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Chikungunya (*Togaviridae*) and Dengue 2 (*Flaviviridae*) Viruses Isolated from *Aedes aegypti* Mosquitoes by qRT-PCR Technique: Xenosurveillance for Arboviruses Circulating in Burkina Faso

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Abstract: In 2016, we conducted an entomological survey in a railway transect between Banfora and Ouagadougou, Burkina Faso. The aim was to evaluate the risk factors for arbovirus epidemics, including vector infection status, in areas representative of the country. *Aedes aegypti* mosquitoes were collected at larval stage from four study sites and reared until adult stage and kept in RNAlater for detection of arbovirus RNA. In the laboratory, the mosquito specimens were screened for dengue virus (DENV) and chikungunya virus (CHIKV) using one step real-time qRT-PCR. We detected one DENV-2 positive pool from Ouagadougou, giving a minimum infection rate (MIR) of 16.67, and 6 CHIKV positive pools, giving a MIR of 66.67 from Ouagadougou, but also in Banfora and Boromo. The qRT-PCR is a useful tool for the surveillance of arboviruses of public health importance in Burkina Faso and may be incorporated into disease surveillance and control programs in Burkina Faso.

Keywords: arboviruses; Dengue; Chikungunya; molecular biology; qRT-PCR; *Aedes aegypti*; Burkina Faso

1. Introduction

Arboviruses are a major public health concern throughout the world and have been responsible for numerous outbreaks and epidemics. Some arboviruses, such as yellow fever virus (YFV), dengue virus (DENV), chikungunya virus (CHIKV), and Zika virus (ZIKV), have rapidly expanded their geographical range across the globe in recent years [1,2]. Currently, more than two billion people are at risk of arboviral disease infections [3]. Dengue is endemic in more than 128 countries according to WHO [4]. The number of dengue cases reported to WHO increased over 8-fold over the last two decades, from 505,430 cases in 2000, to over 2.4 million in 2010, and 5.2 million in 2019 [4]. CHIKV has spread to

almost 40 countries worldwide. CHIKV is distributed worldwide because of the prevalence of the vectors and their efficiency in transmitting the virus. Another possible cause of the spread of CHIKV is travel. Travel patterns have increased the importation of the virus into new geographical regions via viremic people [5]. In sub-Saharan Africa, the arboviral diseases are mainly dominated by dengue epidemics localized mostly in the Western African countries [6–10].

From 1983 to 1986, a study conducted in the western region of Burkina Faso reported a number of arboviruses, primarily flaviviruses, associated with sylvatic *Aedes* species including *Ae. furcifer*, *Ae. luteocephalus*, *Ae. cumminsi*, *Ae. opok,* and *Ae. aegypti* [11]. Since then, in 2016, an outbreak of dengue virus was reported in the human population within Ouagadougou without an incriminated vector [12]. Arboviral infection can be effectively controlled using safe and effective vaccines. Unfortunately, except for the yellow fever vaccine and Rift Valley fever vaccine (available for livestock) no effective vaccine is available for the rest of the arboviruses including dengue [13]. Preventing or minimizing vector–human contact and/or early detection of virus in the vector or humans (for *Ae. aegypti*–borne arboviruses) can be key parameters to preventing or minimizing outbreaks. Detecting the presence of arboviruses in natural vector populations has been attempted several times with inconclusive results suggesting that this approach to arboviruses detection in natural mosquito populations can be challenging [14]. However, to predict the emergence of arboviral epidemics, there is need to establish vector surveillance and control programs that include monitoring and the assessment of the prevalence of virus infection in natural and wild local vector populations. During the dengue outbreak of 2016, the surveillance and control strategy in Burkina Faso consisted of passive detection of symptomatic human cases and spraying insecticides targeting adult *Ae. aegypti*, the presumed vector for DENV in this outbreak. Targeting the aquatic stages of *Ae. aegypti* was included in the national dengue vector control strategy and this consisted primarily of the systematic removal of larval habitats and source reduction. Source reduction ultimately leads to the reduction of the adult vector population and the possibility of vertical transmission, as revealed by several studies of *Ae. aegypti* populations [15–18]. Indeed, adult stages resulting from the development of aquatic stages being infected by their previous mothers through transovarian transmission (TOT) are potential reservoirs for arboviruses and may initiate transmission and outbreaks. The aquatic stages that are not reached by spray campaigns can maintain residual and autochthonous arbovirus transmission especially toward the end of rainy season, when the adult vector abundance is low [19 Therefore, the need to incriminate the vector species responsible for the transmission of a particular arbovirus, and to enhance disease surveillance in the vector population, are both important to developing successful outbreak responses.

Within the genus *Aedes*, Ae. aegypti is being probably the most important species, it has been incriminated in a large number of arboviral outbreaks [1]. Recent studies carried out in Burkina Faso one of which in four cities along a railway transect reported that *Ae. aegypti* was the most common vector species collected in the container in domestic and peridomestic areas [20,21]. The abundance of *Ae. aegypti* is associated with a high risk of transmission of DENV and other arboviruses in Ouagadougou, Bobo-Dioulasso, Boromo, and Banfora cities. All *Stegomyia* indices such as the house index (HI), the container index (CI) and the Breteau index (BI) exceeded the risk level associated with arboviral outbreak [20]. However, the mosquitoes were not processed for presence of arboviruses.

Real time quantitative polymerase chain reaction (RT-PCR) a molecular approach has been used in the recent years to detect the presence of arboviruses in *Aedes* mosquitoes and proved to be a sensitive tool [22, 23]; we used this technique to screen the mosquito samples collected during previous entomological surveillance [20] for their infection profile. The main objective was to extend the entomological surveillance undertaken since 2016 so that the infection rate in the wild population could be computed and the outbreak risk within the study area better understood.

2. Materials and Methods

2.1. Study sites and sample preparation

Specimens of *Ae. aegypti* collected in August 2016 during dengue outbreaks in Ouagadougou (12°21′56″N, 1°32′01″W), Bobo-Dioulasso (11° 10′ 41.16″N, 4° 17′ 30.38″W), Boromo (11° 45′ 0.00″N, 2° 55′ 60.00″W) and Banfora (10° 37′ 60.00″N, 4° 45′ 0.00″W), as previously reported in [22] and [23], were analysed (**Figure 1**). Ouagadougou and Bobo-Dioulasso are urban whereas Banfora and Boromo are more suburban. Briefly, in the four study sites, mosquito larvae were collected from June to November 2016 to establish the stegomyian indices [23]. From each study area and in each sampled house, all containers found inside and outside houses were inspected for the presence of water and mosquito larvae. Each container was recorded by type, presence of water, and presence or absence of larvae. In positive containers, mosquito larvae were collected with a pipette and put in plastic cups containing water, labelled with the house identification number. The larvae were counted and classified by *Culicinae* genus. The mosquito breeding habitats were grouped into five types: plastic containers, metallic containers, terracotta jars, discarded tires, and other containers [23]. Then emerged adults were identified to the genus of species level [26,27] under a binocular (10X magnification). The mosquito breeding habitats were grouped into five types: plastic containers, metallic containers, terracotta jars, discarded tires, and other containers [23]. Larvae were allowed to emerge to adults, which were identified to the genus [26,27] under a binocular microscope (10X magnification). Although other *Aedes* species were identified, such as *Ae. vittatus* (found in very small number), we only analysed *Ae. aegypti* adults for this study. Therefore, females *Ae. Aegypti* mosquitoes were separated into pools according to site and placed in a cryotube containing RNA later (Qiagen RNAlater RNA Stabilization Reagent, 250mL, lot No 151026237, Qiagen Straße 1, 40724 Hilden Germany) and stored at -20°C for further laboratory analysis. A total of 580 females grouped in 33 pools of 10 and 20 mosquitoes ready to be screened for the detection of dengue viruses 2 and chikungunya virus in this preliminary data research article. The Analyzis was focused on DENV2 detection due to the reported results of serological surveys from positive cases detected during the 2016 outbreak in Burkina Faso [23,24]. The others arboviruses (WNV, DENV1, etc.) will be the subject of future analyzis. Samples were prepared for shipment to the Insect Pest Control Laboratory (IPCL), Joint FAO/IAEA Programme of Nuclear Techniques in Food and Agriculture, Seibersdorf, Austria for the RNA extraction and qRT-PCR analysis.
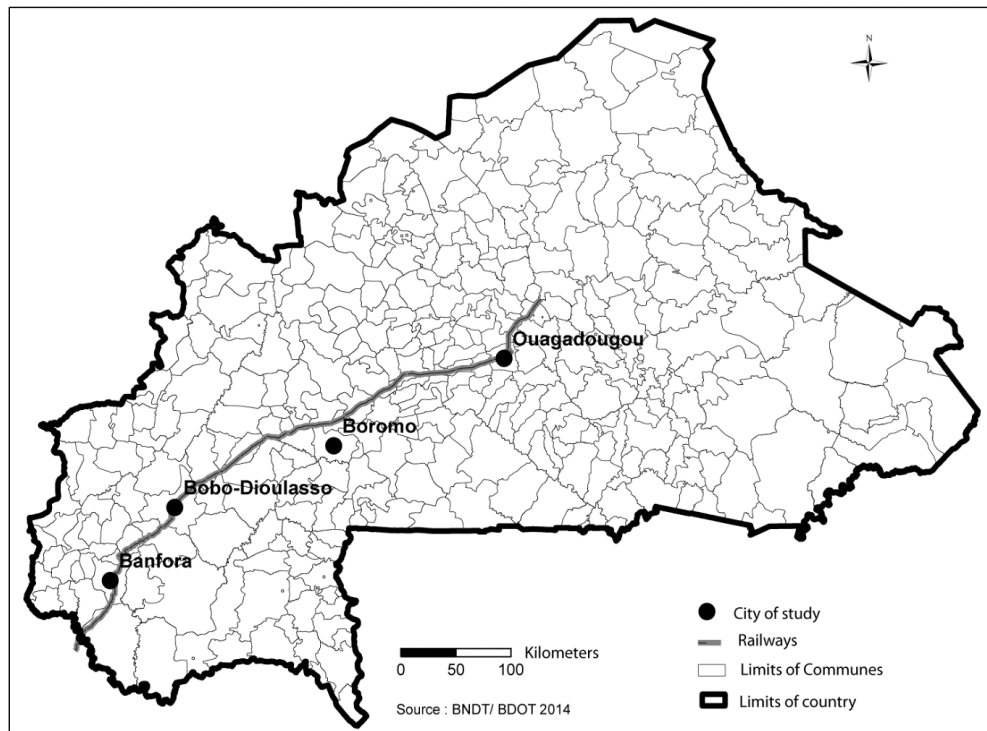
Figure 1. Study sites located on railway transect in Burkina Faso (Banfora, Bobo-Dioulasso, Boromo, Ouagadougou).

2.2. Total RNA virus isolation

Total RNA was extracted from mosquitoes using TRIzol Reagent (Invitrogen, Waltham, MA, USA, Catalog Numbers 15596026 and 15596018) according to the supplier's instructions. Based on the size of the pool, adult mosquitoes were homogenized in 1ml TRIzol using sterile pestle. All pools were individually homogenized by vertexing for 15 s and incubated for 5 min at room temperature to permit complete dissociation of the nucleoproteins complex. Chloroform (200 µL per 1 mL of TRIzol™ Reagent) was added to the mixture, and homogenization was performed by shaking the tubes vigorously for 15s by hand. The mixture was then incubated at room temperature for 2–3 min. The samples were centrifuged at 12,000 g for 15 min at 4 °C. The mixture separates into a lower red phenol-chloroform, and interphase, and a colourless upper aqueous phase. The aqueous phase of each sample was removed and transferred to a new tube containing 500 µL of 100% isopropanol by angling the tube at 45° and pipetting the solution out. This mixture was incubated at room temperature for 10 min and then centrifuged at 12,000 g for 10min at 4 °C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube. The supernatant was removed, and the RNA pellet was washed with 1 mL of 75 % ethanol mixing occasionally by gentle inversion. The samples were homogenized briefly and then centrifuged at 7,500 g for 5 min at 4°C. The supernatant was discarded, and the RNA was then air-dried for 15 min. The RNA pellet was resuspended in 50 µl of RNase-free water by pipetting up and down; then incubated in a water bath or heat block set at 55°C for 10-15 minutes. The quantity and quality of RNA samples were determined using a Synergy™ H1 microplate reader (BioTek, Winooski, Vermont, USA). The RNA samples were serially diluted in ten-fold steps from 10 to 0.0001 ng/µl for a concentration that would consistently give the same amount per well in the qRT-PCR and were stored at − 80 °C.

2.3. Generation of RNA standard for the qRT-PCR assays

The standard curve for DENV-2 virus was conducted according the method described by Tang et al., [25]. In brief, the target region from the 3'UTR was amplified from DENV2 and CHIKV cloned into the pGEM®-T Easy Cloning Vectors (Promega

Corporation, Madison, WI, USA) by primers listed as plasmids (**Table 1**). Theses plasmids were linearized by digestion with *EcoRI* and the target sequences were amplified using the *in vitro* RNA transcription kit (Roche Diagnostics, IN, USA). The plasmids were treated with Fast-Media® Amp LB and the cDNA from four arboviruses were extracted and purified using the QIAprep® Miniprep purification kit (Qiagen). The cDNA thus obtained were used as positive control in all qRT-PCRs. The cDNA was quantified by a NanoDrop™ Spectophotometer (SYNERGY H1, BIOTEK). The copy number of the cDNA was calculated based on the concentration and its molecular weight and 7-fold serial dilutions from 10^2 to 10^8 copies per reaction with known cDNA copy number was used to estimate the virus copy number in infected mosquito samples and then tested in duplicates. Sterile, nuclease-free water was used as a no template control (NTC).

Table 1. Flanking primers used for viral cloning in pGEM T vector as plasmid

Oligo name	Sequence (5'-3')	Size (bp)	Genome position
DENV2_1358F	TAACACCTCACTCAGGRGAAGAG	684	1358-1381
DENV2_1667R	TGGGGATTTTTGAAAGTGACCAATG		3939-3959
CHIKV_645F	GTGCCTACCCCTCATACTCG	553	645–664
CHIKV_1198R	CCGTTGCGTTCTGCCGTTA		1180–1198

2.4. ARBOV RNA detection by qRT-PCR assays

ARBOV detection was performed by quantitative RT-PCR using a C1000 Touch[R] Thermal Cycler machine (CFX96[R] Real-Time System from BIO-RAD). One step real-time qRT-PCR was conducted using ARBO virus-specific primers and TaqMan probes previously reported to detect each specific virus were synthesized by Eurofins Genomics with 5-FAM, HEX as the reporter dye for the probe. The details of the primers and probes sequences and their characteristics are summarized in **Table 2** [26,27]. Using Quantitect Probe qRT-PCR kit (Qiagen), qRT-PCR reactions were performed in a 20 µl volume mixture containing 2 µl of RNA template, 10 µl of 2× QuantiTect Probe qRT-PCR Master Mix, 8 µM of forward primer, 8 µM of reverse primer, 2.5 µM of probe, 0.25 µl of QuantiTect RT Mix and 4.95 µl RNase-free water. The primers and probes were tested in duplicate. qRT-PCR cycling included a single cycle of reverse transcription for 30 min at 50 °C, followed by 15 min at 95 °C for reverse transcriptase inactivation and DNA polymerase activation, and then 45 cycles of 15 s at 94 °C and 60 s at 60 °C (annealing-extension step). The amount of viral RNA in each sample was estimated by comparing the cycle quantification (Cq) threshold values to the standard curve for every qRT-PCR assay. The number of viral copies in each positive sample was estimated by qRT-PCR using absolute quantification by the standard curve method and reported as viral RNA copies/µL (of the eluted RNA).

Table 2. Oligonucleotide primers and fluorogenic probes used in real-time qRT-PCR assay

Oligo names	Nucleotides sequences	Position in ge-nome	Fluorophore	Reference
DEN-2F (forward)	-CAGGTTATGGCACTGTCACGAT-	1605		(26)
DEN-2C (reverse)	-CCATCTGCAGCAACACCATCTC-	1583		
DEN-2 probe	-CTCTCCGA-GAACAGGCCTCGACTTCAA-	1008	HEX/BHQ-1	
CHIKV874(forward)	-AAAGGGCAAACTCAGCTTCAC-	874		(27)
CHIKV961(reverse)	-GCCTGGGCTCATCGTTATTC-	942		
CHIKV899 probe	-CGCTGTGATACAGTGGTTTCGTGTG-	899	FAM/BHQ-1	

2.5. Data analyzis

The real-time data were analyzed using the CFX manager software version 3.1 provided by Bio-Rad. Negative and positive controls were included in all PCR reactions performed. A sample was determined empirically to be positive if the cycle quantification (Cq) value was lower than 36, based on background cross-reactivity of the primers and probes in non-template control reactions. Positive results were determined according to the amplification cycle at which the relative fluorescence unit (RFU) was detected below the cycle quantification.

The Pool Screen 2.0 Version 2.0.3 software were used to calculate the arboviruses minimum infection rate (MIR) with 95% Confidence interval [28]. The MIR uses the assumption that a positive pool contains only one infected mosquito. The statistical analyzis was performed using Fischer exact test. Data were compared by sites and also following to types of area such as urban (Ouagadougou and Bobo-Dioulasso) and suburban (Banfora and Boromo).

3. Results

3.1. Quantification of RNA isolated and ARBOV detection from fields mosquitoes

The RNA concentrations were 11.53 ng/µL and 490.98 ng/µL in pool of 10 and 20 mosquitoes respectively. The ratio of A260:A280 were around 2 value showing a purity of RNA extracted from samples.

Beyond cycle threshold (Cq) of 36 of the amplified RNA samples showing curves were considered very low concentrations and therefore the samples were considered not infected with the targeted virus. Following the protocol described by Tang et al. (25), the purified plasmid was used for each virus and a DNA concentration with a known copy number of 4.7×10^9 was prepared. Subsequently, 10-fold serial dilutions in water were used to prepare seven DNA concentrations with copy numbers ranging from 4.7×10^8 to 4.7×10^2 per ml, which were used to prepare the calibration curves for primer and probe for DENV2. Viral DNA detection was successful for all viruses and the standard curves exhibited linearity over 7 orders of magnitude (**Figure 2**). The correlation coefficient (R^2 value) was 0.998, for DENV2. The calibration curves for CHIKV were previously described per Tang et al., (25), where the correlation coefficient (R^2 value) was 1.000. The regression coefficients (R^2) of 0.998 and 1.000 for DENV2 and CHIKV, respectively indicated that the assay was highly reproducible.

Furthermore, the quantification cycle (Cq) values of DENV2 standard concentration from seven serial dilutions ranged from 11±0.02 to 33.5±0.065 Cq (**Figure 2**). Samples from Ouagadougou tested for the DENV-2 showed an amplification curve with Cq of 32 indicating the samples were infected by DENV2 (**Figure 3**). Otherwise, the quantitative cycles

values of CHIKV standard concentration from seven dilutions ranged from 10±0.05 to 30±0.21 Cq as previously described by Tang et al. [25]. The amplification curves of RNA samples tested for detecting CHIKV started at 28 Cq and reached 35 Cq (**Figure 4**) indicating the presence of the virus RNA at different densities varied based on samples tested. All samples analyzed for CHIKV detection were less than 36 Cq (**Figure 4**), therefore were all considered positive.
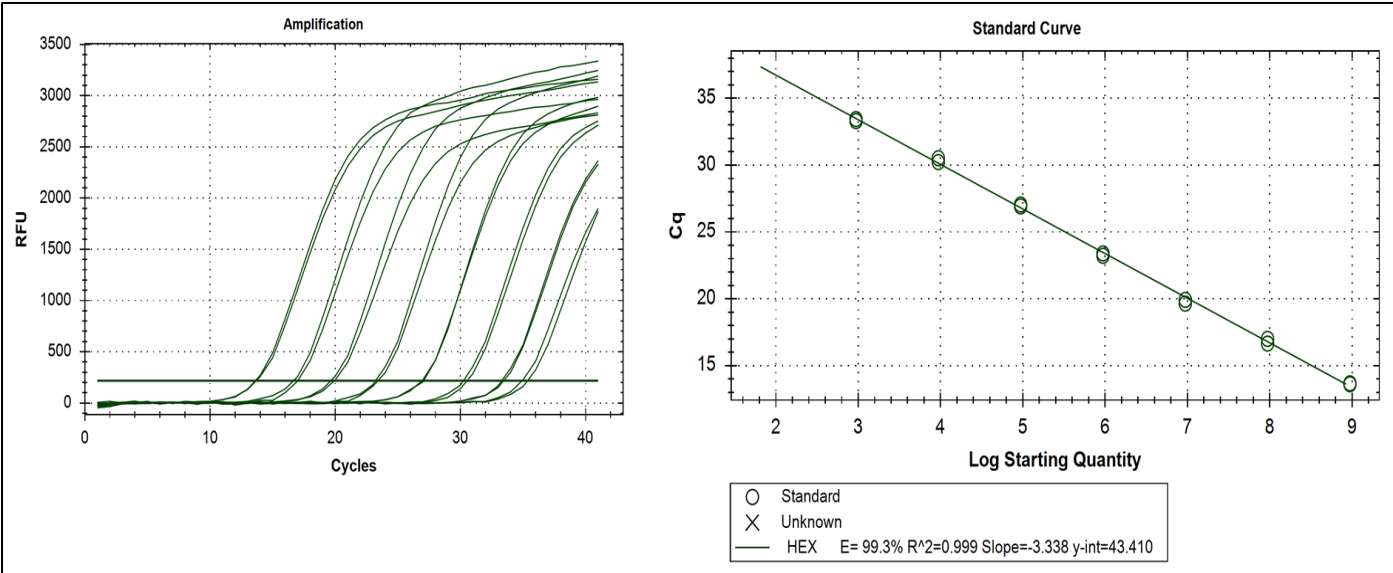

Figure 2. Amplification and standard curves of serial dilution of plasmid containing the sequence targeted by the primers and probes for the qRT-PCR for DENV2 detection. The correlation between the relative florescent unit (RFU) and the quantification cycle (Cq) on the left and between the virus log 10 copy number and the Cq on the right. The regression equations and correlation coefficients (*R*) are given for DENV2 plot.
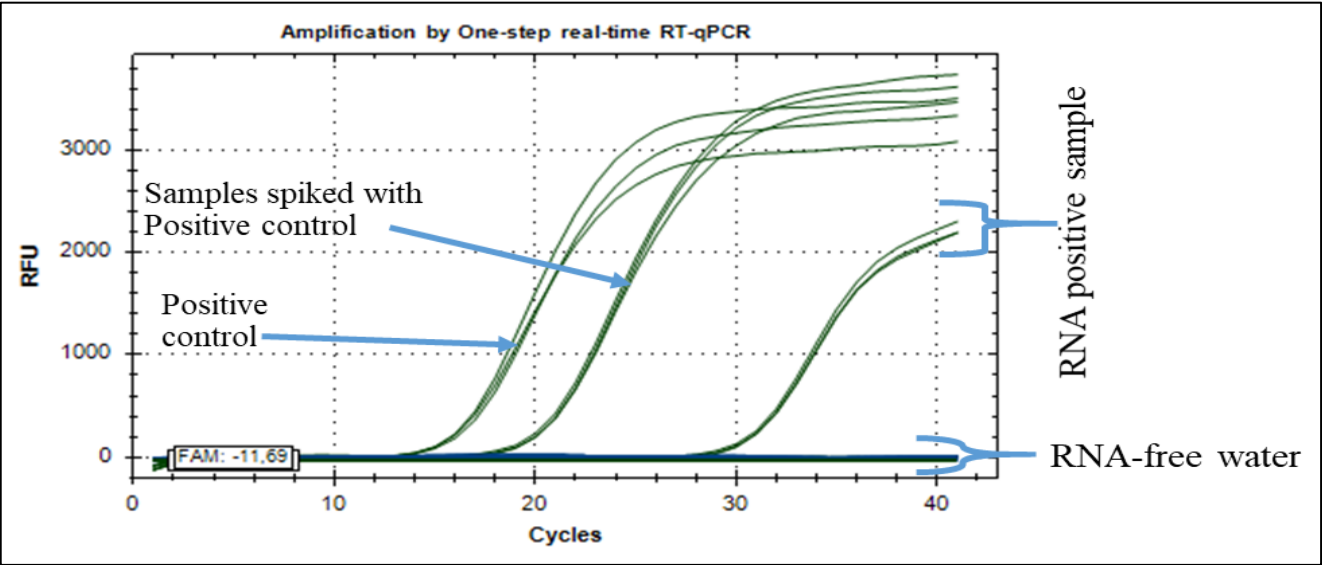

Figure 3. Positive RNA samples from Ouagadougou for detection of dengue virus 2 (DENV2) RNA by one step real-time qRT-PCR assay.
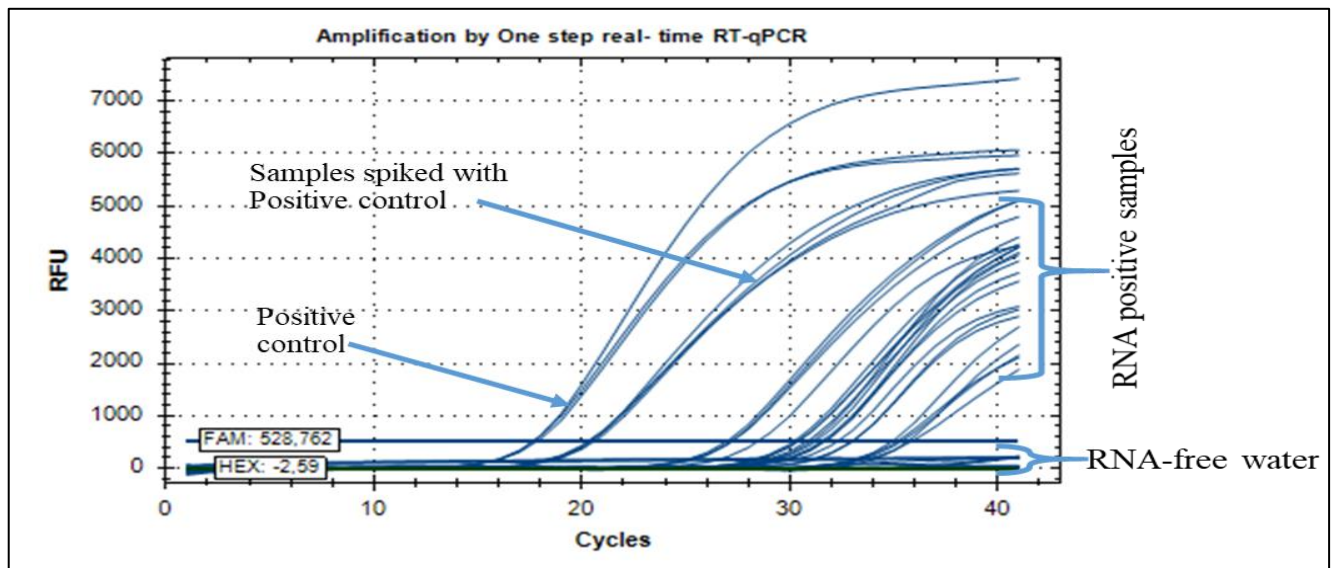
Figure 4. Positive RNA samples from Ouagadougou, Boromo and Banfora for detection of chikungunya virus (CHIKV) by real-time qRT-PCR assay.

3.2. *Spatial distribution and infection rates of arbovirus*

A total of 33 pools of *Aedes* mosquitoes were tested for arboviruses. The mosquitoes were collected in August 2016, corresponding at the time of a dengue outbreak in Burkina Faso. Overall, 580 *Ae. aegypti* were tested (**Table 3**). The qRT-PCR analyzis showed the presence of arboviruses including one pool positive for DENV-2 (3.03%) and six pools positive for CHIKV (18.18%); but density varied depending on locality (**Table 3**). Indeed, the minimum infection rates (MIR) of CHIKV were estimated at 66.67 (95%CI:41.2—72.6) (4 infected pools/60 samples tested); 0 (0 infected pool/140 samples tested); 7.1 (95%CI :4.3—10.2) (1 infected pool/140 samples tested) and 7.1 (95%CI:4.3—10.2) (1 infected pool/140 samples tested) in Ouagadougou, Bobo-Dioulasso, Banfora and Boromo, respectively (**Table 4**). The MIR of CHIKV was significantly higher in Ouagadougou compared to the other locations (P = 0.0219). The MIR of DENV-2 was estimated at 16.67 (95%CI: 14.7—19.4) (1 infected pools/60samples tested) in Ouagadougou while it was not detected in other sites (**Table 3 and 4**). When the study sites were categorised according to their predominant land use type (urban or suburban), MIR differed. In urban areas, the MIRs of DENV-2 was 5 (95%CI :3.7—6.9) and for CHIKV was 20 (95%CI :17.5—26.1), both higher than suburban sites (P<0.01) (**Table 4**). The data also showed a co-infection of arboviruses (DENV/CHIKV) in one pool from Ouagadougou (**Table 3 and 4**).

Table 3. Summary of arboviruses examination of pools (10-20 size) of *Aedes* mosquitoes collected in Burkina Faso

Localities	Number of pools tested	Total number of mosquitoes	Positive pool after qRT-PCR N (%)	
			DENV2	CHIKV
Banfora	7	140	0	1 (14.28)
Bobo-Dioulasso	7	140	0	0
Boromo	7	140	0	1 (14.28)
Ouagadougou	6	60	1 (16.67)	4 (66.67)
Total number	**33**	**580**	**1 (3.03)**	**6 (18.18)**

Table 4. Minimum infection rates (with 95% Confidence interval) of targeted ARBOV screened in *Ae. aegypti* mosquitoes

Locality	DENV2 (95%CI)	CHIKV (95%CI)
Banfora	0	7.1 (4.3—10.2)
Bobo-Dioulasso	0	0
Boromo	0	7.1 (4.3—10.2)
Ouagadougou	16.67 (14.7—19.4)	66.67 (41.2—72.6)
Urban	5 (3.7—6.9)	20 (17.5—26.1)
Sub-urban	0	7.1 (5.4—12.8)

4. Discussion

This study follows on from entomological surveys performed in 2016 in urban and suburban areas along a railway transect from Banfora to Ouagadougou during a dengue outbreak in Burkina Faso. In the first article published from this survey, we found all the larval indices examined had largely exceeded the critical level for outbreaks in the four cities surveyed, suggesting a raised transmission risk for at least DENV, even in suburban areas of Boromo and Banfora [20]. The current data extend the previous conclusions by revealing and quantifying the presence of arboviruses in wild *Ae. aegypti* populations. Indeed, DENV-2, and CHIKV were detected in some pools with pronounced infection rates observed particularly in Ouagadougou for DENV-2. Although CHIKV infection was also detected in Boromo and Banfora, its prevalence was higher in Ouagadougou indicating probably that these viruses were more concomitantly circulating in the capital city than in the other study areas.

Globally the infection features described in *Ae. aegypti* populations confirmed the autochthonous urban transmission of dengue in urban cities as Ouagadougou. They were more or least overlapping with human infections patterns where DENV-2 and DENV-3 serotypes were the most detected in humans [29–32] but exceptionally DENV-4 was reported by Ridde et al.[23] together with DENV-2 and DENV-3. The presence of DENV-2 was also found in the blood of febrile travellers returning from Burkina Faso to France during this period [24].

Our results showed also the occurrence of CHIKV in the vector populations from three of the four sites sampled. This was more prevalent in Ouagadougou than the other areas. No data has previously confirmed their circulation in human patients, probably due to the lack of specific serological tests targeting these viruses during the diagnosis process.

This is the first study confirming the circulation of arboviruses in vector populations both from urban and suburban settings in Burkina Faso. In 1993, sylvatic circulation of arboviruses was confirmed with the isolation of both DENV-2 and CHIKV in the peripheral gallery forest of Bobo-Dioulasso area from wild *Aedes* mosquitoes including *Ae. furcifer*, *Ae. luteocephalus*, *Ae. cumminsi*, *Ae. opok*, and in Bobo-Dioulasso city for DENV-2 from *Ae. Aegypti* [13]. Arbovirus's epidemiology in Africa during the past five years shows an expansion of some arboviruses such as West Nile, Chikungunya, Zika in West Africa [33–35].

Our results confirm a vertical transmission of DENV-2 and CHIKV in *Ae. aegypti* populations from urban and suburban settlements that has been reported by previous studies [17,19,20].]. Vertical transmission (infected female mosquito-infected offspring) is assumed to be a mechanism ensuring the maintenance of the virus during conditions that would be adverse for horizontal transmission (i.e. harsh winters, inter-epidemic stages) [24] and can also potentially accelerate the epidemiology of ARBOV if the surveillance system is not sufficiently reliable. Although it is mainly the horizontal transmission (human-mosquito-human) of arboviruses that determines the epidemiology of the disease,

health authorities should therefore be vigilant for future outbreaks. In addition, one co-infection (DENV / CHIKV) was detected in a pool of Aedes from Ouagadougou highlighting the co-circulation of both ARBOV. As our control system did not target the larval stages assuring the vertical transmission, it can support autochthone transmission if larval indices and house index reach the threshold. Furthermore, the growth of international travel to and from Burkina Faso, particularly of passengers arriving from other arbovirus-endemic countries, should be considered a potential virus reservoir. This situation maintains the circulation of arboviruses ARBOV in Burkina Faso. During the rainy season, this vertical transmission could be of the origin of a re-emergence of at least dengue disease.

5. Conclusions

Our study revealed the presence of DENV-2 and CHIKV, both in urban and suburban areas, isolated from wild *Ae. aegypti* larvae reared through to adult. This highlighted the occurrence of vertical transmission and leads us to suggest the urgent need to implement a vector control programme including larval source reduction in Burkina Faso, instead of only fogging adult *Ae. aegypti* populations with insecticides, which were not evaluated for their field efficacy after intervention. This molecular approach is the first successful investigation using molecular diagnostic in Burkina to detect and quantify arboviruses within wild mosquito populations during outbreak periods. This tool is powerful and suitable and should be adopted by the National surveillance program of ARBOV and can be extended to adult mosquitoes directly collected in the field. The results indicated that this technique is suitable for widescale use and should be adopted by arboviral surveillance and control programs in Burkina Faso.

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