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Francis Zeukeng obtained his BSc. in Biochemistry in 2009 and his MSc. in Biotechnology and Development from the Biotechnology Centre of University of Yaounde I in Cameroon in 2012 working on the epidemiology of concomitant infections between malaria and helminthiasis. As a research fellow, he is in his final year of Ph.D. Research Studies in Biochemistry (Molecular Biology and Biotechnology) between the International Institute of Tropical Agriculture (IITA-Benin) and the Biotechnology Centre of University of Yaounde I. His current research concerns the screening of environmental sources/reservoirs of Mycobacterium ulcerans and the study of its transmission mode(s) from the colonized environment to humans. He is combining field data with experimental studies to understand how humans get contaminated with Mycobacterium ulcerans from the suspected environment and develop Buruli ulcer disease.

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ISID Research Grant Report

Proof of concept study: Are mosquitoes capable of picking-up Mycobacterium ulcerans (Buruli ulcer etiological agent) from their breeding environment and hosting this Mycobacterium in their bodies throughout their developmental stages?

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Introduction

Buruli ulcer (BU) is a neglected emerging disease that has recently been reported in some countries as the second most frequent mycobacterial disease in Humans after tuberculosis. BU is distributed in over 33 countries and is characterized by severe subcutaneous necrotic lesions that lead to chronic opened sores and ulcerations, ultimately affecting the bone in extreme cases (WHO, 2015). The mode of transmission of its etiological agent, *Mycobacterium ulcerans* (MU), remains unclear and its reservoirs are still being uncovered. In Australia, both larvae and adult mosquitoes can harbor MU (Johnson et al., 2007, 2009; Quek 2007; Lavender et al., 2011). However, there is no clear information linking mosquitoes to BU transmission in Africa, the continent with the highest endemicity of this disease. The implication of mosquitoes in the transmission of BU therefore remains a contradictory event with several hypotheses (Johnson et al., 2007; Wallace et al., 2010, 2015; Hoxmeier et al., 2015, Zogo et al., 2015). One hypothesis is that mosquitoes could transmit MU to humans. However, there is no scientific or historic precedent for mosquitoes transmitting a bacterium to host in any diseases system, either directly or mechanically (Merrit et al., 2010). In vector ecology, mosquitoes may serve as biological vectors and hosts for pathogen replication, or, mechanical vectors carrying organisms from hosts to hosts without serving as a site of replication (Wallace et al., 2010). Here, we combined both field surveys and laboratory-based experiments to provide clear evidences on the implication or not of mosquitoes in the transmission of BU. Firstly, we screened the presence of MU in larvae and adult mosquito species collected in BU endemic villages in Southern Benin. Secondly, we further investigated in a laboratory-based experiment the potentials of mosquito's larvae to pick-up MU from their breeding environment and remain colonized through the larval development stages to the adult stage. (vertical transmission of MU by mosquitoes).

Materials and Methods

Ethical considerations: This research which was mainly a laboratory based experiment received administrative clearance from the International Institute of Tropical Agriculture (IITA). In addition, community consents were obtained prior to mosquitoes sampling in the communities.
Study area and sampling of mosquitoes: Field surveys for mosquito collections were conducted during rainy seasons (from 2014 to 2016) in 3 BU endemic communities

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(Agbahounsou, Agodenou and Agongbo) of the Sedje-Denou locality (6°32'N & 2°13'E) in Benin. One BU non-endemic village, Tanongou (10°48'N & 1°26'E) was selected as a negative control village for data comparison. Sedje-Denou stands as the second most BU endemic locality in Benin with a reported prevalence of 450 cases of BU per 100,000 inhabitants (Sopoh et al., 2010). Adult mosquitoes were caught indoors using insecticide spraying technique which is one of the effective methods for collecting indoors resting mosquitoes (WHO, 2006). In addition, mosquito larvae were collected from temporal, semi-permanent and permanent breeding waters using the WHO protocol (WHO, 2003). Mosquitoes caught were morphologically identified and pooled in 10 according to the species (Anopheles *gambiae s.l.*, *Culex quinquefasciatus*, *Aedes aegypti* and *Mansonia africana*)..

Molecular identification of MU in mosquito samples: Genomic DNA was extracted from a total of 7,218 (721 pools) mosquito samples (adult and larvae) using the Phenol/Chloroform extraction method described by Sambrook and Russel (2001). The TaqMan qPCR analysis described by Fyfe et al. (2007) was performed on extracted mosquito DNA samples to detect MU DNA targets (IS2404+IS2606+KR-B) in these samples

Investigations on the capability of mosquitoes to pick and host MU bacteria from larval to adult stages (vertical transmission of MU in mosquitoes): This experiment was carried out in the insectary of the AgroEcoHealth Platform of the International Institute of Tropical Agriculture (IITA-Benin). The laboratory strain *Anopheles gambiae kisumu* and the bacterial strain MU *A*gy99 were used in this experiment.

Experimental infection of mosquito larvae with MU: Mosquito larvae were infected by ingestion of MU-contaminated food. The infection protocol was adapted from Wallace et al. (2010). Six groups (4 tests and 2 controls) of 100 eggs of *An. kisumu* each were distributed for rearing into labeled plastic bowls containing 250 ml sterile water. Prior to introducing eggs into bowls, the breeding/rearing water in test groups received 80mg of Tetramin® Baby Fish Food contaminated with 100µl of MU (2.0 10^5 CFU/ml). The control groups (2 bowls) were constituted in the similar way as test bowls except for the presence of *MU* bacteria in the control bowls. The mixture (eggs-food-MU) were kept in the insectary at 27°C, 75% RH and 12:12 LD for eggs hatching. The first instars larvae progeny (L1) obtained were kept in the contaminated breeding water for ingestion of the bacteria (MU) for 24 hours after which the breeding water was completely replaced with a new MU free breeding water (Water+food only). The L1 larvae were fed with Tetramin® and bred till obtaining the second, third and fourth instars larvae, as well as the pupae and adult mosquitoes. *Monitoring of infected mosquito:* Pools of 10 individuals per developmental stage (egg, L1, L2, L3, L4, pupae, adult) were constituted from test and control bowls and were kept for molecular screening of MU. In addition, we harvested from breeding water the cuticles resulting from the different larval molting phases and preserved them for similar molecular analysis. Finally, the third group of stored samples was constituted of small volumes of breeding water collected during all the larval developmental stages.

Statistical analysis: Data were analyzed using SPSS v.17.0. Non-parametric ANOVA test (Krustal-Wallis) was used to set the difference in means. Pearson logistic regression test was used to establish the correlation between MU bacterial loads and the corresponding "Ct" values. Two standard curves were plotted from serial dilutions of MU strain and the Ct values for *IS2404* and KR-B genes. Based on these standard curves, the cycle threshold (Ct) cut-off was set at less than 35 cycles for *IS2404* and less than 37 cycles for KR-B.

Results and Discussion

Out of 5,240 mosquitoes (adults and larvae) from BU endemic villages and subjected to TaqMan real time quantitative PCR analysis, none was found simultaneously positive to the

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three *MU* targets (*IS2404, IS2606* and *KR-B*), revealing the absence of MU in wild populations of mosquitoes longitudinally caught in the surveyed BU endemic localities of Benin. These findings certainly confirm the low capability of wild mosquito populations to carry MU as previously published by other research teams in Africa (Zogo et al., 2015). However, our data seems to contradict works conducted in Australia which revealed the presence of MU in mosquito samples (Johnson et al., 2007, 2009; Quek 2007; Lavender et al., 2011). Working with larvae collected in BU endemic and non-endemic localities, we also showed that none of the 2,235 larvae tested was positive to MU, suggesting the inability of mosquito larvae to be MU reservoirs. In mimic the environmental conditions of natural mosquito breeding sites, we further analyzed samples through a laboratory designed experimental model to better understand the poor implication of mosquitoes in increased number of BU cases in West and Central Africa. The laboratory experimental model performed in the course of this research revealed that mosquito larvae readily ingest MU and host this bacterium only during the larval developmental stages (L1, L2, L3 and L4) (**Table 1**). Overall, the bacterial load decreased throughout the experiment from the young (1st instars larvae, L1) to the old (pupae and adult stages) developmental stages of *An. kisumu* (**Figure 1**). Results from this laboratory based experiment are consistent with those obtained from the analysis of thousands of wild populations of mosquitoes collected in the endemic locations and which did not show any MU colonization through molecular testing.

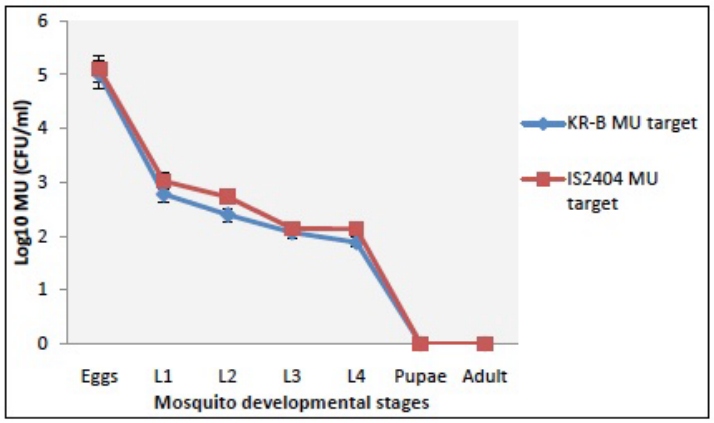

Figure 1: Vertical transmission of MU in mosquitoes; Distribution of average bacterial load during mosquito development stages. L1, 2, 3, and 4 correspond to first, second, third and fourth instars larvae respectively.

At the pupae series of high energy demanding, metabolism taking place in the mosquito certainly affects MU development leading to the clearing of this bacterium both at the end of pupation and at adult stage (**Table 1**). Our research revealed the total absence of MU at both pupae and adult stages, and further highlighting the in-ability of these biting dipterans to act as a good vector/host of MU in a BU endemic environment. Findings published by Wallace et al. (2010) suggested a refractory effect of mosquitoes to MU, a behavior which stands as a natural protective mechanism of mosquitoes to bacterial infections. According to Hoxmeier et al. (2015), the contamination of *Anopheles gambiae* mosquito with MU resulted in disruptions to phospholipid metabolic pathways in the mosquito, especially the use of glycolipid molecules. Moreover, glycolipids are actively involved in signaling and are mediators in cellular and immune processes (Atella and Shahabuddin, 2002). Hence, the disruption in synthesis of this molecule probably has a negative impact on the various interactions between MU cells and *Anopheles,* and the poor capability of mosquitoes to serve as biological vectors for MU. Our findings in addition to confirming these previous assertions also show that hosting of MU by mosquito larvae is very temporal as larvae system is capable of clearing the bacterial load during

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the late developmental stages. The vertical transmission of MU pathogens therefore seems not effective in mosquito populations as documented with several viruses. However, individuals from endemic areas should remain aware and avoid frequent contacts with mosquito's bites by sleeping under mosquitoes bed nets, wearing protective clothing while farming or using clean water for bathing and cleaning (Merrit et al., 2010).

Conclusions

Here, we provided the first longitudinal data on the absence of MU in mosquito specimens (adults and larvae) trapped in BU endemic localities in Benin. Using an experimental model, we also showed the inability of laboratory infected or colonized *An. kisumu* larvae to transfer the bacteria to their pupae and the emerging adults. This low ability of mosquitoes to vertical transmit MU pathogens to their offspring coupled with the absence of MU in field-caught mosquitoes, further highlights the low probability of these biting insects as biological vectors for MU in endemic villages in Benin. Mosquitoes may therefore not be involved in the dissemination of this pathogen from the risk environments to humans in investigated areas. However, further studies should be performed to evaluate their mechanical implication, before completely excluding whether they are involved or not in the transmission cycle of this emerging disease.

Table 1: Vertical transmission of MU in mosquitoes (MU distribution among mosquito development stages, cuticles and breeding waters).

Nature of the samples	Mosquito developmental stages	Distribution of MUmolecular targets		Pool positive/Pool tested	Presence of MU
		Mean Cts (*IS2404-qPCR*)	Mean Cts (*KR B-qPCR*)		
Mosquito	Eggs	19 ± 1.79	21 ± 2.22	4/4	Yes
	L1	27.67 ± 2.66	31.59 ± 3.15	4/4	Yes
	L2	29.92 ± 2.58	33.06 ± 2.98	4/4	Yes
	L3	31.36 ± 2.98	34.33 ± 3.34	4/4	Yes
	L4	31.38 ± 2.20	35.03 ± 1.17	3/4	Yes
	Pupae	NoCt	NoCt	0/4	No
	Adults	37.89	NoCt	0/4	No
Mosquito cuticles	Eggs	NA	NA	NA	NA
	L1	30.72 ± 1.78	36.51 ± 2.09	4/4	Yes
	L2	34.25 ± 2.83	36.82 ± 1.65	3/4	Yes
	L3	34.13	39.53	1/4	Yes
	L4	NoCt	NoCt	0/4	No
	Pupae	38	NoCt	0/4	No
	Adults	NA	NA	NA	NA
Mosquito breeding waters	Eggs	18.43 ± 2.03	21.49 ± 1.63	4/4	Yes
	L1	23.04 ± 3.19	30.61 ± 2.80	4/4	Yes
	L2	22.71 ± 2.59	31.88 ± 2.60	4/4	Yes
	L3	28.4 ± 2.86	33.53 ± 3.00	4/4	Yes
	L4	32.00 ± 2.64	35.94 ± 1.04	3/4	Yes
	Pupae	33.65	35.47	1/4	Yes
	Adults	NA	NA	NA	NA

L1, 2, 3, and 4 correspond to first, second third and fouth instars larvae respectively. Randomly selected specimens from the 4 repetitions of the experiment were subjected to qPCR analysis. Cycle threshold values (Cts) are given in terms of mean +/- SD. The statements Yes or No correspond to the presence or the absence of the bacteria in analyzed samples. NA, stands for Not applicable. The bacterial loads did not vary significantly among the developmental stages ($p<0.05$).

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