In two population-based studies, 22.3% and 23% incidences of shigellosis in Nairobi, Kenya were reported[46,47]. *Aeromonas* associated diarrheal study in Kenya reported 0% isolation of this pathogen from less than 5 years old children with diarrhea[32]. However *Aeromonas Hydrophila* was isolated from River Njoro in Kenya in 2012[48]. Another study in India reported 17.7% prevalence of *Aeromonas hydrophila* from patients with diarrhea[49]. The assumption was, there could be other more bacterial causing diarrheal illness. Therefore, this study was designed with an aim of determining if there could be other more bacterial pathogens causing diarrhea in addition to the ones that are documented.

In Africa, including Kenya, the diagnosis of diarrheal illness is based on clinical and laboratory investigations of pathogens causing diarrhea. Clinically, the doctor investigates the patients based on the presenting syndromes i.e. acute watery stool, three or more episodes of diarrhea per day or bloody diarrhea by examining the rectum. Laboratory investigations involve two aspects i.e. the use of simple microscopy technique on a stool sample to be able to identify protozoa or helminthes causing diarrhea. The second laboratory diagnosis is microbiological culture of stool sample to be able to identify bacteria causing diarrhea.

In treatment of different bacterial diseases, antibiotics such as tetracycline, doxycycline, erythromycin and streptomycin are generally used[50]. Antimicrobial resistance is one of the most important public health problems that directly relates to disease management and control[51]. Resistance to antibiotics is an increasingly common problem in human medicine. Its management is the subject of urgent debate. Efforts to reduce this resistance are based on the assumption

that it is maintained in bacterial populations as a result of exposure to antibiotics, and restricting the use of antibiotics[52]. With few exceptions, the major classes of antibiotics used to manage infections with enteric bacteria include the beta-lactams (penicillin derivatives), carbapenems, the fluoroquinolones, the aminoglycosides, and TMP-SMZ. Because most *Enterobacter* species are either very resistant to many agents or can develop resistance during antimicrobial therapy, the choice of appropriate antimicrobial agents is complicated[53,54]. In Kenya there are diverse commonly prescribed antibiotics for treating infections caused by enteric bacteria. These include, tetracycline, gentamicin, doxycycline, norfloxacin, ciprofloxacin, streptomycin, chloramphenical, cotrimoxazole, Nalidixic acid, Ampicilin, cefotoxime e.t.c[47]. Antibiotics such as tetracycline, doxycycline, norfloxacin, ciprofloxacin and streptomycin are used with adjunct in rehydration therapy and are critical in the treatment of septicemia patient[50,55,56]. Resistance to many of these drugs in general *Vibrio* pathogens have been reported in various African countries including Kenya[51, 57,58,59,60,61,62]. Antibiotic resistance of bacterial pathogens isolated from childhood diarrhea in four provinces of Kenya reported resistance of 100% of Vibrio pathogens to Nalidixic acid and chloramphenicol; sensitivity of 100% to ciprofloxacin and tetracycline and resistance of 50% to ampicillin[60].

Studies carried out for more than 10 years ago have reported resistance of *Salmonella* strains to commonly prescribed antibiotics in some parts of Kenya[34,35,36,37,38,39,40,41]. A study carried out in Kenya reported *Salmonella l*evel of resistance ranged from 44.4% to 75% for ampicillin; 22.2% to 50% for chloramphenicol; 33.3% to 66.7% for tetracycline; 0% to 25% for nalidixic acid and sensitivity of 100% to ciprofloxacin[60]**.** Treatment of shigellosis by appropriate antimicrobial agents has proven efficacious in shortening the duration of fever, diarrhea and toxaemia and apparently in reducing the risk of lethal complications as well[63]**.** The increase in the number of *Shigella* isolates resistant to most of the antibiotics (sulfonamides, tetracyclines, ampicillin, and trimethoprim-sulfamethoxazole (TMP-SMX) available in countries where the choice of treatment is limited[34], represents an important health problem. The World Health Organization (WHO) recommends that all suspected cases of shigellosis based on clinical features be treated with effective antimicrobials (antibiotics)[64]. If S*higella* dysenterea has been isolated, Beta-lactams (Ampicillin, amoxicillin, third-generation cephalosporins (cefixime, ceftriaxone), and pivmecillinam); Quinolones: (Nalidixic acid, ciprofloxacin, norfloxacin, and ofloxacin); Macrolides (Azithromycin) and others (sulfonamides, tetracycline, cotrimoxazole, and furazolidone) can be used for treatment. Resistance of this organism against antibiotics is has been reported in Kenya[12]. A study carried out among children in four provinces of Kenya reported sensitivity of 75% to 100% of *Shigella* pathogen to ciprofloxacin and nalidixic acid; 50% to 100% to chloramphenicol; 20% to 100% to tetracycline and resistance of 33.3% to 80% to ampicillin[60].

Rationale

From the literature, diarrheal illness has been studied extensively among children under five years, however, little information is known among the population over five years. Furthermore, different levels of resistances and sensitivities of these organisms against commonly prescribed antibiotics have been reported in some parts of Kenya[12,60,61,62]. However, little information has been reported in the current study site. Therefore, this study was designed to determine etiological agents causing diarrheal illnesses among individuals of five years and above and determine antibiotic susceptibility profile of these enteric pathogen and advice the clinicians on the appropriate antibiotics to use for managing diarrheal patients.

METHODS

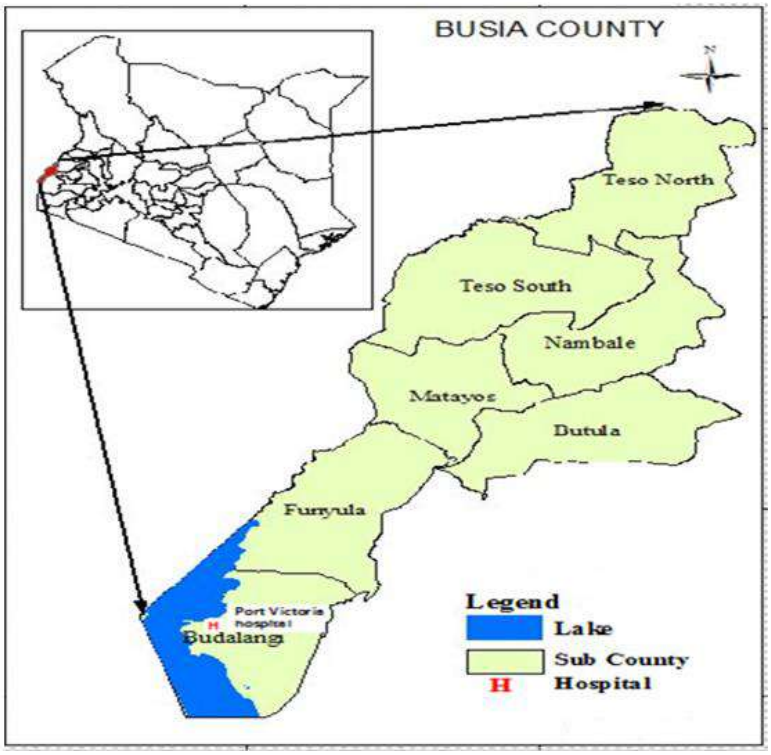
Study design

This was a cross sectional study involving patients presenting at Port Victoria hospital with three or more episodes of diarrhea per day during rainy season (April, May, June) and (August and September). A total of 100 samples were collected every year within these five months, 20 samples per months and an average of 1 sample per-day.

Study site

The study took place at Port Victoria Sub-county hospital (Figure 1) in Busia County, Kenya. This health facility borders Lake Victoria and located about 2 km away**.** Majority of residents are fisher folk from Luo and Luhyia communities. The hospital offers the following services: Antenatal, Antiretroviral Therapy, Basic comprehensive Emergency Obstetric Care, Caesarean, Curative In-patient Services, Curative Outpatient Services, Family Planning, Growth Monitoring and Promotion, HIV Counseling and Testing, Immunization, Integrated Management of Childhood Illnesses, Prevention of Mother to Child transmission of HIV, Radiology (e.g. X-ray, UltraScan, MRI, etc.), Tuberculosis Diagnosis, and Treatment, Laboratory and pharmaceutical services. Among the most diagnosed diseases are Malaria, HIV, diarrhea, pneumonia, tuberculosis etc. The climate of the area is warm and hot with two distinct seasons, dry and rainy.

FIGURE 1: ***A map of Kenya showing Port Victoria Sub-county hospital***



Source: https://www.google.com/search?g=kenya+busia+county+map

Inclusion criteria

Patients with diarrhea of three or more episodes per day, who are five years and above

Patients willing to consent to participate in the study.

Exclusion Criteria

Patients with diarrhea of less than three episodes per day and patients below five years of age Patients unwilling to consent to participate in the study.

Sample determination

The formula by fisher *et al*. 1998 was used to determine the minimum number of diarrheal patients to be sampled in this study. We suspected diarrheal illness is common perhaps 50% suspected cases during rainy season. The formula was

$n=Z_1\text{-}\alpha/2^2P(1\text{-}P)\ d^2$

where;

n=Sample size$Z_1\text{-}\alpha/2^2$ =1.96P= Expected proportion in population (50%)

d=Absolute precision.

Diarrheal causing pathogen isolation rate was assumed to be 50% and level of significance was 5%. Therefore, P=0.5, d=0.05 (5% absolute precision).

Therefore, $n=1.96^2 \times 0.5 \times (1\text{-}0.5)/0.05^2$ =384.16 patients. This was rounded to 400 stool samples from the same number of patients.

Procedure for Study Participants

All patients attending Port Victoria Sub-County Hospital and who met the inclusion criteria were sampled during rainy seasons (April to June) and August to September. A total of 100 samples were collected every year within these five months, 20 samples per months and an average of 1 sample per-day. The study purpose was explained to the patients. Those who accepted to participate in the study were requested to sign consent for recruitment.

A questionnaire was used to collect socio-demographic information such as age, gender, area of residence and occupation. Guardians/parents of minors/children were interviewed on their behalf. Patients were recruited from the outpatient clinic. We defined any diarrhea as the passage of three or more liquid stools within 24 hours, or any number (more than three times) of loose stools within 24 hours as per

WHO definition. Stool samples were collected on the same day the patient arrived at the hospital with episodes of diarrhea. Identification of bacterial pathogens and antimicrobial susceptibility was conducted following standard microbiological processes.

Specimen Collection

Stool / rectal swabs were collected from patients presenting to the hospital with diarrhea during rainy season using sterile spatula and placed in sterile polypots. These were transferred into Carry Blair transport medium (Thero Fisher Scienific Inc, USA). The specimen tubes were transported in cooler boxes from the health facility to KEMRI, CIPDCR– Busia laboratories within 48 hours. After stool processing, the culture plates were decontaminated before disposal. The study subjects benefited from free diagnosis.

Stool Processing

Microscopy Procedures

Microscopy is a technique that involves the evaluation of a stool sample under Columbus microscope (Olympus BH-2, Optical Co. ltd, Japan) to determine the presence of ova and parasite that infect the lower digestive tract, causing symptoms such as diarrhea. Most parasites have more than one form through which they develop. Many have a mature form and a cyst and/or egg (ova) form. A small amount of the stool specimen was placed onto a microscope slide. Two drops of saline were added and mixed well and placed under a microscope. This was examined at X40 for presence or absence of ova or cyst**.**

Microbiological Procedures

A loopful of stool sample was inoculated into alkaline peptone water; pH 8.2 and incubated for 8 hours at 37ºC. Subculture was done on TCBS (Central drug house ltd, India), XLD (Hardy diagnostics, USA) and Mackonkey (Oxoid, UK) and further incubated at 37ºC for 24 hours. Direct culture was carried out on TCBS, XLD, Campylobacter blood free agar (Oxoid, UK), and enrichment in Selenite F medium (Himedia laboratories PVT ltd India) to isolate the bacterial pathogens. Rectal swabs were processed in the same manner.

Identification of Bacterial Pathogens

Standard methods for the identification of both Enterobacteriacae and non-entrobacteriacae was followed (Edward and Ewing). These included colony morphology on TCBS, XLD and Mackonkey; gram stain reaction and other biochemical tests like motility in saline, oxidase production and susceptibility to vibriostatic compound O/129 at 10 and 150 micrograms (Oxoid, USA). Further identification was done on API20NE and API20E. (API bioMerieux, Sa, France).

Isolation and Characterization of Isolates

Within a maximum of 2 hours upon arrival at the KEMRI laboratories, the stool samples/rectal swabs were plated on TCBS using cotton swab and left to incubate overnight at 37°C. In the second day, TCBS plates were examined for growth of typical yellow or blue-green colonies. Further incubation was done for an additional 24 hours if no growth was identified.

Sensitivity to vibrio-static agent O129

Typical colonies were inoculated on blood agar with a sterile wire loop. Filter discs containing the vibrio-static agent O129, 10 µg and 150 µg, respectively were added to the agar surface. The resultant was incubated at room temperature for 30 minutes and then overnight at 37°C.

Oxidase test

On day 3, an oxidase strip was placed in a clean Petri dish. Using a sterile applicator stick, colonies from the TCBS agar plate were taken and rubbed in the strip. The immediate development of deep blue or purple colour was a positive test result. If there was no colour change or weak colour change after 10 seconds, this was considered a negative test result. V. cholerae O1 **NCTC** 11218 as positive control and Escherichia coli **NCTC** 10418 as negative control were used.

Motility Test Procedure

Motility refers to ability of bacteria to change direction. This is important when bacteria require moving away or towards repellents or attractants respectively. Motile bacteria are effective root colonizers and can swim towards root exudates or other nutrient gradients earlier than non-motile bacteria. A colony of a young (18 to 24 hour) culture growing on agar medium was touched with a sterile needle. This was stabbed down the center of the tube to about half the depth of the medium. This was then incubated at 35ºC-37ºC and examined daily for up to 7 days. Positive motility test was determined if organisms spread out into the medium from the site of inoculation.

A negative motility test was determined if the organisms remained at the site of inoculation. These Organisms were again placed on a microscope glass slide with a drop of distilled water; a similar slide was set as a control by adding a drop of saline. A small loop of culture was emulsified in saline using separate wires and this was covered by a glass cover slip. This was examined under x40 and oil immersion

lenses and the presence/absence of motility were confirmed.

Agglutination Test Procedure

Agglutination is the clumping of cells such as bacteria or red blood cells in the presence of an antibody or complement. An agglutination test is carried out on slides and is called slide agglutination tests. Serotyping of bacteria is a good example of slide agglutination tests. For bacterial serotyping, a saline emulsion of the bacterial colony is made on a glass slide and a drop of specific antiserum is added and mixed. Antibodies bind to the antigens on the surface of bacteria and aggregate them resulting in visible clumps. One drop of saline and one drop of anti-sera was placed on microscope glass slide. The test organism was then emulsified (flame between each drop). The slide was rotated for 30 seconds and the presence of visible agglutination recorded. The presence of agglutination in the anti-sera denoted a positive reaction. Non- agglutination was observed in the saline portion.

Drug Susceptibility Testing Procedure

A susceptibility analysis is a test that determines the "sensitivity" of bacteria to an antibiotic. It also helps the doctor determine which drugs are likely to be most effective in treating the infection. A sterile wire loop was used to inoculate pure colonies of bacterial isolates onto Muller Hinton agar (Himedia Laboratories PVT Ltd, India) plate. About three to four different antibiotic disks was spread on each agar plate containing bacterial inoculums. The plate was sealed using a parafilm and incubated for 12-24 hours at 37°C. Antibiotic inhibition zones were taken using a ruler.

The results noted down was interpreted either as sensitive or resistant depending on the standard measurement of a specific antibiotic disks.

Five antibiotics were used for susceptibility testing, Chloramphenicol, Ciprofloxacin, Ampicillin, Nalidixic acid and tetracycline.

Data Analysis

Collected data was cleaned and entered into a Microsoft excel for Windows database developed for this purpose. Analysis was done by SPSS version 20. Univariate analysis was carried out to summarize socio-demographic factors and other frequencies. Descriptive statistics was done to determine the frequency of bacterial pathogens from stool samples.

Ethical considerations

Ethical approval for this study (KEMRI/SSC 1503) was sought from the national ethical review committee at KEMRI. The study set-up and purpose were explained to the study participants/caregivers after which written informed consent was sought. For confidentiality purposes, patients' names were not used on labels, on forms, or elsewhere. Instead unique patient identification numbers were used for identification of samples.

RESULTS

Out of 400 participants (male, 187 and female, 213) had the median age of 20 years with a range of 5–60 years. A total of 171(42.8%) patients had no formal education, 123 (30.8%) had attained primary education, 86 (21.8%) had secondary education and 20 (5.0%) had gone to college and university (tertiary education). 200 (50%) patients were involved in fishing, 50 (12.5%%) were small scale traders, 20 (5%) were small scale farmers and 30 (7.5%) were fishing at the same time students and 50 (12.5%) students and 50 (12.5%) pupils ***(Table 1)***. The study sought to determine the spectrum of bacterial pathogen causing diarrhea among patients visiting Port Victoria hospital facility along Lake Victoria. From the findings, Non O1 *Vibrio Cholerae* and *Salmonella* pathogens seem to cause majority of diarrhea than the rest ***(Table 2)***.

TABLE 1. ***Socio-demographic Information of Participants***

Age (years)	Gender		Total (%)
	Male	**Female**	
5 – 12	78 (19.5)	72 (18.0)	150 (37.5)
13 – 17	54 (13.5)	60 (15.0)	114 (28.5)
≥ 18	55 (13.8)	81 (20.3)	136 (34.0)
Total	**187**	**213**	**400(100.0)**
Education			
No Education	71 (17.8)	100 (25.0)	171 (42.8)
Primary	59 (14.8)	64 (16.0)	123 (30.8)
Secondary	50 (12.5)	36 (9.0)	86 (21.5)
Tertiary	7 (1.8)	13 (3.2)	20 (5.0)
Total	**187**	**213**	**400**
Occupation			
Fishing	111 (27.7)	89 (22.3)	200 (50.0)
Small scale traders	35 (8.7)	15 (3.8)	50 (12.5)
Small scale farmers	6 (1.5)	14 (3.5)	20 (5.0)
Fishing and students	21 (5.3)	9 (2.2)	30 (7.5)
Students	22 (5.5)	28 (7.0)	50 (12.5)
Pupils	27 (6.8)	23 (5.7)	50 (12.5)
Total	**222**	**178**	**400**

TABLE 2. ***Number (%) of Bacteria Pathogens Isolated and Parasites Identified n=317***

Bacterial Pathogens	Frequencies n (%)
Non O1 Vibrio Cholerae	98 (30.9)
Salmonella species	97 (30.6)
Shigella species	47 (14.8)
Aeromonas species	38 (12.0)
Aeromonas and Giardia lamblia	3 (0.9)
Giardia lamblia and Schistosoma mansoni	11 (3.5)
Giardia lambla	6 (1.9)
Others	3 (4.4)

Sensitivity patterns of *bacterial species* to commonly prescribed antibiotics were determined. out of 98 non O1 *Vibrio cholerae* isolates, 86.7% were sensitive to chloramphenicol; 76.5% to ciprofloxacin; 65.3% to Tetracycline; 63.3% to Ampicilin and 53.1% to Nalidixic acid. Sensitivity patterns of *Salmonella* species to commonly prescribed antibiotics were also determined. out of 97 *Salmonella* species isolates, 80.4% each were sensitive to chloramphenicol and ciprofloxacin; 79.4% to Tetracycline; 50.5% to Ampicilin and 70.1% to Nalidixic acid in. Out of 47 *Shigella* species isolates, 76.6% were sensitive to chloramphenicol; 74.5 to ciprofloxacin; 61.7% to Ampicilin; 70.7 to Nalidixic acid and 70.2% to Tetracycline. Sensitivity patterns of *Aeromonas species* to commonly prescribed antibiotics were determined. Majority of the bacteria responded well to Chloramphenicol, Ciprofloxacin and Nalidixic acid and poorly to ampicillin. Out of 41 Aeromonas species isolates, 73.2% were sensitive to chloramphenicol; 73.2% to ciprofloxacin; 53.7% to Ampicilin; 70.7 to Nalidixic acid and 68.3% to Tetracycline ***(Table 3)***. A total of 317 organisms (283 bacteria, 31 parasites and 3 others) were identified from 303 patients. These were: 98(30.9%), Non O1 *Vibrio cholera;* 97(30.6%), *Salmonella species;* 47(14.8.0%), *Shigella species;* 38(12.0%), *Aeromonas species; 6(1.9%), Giardia lambla.* 3(0.9%), others. About 3(0.9%) had a mixed infection of

Aeromonas species and *Giardia lamblia* and 11(3.5%), *Giardia lamblia* and *Schistosoma mansoni* ***(Table 2)***. Sensitivity patterns of *bacterial species* to commonly prescribed antibiotics were determined. out of 98 non O1 *Vibrio cholerae* isolates, 86.7% were sensitive to chloramphenicol; 76.5% to ciprofloxacin; 65.3% to tetracycline; 63.3% to ampicilin and 53.1% to nalidixic acid. Sensitivity patterns of *Salmonella* species to antibiotics were as follows: out of 97 *Salmonella* species isolates, 80.4% each were sensitive to chloramphenicol and ciprofloxacin; 79.4% to Tetracycline; 50.5% to ampicilin and 70.1% to nalidixic acid. Out of 47 *Shigella* species isolates, 76.6% were sensitive to chloramphenicol; 74.5 to ciprofloxacin; 61.7% to ampicilin; 70.7 to nalidixic acid and 70.2% to Tetracycline. Sensitivity patterns of *Aeromonas species* to commonly prescribed antibiotics were determined. Majority of the bacteria responded well to chloramphenicol, ciprofloxacin and nalidixic acid and poorly to ampicillin. Out of 41 *Aeromonas* species isolates, 73.2% were sensitive to chloramphenicol; 73.2% to ciprofloxacin; 53.7% to ampicilin; 70.7 to nalidixic acid and 68.3% to tetracycline ***(Table 3)***.

TABLE 3. ***Susceptibility Pattern for Bacterial Isolates***

Non O1 *Vibrio cholerae* species (%). n=98	
Antibiotics	**Sensitive**
Chloramphenicol	85 (86.7)
Ciprofloxacin	75 (76.5)
Ampicillin	62 (63.3)
Nalidixic acid	52 (53.1)
Tetracycline	64 (65.3)
***Salmonella* species (%). n=97**	
Chloramphenicol	78 (80.4)
Ciprofloxacin	78 (80.4)
Ampicillin	49 (50.5)
Nalidixic acid	68 (70.1)
Tetracycline	77 (79.4)
***Shigella* species (%). n=47**	
Chloramphenicol	36 (76.6)
Ciprofloxacin	35 (74.5)
Ampicillin	29 (61.7)
Nalidixic acid	39 (83.0)
Tetracycline	33 (70.2)
***Aeromonas* species (%). n=41**	
Chloramphenicol	30 (73.2)
Ciprofloxacin	30 (73.2)
Ampicillin	22 (53.7)
Nalidixic acid	29 (70.7)
Tetracycline	28 (68.3)

DISCUSSION

The study was designed to determine pathogens isolated from patients with diarrhea who were five and more years living along Lake Victoria region of Kenya. In this study, the most affected were young individuals aged between 5 and 12 years. This is because this age bracket have poor personal hygiene including not washing hands before and after eating[65]. In this study, individuals with no or lower education were more likely to have diarrhea when compared with individuals with higher education. This finding was similar with other studies, where the prevalence of diarrhea varies according to education. Children whose mothers didn't know how to read and write[66] had relatively high diarrhea cases than those who knew how to read and write. Thus education provides the knowledge on the rules of hygiene and clean feeding[67,68].

Majority of the studied group regardless of the gender were fishermen and women. Exposure to drinking of contaminated untreated water directly from Lake Victoria[45] during fishing might have contributed to transmission of known enteric pathogens which are passed through contaminated food and water. In this study, four types of bacterial pathogens were isolated and two parasitic pathogens were identified. These bacterial pathogens were Non O1 *Vibrio cholerae, Salmonella species, Shigella species and Aeromonas species.* As much as the diarrheal infection is a common problem worldwide[69], the isolation of the first three bacteria from patients with diarrhea have been reported in previous studies including Kenya[24,43,60,70,71,72].

In this study Non O1 *Vibrio cholera* 98(30.9%) were the leading isolates causing diarrhea in the studied population. The ecosystem of Non O1 *Vibrio cholerae* being Salty and brackish environment[73] contradicts the existence of the bacteria in fresh waters of Lake Victoria. However, human activities may alter water ecosystems[74]. Lake Victoria is well characterized with extensive fishing[75] and this is evident from this study that majority of diarrheal patients were fishermen and women. Pollution of the Lake Victoria with discharge of raw sewage dumping of domestic and industrial waste, fertilizer and chemicals from farms which in turn increase the invasive water hyacinth[76] may alter the salinity of waters thus favoring the existence of these bacteria. Evaporation throughout the year due to high temperatures along Lake Victoria may lead to relatively increased salinity in freshwater bodies. In addition non O1 *Vibrio cholera* possess additional virulence factors that enable it to survive in various ecosystems[77]. Although, studies carried out in other places of Kenya have reported the isolation of serotypes of *Vibrio cholera* pathogen from stool of both children and adults[45,60,24,62].

The prevalence of these *Vibrio* species was lower especially among the children compared to the current study which constituted both children and adults. The prevalence difference could be due to groups of study participants where the previous obtained samples from children and the current from both the children and adults. Other studies in Kenya have reported isolation of this pathogen from environmental samples[61,62]**.** Another study carried out in Nigeria reported lower isolation of non O1 *Vibrio cholera* from only adult patients with acute diarrhea compared to the current[14] which studied all patients with three or more episodes of diarrhea per day excluding patients with two or one episodes of diarrhea per day.

Salmonella typhi causes typhoid fever which is spread by eating or drinking food or water contaminated with the feces of an infected person. *Salmonella species* is associated with poor sanitation and poor hygiene[78]. Port Victoria being among the areas in Kenya with poor sanitary condition may have contributed to existence of these bacteria. The finding of this investigation also confirms earlier observations of the isolation of *Salmonella* from patient with diarrhea elsewhere[79,80,81]. In this study, Salmonella species was the second prevalent organism. In comparison with other studies carried out elsewhere, the prevalence of this organism in other studies carried out in Kenya[45,39,40,43,60,] Korea[18] and Ethiopia[81] were lower compared to the current study. This difference could exist because previous studies considered characterization of S*almonella* species into specific serotypes compared to the current which did not characterize *Salmonella* species into various serotypes types.

Shigella species causes human dysentery in man[82,83.] Report from the World Health Organization is that, the bacteria leads in the cause of diarrhea in human[84] worldwide. In this study, *Shigella* species was third affecting 47(14.8%) patients presenting in the health facility with diarrhea**.** *Shigella* species has been isolated from stool of patients with diarrhea in Masaai communities in Kenya[70]. Consumption of meat by Masaai communities and fish by the participants in the current study might have served as associated factors for *Shigella* infection since this pathogen is known to be zoonotic in nature. Other previous studies have also reported *Shigella species* to cause travelers' diarrhea[85,26,86] , however the current study reports *Shigella* species to cause diarrhea in the domestic population who did not have a history of travel for the last one year. Isolation of *Aeromonas* species from clinical samples is of great concern. This bacterium was first reported from a river sample in Kenya[48]. However, in this study was least isolated from patients with diarrhea. In other previous studies carried out elsewhere, this bacterium was isolated from travelers with diarrhea, healthy carriers[72] and immune-compromised individuals due to HIV[87,88].

However, in the current study the patient had no history of travel for the last one year and their HIV status was not determined. Its isolation in the current study is linked to the fact that majority of patients were fishermen and women who come into contact with and drink water directly from the lake during fishing. This might have served as a risk factor for its transmission as evidenced by the fact that, *Aeromonas species* had been isolated from a fresh water river samples in Kenya[48] and elsewhere[89]. In the current study, all isolated pathogens were subjected to five commonly prescribed antibiotics. Sensitivity pattern showed varied sensitivities to all tested antimicrobials. Hence, there was variation in drug sensitivity patterns among all isolates compared to other studies carried out elsewhere. As much as over 50% of all the isolates were sensitive to all tested antimicrobials. The resistance shown by these pathogens still poses a threat in their management. In the current study, the predominance of Non O1 *Vibrio cholera* from diarrheal patients probably reflects a high prevalence in the community.

Tetracycline and ciprofloxacin among others have been generally considered as the drug of choice for the treatment of cholera. However, the resistance profiles indicate that these antibiotics will be less effective for treating non-01 *V. cholera* infections. In deed comparing with another previous study in Kenya[61], the resistance of this pathogen against tetracycline in the current study is slightly higher than the previous [61]. However the resistance of ciprofloxacin against *Vibri*o species was lower in the current study than the previous because this drug is expensive for the population in the studied site and it is not purchased over the counter[61]. In another study conducted along Lake Victoria, Kenya more than 10 years ago showed that, all *Vibrio* species[45] isolated were susceptible to all antibiotics that were tested in this study. This difference in antibiotic susceptibility is that the previous study considered patients who had only bloody diarrhea while the current considered all the patients with diarrhea. To add on this, the previous study was carried out more than 10 years ago of which resistance might have developed along the way. In another study carried out elsewhere[90], *Vibrio* species reported lower resistance to tetracycline and ciprofloxacin, perhaps because it was not the drug of choice to treat infection caused by this pathogen. In the current study, resistances of *Vibrio Cholerae* Non O1 was slightly more against Nalidicic acid and slightly lower against Ampicillin compared to the previous study, 30% and 42.5% respectively in China[90]. This difference in susceptibility could be due to geographical location of the current and previous studies as it is ascertained by other studies that resistances of bacteria are different basing of geographical distribution[91].

The current study identified and determined susceptibilities of *Salmonella* species against various antibiotic. Generally, susceptibilities to chloramphenicol, ciprofloxacin and tetracycline were excellent, while susceptibility of Nalidixic acid and Ampicilin were fairly good. However, resistances of some isolates to these antibiotics in the current study are of great concern especially in the studied individuals, because these may rise with time. The current study noted some differences in all the five antibiotics tested with previous study carried out in Kenya[37] where by all isolates of *Salmonella* species from sixteen adults were susceptible to ciprofloxacin, chloramphenicol and nalidixic acid while 82% of the isolates from 22 children were resistant to ampicillin, tetracycline and chloramphenicol. This difference could be explained by the fact that the previous study considered fewer blood culture samples compared to the sample size of stool cultures in the current study. There was also variation in antibiotic resistance and susceptibilities of all these tested antibiotics against *Salmonella* isolates between the current study and the previous study in Kenya[38]. The difference could be due to the fact that the previous study obtained its samples from an outbreak which probably had different species of *Salmonella* from the current. In another study carried out in Kenya[41], all *Salmonella* isolates were sensitive to Ciprofloxacin while in the current study, some isolates were resistant to this drug. This is because previous study was carried out more than 10 years ago of which resistance might have developed along the way. The current study shows that 77(79.4%) and 49(50.5%) *Salmonella* isolates are sensitive to Tetracycline and Ampicillin while the previous study shows all to be resistant[92]. The previous study obtained samples from poultry while the current from human. About 93.1% of *Salmonella* species were resistant to both Tetracycline and Ampicillin in a previous study[93]. The previous study considered both samples from both human and animals while the current samples were from only human. On the other hand, study conducted in India reported that 78.4% *S. typhi* showed resistance to chloramphenicol which is conventionally used to treat typhoid fever[94], the current study shows opposite results in that 80.4% are sensitive to chloramphenicol which is again frequently prescribed in the treatment of typhoid cases.

In this study, testing of *Shigella* species against five antibiotics has revealed various susceptibility and resistance patterns. Both patterns have revealed differences in the current and previous studies in Kenya. In the current study susceptibility of this pathogen is lower to ciprofloxacin and higher to ampicillin and tetracycline than the previous study[24].

In the current study susceptibility of this pathogen is lower to ciprofloxacin and nalidixic acid and higher to chloramphenicol, ampicillin and tetracycline than the previous study[45,60]. The differences between the current and the previous studies is that, the previous studies obtained their samples from children while the current considered both children and adults. As the antimicrobial sensitivity test results imply, 74.5% and 83.0% of the *Shigella* species isolates to be sensitive to two antimicrobials: ciprofloxacin and nalidixic acid respectively. Differently, results have been reported from Ethiopia where 100% *Shigella* species isolates were found sensitive[81] and 96.4% *Shigella* species isolates in China were resistant to nalidixic acid[95]. Sensitivity to ciprofloxacin in the present study is slightly lower than the result found from Gondar University where 91.1% were found to be sensitive[96]. In comparison to a previous study in Tehran, the percentage of resistance of *Shigellla* species against ampicillin is slightly lower with the current study which was 10% [97]. In the present study only 10(21.3%) of the isolates are resistant to tetracycline and this does not agree with related studies in Iran, India, Chile, and Nepal whose resistance was more than 50.0%[47,98,99,100,101.] Study done in India found all *Shigella* species isolates sensitive to Chloramphenicol[102], while in the current study, 36(76.6%) were sensitive and 6(12.8%) resistant. The findings of the current study do not agree with the previous Uzbekistan where some strains of *Shigella flexneri*, 28(90.4%) and *Shigella sonnei* 1(4.8%) were resistant[103]. The resistance differences between the current and the previous studies could be due to heritable factors that determines intestinal environment[87] thus heritable factors of the studied individuals in the previous studies are different from the individuals in this study.

More than a half of the isolated *Aerononas species* were susceptible to all antibiotics except Ampicillin whose susceptibility was almost half. Unlike in the previous study whose all the isolates were susceptible to Ciprofloxacin[89], only 30(73.2%) in the current study were susceptible. This difference could be attributed by the fact that the previous study considered specimen from environment and clinical sources while the current concentrated on the clinical samples only. Susceptibility of *Aeromonas* species to Tetracycline in the current study was lower than in the previous study 94.4%[89]. The data from the present study indicated 53.7% of *Aeromonas* species being susceptible to Ampicillin a situation that does not concur with results from sensitivity pattern of the previous study that showed varied sensitivities to all tested antimicrobials[104]. The previous study had 88% some biotype resisting ampicillin and 100% other biotype susceptible. The reason for varied susceptibilities in the previous study was that resistant biotype are frequently isolated than the susceptible ones a situation that could be related to our case. As much as in the current study indicate *Aeromonas* species as the least isolated pathogen from patients with diarrhea still some 17.1% and 14.6% were resistant to chloramphenicol and Nalidixic acid respectively the reason of which could be these antibiotics are still commonly prescribed in typhoid cases.

Limitation of the study

This study did not consider HIV status of the participants because the study was interested to know the type of organisms that cause diarrhea regardless of whether patients had HIV or not. It did not also analyze antibiotic testing in relation to sex and age rather it generalized the analysis. The study also did not determine whether diarrhea was caused by the virus to check the 97 samples without parasite or bacteria whether they had viruses or not.

CONCLUSION

Bacterial isolates in this study responded differently to antibiotics as compared to other previously done studies. The susceptibilities expressed were fairly good; however, some of the resistances shown pose a threat to public health, especially in unexposed populations. With the observed trends, bacterial species in different geographical settings have responded differently to antibiotics. It may be beneficial for clinicians to consider antibiotic susceptibility testing prior to management of patients to slow down resistance development.

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EAHRC
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ORIGINAL ARTICLE

Etiology Agents and Antibiotic Susceptibility profile of Cerebrospinal Meningitis: Retrospective Description of Surveillance Data in Rwanda

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ABSTRACT

Background: Cerebrospinal meningitis is an important cause of morbidity and mortality globally, but its highest burden is in sub-Saharan Africa. Surveillance is critical to assess the burden of the disease and provide the necessary information to mitigate the impact. The aim of this study is to assess the cases of Cerebrospinal meningitis occurring across the Rwanda in order to determine where the burden of illness lies, the causative agents and effective treatments.

Method: A retrospective study was carried out from January 2010 to December 2016. 173 cerebrospinal fluid samples from 25 Districts Hospitals in Rwanda were collected from patients suspected of meningitis. Demographic characteristics, region and season, etiological agents, antimicrobial susceptibility variables were analyzed with SPSS, version 22.

Results: Out of 173 suspected cases, 68 were positive for cerebrospinal meningitis. Of the positive cases 35 (56.1% were male) and 17 (25%) were under 5 years of age. The majority of cases 27 (37.5%) occurred between June and September and 22 (32%) were found in the eastern region. In this sample 32 (47.1%) of the confirmed cases were caused by *Streptococcus pneumonia*, followed by *Neisseria meningitidis* 17 (x/y; 25.1%) and *E. coli* with 3 (4.5%). Two different fungi, Cryptococcus *neoformans* and *Candida albicans* represented 3 (4.4%) of causative agents. The most common treatment was Ceftriaxone which was the most active/sensitive to all bacterial agents.

Conclusion: Children under 5 years of age are the most affected, and the higher prevalence occurs during the dry season in the eastern region. *Streptococcus pneumoniae* was the leading cause of meningitis, and ceftriaxone antibiotic was the most active to the majority of identified bacteria.

Key words: Antibiotics, Cerebrospinal meningitis, Retrospective data, Rwanda, Susceptibility.

INTRODUCTION

Meningitis is the inflammation of meninges affecting the arachnoid, and subarachnoid that occurs in response to infections or non-infections causes. Infectious causes of meningitis include bacteria, viruses, fungi, parasites. Non-infectious meningitis may be due to sarcoidosis, systemic lupus erythematous, tumor, and leukemia. [1] Bacterial meningitis is a very common and fatal disease causing a significant morbidity and mortality globally. According to the World Health Organization (WHO) 1.2 million cases of bacterial meningitis are estimated to occur every year with 170,000 deaths worldwide. [1, 2] In 2010, the WHO reported that 15,000 deaths from bacterial meningitis occurred in Europe, 18,000 in America, 20,000 in Africa, and 73,000 in South East Asia[1]. The Africa meningitis belt includes parts of 26 countries and within this zone; the burden of the disease is usually the highest in the world, with an incidence rate of 7000 to 18000 annually. There is a clear seasonal pattern of the disease, with most cases being seen in the first 6 months of the year, specifically in the dry season. [3] Studies report that airborne dust[3] and higher temperatures[3] increase the risks of transmission. Newborns, people

living in low-income countries, and those with immune-compromised conditions are at risk for high mortality and morbidity. [4, 5] The disease circulates through direct contact in the form of person to person contact, through kissing, droplets, sneezing and coughing. [5] Morbidity and mortality associated with bacterial meningitis is increased by the presence of antimicrobial-resistant organisms and incomplete knowledge of the pathogenesis [6]. Suspected bacterial meningitis is a medical emergency and immediate steps must be taken to establish specific diagnosis and treatment.

Most cases of bacterial meningitis are caused by *Streptococcus pneumonia, Streptococcus agalactiae, Neisseria meningitides, Listeria monocytogenes*, and *Hemophilus influenza B.* [7, 8, 9] Bacterial meningitis reaches the subarachnoid space through the hematogenous route or may directly reach the meninges with a parameningeal focus of infection.[8, 9] Bacterial meningitis can cause brain damage, hearing loss, limb amputation, learning disabilities, and even death. Fungi can cause severe infections but are much less frequent than bacterial or viral infections. [9, 10] The most common causes of fungi meningitis are *Cryptococcus neoformans, Candida albicans, and Aspergillus* species especially, in immunocompromised patients.[10] *Cryptococcus neoformans* is of medical importance as it is capable of crossing the blood-brain barrier and causing meningitis in both immunocompetent and immunocompromised individuals. [11, 12] Permanent neurological sequelae such as hearing loss, mental retardation, deafness, blindness, seizures disorders, and behavioral changes may occur up to 50 % of survivors even after receiving antimicrobial therapy.[13] Prevention of bacterial meningitis can be achieved through vaccinations. Effective vaccines are available against the most common causative agents. These include *H. influenza type b, Streptococcus pneumonia, and Neisseria meningitides* serogroups: A, B, C, W135, and Y [14]. In the event of infection then early diagnosis and proper case management including prompt treatment with appropriate antibiotics is needed to mitigate poor outcomes.

The African meningitis belt region stretches from Senegal in the west to Ethiopia in the east. However, the burden goes beyond this area. The causative agent of meningitis, the mortality and morbidity vary by age and geographical location. Rwanda is not included in meningitis regions. Nevertheless, cases of suspected meningitis have been reported. We therefore, conducted this retrospective study to understand the disease dynamics in the Rwandan setting. Specifically, we determined the bacterial causative agent, antibiotic susceptibility, and the spatial distribution of the affected age groups with the aim of providing evidence to support the establishment of effective preventive measures.

METHODS

In this study we retrospectively analyzed 173 Cerebrospinal Fluid (CSF) samples that had been collected from 25 District Hospitals in Rwanda between the periods of January 2010 to December 2016. All 173 cerebrospinal fluid samples were collected from patients suspected of meningitis, based on clinical signs and symptoms for bacterial meningitis, which included nausea and vomiting, headache and stiff neck, confusion, and loss of hearing.

Sample Collection

The CSF samples were obtained using standard guidelines for sterile clinical procedures. Before lumbar puncture procedure, the patients were made to lie down at an appropriate position, the skin was disinfected along a line drawn between the crests of the two ilia with 70% alcohol and iodine and allowed to dry completely .The clinician then injected the spinal needle into the skin between the 4 and 5th lumbar vertebral spines with the bevel of the needle facing up.

As soon as the needle was in position, the CSF pressure was measured and a sample of 3 to 4 ml of the fluid was collected in two sterile screw tubes for testing .Specimens were allowed to stand on the table for five minutes and inspected for colour change or 'cobweb' formation and subsequently delivered to the laboratory within half an hour after sample collection. One container was analyzed in the bacteriology laboratory in which the sample was collected and the second was sent to the National Reference Laboratory for confirmation and quality control

CSF Sample Processing

The initial processing of CSF was started by recording the volume of CSF and its macroscopic appearance. The CSF was spun to sediment cells and bacteria. The films of sediments were stained by Gram's stain technique and examined microscopically.

Culture

Samples were processed according to the standard microbiological procedures by inoculating on blood agar, chocolate agar plates, and incubated at 35ºC to in presence of 5% Carbone dioxide for the growth of fastidious bacteria, MacConkey agar, and sabouraud dextrose agar incubated aerobically at 35º C-37ºC. After 18-24 hours of incubation, the plates were observed for bacterial growth; those that showed no growth were further incubated for another 24 hours. The plates were examined for the presence of bacterial growth, and isolates were identified by standard microbiological methods including but not

limited to colony morphology, staining, serological typing, and biochemical test [16]. The disc diffusion method was performed for the antibiotic sensitivity on pure plates for penicillin, oxacillin, ceftriaxone, erythromycin, and trimethoprim-sulfamethoxazole. The selection of the panel of antimicrobials used for testing was based on the guidelines from the Clinical and Laboratory Standards Institute.[16] The inhibition diameter zones were measured around antibiotics disc were measured and interpreted as sensitive, intermediate, or resistant (for the details refer to the Clinical and Laboratory Standards Institute antimicrobial susceptibility testing, CLSI) .[16]

Secondary data obtained using the methods described were analyzed and presented to map the dynamic of cerebrospinal meningitis cases identified in Rwanda from 2010 to 2016. Note that the authors conducted the methods during this period.

Data were retrieved from National Reference Laboratory logbook and entered into Microsoft excel spreadsheet. Percentage and Chi-square were calculated using SPSS, version 22. Demographic characteristics, region and season, etiological agents, antimicrobial susceptibility variables were extracted. Using MS-Excel 2013, we analyzed data in terms of person (age and sex), place (district), time (months and years), and types of causative organisms identified among confirmed. Results were illustrated by graphs and frequency distributions generated for key variables.

Ethical consideration

Initial the data have been collected for therapeutic purpose but in order to improve the quality of surveillance, we have shared the data. To ensure confidentiality, we used coded patient identification numbers in place of names. Data held on computers were encrypted with a password.

RESULTS

The data below presents the results according to the age, region, bacterial profile, antibiotic sensitivity of each of the 68 positive cases that were identified.

Age distribution of cases

The highest proportion of cases was amongst patients under 5 years of age (17/68; 25%) followed by 6-10 year olds (14/68; 20.6%) ***(Table 1)***.

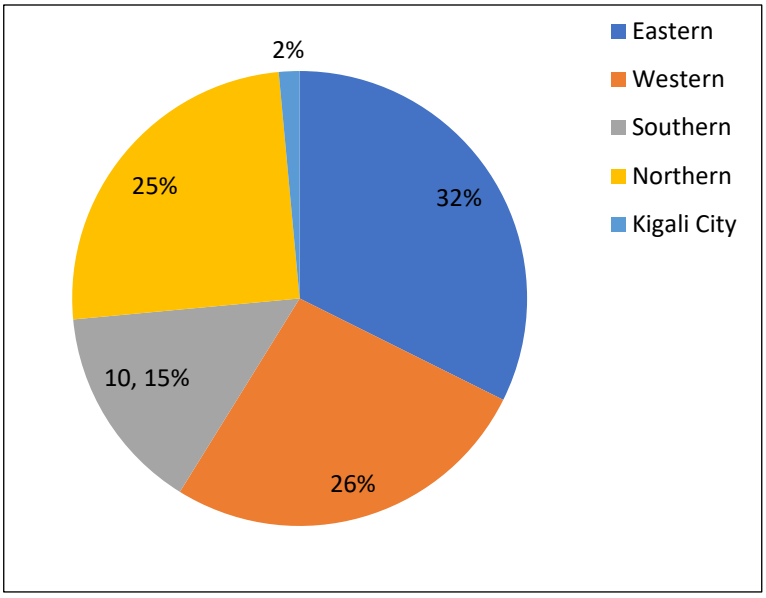
Geographical distribution of cases

The highest proportion of cases came from the eastern province (22/68; 32%) followed by the western province with a frequency (18/68; 26 %). ***Figure 1*** illustrates the geographical spread of the 68 cerebrospinal meningitis cases across the provinces in Rwanda

TABLE 1. ***Distribution of the confirmed positive cases according to the age***

Age	Frequency	Percentage
Under 5 years	17	25.0
6 - 10	14	20.6
11 - 15	9	13.2
16 - 20	10	14.7
21 - 25	2	2.9
26 - 30	3	4.4
31 - 35	5	7.4
36 - 40	1	1.5
41 - 45	4	5.9
Over 45	3	4.4
Total	**68**	**100**

FIGURE 1. ***Distribution of positive meningitis according to the provinces in Rwanda***



Etiology agents of cases

The most common pathogens isolated responsible for meningitis in this sample was *Streptococcus pneumonia* (32/68; 47.1%), followed by *Neisseria meningitis* W135 (17/68; 25.1%) and *E. coli* (3/68; 4.5%). The 16 (23.3%) remaining cases were caused by other pathogens including fungi *Cryptococcus neoformans* and *Candida albicans* ***(Table 2)***.

Antibiotic susceptibility profile

Antimicrobial susceptibility pattern of isolated bacterial pathogens was performed by Kirby Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute[16]. Inoculum was prepared by picking parts of two or three identical colonies with a sterile wire loop. This was suspended in sterile peptone water (broth) and incubated up to two hours to allow organisms to reach their log phase of growth. The density of suspension to be inoculated was determined by comparison with the opacity standard on McFarland 0.5 Barium sulphate. Sterile swab was dipped into the suspension of the isolate in the peptone water, squeezed free from excess fluid against the side of bottle and spread over the Mueller –Hinton agar plate. The test organism and the standard control from the broth were spread evenly over the surface of the Mueller –Hinton agar using sterile cotton wool swabs. Sensitivity discs for appropriate drugs were placed onto the media and incubated at 37°C for 24hrs. After 24 hours each plate was examined and growth zones were measured to the nearest millimeter, using sliding caliper which was held at the back of the inverted media plate. The results of drug susceptibility of tested antibiotics to the isolates are presented in ***(Table 3)***.

The season

We found that in this sample the largest proportion of cases occurred prevalence of in the summer between June and September in Rwanda ***(Figure 2)***.

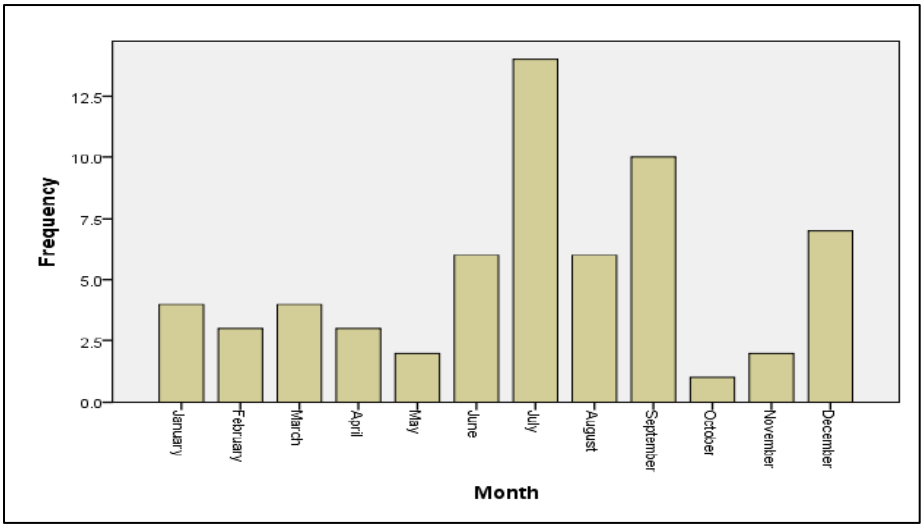
TABLE 2. ***Distribution of isolated pathogens among cerebrospinal meningitis confirmed cases***

Bacteria	Frequency	Percentage
S. pneumonia	32	47.1
N. Meningitidis W135	17	25.1
E. coli	3	4.5
S. aureus	3	4.5
C. neoformans	*2*	*3*
H. influenza B	*2*	*3*
L. monocytogenes	*2*	*3*
C. albicans	*1*	*1.4*
H. para influenza	*1*	*1.4*
P. aeruginosa	*1*	*1.4*
E. cloacae	*1*	*1.4*
S. xylosus	*1*	*1.4*
S. lugdunens	*1*	*1.4*
S. enteritidis	*1*	*1.4*
Total	**68**	**100**

TABLE 3. ***Susceptibilities of isolated Bacteria to antibiotics (in mm)***

Antibiotics/Resistance	*S. pneumoniae*	*N. meningitis w135*	*E. coli*	*H. influenza B*	*S. aureus*
Penicillin	60.1	55.3	68.5	71.3	74.5
Oxacyllin	80.5	92.8	75.6	80.6	71.1
Ceftriaxone	95.5	98.6	92.8	97.6	98.5
Erythromycin	87.6	89.7	90.8	92.7	97.7
Cefotaxime	87.7	96.7	98.9	99.3	86
Trimethoprim	92.5	97.4	98	75.3	80.4
Sulfamethazole	76.1	83.2	88.5	91.5	87

FIGURE 2. ***Prevalence of Cerebrospinal meningitis according to the seasons***



DISCUSSION

Meningitis is a serious infection and is associated with considerable mortality and morbidity in various parts of the world, with sub Saharan African countries bearing the highest burden of disease. Rwanda is located in eastern African and borders the African meningitis belt. Surveillance of meningitis is crucial to ascertain the real situation of cases and to provide evidence needed for treatment and prevention strategies. This study found that bacterial agents (specifically *Streptococcus pneumonia*) were the most common causes of meningitis in a sample of 68 cases obtained from across the country between 2010 and 2016 and sensitivity to antibiotics remained high. In this sample of confirmed cases, 56.1% were males, and amongst those under 5 years of age (17/68; 25%), finally cases were mainly recorded between June and September. Studies from other regions around the world have also found a higher prevalence of meningitis amongst males. [17] A study of the characteristic of bacterial meningitis in four major tertiary care hospitals in Bangladesh during 2003 and 2004 showed that out of 1841 cases, 71% (1307) were male [19]. Similarly, studies conducted in Ethiopia also reported a higher incidence in males. [17] In our study most cases were amongst children less than 10 years of age, which is similar to findings reported in a study conducted in North Ethiopian during 2001- 2002. [17, 18] Bacterial meningitis is known to affect children[16]. Mortality amongst this age group from this cause is also high [1, 2, 16] therefore highlighting the importance of early childhood immunization [1, 4] and rapid identification and treatment strategies to mitigate the impact of the disease. Our study found that most of the 68 cases occurred during the dry season starting from June till September, and the regions of the country (eastern and western provinces) which are the driest were also the most represented amongst the cases. Other studies have also noted that the climate, specifically the dry season, can lead to higher case numbers.[20] The dryness of the season provides the conditions for destruction of the mucosal defenses thus making individuals more susceptible to meningitis.[20]

According to the pathogens responsible for meningitis, in this study the majority of cases were had a bacterial etiology; specifically *Streptococcus pneumonia* then *Neisseria meningitides* W135. The predominance of these two bacterial agents was also noted as most common in the United States and across other countries globally. [1] A possible reason for the predominant prevalence of pneumococcal meningitis could be an auto-infection from colonizing bacteria in the nasopharynx. [22] Auto-transmission from the nasopharynx into the meninges is therefore highly possible especially during the dry season when cracks and injuries tend to occur in the nasopharynx. With the introduction of the pneumococcal vaccine, this prevalence will be reduced in the future as reported in other countries. [14] In this small sample of cases we only found two cases of *H. influenzae B*, which used to be the predominant causative agent. [20] It is likely that this change was caused by the introduction of Hib vaccines in Rwanda. The impact of the Hib vaccine on the reduction of meningitis has been reported in other countries such as Turkey. [22, 23, 24] In this study we identified two cases of *Cryptococcus neoformans* and one case of *candida albicans*. It is possible that the cases were amongst patients living with HIV. The prevalence of HIV in Rwanda is 3% [25] and meningitis caused by fungi has been reported in other studies amongst those who are immunosuppressed, specifically amongst people living with HIV.[26]

All the bacterial isolates from this study remained susceptible to antibiotics. This findings in some way supports the presumptive treatment of bacterial meningitis prior to identifying the infectious agent. However continued care needs to be taken to monitor drug resistance patterns and alter treatment recommendations accordingly. A low sensitivity rate has been observed to penicillin ranging from 60.3; 55.3, 68.5; 68.2 for *S. pneumoniae*, *N. meningitidis* W135, *E. coli*, and *S. aureus*. Those results are similar to others obtained in Madagascar[27].

Limitations of this study

The data described is based on reported cases which may not be accurately representative of numbers and distribution of bacterial meningitis cases in Rwanda. Also, this study the relationship between different variables was not considered.

CONCLUSION

Meningitis is a global health problem and whilst Rwanda is not within the African meningitis belt, cases have been reported. Children under 5 years of age are the most affected and the higher prevalence occurs during dry season, and within the eastern region. *Streptococcus pneumoniae* was the leading cause of meningitis in this sample and ceftriaxone antibiotic was most active to the majority of identified bacteria. Broad surveillance needs to be conducted to continually assess the nationwide prevalence of meningitis and to document and identify any emerging antimicrobial resistance patterns circulating in Rwanda. Additional studies should consider analyzing the relationship between age, month and location to specific type of pathogens should be analyzed.

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EAHRC
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ORIGINAL ARTICLE

Low exposure to *Plasmodium falciparum* and Acquisition of Antibodies to Circumsporozoite Protein Antigens in Individuals Living in Western Highlands of Kenya with Unstable Malaria Transmission

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ABSTRACT

Background: Malaria continues to be a major public health concern despite the concerted efforts to eliminate it. Antibodies to *Plasmodium falciparum (P. falciparum)* antigens are involved in prevention of infection and disease with some of the antigens being targeted as lead candidates in vaccine development. However, most of the studies have been done in malaria endemic areas with little information available on exposure and acquisition of protective antibodies in areas of low and unstable malaria transmission. This study sought to determine whether the extent of exposure to *P. falciparum* affect acquisition of protective antibodies by measuring antibody levels and determine the prevalence of circumsporozoite protein (CSP) and crude schizont extract (SE) in individuals living in an area with low unstable malaria transmission in Western Highlands of Kenya.

Methods: Sixty plasma samples from individuals living in an area of low unstable malaria transmission in Western Highlands of Kenya were randomly selected and categorized into three age groups: <8years (n=25), 8-18years (n=21) and >18years (n=14). Antibodies levels in plasma were measured by Enzyme Linked Immunosorbent Assay (ELISA) and compared to known positive and negative control plasma samples. Prevalence and levels of antibodies produced against circumsporozoite protein and schizont extract antigens were compared between age groups.

Results. The prevalence of antibodies ranged from 0% to 14.29% at arbitrary units (AU)>2 for the two antigens. The antibody prevalence did not significantly increase with age ($P>0.05$) but correlated with CSP and SE antigens ($r=0.5977$; $P<0.05$).

Conclusion This study highlights antibody responses to CSP and SE antigens in individuals living in low and unstable malaria transmission. The levels of antibodies were generally low across all age groups and there were no significant differences among age groups. Longitudinal study on more antigens is needed to inform exploration of multi-antigen vaccines and also adopt several control measures including Epidemic Preparedness and Response (EPR).

Key words: Malaria, Circumsporozoite protein, Schizont extract, ELISA, Plasmodium falciparum, IgG antibodies, Kenya

INTRODUCTION

Malaria is a major global health problem and more so in sub-Saharan Africa. It is one of the most important infectious diseases worldwide, causing an estimated 214 million clinical cases and 438,000 deaths annually[1]. Sustainable control of malaria would be enhanced if effective vaccines were available for use in combination with the available control measures including insecticide treated nets (ITNs), long lasting treated nets (LLTNs), indoor residual spraying (IRS) and epidemic preparedness and response (EP)[2]. Factors that hinders development of malaria vaccine include the complexity in life cycle of

malaria parasite which involve a succession of stages in the human and the ability of parasite to utilize a number of immune evasion mechanisms[3].

Malaria is a leading cause of morbidity and mortality in Kenya with about 27 million people (70% of the country's population) at risk of infection[4]. In Kenya, malaria distribution is not uniform due to geographical differences in altitude, rainfall and humidity. These geographical factors influence the transmission patterns as they determine the vector densities and biting intensity where high ambient temperature shortens sporogonic cycle of the mosquito parasite hence shorter duration of the gonotrophic cycle. Four epidemiological zones are determined in Kenya including; endemic areas (with stable malaria transmission in altitudes of 0-1300M at Kenyan coast and around lake Victoria), seasonal transmission zones (arid and semi-arid), low risk zones (central islands of Kenya including Nairobi) and epidemic zones which covers Western highlands of Kenya with seasonal transmission with considerable year to year variation[5]. The epidemic phenomenon is attributed to increase in minimum temperature during long rains period which favours and sustains vector breeding. The whole population is at risk during epidemics and fatality can be up to ten times greater than in the areas with stable malaria transimission[6].

Immunoglobulin-G (IgG) antibodies are produced against a number of malaria vaccine candidate antigens including the pre-erythrocytic antigen CSP[7]. These antibodies have been associated with some degree of protection from clinical malaria in areas of stable transmission. However, most of information to date is based primarily on observations of naturally infected individuals living in areas of stable malaria transmission with little information in areas of unstable transmission. Residents of such areas could develop partial protection against severe malaria morbidity with increasing age[8]. Children under the age 5 years are at greater risk of infection with *P. falciparum* in malaria holoendemic areas with older children and adults developing partial immunity to malaria. However, this is not the case in areas of low and unstable malaria transmission.

Protective immunity to *P. falciparum* malaria is slowly acquired after several infections and is dependent on the intensity and duration of the individual exposure to the parasite[9]. The target antigen of the protective immunity is the CSP, a major polypeptide covering, which consists of conserved central region of repeating tetramers (B-cell determinants) that is the primary target of humoral response[10]. Indeed, sterile protection has been achieved in experimental settings using high doses of irradiated sporozoites, making the pre-erythrocytic stages of *Plasmodium* a focus of the malaria vaccine effort[11]. Subunit vaccines based on circumsporozoite protein (CSP), such as RTS, S, are at the center of this endeavor.

Antibodies to Circumsporozoite protein confer some protective immunity in individuals in malaria endemic areas. Although sporozoites are not associated with clinical symptoms, this is a time when parasite numbers in the host are low and their eradication can completely prevent infection[12]. As much as the protective mechanisms have not been completely fully understood, they are postulated to partly involve humoral immune responses elicited by pre-erythrocytic antigens as a result of repeated sporozoite infection[13]. It has also been suggested that IgG and its subclass antibodies to CSP could be important surrogate markers of resistance to infection and reduced malaria morbidity in endemic areas of Africa[14].

Previous studies have determined that antibodies to schizont extract and CSP occur frequently in individuals living in malaria endemic areas[15]. The antibody levels and prevalence are also proposed to be indicators of malaria exposure and transmission[16]. Most of the studies have been done in areas of high and stable malaria transmission and little done in areas of low and unstable malaria transmission. The present study aimed to assess age dependent immune response against SE and CSP among individuals living in an area with low unstable malaria transmission.

METHODS

Study site

The study was conducted in a highland area of Kamasai in the western highlands, Kakamega County, bordering Nandi County, Kenya, where malaria transmission is low, unstable and seasonal. The site is located at an altitude of between 1734 and 1822M with an approximate population of 3000 as of 2009 census. There are usually two rainy seasons with short season in October and long rains between March and May. Malaria transmission at the site is low and unstable during long rains. It is usually episodic with local outbreaks of malaria with high rates of morbidity and mortality having occurred in the past[17]. Both *P. falciparum* infection and *Plasmodium malariae* infection have been documented in the area[18].

Study population

The study was carried out in the month of May when transmission was expected to be at peak. Study participants were males and females of all ages who had not moved out of the study area for at least six months by the start of the study and who were residents of the area during the study. This was to minimize the confounding effects of travel and acquisition of infection in nearby lowland areas where malaria is endemic. The enrolment of the participants was

done by study assistants recruited from the locals and signs and symptoms recorded by a trained clinician.

Inclusion and exclusion criteria

The participants included in the study were male and female age not limiting, residents of the area who had not moved out of the study site six months prior to commencement of the study and were willing to take part in the study by signing the written consent. Individuals who had haemoglobin values below 8gHb/dL and were evidently ill according to the assessment of the clinician were excluded from the study. Also those who declined or whose parents/guardians declined to sign the written consent were excluded.

Sample size determination

Sample size was determined using StatsToDo online statistical software for estimating correlation coefficient (www.statstodo.com). With the following parameters:

Probability of type 1 error (α) =0.05

Expected correlation coefficient (ρ) = 0.32

At power (1-β) = 0.8 (80%)

This gave a sample size of 59

The assumption was that approximately 5% declined to give consent. Since the study involved a one-time point sample collection and only those who had not moved out of the study site for at least six months were eligible, migration after sample collection would have no effect on the results. The number was adjusted to 60 participants in the study site.

Study Design and Data Collection

The study was cross-sectional and was part of a larger study which involved one time point collection of blood and plasma samples. Prior to sample collection, signs and symptoms of malaria were recorded. Thick and thin smear microscopy was used to diagnosis malaria. Haemoglobin levels were measured using photometry (HaemoContol EKF Diagnostics, and Barleben, Germany). Sixty plasma samples (n=60) were randomly selected from the samples of the larger study and used in the present study. The samples were categorized into three (3) groups based on the age; <8years (n=25), 18years (n=21) and >18years (n=14) to assess the effect of age on IgG responses. Seven plasma samples (n=7) from North American individuals who were never exposed to malaria were used as negative controls. Thirty (30) pooled plasma samples from adults living at Asembo Bay in Nyanza, an area of stable malaria transmission was used as positive control. Measurement of IgG responses to crude schizont extract (SE) and circumsporozoite protein (CSP) was done by Enzyme Linked Immunosorbent Assay (**ELISA**).

Ethical consideration

Since this study involved both adults and minors, written informed consent was obtained from the study participants or, in the case of minors, from their parents or guardians. The consent forms were signed by participants or their parents/guardians before witnesses. The forms were translated into the local language, Kalenjin (Nandi) for participants and parents/guardians who could not understand English. The samples from the participants were assigned arbitrary numbers to conceal the identity of the participants. Study participants who tested positive for malaria were treated for free at the Health Centre using the recommended line of malaria treatment. Ethical review and approval for this study was obtained from Kenyatta University Ethical Review committee and Ethical Review Committee of Kenya Medical Research Institute.

Laboratory Procedures

The presence of antibodies to CSP was tested by using recombinant central repeat sequence peptides of CSP consisting of five Asparagine-Alanine-Asparagine-Proline $(NANP)_5$ repeat tetrapeptide to which individuals from areas where malaria is endemic demonstrate IgG responses. Antigens for schizont extract (SE) were obtained from malaria culture using *P. falciparum* from 3D7 parasite isolates[19]. We measured Immunoglobulin G (IgG) antibodies by enzyme-linked immunosorbent assay (ELISA).[20] We coated separate microtitre plates (Nunc-Immuno™Microwell™96 well solid, SIGMA) with 50µl of Circumsporozoite peptide and crude schizont extract separately dissolved in 1x tris-buffered saline (TBS) to a concentration of 10µg/ml. Following overnight incubation at 4°C, we washed the plates 4× with TBS 0.05% Tween 20 using an automated plate washer and blocked for nonspecific binding with 5% (wt./vol) bovine serum albumin (BSA) in TBS. We added 50µl of plasma samples diluted to 1:100 in 5% BSA in TBS to wells in duplicates and incubated for 2 hours at room temperature. After washing with washing buffer, we added 50 µl of alkaline phosphatase-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1:1,000 in 5% BSA in TBS and incubated for 1 hour. After extensive washing (6×) with washing buffer we added p nitrophenyl phosphate substrate as per the manufacturer's instructions (Sigma, S0942 St. Louis, MO). After colour development we measured the optical density (OD) at 405 nm on a 96-well ELISA plate reader (Molecular Devices, Sunnyvale, CA). We tested all samples in duplicates. We used pooled plasma from 30 individuals from malaria endemic Lake Victoria region as positive control on each plate to achieve Standardization of the plates. We determined the background from the wells with no plasma (blanks). We deducted the background from the mean OD of each sample and a cut-off threshold of positivity was determined as the mean plus 3 standard deviations from the seven negative control plasma samples

(Canadians) which we included in each assay. We expressed the IgG antibody levels in arbitrary units (AU). We calculated the numbers of AU by dividing the optical density of a sample by the mean optical density plus 3 standard deviations (SD) for plasma from seven Canadians who had never been exposed to malaria. We determined two thresholds; AU>1 for low level antibody responses and AU>2 for high level antibody responses.

Data Analysis

Microsoft Office Excel 2007 was used in the initial processing and management of data and further analysis was done using GraphPad Prism 6 statistical software (Prism v6.0, GraphPad Software Inc., La Jolla CA). Wilcoxon rank sum test was used to determine summary statistics for the age groups. Kruskall-Wallis test was used to assess differences in the antibody responses in the various age groups. The non-parametric tests were used since the IgG response data was not normally distributed. The spearman rank correlation test was used to analyze the relationship between IgG responses to SE and CSP. Results were considered significant at $P<0.05$.

RESULTS

Microscopy results from peripheral blood smears of participants at the time of sampling demonstrated that the individuals were non-parasitemic and asymptomatic. Measurement of haemoglobin (HB) levels revealed that 53(88.3%) of participants had HB levels above the cut-off of anaemia at their various age groups. The proportion of antibody-positive individuals at a cut-off of AU> 1 (low level antibodies) was relatively low for both Schizont extract (SE) and circumsporozoite protein (CSP). The results showed that 11(18.3%) were IgG positive for SE while 12(20.0%) were IgG-positive for CSP. There was no difference in proportion of IgG-positive individuals to CSP and the proportion of IgG-positive individuals to CSP and SE (P=0.05). When the cut-off was set at AU>2 (high level antibodies), the proportion of positive individuals dropped to 4(6.7%) for both SE and CSP ***(Table 1)***. The total IgG levels to schizont extract (SE) ranged from 0.140-6.260 AU with median level of 0.575 AU and mean of 0.8602 AU in the 60 individuals of the study population. On the other hand, the total IgG to circumsporozoite protein ranged from 0.020-4.22 AU and the median level was 0.530 AU and mean of 0.8075 AU in the 60 individuals ***(Table 2)***. ***Figure 1*** depict correlation between IgG levels to SE and CSP in the study sample (r=0.5977; P<0.001).

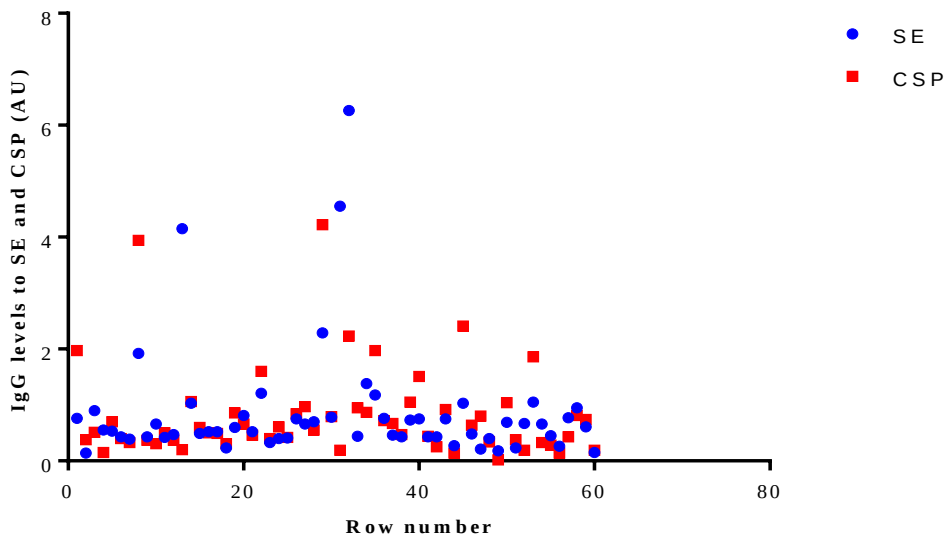
TABLE 1. ***Proportion of antibody-positive individuals to Plasmodium falciparum Schizont extract and Circumsporozoite protein at a cut-off of AU>1 and AU>2***

Antigen	Number (N)	IgG Positive at AU>1		IgG Positive at AU>2	
		Number (n)	Percentage (%)*	Number (n)	Percentage (%)*
SE	60	11	18.3	4	6.7
CSP	60	12	20	4	6.7

TABLE 2. ***Study Participants' Median and Mean IgG levels (AU) to Plasmodium falciparum Antigens***

Parameter	Antibody response to SE (AU)	Antibody response to CSP (AU)
Number of participants (N)	60	60
Minimum	0.1400	0.0200
25% percentile	0.4300	0.3475
Median	0.5750	0.5300
75% percentile	0.7775	0.9075
Maximum	6.2600	4.2200
Mean	0.8602	0.8075
Std. deviation	1.0492	0.8110
Std. error of mean	0.1354	0.1047

FIGURE 1. ***Correlation of antibody levels between schizont extracts (SE) and Circumsporozoite protein (CSP) for all participants (n=60) in an area of unstable malaria transmission.***



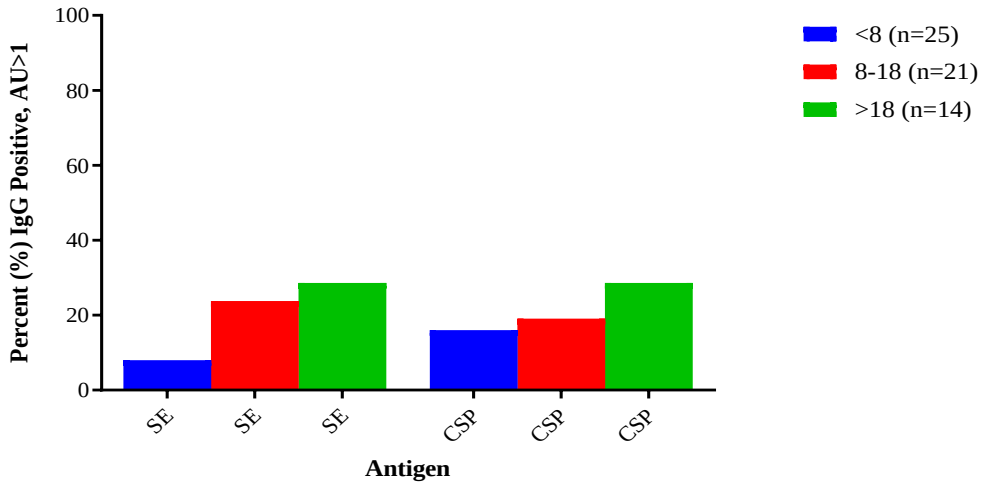
IgG levels were expressed as optical density (OD) in arbitrary units (AU). Correlation coefficient (r=0.5977), 95% confidence interval (0.3988 to 0.7428) and two-tailed P value of <0.001.

Analysis of frequency of IgG to *P. falciparum* by age group is depicted in ***(Figure 2)***. When antibody-positive cut off was set at AU>1 (low level antibodies), 2 (8.0%) of participants aged <8 years, 5(23.8%) of participants aged 8-18 years and 4(28.6%) of participants above 18 years were IgG-positive to SE. the respective proportion of IgG-positive participants to CSP at cut-off of AU>1 was 4(16%), 4(19.04%) and 4(28.6%) for participants of age groups <8 years, 8-18 years and > 18 years. The antibody frequency for individuals aged >18 years was the same for both SE and CSP ***(Figure 2)***.

When the threshold for antibody positivity was considered at AU>2 (High-level antibodies) the proportion of individuals with positive antibody dropped to 6.7% for both SE and CSP. No antibody against SE and CSP was detected in younger age group when the cut-off point was set at AU>2. Interestingly, at cut-off of AU>2 the frequency of IgG to CSP was much lower in the individuals aged above 18 years (7.14%) than in individuals aged 8-18 years (14.29%) though not significantly different (p>0.05).

Similarly, the IgG frequency in age group >18 years at cut-off of AU>2 was lower to CSP (7.14%) than to SE (14.29%). However, the difference was not significant (P>0.05) ***(Figure 3)***. There was no significant association between median IgG levels and age ***(Table 3)*** When the median levels of IgG antibodies to SE were compared to CSP among the three age groups, the levels to CSP were slightly higher than IgG levels to SE. However, the difference was not significant (P>0.05) ***(Figure 4)***.

FIGURE 2. ***Prevalence of total IgG antibodies, determined by ELISA for P. falciparum Schizont extract (SE) and Cicumsporozoite Protein (CSP) in participants of different age groups in an area of unstable malaria transmission.***

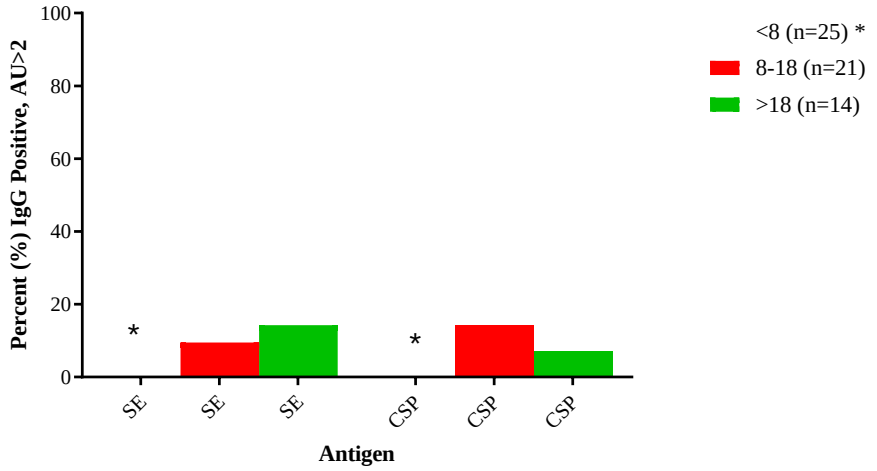


The frequencies were expressed in percentage (%) when the cut-off of AU>1 was considered antibody-positive (low-level antibodies)

TABLE 3. ***Median Levels of IgG Antibodies to Plasmodium falciparum Schizont Extract and Circumsporozoite Protein by age groups AU>1***

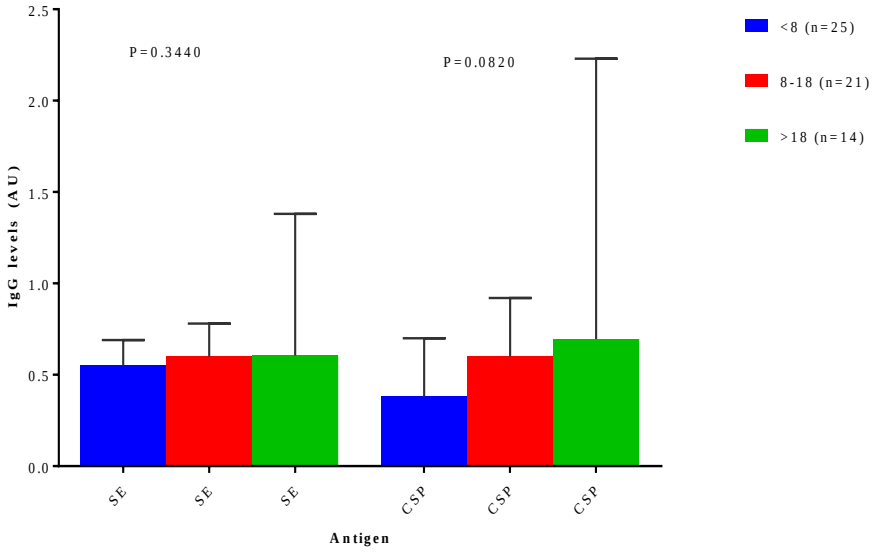
Antigen	Age (Years)	Number of Participants (N)	Median level (Range)	95% CI of Median
	<8	25	0.5500 (0.1400-1.050)	(0.3721-0.6140)
SE	8-18	21	0.6000 (0.2300-4.150)	(0.4700-0.7800)
	>18	14	0.6050 (0.2700-6.260)	(0.4300-1.3800)
	<8	25	0.3800 (0.0200-1.970)	(0.3300-0.7000)
CSP	8-18	21	0.6000 (0.2000-4.220)	(0.4600-0.9200)
	>18	14	0.6950 (0.1400-2.230)	(0.2500-1.5100)

FIGURE 3. ***Prevalence of total IgG antibodies, determined by ELISA for P. falciparum Schizont extract (SE) and Cicumsporozoite Protein (CSP) in participants of different age groups in an area of unstable malaria transmission.***



The frequencies were expressed in percentage (%) when the cut-off of > 2 AU was considered antibody-positive (high-level antibodies). *= 0% prevalence of IgG antibodies.

FIGURE 4. ***Relationship between IgG levels to schizont extract (SE) and circumsporozoite protein (CSP) among participants of different age groups in an area of unstable malaria transmission.***



IgG levels were expressed as optical density (OD) in arbitrary units (AU), error bars represent standard error of the mean (SEM). There was no significant difference when medians were compared by Kruskal-Wallis Test (P>0.05).

DISCUSSION

Previous studies have documented that the presence of high-level IgG antibodies to pre-erythrocytic antigens especially to CSP correlates with protection from *P. falciparum* infection in adults and clinical malaria in children[7]. Data from the present study show that generally a very small proportion of individuals living in the study area had high-level IgG antibodies to the two pre-erythrocytic *P. falciparum* antigens, CSP and schizont extract. This percentage is relatively higher than 3.3% documented in an earlier study in the same area[21]. This difference may be partly due to timing in sample collection. All the same, the results of the present study demonstrate that the prevalence of high-level IgG antibodies in an area with unstable malaria transmission is extremely low as compared to documented results (44.3%) for an area of stable malaria transmission[21]. These findings suggest in part that the intermittent risk of clinical malaria in individuals living in an area of unstable malaria transmission may relate to the low or absence of high-level IgG antibodies to pre-erythrocytic antigens particularly CSP. Low levels of antibodies to crude schizont extract indicate low exposure to *P. falciparum.* As much as presence of high-level IgG antibodies to pre-erythrocytic antigens may not completely prevent blood-stage infection, it may control the parasite density and the clonality of parasitemia by reducing the invasion of *P. falciparum* sporozoites into the liver or by impairing progressive development of parasites in the liver. This notion is supported by documented results in earlier studies that, an increased level of anti-CSP antibodies is associated with decreasing parasite densities in asymptomatic adults in malaria endemic areas[22]. Reduced parasitemia density may also reduce haemolysis of erythrocytes thereby resulting in high haemoglobin levels. Findings in the present study document reduced prevalence of anti-CSP and anti-SE antibodies. This may in part explain the absence of protective immunity among individuals in the study area resulting in frequent epidemics.

The present study generally reports very low IgG antibodies levels to both schizont extract and circumspozoite protein in highland area with low unstable malaria transmission. The levels compared to documented data from areas of high transmission are extremely low. Documented information associates high levels of antibodies to CSP and other pre-erythrocytic antigens such as MSP, LSA and TRAP with protection against clinical malaria[20]. The low levels IgG antibodies to CSP and SE reported in the present study suggest in part why the highland areas are prone to epidemics attributed to low exposure to malaria parasite. The findings in the present study that there is a correlation between IgG antibodies levels to the two antigens conform to documented data of strong correlation in expression of antibodies to pre-erythtrocytic antigens especially CSP, MSP, LSA and TRAP in malaria holoendemic areas[20]. The results in the present study suggest that protection against malaria may be as a result of antibodies to pre-erythrocytic antigens and that repeated exposure and infection appears to be the major determinant of high levels of IgG antibodies to these antigens. The mechanism of association of antibodies to different pre-erythrocytic antigens in protection is not clearly understood. However, one potential mechanism for antibody related protection is the combined effects of antibodies that target different steps during the pre-erythrocytic phase of infection life cycle.

Circumsporozoite protein is important in adhesion of the sporozoite to the basolateral membrane of the hepatocyte[23] and monoclonal antibodies to CSP inhibit parasite invasion of hepatocytes[24]. Thrombospondin related adhesive protein (TRAP) is essential for sporozoite gliding motility[25] and hepatocyte invasion and antibodies to TRAP have also been shown to prevent sporozoite invasion of hepatocytes[26]. The low levels of IgG antibodies to pre-erythrocytic antigens particularly CSP in highland areas of Western Kenya therefore is attributed to infrequent exposure to malaria parasites hence lack of protective immunity. This low and infrequent exposure is evidenced by low levels of antibodies to schizont extracts in the present study. The degree of natural exposure to malaria required to attain high levels of IgG antibodies to pre-erythrocytic antigens is not known. These high levels of IgG are usually attained by some children in malaria holoendemic areas thereby developing immunity but not others. However, findings of the present study present very low levels of anti-CSP and anti-SE IgG antibodies which may in part be attributed to very low degree of natural exposure to malaria.

In highland areas of Africa, older children and adult continue to be at risk for clinical malaria, although the risk is somewhat lower than that for young children[27]. This is in contrast with areas with stable, high level malaria transmission, where clinical immunity to malaria develops by the age of 3 to 5, with intermittent episodes of mild malaria and very few episodes of malaria occurring after this age[28]. Results from the present study demonstrate very low prevalence of high-level antibodies to both CSP and schizont extract in individuals of all ages. These frequencies are generally low in comparison to results documented for studies done in areas of stable malaria transmission where over 50% individuals show high level IgG antibodies to pre-erythrocytic antigens[20].

Several previous studies have demonstrated that high levels of IgG antibodies to multiple pre-erythrocytic antigens are associated with protection against *P. falciparum* infection in adults living in malaria-holoendemic area[7]. Majority of this protection is associated with high-level IgG antibodies to

CSP and LSA not assessed in the present study[20]. The findings of the present study suggest that the persistent risk of clinical malaria in older children and adults in areas of unstable transmission may be as a result of low prevalence of high-level IgG antibodies to pre-erythrocytic antibodies especially CSP.

The immunity to malaria that develops with age in areas of stable malaria transmission is not completely characterized. However, several studies have documented a correlation with protection from clinical malaria for anti-CSP IgG antibodies assessed in this study and IgG antibodies to other antigens not tested in the present study such as LSA-1, TRAP, AMA-1, MSP-1, MSP-2, MSP-3 and GLURP[7,29,30,31,32]. These previous studies confirm the association of age dependent acquisition of anti-malaria antibodies with cumulative exposure to malaria in areas with high and stable malaria transmission. However, little comparable information is available for individuals residing in areas of unstable, epidemic-prone malaria transmission. A study conducted in Tanzania dwelt majorly on blood-stage antigens including MSP-1_{19} MSP-$_2$, AMA-1 and glycosylphosphatidylinositol (GPI) across a wide range of ages and transmission intensities. The Tanzanian study reported that antibody isotypes for the tested antigens relate to age and transmission intensity[33]. The present study dwelt on pre-erythrocytic antigens which the Tanzanian study did not test. The key finding of the present study is that highland areas of western Kenya with unstable malaria transmission present extremely low levels of IgG antibodies as compared to documented data of areas of stable malaria transmission. Furthermore, the results of this study indicate that there is no significant difference in IgG antibody levels to CSP and schizont extract in the study area. Although results of the present study indicate that the levels of IgG antibodies to CSP and schizont extract increase with age, adults have very low levels of anti-CSP and anti-SE IgG antibodies suggesting why adults are also at risk of malaria in areas of unstable malaria transmission. The association of IgG antibodies to CSP tested in this study with protection from *P. falciparum* and disease and the striking low or absence of high-level antibodies in the study area, suggests that these responses may be important in protection against infection and disease. The idea that immune response to CSP is important for development of clinical immunity against malaria is supported by the success of CSP-based RTS, S vaccine in inducing protection from clinical malaria due to *P. falciparum* in children. Indeed, the first results of phase 3 trial which was launched in 2009 in seven African malaria-endemic countries; Gabon, Ghana, Kenya, Malawi, Burkina Faso, Tanzania and Mozambique showed that RTS,S reduced the risk of children experiencing clinical malaria and severe malaria by 39% and 29% respectively[34]. The vaccine is now undergoing pilot implantation in 3 to 5 settings in sub-Saharan Africa[35]. Therefore, the low levels of IgG antibodies to CSP and schizont extract in the area of low and unstable malaria transmission reported in the present study could be part of the reason for impaired clinical immunity in adults resulting in epidemics.

CONCLUSION

This study highlights the antibody responses to CSP and SE antigens in individuals living low and unstable malaria transmission. The levels and prevalence of IgG antibodies response to SE and CSP were generally low across all age groups and there were no significant differences among age groups. Longitudinal study on more antigens is needed to inform exploration of multi-antigen vaccines and other malaria control measures including Epidemic Preparedness and Response (EPR).

Competing interest

The authors declare that there are no competing interests. The publication of this article was approved by the dean School of Pure and Applied Sciences, Kenyatta University.

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EAHRC
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ORIGINAL ARTICLE

Candida albicans infection among HIV positive and HIV negative women- Case study at Butare University Teaching Hospital (CHUB), Southern province of Rwanda

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ABSTRACT

Background: *Candida albicans* still major leading global health challenge especially in immunocompromised individual particularly living in low-income countries with limited access to the antifungal drugs. Though the incidences and prevalence of opportunistic infection have been reduced due to the availability and strict adherence to the antiretroviral therapy, candidiasis remains the most frequent fungal infection with high morbidity and mortality and It is still a neglected topic in the research sectors of developing world including Rwanda. This study aimed to determine the prevalence of *candida albicans* infection among HIV positive and HIV negative women attending Butare University Teaching Hospital in the southern province of Rwanda.

Methods: A descriptive cross-sectional study was conducted with 306 female participants aged between 17-64 years old. Conventional sampling method was used to obtain the desired population and an interviewer structured questionnaire was administered to gather social demographic information and associated risk factors. both urine and a drop of blood for HIV were requested for testing. HIV testing followed the national HIV testing policy.

Results: Among 306 women participated; the average age range was 30-40 ages. The overall prevalence of *candida albicans* was 10.5% and was highly isolated in HIV positive women 21(13.7%) than in HIV negative women 11(7. 2%). Pregnancy and antibiotics usage were statistically significant associated with candidiasis with p-value <0.05 while age was not statistically associated with *candida albicans* infection with p-value>0.05.

Conclusion: The availability and strict adherence to antiretroviral therapy to people living with HIV have tackled the opportunistic infections like candidiasis**.** However, poor hygiene still the major health challenge for both groups. Hence, improved hygiene and early treatment with an improved diagnostic test to the risk population should highly be importance.

Key words: Prevalence, Candida *albicans* infection, Butare University Teaching Hospital.

INTRODUCTION

Fungal infections continue to pose significant morbidity and mortality due to the increasing number of at-risk population across the world particularly in developing countries[1,2]. Globally, an estimated 37.9 million people were living with HIV in 2018[3]. HIV/AIDS officially recognized as epidemic since the 1980s with the increase of immunocompromised individual that has exposed them to numerous opportunistic infections including candida infection of fungal aetiology and accompanied with massive population death[4,5]. Candida species including *C. albicans, C. tropicalis, C. glabrata, C. kruzei, C. dublinensis, C. guilliermondii, C. parapsilosis, C. kefyr, and C. pelliculosa* are the most frequent fungal species of the human microbiota and these species asymptomatically colonizes many areas of the body, predominantly the gastro-intestinal and genital-urinary tracts of healthy individuals[6,7]. However, in the condition that could impair the host immune system, C.

albicans can change from normal commensal state to a pathogenic state by penetrating the mucosal surface and cause a deep-seated invasive and life-threatening infection[8]. The pathogenic state of *C. albicans* is highly associated with its ability to undergo phenotyping switching from different cellular form like yeast cell to hyphae cell depending on environment growth conditions[9,10,11].

C. albicans remains the most frequent pathogenic candida species responsible for almost fungal morbidity and mortality in the developing world and causing the increased cost of care and duration of hospitalization[12]. The non-albicans candida species have significantly emerged to cause infection in patients living with HIV due to the fact that these non-albicans species are less susceptible to the commonly used antifungal drugs which hinder their effective treatment[13]. Although *C. albicans* remains the dominant species causing Blood Stream Infection, the frequency of occurrence varies throughout the world from a low of 37% in Latin America to a high of 70% in Norway[14]. The recent global incidence was estimated to be 700,000 cases of invasive candidiasis and reported to have a mortality rate of 38% to 44% in developing countries with resource-limited settings where conventional diagnostics test is slow, expensive or delay to access the antifungal drugs in all countries and a high burden of HIV coinfection[15].

In Africa and the Middle East, *Candida albicans* is highly isolated to cause invasive and superficial candidiasis with the prevalence of 55.18% and 76.92% respectively.[16]This was mostly seen in Sub-Saharan Africa and central Africa where *Candida albicans* infections rate was (22.74%), followed by the South African region (22.44%),. With regard to *C. albicans* invasive infections still prevalent in Sub Saharan and central Africa at (12.34%), followed by the Middle East (6.05%)[16]. Vaginal *C. albicans* infection has been implicated most often in a number of candida infections which affects approximately two-thirds of the women of reproductive age globally. Candida may become worse in a patient with the compromised immune system, such as HIV/AIDS patients or those that are on immunosuppressive therapies, broad-spectrum antibiotics users and pregnant women, this is a potentially life-threatening leading to systemic fungal infection[17].

In Rwanda, Candida infection and other fungal aetiology disease are still under-diagnosed with routine diagnostic tests and rarely considered as a global health challenge particularly in immunocompromised individuals that could result in drug resistance. Moreover, there is limited data with no published information mainly in research sectors oriented on candidiasis highlighting the burden of the aforementioned infection in HIV positive or HIV negative individuals that could contribute to the ineffectiveness of treatment if not properly managed. Thus, this study is aimed to determine the prevalence of C*andida albicans* infection among HIV positive and HIV negative women attending Butare University Teaching Hospital in the southern province of Rwanda.

METHODS

Design and Study area

This was a hospital-based descriptive cross-sectional study conducted from May to September 2019 at Butare University Teaching Hospital. It is located at Mamba, Butare Cell, Huye District in the Southern province of Rwanda, a National Referral Hospital which serves more than 3,772,230 people from Southern Province and others from different locations of Rwanda.

Sample size determination & Sampling

A convenience sampling strategy was used for participant's selection. By referring to the prevalence of Candida albicans 27.5% in the previous study conducted in Uganda[18]. The minimum sample size was calculated using Fisher's formula $\mathbf{n=z^2*p(1-p)/d^2}$ which yields a minimum sample size of 306[19]. Where **n**=sample size, **z**= **z** statistic for the level of confidence i.e.1.96, **P=**expected prevalence (27.5%) and **d=**allowable error i.e. 0.05.

Data collection and processing

Participants meeting the inclusion criteria and willing to participate in the study were conveniently recruited from the ART (Antiretroviral Therapy) clinic and OPD (Outpatients Department) service. The study was mainly intended to isolate the pathogenic microorganism, therefore those who were using the antifungal treatments were excluded from the study.

The informed consent forms were given together with a semi-structured questionnaire to gather demographic information. The participants from the ART clinic were given urine containers and requested to bring urine sample whereas those from OPD department were requested to give blood for HIV status testing as an addition to urine samples. The HIV status testing followed the national HIV testing policy by use of Alere® HIV combo for screening and Stat Pak for confirmation. The participants confirmed positive HIV test result was counselled by Hospital counsellors of the ART clinic. All 306 urine samples were streaked over Sabouraud Dextrose Agar media -SDA and incubated aerobically at 37°C for 3-4 days. Yeast growths were then identified by colonial morphology characteristic of Candida on above-mentioned media. For confirmation of *Candida albicans* using germ tube test, a single colony from SDA was

isolated, transferred and emulsified in human serum for 4 hours at 37ºC.

Data analysis method

The data were entered into Microsoft Excel (Microsoft Corp, Santa Rosa, CA, USA) and exported for statistical analysis in IBM SPSS Statistical developer version 21. Relative frequency of demographic characteristics was summarized using descriptive statistic. Correlation of dependent and independent variables was analyzed using Chi-square test. The level of statistical significance was set at 95% confidence level where p-value <0.05.

Ethical considerations

Ethical approval was obtained from the Institution Review Board, University of Rwanda/ College of Medicine and Health Sciences and Ethical Committee of Butare University Teaching Hospital with reference numbers CMHS/IRB/219/2019 and RC/UTHB/057/2019 respectively. The participants were explained about the study, only participants with signed informed consent form were enrolled in the study. Moreover, Ethical clearance was distributed to the head of the respective department.

RESULTS

The study included 306 women participants of the average age range of 31-40 years old of which 153 were HIV positive and 153 were HIV negative women from ART clinic and OPD department respectively (table1). *Candida species* were isolated in 59(19.2%) out of total population which account 32(54.2%) of germ tube formers isolated as *Candida albicans* and 27(45.7%) germ tube test negative isolated as *Candida non-albicans (*data not shown*). C. albicans* was highly isolated among ART participants 21(13.7%) compared to OPD participants 11(7.2%) ***(Table 1)***. The overall prevalence of *C. albicans* was 10.5% while Candida non-albicans accounted for 8.8% ***(Table 1)***. However, *Candida albicans* was significantly associated with antibiotics usage and pregnancy status in both HIV positive and HIV negative women with p-value <0.05 ***(Table 2)***.

TABLE 1. ***Demographic characteristic of study population in relation to Candida species***

		Microorganisms isolated			
Variables	n	***C. albicans* (%)**	***C. non albicans* (%)**	**Negative (%)**	***p*-value**
Age, years					
<20]	7	0(0%)	1(14.3%)	6(85.7%)	
[21-30]	87	16(18.4%)	8(9.2%)	63(72.4%)	
[31-40]	123	7(5.7%)	14(11.4%)	102(82.9%)	0.084
[41-50]	55	7(12.7%)	2(3.6%)	46(83.6%)	
>50	34	2(5.9%)	2(5.9%)	30(88.2%)	
HIV status					
Positive	153	21(13.7%)	8(5.2%)	124(81%)	0.002
Negative	153	11(7.2%)	19(12.4%)	123(80.4%)	
Total	306	32(10.5%)	27(8.8%)	247(80.7%)	

TABLE 2. ***Distribution of Candida albicans among study population associated with pregnancy and antibiotics usage status***

Variables			**Participants n**	***C. albicans (%)***	**P-value**
HIV Positive	Pregnant	Yes	5	3 (60%)	0.002
		No	148	18 (12.2%)	
	Antibiotics Usage	Yes	19	7 (36.8%)	0.002
		No	134	14 (10.4%)	

HIV Negative	Pregnant	Yes	29	8 (27.6%)	0.001
		No	124	3 (2.4%)	
	Antibiotics Usage	Yes	20	5 (25%)	0.001
		No	133	6 (4.5%)	

DISCUSSION

Candida albicans infection remains a major public health concern particularly among immunocompromised individuals in developing countries with limited resources including Rwanda. Understanding its prevalence and associated risk factors can be a major contribution to the effective control and preventive measures. The present study revealed 10.5% as the prevalence rate of *Candida albicans* in the entire study population and was the most isolated species considered with other *Candida species*. This is in accordance with the works reported elsewhere in a different study which stated that *C. albicans* is the most frequent colonizer and responsible for almost clinical manifestation of candidiasis[20,21].

The prevalence of 10.5% in this study is low compared to 22.3% and 32.4% that reported among women in Kigali University Teaching Hospital, Rwanda and Lagos Teaching Hospital, Nigeria[20,22]. This reduction of prevalence could be attributed to the fact of availability of antiretrovirals to the patients, strict adherence to their antiretroviral regimen and sensitive sampling method (vaginal swabs) used.

Considering the prevalence among the study populations; *C. albicans in* HIV positive women was high 13.7% compared to 12.5% and 9.7% of the seropositive population in Nigeria[23,24]. This could be due to the fact that they included both HIV positive male and female who were on antiretroviral therapy with the exclusion of the associated risk factors. Contrary, it was low compared to 22.3% and 50% reported among seropositive patients in Kigali and India respectively[13,22].

As observed in the present study, the prevalence of *Candida albicans* among the study population in relation to pregnant status was similar to 34.3% reported among pregnant women in a federal medical centre, Nigeria[20]. This was supported by the facts that, pregnancy could be a risk factor which increases the possibility of getting candida infections due to the increases of the sensitivity of vagina to infection and increase of reproductive hormones that elevate the amount of glycogen in the vagina which can suppress the normal vaginal environment[20,25]. The 21-30 age groups were shown to be more susceptible to candida infection compare to other groups ***(Table 1)***. This work agreed with that reported in the previous studies which showed that 20-29 age groups had a higher prevalence of candida colonizers with predominant aetiology of *C. albicans* to cause vaginal candidiasis[26]. This could be attributed to the fact of the active age group with the highest risk of pregnancies, indulgence in family planning pills and immunosuppression due to HIV/AIDS. Contrary, the population aged 46 years or above have reached menopause and have shown to develop resistance to candida infection due to the fact that, they are less or not sexually active, not using contraceptive drugs to prevent pregnancy and they have increased vaginal immunity as they have reduced the level of estrogen and corticoid[21].

With the regards to the antibiotics usage facts, the prevalence of *C. albicans* among antibiotics users living with HIV was 36.8% and 10.4% for antibiotics non-users while the prevalence of C. albicans among antibiotics users and non-users who were HIV negative was 25% and 4.6% respectively. These results disagreed with that of reported among women in primary health care centres of Jos, Nigeria that, the broad-spectrum antibiotics users accounted for 16% and 33.6% respectively as the risk for vaginal candidiasis[20,26]. The fact of using antibiotics can be a predisposing factor that leads to candida colonization but the absence of this factor does not necessarily defend against Candida colonization[25].

CONCLUSION

Low prevalence of *Candida albicans* infection was significantly observed compared to that conducted in Rwanda in 1995. Pregnancy and antibiotics usage was revealed to significantly contribute to candida infection. Thus, regular and early medical checkup and a regular public education on the importance of personal hygiene and proper use of antibiotic should reduce disease burden. Further research should be conducted to explore more about *C. albicans* infection and burden of the fungal etiological agent in women by using different sampling method and improved diagnostic test.

Limitation

This study was limited by small sample size and less sensitive samples used which might have interfered with the accuracy of the results.

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ORIGINAL ARTICLE

Role of Gamma Glutamyltransferase and Alkaline Phosphatase Assay in Enzymatic Panel for Hepatobiliary Function in Patients Attending Kibungo Hospital, Rwanda

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ABSTRACT

Background: Liver complications show specific processes like hepatoxicity associated with drugs, primary neoplasm, or hepatotropic virus infections. Different markers based on laboratory testing help to diagnose and monitor liver related conditions. Mostly used tests are classed in a set known as hepatic panel or liver profile mainly consisting of body enzymes.
Objective: The objective of the present study was to ascertain the role of gamma glutamyltransferase (GGT) and alkaline phosphatase (ALP) assay in enzymatic panel for hepatobiliary function assessment among patients attending Kibungo hospital.
Methods: Two hundred twenty-five clients were included in the study. Demographic data were collected from December 2016 to March 2017. Blood sera were also collected and tested for serum GGT and ALP levels. Statistical package for social sciences (SPSS) was used in data analysis.
Results: Seventy-four point seven per cent of clients had normal GGT whereas 63.1% had normal ALP. The 0.9% of clients comprised low levels of ALP. The means were 53 and 153 U/L for GGT and ALP, respectively. Fifty per cent of alcohol consumers' population had elevated GGT and ALP. An increase of 69.2 and 61.5% for GGT and ALP, respectively was observed in smokers' population. The subpopulation of hepatitis C virus (HCV) was the most with elevated GGT and ALP levels. In HIV population, serum GGT and ALP were raised at 31.1 and 37.8%, respectively. In fact, hepatitis B virus (HBV), HCV, and HIV patients are clinically considered as immuno-compromised people. Alcohol consumption and smoking were also found to increase GGT and ALP concentrations. In addition, GGT and ALP levels were simultaneously elevated in 19.6% of the clients, indicating the frequency of cholestatic liver disease.
Conclusion: Elevated GGT and ALP revealed the occurrence of cholestasis among study participants due to factors that elevate serum GGT and ALP levels as a result of dysfunctional liver conditions. In hospital laboratories, GGT and ALP should always be included in the panel of tests for screening and bio-monitoring liver related conditions in Rwanda.

Key words: Cholestatic liver disease; Kibungo hospital; Enzymatic panel; Alkaline phosphatase; Glutamyltransferase

INTRODUCTION

Gamma glutamyltransferase (EC 2.3.2.2) catalyzes the transfer of gamma-glutamyl functional groups from substances like glutathione to an acceptor that may be an amino acid, a peptide or water forming glutamate. Alkaline phosphatase (EC 3.1.3.1) is a ubiquitous metaloenzyme that catalyzes the breakdown of monophosphate in the body by hydrolysis reaction. Both GGT and ALP are mostly produced in the liver[1]. Complications of the liver usually show specific processes like hepatoxicity associated with drugs, primary neoplasm, or hepatotropic virus infections[2].

Liver diseases include viral hepatitis, alcoholic hepatitis and cirrhosis. Cholestatic liver diseases are primarily risks for cirrhosis. These diseases progress slowly in patients and may result in hepatocellular carcinoma (HCC). The long-term consequences of hepatobiliary and cholestatic diseases are major contributors to global mortality[1,3].

Apart from laboratory tests for parameters which may change in patients with liver and biliary diseases,[4] the most sensitive and complementary biomarkers for such diseases are serum liver enzymes[5]. The physiological applications of GGT are transport function. For instance, the transport of amino acids through reactions sequence to form a gamma-glutamyl cycle[6]. GGT is also most important for the availability of the cysteine whose cycle of reactions lead to the glutathione synthesis[7]. For the clinical use, GGT was investigated and accepted as a liver function test 50 to 60 years back. For example, it helps in hepatitis C and alcoholic-related liver diseases diagnosis. Other conditions like type 2 diabetes and obesity were associated with nonalcoholic steatohepatitis (NASH) as they share many clinical features with alcoholic liver disease; and in most patients with this condition, GGT is increased[8-10]. The GGT increase was attributed to serum GGT release into the circulation as the result of bile acids acting on the cell membrane. The induction of GGT may also be ascribed to some extent specific for alcohol related disease. Therefore, hepatic GGT is increased in some types of liver diseases and the increase in serum GGT is not easily caused by the release of the enzyme from damaged cells[11].

As the body mechanism, the response of the liver to any obstructive biliary tree induces hepatocytes to synthesize ALP and newly formed coenzyme is released from the cell membrane by the action of bile salts and enters the blood circulation to increase the enzyme's serum activity. This ALP increase tends to be noteworthy in extrahepatic obstruction than in intrahepatic obstruction and it is greater in the more complete obstruction. Serum ALP activity may reach ten up to twelve times the normal range and usually return to baseline on surgical removal of the obstructed part. The same raise is observable in patients with primary liver cancer or widespread secondary hepatic metastases. The increase of ALP at least greater than 2 folds the normal range may predict transplant-free survival rates of patients with primary biliary cirrhosis[12]. Several hepatic tests are used to improve the detection of liver diseases. They basically differentiate clinically suspected disease and determine how severe the liver is damaged[13]. Liver enzymes are commonly elevated in patients with liver diseases and therefore reflect the status of liver damage[14]. Consequently, physicians use significant elevations of liver enzymes GGT and ALP levels as biomarkers of cholestatic predominant diseases, like obstructive biliary tree disease and biliary cirrhosis, leading to chronic liver failure[15]. Unfortunately, no study about the use and diagnostic role of these enzymes has been done in Rwanda. Although GGT and ALP test reagents are ordered like other liver enzymes, they are rarely included in the enzymatic panel for liver function assay in many hospital laboratories. However, results for this couple of tests should usually be analyzed together with other liver function tests in order to interpret in a wide diagnostic spectrum. Thus, the present study was carried out to determine the role of GGT and ALP assay in enzymatic hepatic panel when assessing the liver and biliary function among patients attending Kibungo hospital, in Rwanda.

METHODS

Kibungo hospital

Kibungo hospital was built in 1932 by China and it is among the oldest medical facilities in Rwanda. It is located in the Eastern Province of Rwanda, Ngoma District and was formerly known as a District hospital. Today, Kibungo hospital is under the Ministry of Health and was made a referral hospital and named Kibungo Referral Hospital. The hospital has a well-equipped modern laboratory bloc that makes possible for the hospital to meet international standard testing requirements. The hospital serves more than 15 health centers of all Ngoma District's Sectors.

Ethical consideration

The study proposal was presented to ethic committees of both INES Ruhengeri and Kibungo hospital. Ethical approvals were obtained from both institutions' ethics committees.

Inclusion criteria

Clients who attended Kibungo hospital laboratory during the study period and requested for any clinical chemistry laboratory test for liver conditions assessment were requested to participate in the study. Patients who met these criteria and who voluntarily consented to participate in the study were enrolled in the study. The participation of patients less than 18 years old was accepted after consent from their parents/guardians.

Exclusion criteria

Patients who were excluded from participation in the study included those who attended the laboratory for tests other than clinical chemistry; those who refused to consent for the participation and patients less than 18 years old, who did not have consent from their parents/guardians.

Sampling methods

Simple random probability sampling technique was adopted during the determination of the sample size for this study.

Demographic data and risk factors

The questionnaire was used to collect demographic, viz. sex, age, weight and marital status.

To ensure the confidentiality of clients' information, only study numbers, and not names, were used on the data collection tools. Imperative risk factors that elevate GGT and ALP levels were assessed via the oral interview with the patient prior to blood draw. Short answer ended questions were used in risk factors assessment. Data were recorded in raw database on daily basis.

Serum GGT and ALP levels determination

Whole blood was collected and separated using clinical laboratory centrifuge (Z100A) to obtain serum. During the entire study, 225 blood sera were tested for GGT and ALP activity. The GGT and ALP levels were automatically generated by the chemistry analyzer Roche Cobas C311.

Statistical analysis

Results for GGT and ALP were grouped in classes. The interval was determined by subtracting the lower reference limit from the upper limit. ALP/GGT ratio was used to test the distribution of results using one sample Kolmogorov-Smirnov test. Basing on those results, the classification of results as normal, low or high (elevated) was done. Six (6) classes were formulated for each enzyme levels as follows (a) for GGT: < 5, [5-61], [62-118], [119-175], [176-232], and > 232; (b) for ALP: < 40, [40-129], [130-219], [220-309], [310-399], and > 399. The class intervals were 56 and 89, for GGT and ALP, respectively. The variability of GGT and ALP levels was studied based on reference limits for both enzymes. Obtained results were also interpreted according to risk factors. Karl Pearson's correlation analysis was used to associate risks factors with enzymes' elevation. From GGT and ALP levels, simultaneously elevated levels were scrutinized. Data were statistically analyzed using statistical package for social sciences (SPSS), version 24. $P < 0.001$ was considered as significant.

RESULTS

Demographic data and risk factors

Demographic data and risk factors for participants are presented in ***Table 1***. The females were in higher number compared to males, and most of the studied population, about two third, was married. Risk factors were assessed for all participants. Most of the population (around 80-90%) do not smoke, not consume alcohol, and do not have viral hepatitis. Sixty seven percent (67%) of them do not have HIV.

TABLE 1. ***Demographic data and risk factors***

Description		Number	Percentage
Sex	Female	121	53.8
	Male	104	46.2
	Total	225	100.0
Marital status	Infant	14	6.2
	Single	58	25.8
	Married	150	66.7
	Widow	3	1.3
	Total	225	100.0
Alcohol consumption	No	183	81.3
	Yes	42	18.7
	Total	225	100.0
Smoking	No	212	94.2
	Yes	13	5.8
	Total	225	100.0
Viral hepatitis	None	202	89.8
	HBV	6	2.7
	HCV	17	7.6
	Total	225	100.0
HIV status	No	151	67.1
	Yes	74	32.9
	Total	225	100.0

Serum GGT and ALP levels among participants

Serum levels for GGT and ALP among participants are presented in ***Table 2***. For GGT, most of the patients had levels falling within the normal range. No patient was found with levels below normal range, however, 25.3% had levels above limit. Similarly, for ALP, most of the participants had levels within the normal range, with 0.9 and 26% below and above normal ranges, respectively.

TABLE 2. ***GGT and ALP levels***

GGT (U/L)	Frequency	Percent	ALP (U/L)	Frequency	Percent
<5	0	0	<40	2	0.9
[5-61]	168	74.7	[40-129]	142	63.1
[62-118]	36	16.0	[130-219]	46	20.4
[119-175]	13	5.8	[220-309]	13	5.8
[176-232]	1	0.4	[310-399]	11	4.9
>232	7	3.1	>399	11	4.9
Total	225	100.0	Total	225	100.0

GGT and ALP according to alcohol consumption

The serum GGT and ALP levels in drinkers' subpopulation were determined ***(Tables 3 and 4)***. In both cases, 50% of the clients consuming alcohol were found to have higher values of both enzymes. No client found to have levels below normal range for GGT, whereas 1 was below average level for ALP.

TABLE 3. ***GGT levels among patients according to their sub populations***

GGT Levels (U/L)	Alcohol consumers	Cigarette smokers	Viral hepatitis patients			HIV patients
			HBV	HCV	Total	
<5	0	0	0	0	0	0
[5-61]	21	4	4	8	12	51
[62-118]	13	5	1	5	6	13
[119-175]	5	1	1	1	2	8
[176-232]	0	0	0	0	0	1
>232	3	3	0	3	3	1
Total	42	13	6	17	23	74

TABLE 4. ***ALP levels among patients according to their sub populations***

ALP Levels (U/L)	Alcohol consumers	Cigarette smokers	Viral hepatitis patients			HIV patients
			HBV	HCV	Total	
<40	1	0	0	0	0	0
[40-129]	20	5	1	5	6	46
[130-219]	9	2	1	5	6	17
[220-309]	4	2	3	3	6	4
[310-399]	3	1	1	1	2	3
>399	5	3	0	3	3	4
Total	42	13	6	17	23	74

GGT and ALP according to smoking

GGT and ALP levels according to smoking were investigated and 69.2% had elevated GGT levels ***(Table 3)*** while 61.5% had raised ALP ***(Table 4)***. No patient was found with levels below normal ranges for both enzymes.

GGT and ALP according to viral hepatitis

In the current study, 2.7% participants had HBV infection and 7.5% had HCV infection. Among patients with HBV and HCV infections, none had levels below normal range for both GGT and ALP enzymes. Among HBV patients, 33.3% and 83.3% had high levels for GGT and ALP, respectively. In HCV patients, 52.2% and 70.6% had elevated levels for GGT and ALP, respectively (***Table 3 and 4)***.

GGT and ALP according to HIV status

The levels of GGT and ALP according to HIV status were assessed and 68.9% and 62.2% were in normal ranges for GGT and ALT levels, respectively. No clients were found to have levels below normal ranges ***(Table 3 and 4)***.

DISCUSSION

The present study was carried out to highlight the role of GGT and ALP assay in enzymatic hepatic panel when investigating the liver and biliary function among patients attending Kibungo hospital. The target population for the present study was 673 but data were collected from 225 participants who were chosen randomly. By considering the population and sample size, 1 participant in the study represented 3 clients in the target population.

Levels of GGT and ALP vary from person to person, depending on demography and factors like alcohol consumption, tobacco use, viral hepatitis, and living with HIV. Previous studies have shown that alcohol consumption, cigarette smoking, viral hepatitis, and HIV infection are associated with elevation of GGT and ALP levels[16-19]. It has also been established that serum GGT and ALP are predominantly biomarkers of cholestatic disease[20]. The normal ranges for GGT and ALP are within 5-61 and 40-129 U/L, respectively[1]. In the present study serum levels for GGT and ALP among participants were analyzed and values within classes surpassing the normal reference limits were found to be high whereas values less than normal limits were low for both GGT and ALP.

The normal distribution was significant ($P < 0.001$) as shown by the ratios of ALP to GGT, which were used to test that distribution. High levels of ALP were observed in infants and males. ALP levels also correlated with the weight of participants. Demographically, ALP levels are higher in males than females and the levels correlate with the weight of the person. ALP levels are also high in neonates and children with accelerated bone growth[21]. In this study, classes formed served to find abnormal values of studied enzymes. High levels were classed in 4 classes as it specifies the degree of enzyme levels elevation. For example, participant in the 6th class had elevated levels greater than 3 times.

The means levels for 225 clients were 53 for GGT and 153 U/L for ALP. The mean level of GGT was within the reference limits but tended to surpass its peak. In contrast, the mean level of ALP exceeded its upper limits. Results were significant and basing on averages, participants were probably having some of the factors, which result in elevation of ALP levels. Indeed, all forms of cholestasis were dominant among participants. King and Armstrong[22] evaluated ALP among subjects and the elevation was significant in patients with liver diseases. The mean of levels in statistical distribution viewed the normality and abnormality of GGT and ALP, respectively. 0.9% of participants had low ALP levels. Zinc is a coenzyme of ALP catalyzed reactions, and persistent low levels of ALP may mean low zinc levels of serum[20]. Despite hepatobiliary diseases, other disorders in the body can interfere in the levels of ALP. To analyse ALP with GGT together as a couple test may help to detect cholestasis among participants.

According to Wolf,[23] in alcoholic liver disease GGT raises 8-20 times the upper limits and persistence elevation is an indicator of cirrhosis. In the present study the serum GGT level was high in 50% clients consuming alcohol. The same percentage (50%) for elevated ALP levels was obtained for drinkers' population. GGT and ALP were significantly elevated ($P < 0.001$). The high alcohol intake can lead to chronic liver failure, a condition requiring liver transplantation in most cases as the prognosis for chemotherapy is frequently worse[24,25]. Drinking alcohol may therefore be associated with hepatobiliary disease.

ALT and GGT levels in smokers were evaluated and both GGT and ALP levels in smokers were found to be significantly elevated. Similar findings were also reported by Boonstra et al[26] and Wannamethee and Shaper[19]. Levels of GGT and ALP correlate in liver disease principally of cholestatic type[27,28]. Mohammad[29] demonstrated a relationship of elevated serum GGT levels to cigarette smoking, and the significantly increased serum GGT in smokers seemed to increase the harmful effects of cigarette smoking on the liver.

Viral hepatitis often results in liver injury and can lead to chronic liver disease[18]. In the present study higher levels above normal ranges were observed for ALP compared to GGT among HBV and HCV patients. Elevated hepatic enzymes were mostly found among patients with HCV

infection. HBV population had less elevation of these enzymes and results were statistically significant. In previously reported studies viral hepatitis was correlated with elevation of GGT and ALP[14,30]. Cholestatic liver disease in viral hepatitis patients was confirmed. Regular assessment of GGT and ALP in this population can help in monitoring the prognosis.

In this study, 32.9% were HIV patients and clinically considered immuno-compromised. Data showed that 31.1% and 37.8% had high GGT and ALP levels, respectively. The elevation was significant, and ALP was more elevated than GGT with the difference of 6.7%. These findings are in agreement with those reported by Markowitz et al[17], who associated HIV infection with high levels of GGT and ALP. Similarly, HIV infection and HIV and HCV coinfection has also been associated with GGT and ALP elevation[16,17]. Being HIV positive is decidedly a considered risk factor for elevating GGT and ALP levels. Indeed, cholestatic liver disease is frequent in HIV population hence GGT and ALP should be used in biomonitoring of HIV infection progression among patients.

Elevated serum GGT and ALP levels indicate the predominance of cholestatic hepatobiliary disease[20]. According to Friedman *et al.*,[28] increased levels in serum ALP and GGT were associated with liver disease of intra and extra hepatic cholestasis as well as in destruction of hepatocytes membranes. In this study, 19.6% of clients had a simultaneous elevation of GGT and ALP levels. In clinics, this elevation hints at the malfunction of hepatobiliary system among clients.

Among subjects with uniformly elevated GGT and ALP in the present study, 27% were infants. It has been reported that neonates and infants up to one year likely experience high GGT and ALP levels[22,31]. They were suspected to have maternal jaundices that should be cleared in 2 to 3 weeks of birth. Simultaneously, elevated GGT and ALP levels showed biliary disorders of birth among infants and children less than 1 year.

In 44 participants with high levels of studied enzymes, 36 were adults. Twenty adults were males whereas 16 were females. Thirteen were alcohol consumers, 6 were smokers, 2 were HBV positive, 7 were HCV positive, and regrettably among participants with simultaneously elevated GGT and ALP, 20 were HIV patients. High levels of both GGT and ALP were found among some of the study. Elevated enzyme levels were frequent in four classes above the normal reference limits for both GGT and ALP. D'Agata and Balistreri[20] observed that in cholestatic conditions, there is an accumulation of compounds whose excretion fails as a result of biliary tree obstruction. GGT and ALP require a clear biliary tree for elimination, and they will simultaneously be elevated in this condition rather than in hepatocellular injury. Among the clients with simultaneously elevated GGT and ALP levels 19.6% had cholestasis, and this percentage is similar to the frequency of cholestatic liver diseases among clients of Kibungo hospital. GGT and ALP can help in diagnosis and monitoring of the conditions related to liver function.

CONCLUSION

The role of GGT and ALP assay in enzymatic panel for hepatobiliary function assessment was studied. Alcohol consumption, smoking, viral hepatitis, and HIV were found to be associated with GGT and ALP elevation. Simultaneously elevated GGT and ALP, among clients of Kibungo hospital, were strong indicators of both the cholestatic liver disease and the frequency of cholestasis among clients of Kibungo hospital. GGT and ALP should always be included in the panel of tests for screening and bio-monitoring liver related conditions in Rwanda since statistics showed these tests as significant predictors of liver related conditions.

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ORIGINAL ARTICLE

Combined Effects of *Carica papaya* Seeds with Albendazole on Adult *Pheritima posthuma*

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ABSTRACT

Background: *Carica papaya* seed is commonly used as herbal remedy for intestinal worms. This study investigated the combined effect of *Carica papaya* seed extracts and albendazole on adult *Pheretima posthuma*.
Methods: Cold percolation method was used to extract bioactive compounds in dry powdered seeds of *Carica papaya* using water, ethyl acetate and petroleum ether as solvents. Individual extracts and combinations of the extracts with albendazole were assayed for anthelminthic activity in which the time of paralysis (P) and time of death (D) of the worms were determined.
Results: Aqueous extract gave highest extractive yield of 19.8% followed by the ethyl acetate extract at 17.2%. Moreover, the individual aqueous extract and that combined with albendazole paralyzed the worm within shorter time (41.00 and 42.00 minutes respectively) compared to the conventional drug albendazole alone (48.67 minutes). Conversely, Albedazole killed the worms faster than any other extract in just 1.18 hours. Albendazole combined either with water or ethyl acetate killed the worm at 24th hour. Combined albendazole with petroleum ether extract could not kill the worm even after 24 hours.
Conclusion: From this study, combining the *C. papaya* seed extracts with albendazole did not shorten paralytic and anthelminthic activity on adult *Pheretima posthuma*. *Carica papaya* seeds extracts should rather be used individually and best when extracted in water.

Key words: Antihelminthic, *Carica papaya*, Bioactive

INTRODUCTION

Globally, soil transmitted helminthes affect over 1.5 billion people and it accounts for loss of about 200,000 human lives annually[1]. Whipworms (*Trichuris trichiura*), Roundworms (*Ascaris lumbricoides*), and Hookworms (*Necator americanus* and *Ancylostoma duodenale*) are helminthes of medical importance and are mainly transmitted through contaminated soil, water, food, and contact with animals' dung[2]. They are responsible for infections and complications such as; weakness, loss of appetite, [3] reduced weight gain and decreased productivity, aggravating malnutrition, retarded growth and development, retarded cognitive development, anemia, abdominal pains and diarrhea in the affected host[4]. To date, the conventional gold standard for the treatment of soil transmitted helminthiasis has been the use of Benzimidazole (Albendazole and Mebendazole) drugs[5]. However, with the outgrowth of Anthelmintic Resistance (AR) in the livestock industry due to years of mass drug administration and misuse of chemical anthelmintic drugs, soil transmitted helminthiasis is gradually re-emerging[6]. Besides, majority of patients (80%) in poverty stricken economies like Uganda, can hardly afford the conventional drugs hence depending mostly on herbal or medicinal alternatives[7]. *Carica papaya* (Linn.), commonly known as papaya is a fruit crop cultivated in tropical and subtropical regions, and is well known for its nutritional benefits and medicinal applications[8]. Several

studies have revealed the anthelmintic potentials of *C. papaya* on gastrointestinal worms.

For instance, a study by Wasswa and Olila[9] revealed the effective dose (ED50) of *Carica papaya* seeds on *Ascaris suum* at 12.5 mg/ml. Likewise, in comparison to albendazole, *C. papaya* seeds was found to be three times more effective on adult *Pheritima posthuma*[10] and mixed gastrointestinal nematodes[11]. Still, no study has made an attempt to consider the effect of combining the papaya seed extract with a common drug Albendazole. Given the far limited number of anthelmintic drugs in market together with hardly new compounds, this study investigated the possibility of a combination treatment approach by combining papaya seed extracts with Albendazole (5-propylthio-2-benzimidazolecarbomate) on adult earth worms (*Pheritima posthuma*). This could contribute to the knowledge base for a sustainable alternative against soil transmitted helminthiasis.

METHODS

Test Worms and Control Drug

The study used adult *Pheritima posthuma* (earthworms) obtained from moist soils around Lake Victoria in Entebbe, Uganda (0.0436ºN, 32.4418ºE). The worms were authenticated at the Helminthology section of National Animal Disease Diagnostics and Epidemiology Centre (NADDEC), Entebbe, Uganda. The choice of the earthworms was based on their anatomical and physiological resemblance with the gastrointestinal roundworm parasites of human beings[12]. Albendazole tablet (400 mg) manufactured by Beximco pharmaceuticals (Gazipar, Bangladesh) was used as a control drug.

Collection of plant materials

The fresh seeds were collected from ripe *Carica papaya* fruits and washed with clean water to remove dirt. The seeds were then dried in the hot air oven (Memmert, Schwabach, Germany) at temperature of 50°C for 24 hours and later coarsely powdered using a homogenizer prior to extraction.

Extraction procedure

Dry powdered seeds was extracted by cold percolation method according to Parekh & Chanda et al, [13] with minor modifications. Petroleum ether, ethyl acetate and distilled water were used as solvents to maximize phytochemical extraction due to varying polarity. Briefly, 5 g of dried powder was added to a 250 ml conical flask and Petroleum ether was added to the conical flask (Phyrex, Werthiem, Germany) with sample to the 100 ml mark, the flask was sealed with aluminum foil, and kept on a rotary shaker (Ika, Staufen, Germany) at 120 rounds per minute (rpm) for 24 hours. The solution was filtered through eight layers of muslin cloth, centrifuged (Beckman, Indianapolis, USA) at 5000 rpm for 15 minutes and the supernatant was collected and the residue was evaporated to dryness using an oven (Memmert, Schwabach, Germany). Each of the dry residue was transferred to 100 ml of individual solvent (Distilled Water and Ethyl Acetate) and was kept on a rotary shaker at 120 rpm for 24 hours. After 24 hours, the solution was filtered through eight layers of muslin cloth, centrifuged at 5000 rpm for 15 minutes and the supernatant was collected. Each of the filtered solution was left on petri dishes to evaporate. Extractive yield of each extract was calculated and later each of the three extracts were stored at 4°C for anthelminthic assay.

Anthelminthic assays

The individual and combined anthelminthic activity of *Carica papaya* seed extracts was carried out as described by Ajaiyeoba et al. [14] with minor modifications. Extracts, control drug and their respective combinations were administered through contact. Two worms were fully immersed in a 9 cm radius petri dish containing 10 ml of each of the extracts described above, or a combination of the extracts and albendazole. For this study, a uniform concentration of 10 mg/mL was empirically used. This was done in triplicates for all the samples and the time for induced paralysis (P, in minutes) was taken when no movement of any sort could be observed, except when the worms were shaken vigorously. Time of death of worms, if death occurred (D, in minutes) was recorded after ascertaining that worms neither moved when shaken vigorously nor when pricked with a needle. Albendazole (10 mg/ml) was included as reference/control drug, while distilled water was included as the negative control.

Statistical analysis

Tableau Software v2019.4 was used in data presentation. All experiments were repeated at least three times and the Standard Error of the Mean (SEM) determined. The means were compared using SPSS software *v16.0* in which a one-sample T-test was performed to determine the significance of the anthelmintic potentials of *Carica papaya* seeds.

Ethical consideration

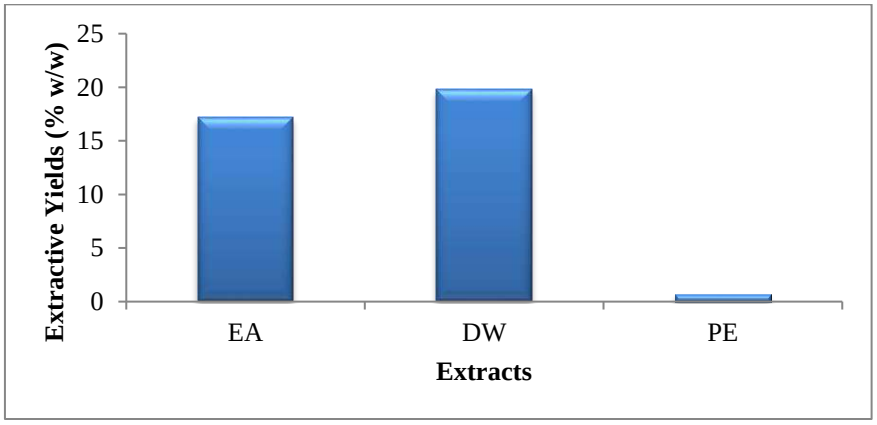
Ethical approval and permission to conduct this study was obtained from the Bugema University Research Ethics Committee, Kampala, Uganda (Ref. BU-REC/2019/U09).

RESULTS

Extractive yields

The extractive yield of *Carica papaya* seeds across three different solvents is presented in ***Figure 1***.

FIGURE 1. ***Extractive yield in different solvents***



EA- Ethyl acetate DW-Distilled water PE-Petroleum ether

Distilled water presented with the highest yield (19.8%) as compared to ethyl acetate and petroleum ether extracts. Ethyl acetate gave about 17.2% while petroleum ether presented with the least extractive yield (1%).

Anthelminthic Activity assay

The anthelminthic potentials of 10 mg/mL *Carica papaya* seed extracts and extract-drug combinations are presented in ***Table 1***. The mean paralysis time for distilled water extract and its combination with Albendazole were found to be shorter than that of the control drug Albendazole ($p<0.05$), indicating that they are more effective in paralyzing the worm than Albendazole alone. Even though, individual ethyl acetate and its combination also had shorter paralysis time than Albendazole, this was not statistically significant ($p>0.05$) and the rest of the extracts were less promising compared to the control drug (Mean Paralysis Time > 46.67±4.16). With respect to the time of death, neither individual petroleum ether extract nor its combination could kill the worms even after 24 hours whereas, the ethyl acetate extracts (individual extract) could kill the worms within a few hours (2.10±0.10). Surprisingly, combining *C. papaya* extract with Albendazole delayed anthelminthic activity by an average of 12 minutes ($r=1.00$, $p=0.001$). A similar trend was observed in distilled water extract and its combination with albendazole.

TABLE 1. ***Anthelminthic activity of Carica papaya seed extracts.***

Test sample	Paralysis Mean Time (Minutes, SD)	Death Mean Time (Hours, SD)
EA	44.67±2.31	2.10±0.10†
DW	41.00±1.73†	24.00±0.19†
PE	107.33±7.37†	No death after 24 Hours
EA+ Albendazole	46.67±1.55	24.00±1.00†
DW+ Albendazole	42.00±1.73†	24.00±0.62†
PE+ Albendazole	100.33±4.04†	No death after 24 Hours
Albendazole	48.67±4.16	1.18±0.10†

EA- Ethyl acetate extract, DW-Distilled water extract, PE-Petroleum ether extract, †-p<0.05.
All values expressed as Mean ± Standard Error of Mean (n=3)

DISCUSSION

Here we found that combining individual extract of *Carica papaya* seeds with Albendazole decreases their anthelminthic activity on adult *Pheritima posthuma*. One inherent weakness of this study is that a fixed dose (10 mg/mL) was used instead of varying the concentration of the extracts. Besides the extract used was crude in comparison to pure control drug, Albendazole of the same concentration and this could have affected the activity of the bioactive compounds in the test sample. However, the cold percolation approach of extraction enabled maximum extraction of bioactive compounds across a wide range of polarity and as well mitigated the loss of the volatile components on heating. Across all individual extracts, the time for paralysis and death of *Pheritima posthuma* in this study (41.00-107.33 minutes and 2.1-24 hours respectively) were longer than in previous study[15] with paralysis and death

time of <35 minutes and < 1 hour respectively. The variation in paralysis and death time in this study could be due to lower concentration, 10 mg/ml as compared to the 25-100 mg/ml concentration range in the above-mentioned comparative study. Similarly, a number of other documented studies have demonstrated the anthelminthic activity of *Carica papaya*, [9,10,16–19]. However, this study advanced to report the effect of combining the individual extracts with control anthelminthic drug, albendazole. *Carica papaya* seeds contain several bioactive compounds with the main ones being enzyme papain and benzylisothiocyanate.[20]. In addition, the seeds are rich with other phytochemicals like tannins, alkanoids, oxalates and trypsin inhibitors[21]. The presence of tannins in *Carica papaya* seeds is the chief reason for its anthelminthic potentials, possibly because tannins can bind onto glycoproteins on the cuticle of the worm to induce necrosis thereby causing death of the worm[22]. Thus, suggesting the possible use of extract of such seeds as de-wormers. However, the individual extract in combination with control drug showed decreased activity. This could be due to a number of reasons ranging from target incompatibility as well as inhibitory effects[23]. The study thence suggests that seeds of *Carica papaya* should be used individually if effective action against the worms is to be achieved. Furthermore, it is needful that anthelminthic bioactive compounds be purified, and structure elucidated for an accurate evaluation of antagonistic activity and its mechanism of action. Toxicity studies of the individual seed extract should as well be executed to establish the safety indices of such extracts.

CONCLUSION

From this study, combining the *C. papaya* seed extracts with albendazole did not shorten their paralytic and anthelminthic activity on adult *Pheritima posthuma. Carica papaya* seeds extracts should rather be used individually and best when extracted in water.

Conflict of Interest

The authors declare no conflict of interest related to this study

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ORIGINAL ARTICLE

Plants extract concoction from *Capsicum annuum, Citrus sinensiss, Tagetes minuta* and *Allium sativum*: A potent killer of bedbugs

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ABSTRACT

Background: *Cimex lectularius* Linnaeus and *Cimex hemipterus* L. (Bedbugs) are hematophagous ectoparasites which are a nuisance and public health menace. In recent past, bed bug infestation has been on the rise which is attributed to the emergence of resistance to commercial insecticides, particularly pyrethroids. Therefore, the aim of this investigation was to evaluate the insecticidal properties of aqueous extracts of different plants on bed bugs.
Methods: Aqueous extracts separately obtained from four different plant materials; ripe fruits of *Capsicum annuum* L. (hot pepper), fruit peelings of *Citrus x sinensis* L. (citrus: orange), leaves of *Tagetes minuta* L. (Mexican marigold) and bulbs of *Allium sativum* L. (garlic**)** were studied against bedbugs in a controlled laboratory setting and their effect based on chemotaxis and mortality observed. The extracts were further tested in a field trial in seven villages randomly selected from Idakho central ward, Ikolomani constituency, Kakamega County, Kenya. The villages were assigned arbitrary identity as V_1, V_2, V_3, V_4, V_5, V_6 and V_7. Twenty households confirmed to be infested with the bedbugs were selected randomly from each sample village. Each village received specific treatment; V_1, V_2, V_3, V_4 and V_5 were treated with 5ml of 10mg/ml of extracts from *Capsicum annuum*, fruit peelings of *Citrus x sinensis*, leaves of *Tagetes minuta*, bulbs of *Allium sativum* and a concoction of the four extracts at the ratio of 1:1:1:1 respectively. Village V_6 was treated with commercial potent synthetic insecticide (Bedlum® 200SL, Jiansu Lanfeng biochemical, China with acetamiprid as active compound) as a positive control and village V_7 was treated with distilled water as a negative control.
Results: The laboratory findings showed that the bed bugs had negative chemotaxis to *T. minuta* and *A. sativum* and also showed some direct mortality. *Capsicum annuum* and *C. sinensis* had a direct killing effect on the bedbugs. The combination of extracts from the four plants showed 100% mortality within the shortest time of 10 seconds as compared to individual plant extracts. The findings from field trials showed higher perception of bedbug management of 90% for a concoction of the four extract as compared to individual plant extracts: 55%, 70%, 40% and 45% for *C. annuum C. sinensis T. minuta* and *A. sativum* respectively. Both laboratory and field trial findings showed that a concoction of the four named plants is a potent insecticide and can be used in bedbug management. There is however need to carry out more research to ascertain other active compounds in the extracts and their mode of action.

Key words: Potent, *Cimex lectularius*, *Cimex hemipterus*, *Capsicum annuum*, *Citrus x sinensis*, *Tagetes Minuta*, *Allium sativum* Bedbug management, Concoction, mortality, Chemotaxis

INTRODUCTION

Bedbugs (*Cimex lectularius* L. and *C. lectularius* L.) are found throughout the world and are blood sucking ectoparasites that feed mostly on human blood but may also attack other animals such as birds and bats[1]. In Africa, tropical bedbugs (*C. hemipterus L.*) are distributed throughout warm zones, whereas temperate ones, (*C. lectularius L.*) are common in the rest of the Africa[2]. They attack normally at night with painless bites but fluids that are injected often produce painful welts on the skin with itching that may be severely irritating[3]. The bedbug may be associated with human pathogens, but no study has clearly proved they can transmit the pathogen to human beings[4,5]. They multiply very quickly as they feed, and the problem become progressively worse. Infestation with bedbugs was initially associated with poverty but has in recent past found their way into social amenities and transport facilities such as hotels, hostels, cruise ships, airplanes, trains, schools, and long- term care facilities making the bedbugs to spread to

many households especially those in the urban areas [2,6,7]. The bedbugs have given many people sleepless nights and due to social stigma associated with them, many people do not seek professional assistance and try to eliminate them by themselves. This has led to uncontrollable spread of the bedbugs. The bedbugs have developed resistance to many known insecticides especially pyrethroid insecticides proving very hard and costly to manage[8,9]. According to research by a PhD student David Lilly of the university of Sydney, the resistance of the bed bugs is due to development of a thicker cuticle which the insecticide could not penetrate[10]. Currently bedbugs have proven to be a challenging pest to contain or manage and it becomes the subject of significant research and public health concern than ever before[7]. The present study explored the use of cost-effective pest control tools based on new classes of plant-based insecticide with novel mode of action to effectively manage the bedbug infestation in the future. Although the history of using plant extracts as insecticides has not been mapped very well, there are various existing historical sources that in Europe, the use of some plants in protection against insects' dates back more than 3000 years. Primarily, various modified parts of some aromatic plants and their extracts were used, particularly as repellents against troublesome insects such as ectoparasites. Plants were also used to protect stored harvests or foods against storage pests[11,12].

Plants are considered as a rich source of bioactive chemicals and they may be an alternative source of insect pest control agents. Natural products are generally preferred because of their less harmful nature to non-target organisms and due to their ease for biodegradability[13]. The present study used extracts of plants which have history of use as insecticides or pesticides. For instance, *Allium sativum* repels root maggots, cabbage loopers, peach tree borers as well cockroaches. On the other hand, *Tagetes minuta* repels most insects, *C. annuum* has direct killing effect on caterpillars, aphids, flies, ants and other insects while *C. sinensis* peelings have direct killing effect on mosquitoes[14]. However scanty information is available on use of plant extracts on bedbugs. The present study sort to determine whether a concoction of plant extracts with known effects to insect pests may be used to manage bedbugs.

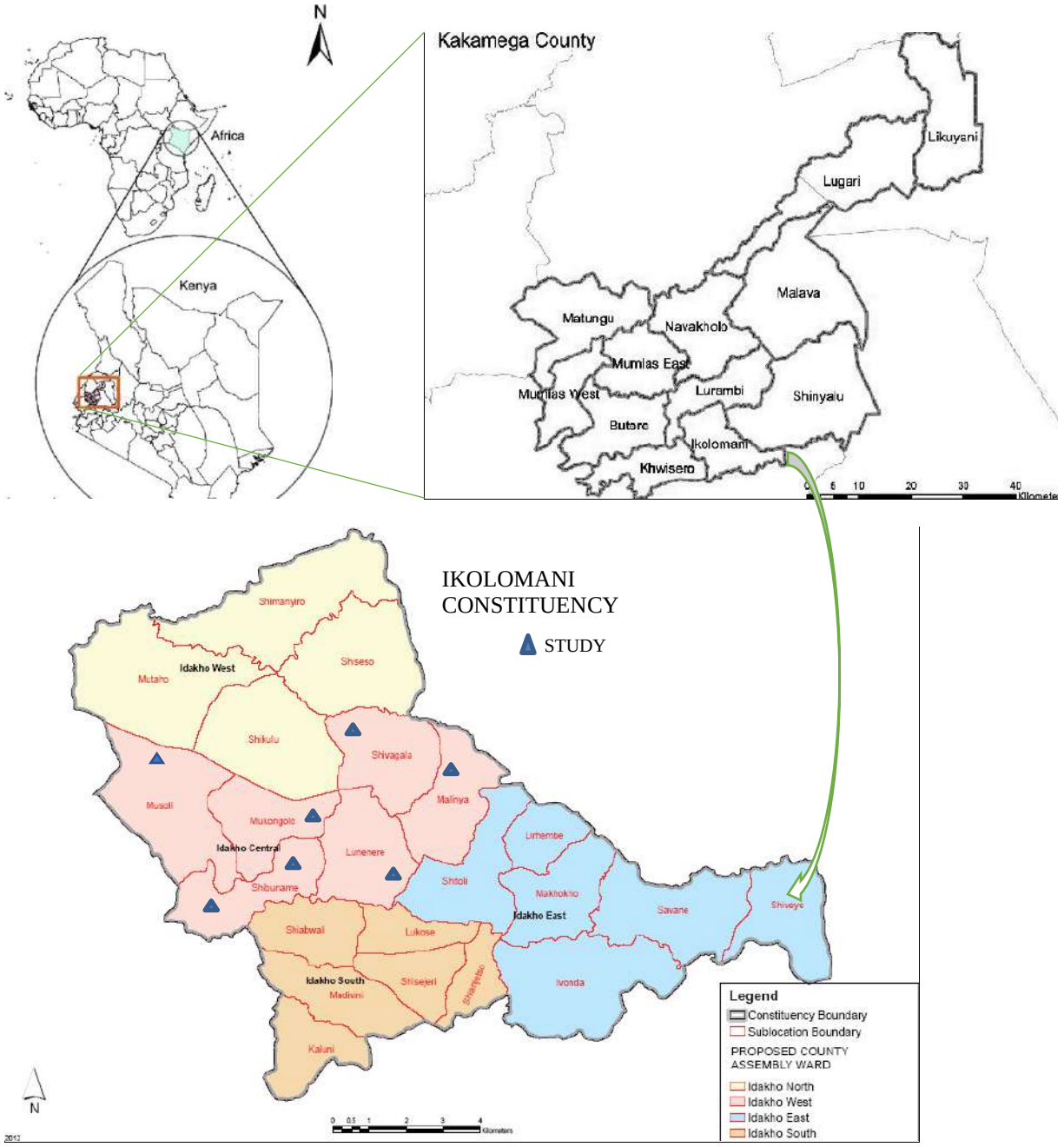
METHODS

The study involved laboratory experiments and field trials of the plant extracts from ripe fruits of *Capsicum annuum*, fruit peelings of *Citrus x sinensis*, leaves of *Tagetes minuta*, bulbs of *Allium sativum* and a concoction of the four extracts on bedbugs.

Study participants

Study participants in the field trials were residents of seven villages in Idakho central, Ikolomani constituency, Kakamega South Sub-county, Kakamega County, Kenya ***(Figure 1)***. Idakho central ward has approximate an area of 39.6 square km and an estimated population of 31,134 people with approximately 3,000 households based on 2009 Kenya national Bureau of statistics (KNBS) census. The ward has 26 villages with an approximate of 200 households each. Houses are commonly two roomed, semi-permanent and mud walled. Recruitment of participants was done by use phone call through a specific line that was displayed on posters throughout the targeted villages. Selected participants were those who made phone calls and were willing individuals who admitted that indeed their households were infested with bedbugs and were staying in 2-roomed and mud-walled semi-permanent houses. This was followed by confirmation by the researcher for bed bug infestation based on inspection for indicators such as detecting the pests themselves or their signs which include; blood spots, fecal spots, egg cases and shed skins (exuviae) on beds, mattress folds furniture and cracks on the walls. The recruited participants signed written consent that they were heads of their households and gave express authority for the study.

FIGURE 1. ***Map of the Study Site: Idakho Central Ward, Ikolomani Constituency, Kakamega County, Kenya***



Map of study site adopted and modified from ikolomanihoney.wordpress.com

Ethical approval

Permission to carry out the study was granted by Public Health Department Kakamega County and National Commission for Science, Technology and Innovation (NACOSTI) Kenya.

Plant material extraction

In the present study four different plants: *Capsicum annuum*, *Citrus x sinensis*, *Tagetes minuta* and *Allium sativum* were used. *C. annuum*, *C. sinensis* and *A. sativum* were bought from Kakamega Municipal Market. Leaves of *T Minuta* collected from Bukura Agricultural Training Centre Farm, Kakamega County, Kenya. Specific parts of each plant were carried to Masinde Muliro University of Science and Technology Biology Laboratory. They were washed with distilled water and processed as explained in the procedures. Extraction was done separately for each of the four plants as follows:

Capsicum annuum: One kilogram of ripe hot pepper were chopped into tiny pieces and boiled in 3 litres of water for 30 minutes. The extract was strained and further processed by soxhlet apparatus to concentrate the extract. The extract was left to cool and 10g of soap was added to improve dispersion as an emulsifier and adherence to surface[15]. The contents were kept in sealed container at 4ºC to be used for further experimental study.

***Tagetes minuta*:** One kilogram of freshly gathered whole plant was crushed using mortar and pestle and boiled in 3 litres of water for 30 minutes and the resultant contents were subjected to similar procedures as in the case of hot pepper.

***Allium sativum*:** Transparent covering were removed from garlic gloves.1kg of garlic cloves (bulbs) were crushed to fine paste and soaked in 3 litres of cold water for 3 days, strained and concentrated. The content was then sealed in a container at 4ºC.

***Citrus x sinensis*:** The fruits were peeled and the rinds (peelings) used for extraction. Much of the white inner portions were removed from the rinds. 1kg of the processed rinds was boiled in 3 litres of water for 10 minutes. The contents were covered and left for 24 hours to allow the rinds to soak creating an oil suspension. Soap was also added as dispersion agent and emulsifier. The extract obtained was also kept in sealed containers at 4ºC.

Laboratory experiments

Adult blood engorged bedbugs were collected from an infested area particularly in the mattress folding and bed joints and identified by an entomologist. The bedbugs were maintained in the laboratory at 25 ± 2ºC and 75 ± 5% RH (Relative Humidity) according to Winston and Bates procedure[16]. Two chambered glass cages (15cm × 10cm × 6cm in length, width and height respectively) with the two chambers separated by a controlled perforated mesh were used to test the action of the plant extracts on the insects. Seven different groups of bed bugs were used in this experiment. Five groups were experimental while two groups were controls i.e. positive and negative controls. Ten (10) bed bugs were put in one chamber of each cage and their movement controlled. In the experimental groups, the bed bugs were treated directly (topically) with approximately 5ml of 10mg/ml of extracts from *Capsicum annuum*, *Citrus x sinensis*, *Tagetes minuta*, *Allium sativum* and a concoction or combination of the four extracts (1:1:1:1) for Group 1, 2, 3, 4 and 5 respectively. Groups 6 and 7 insects were treated topically with 5ml of distilled water and 5ml of 10ml/l of commercial insecticide (Bedlum® 200SL, Jiansu Lanfeng biochemical, China with acetamiprid as active compound) as negative and positive controls respectively. Each treatment was done in duplicates. The action of the extracts and average time taken for 100% chemotaxis or 100% mortality was noted. Chemotaxis was noticed when the bed bugs moved to the second chamber. Mortality was when the insects were immobilized and raised the hind part up.

Field trial study

The extracts were tested in a field trial study in seven villages randomly selected from Idakho central ward, Ikolomani constituency, Kakamega County, Kenya. The villages were assigned arbitrary identity as V_1, V_2, V_3, V_4, V_5, V_6 and V_7 to conceal the village and household identity. Twenty households confirmed to be infested with bed bugs were selected randomly from each sample village. Each village received specific treatments where V_1, V_2, V_3, V_4 and V_5 were treated with 10mg/ml extracts from *C. annuum*, *C. sinensis*, *T. minuta*, *A. sativum* and a concoction or combination of the four extracts respectively. Village V_6 was treated with 5mg/ml of commercial synthetic insecticide (Bedlum® 200SL, Jiansu Lanfeng biochemical, China with acetamiprid as active compound) as a positive control and village V_7 was treated with distilled water as a negative control. The furniture, crevices, mattress folds and beds were thoroughly sprayed with the extracts and entire house fumigated. The treatments were done in the mid-morning and doors and windows closed for 5 hours. A follow up round of treatment was done after 10 days to take care of the bed bugs that may have escaped the first round of treatment. Afterwards, the individuals from the various households were interviewed separately on the perceived effectiveness of the treatment their household received based on the bed bug infestation indicators mentioned earlier. Their response was noted as effective, not effective or not sure.

Data analysis

Data was managed using Microsoft excel (Microsoft company) and data analysis done by Graphpad prism 7 (Graphpad inclusions). Chi-square test was used to analyze the time taken by each treatment to cause mortality or

chemotaxis. Chi-square test was also used to analyze the responses from field trial experiments.

RESULTS AND DISCUSSION

From the laboratory experiments, the time taken for 100% mortality in each treatment was analyzed ***(Table 1)*** and the time taken for 100% movement of the bedbugs to the second chamber (chemotaxisis) was also analyzed ***(Table 2)***.

TABLE 1. ***Time of action for plant extracts to cause 100% mortality on bedbugs***

Groups	Name of plant/treatment	Part of plant used	Quantity and Concentration in mg/ml	Time for 100 % mortality (in seconds)
Group 1	*Capsicum annuum*, ,	Ripe fruits	5ml of 10mg/ml	23± 0.011
Group 2	*Citrus x sinensis*,	Rinds/peelings	5ml of 10mg/ml	15± 0.004
Group 3	*Tagetes minuta*	Whole plant	5ml of 10mg/ml	320± 0.033
Group 4	*Allium sativum*	Bulbs (gloves)	5ml of 10mg/ml	300 ± 0.027
Group 5	*Capsicum annuum* + *Citrus x sinensis* + *Tagetes minuta* + *Allium sativum*	Mixture/concoction of the extracts	5ml of 10mg/ml in the ratio 1:1:1:1	10± 0.001
Group 6	Distilled water	Negative control	5ml	-
Group 7	Bedlum® 200SL insecticide with acetamiprid as active compound	Positive control	5ml of 10ml dissolved in a litre of distilled water	9± 0.002

TABLE 2. ***Time of action for plant extracts to cause 100% motility (Negative chemotaxis) on bedbugs***

Groups	Name of plant/treatment	Part of plant used	Quantity and Concentration in mg/ml	Time for 100 % motility (in seconds)
Group 1	*Capsicum annuum*	Ripe fruits	5ml of 10mg/ml	00± 0.011
Group 2	*Citrus x sinensis*	Rinds/peelings	5ml of 10mg/ml	00± 0.004
Group 3	*Tagetes minuta*	Whole plant	5ml of 10mg/ml	15± 0.035
Group 4	*Allium sativum*	Bulbs (gloves)	5ml of 10mg/ml	13 ± 0.021
Group 5	*Capsicum annuum* + *Citrus x sinensis* + *Tagetes minuta* + *Allium sativum*	Mixture/concoction of the extracts	5ml of 10mg/ml in the ratio 1:1:1:1	00± 0.001
Group 6	Distilled water	Negative control	5ml	-
Group 7	Bedlum® 200SL insecticide with acetamiprid as active compound	Positive control	5ml of 10ml dissolved in a litre of distilled water	0± 0.002

The laboratory findings showed that the bed bugs had negative chemotaxis to *T. minuta* and *A. sativum* and also showed some direct mortality with *A. sativum* taking the shortest time of 13 seconds compared to 15 seconds taken by *T. minuta* for chemotaxis. Extracts from *C. annuum*, *C. sinensis* and the concoction of the extracts did not cause chemotaxis. On the other hand, the concoction of the four extracts killed the bedbugs within the shortest time of 10 seconds as compared to time taken for individual extracts; 23 seconds, 15 seconds, 320 seconds and 300 seconds for *C. annuum*, *C. sinensis T. minuta* and *A. sativum* respectively. The results obtained in the field trial experiments were based on individual responses based on the effectiveness of the plant extract treatment given to their household in managing the bed bugs ***(Table 3)***.

TABLE 3. ***Responses on interviews from field study on the on the perception of effectiveness of the plant extracts on management of bedbugs***

Village	Name of plant/treatment	Number of households	response			
			Effective	Not effective	Not sure	% response on effectiveness of the treatment
V_1	*C. annuum*	20	11	9	0	55
V_2	*C. sinensis*	20	14	2	4	70
V_3	*T. Minuta*	20	8	4	8	40
V_4	*A. sativum*	20	9	5	6	45
V_5	*C. annuum* + *C. sinensis* + *T. Minuta* + *A. sativum*	20	18	0	2	90
V_6	Bedlum® 200SL insecticide with acetamiprid as active compound	20	17	0	3	85
V_7	Distilled water	20	0	19	1	0

The findings from field trials showed higher perception of effectiveness of bedbug management of 90% (18 out of 20 households) when a concoction of the four extract was used as compared to individual plant extracts with perception of effectiveness of 55% (11 out of 20 households), 70% (14 out of 20 households), 40% (8 out of 20 households) and 45% (9 out of 20 households) when *C. annuum C. sinensis T. minuta* and *A. sativum* were used respectively. Results from laboratory experiments revealed that extracts from *C. annuum, C. sinensis* have direct killing effect. This could be strongly attributed to active compounds they possess. Extracts from *C. sinensis* rinds contain limonene as the main component which is a strong insect repellant and also paralyses insects like fleas[17]. It is further documented that its neurotoxic effect characterized by hyperactivity followed by hyperexcitation leads to rapid knock down and immobilization of the insects. Furthermore, extracts from *C. sinensis* rinds contains linalool which is a pest killer identified as an inhibitor of acetylcholinesterase[18]. However, their effect on bedbugs is not well documented and therefore this study provides the basis of use of extract from *C. sinensis* rinds as an ingredient in management of bedbugs. *C. annuum* on the other hand contain capsaicin (8-Methyl-N-vanithyl-6-nonenamide) and other several compounds called capsaicinoids as active compound which are irritants with chocking effect to most animals including insects[19]. In insects, capsaicin causes metabolic disruption, membrane damage and nervous system failure with physical repellent action[18]. According to previous reports, capsaicin has broad-spectrum insecticidal activity against many species of insects, e.g., stored product beetles (*Sitophilus zeamais* and *Tribolium castaneum*) as well as *Bemisia tabaci*[20]. The present study documents that *C. annuum* cause direct killing effect to the bedbugs as observed in the laboratory experiment. The present study revealed that bedbugs are negatively chemotactic to *A. sativum* and *T. minuta* indicating that the extracts have substances that are repulsive to bedbugs. When crushed chopped or blended, garlic bulbs contain an amino acid which undergo biotransformation to a substance called allicin which has a characteristic odor that repel insects[21]. This could probably be the cause of movement of the bedbugs away from the point of application of the extract as observed in the laboratory experiment. Notable activity of *A. sativum* has been reported against a number of insects including dipteran[22], lepidopteran[23, 24] and hemipteran pests amongst others[25]. *Tagetes minuta* on the other hand contains chemical compounds such as thiopenes, flavonoids, carotenoids, and triterpenoids in their seeds, leaves, roots and other parts of the plant which are offensive to insects as well as nematodes[26]. Efficacies of *Tagetes sp*. extract have been reported against blood-sucking parasites such as *Tribolium castaneum*[27]. This effect could probably be responsible for negative chemotaxis to bedbugs in the observed in laboratory experiment. When all the four extracts were mixed to form a concoction, it had direct killing effect which took the shortest time possible as compared to extracts from individual plants. This could be as a result of combined effect of the active compound in the extracts of the four plants. Previous studies have reported effectiveness of a combination of plant extracts in management of bedbugs. For instance, a mixture of extracts from *Azadiracta indica, Mangifera indica, Polyalthia longifolia, Annona squamosa, and Ficus bengalensis* was reported be an effective killer of bedbugs within the shortest time possible[13]. The efficiency of an insecticide is determined by time of action and therefore the effect observed with the concoction plant extracts suggests that it is an effective plant-based insecticide in the management of bedbugs. The results from the field trial experiment also reveal that a concoction (combination) of the extracts from the four plants is most effective in management

bedbugs as opposed to single plant extract. Therefore, based on the findings from the laboratory experiments and field trials, the present study documents that a concoction (combination) of plant extracts of *Capsicum annuum*, *Citrus sinensis*, *Tagetes minuta* and *Allium sativum* is effective in management of bedbugs.

CONCLUSION

Plants contain numerous primary and secondary bio-active molecules used to protect them from their natural enemies predominantly insects and pathogens. Plant extracts are often easily accessible, affordable, user and eco-friendly in nature, and they have become more attractive alternative insecticides to resource-limited settings[28]. The results of the present study apparently reveal that a combination of the candidate plants extracts has remarkable insecticidal activity against bedbugs. Bedbugs have since developed resistance against most of the conventional insecticides, particularly of pyrethroid origin which is considered to be one of the main factors in the recent resurgence of bedbugs. In this context a concoction of plant extracts from *Capsicum annuum*, *Citrus sinensis*, *Tagetes Minuta* and *Allium sativum* has proved effective and could play a pivotal role as a potent bedbug control agent in the near future. However, this finding warrants further investigations to elucidate the responsible bio-active principles, mode of action and safety. Furthermore, adequate efforts have to be made by involving pertinent stakeholders in order to formulate and commercialize the plant extract concoction-based insecticide.

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