



Research Article

Distribution and Molecular characterization of Blackflies (*Simulium damnosum*) infected with *Onchocerca volvulus* along river Zururu Malalin Gabas Kaduna

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ABSTRACT

Blackflies (*Simulium damnosum*) found along fast-flowing rivers and streams is the vector of the disease-causing organism, *Onchocerca volvulus* which is transmitted through the bite of the fly during blood-feeding on humans resulting in ultimately irreversible blindness and debilitating skin disease which brings about the restriction of social activities and economic development in areas of agricultural activities located near fast-flowing streams and rivers. This study was aimed at determining the distribution and molecular characterization of blackflies infected with *Simulium damnosum* and the physicochemical parameters of the water along river Zururu Malalin Gabas Kaduna. About 150 blackflies were collected from the sampling station, river zururu which is a tributary that terminates into river Kaduna at Malalin gabas Kaduna, using a hand scooping net and preserved using 80% ethanol from October 2015 to November 2016. The 150 flies collected were separated into pools of 50 for DNA extraction. Purified DNA obtained was subjected to PCR analysis and amplified using the 12SrRNA forward and reverse primers for the *Simulium* species and the 0-1500V forward and reverse primers for the *Onchocerca volvulus*. The gene amplification using the 12SrRNA generated a band size of 196bp for the blackfly species and the 0-1500V generated a band size of 190bp for the *Onchocerca volvulus*. Sequencing and BLAST of the PCR products using the 12SrRNA confirmed the following identities. *Simulium damnosum* (93%), *S. maklarini* (90%), *S. laterale* (90%), and *S. middlemissae* (78%). The results from these studies show without any doubt that there is a presence of blackflies actively feeding in the area and are infected with *Onchocerca volvulus*, and also their distribution is correlated with physicochemical parameters of the river waters.

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INTRODUCTION

Black flies (*Simulium damnosum*) breed along streams, river banks and waterfalls in the tropical and subtropical areas of Africa and Asia (Adeleke *et al.*, 2011). They are the vectors of the parasitic worm *Onchocerca volvulus* (*O. volvulus*), which causes a disease condition known as Onchocerciasis (Wilson and Post, 1994). *Onchocerca. Volvulus* can live in the human body for up to 14 years. Onchocerciasis is a chronic parasitic disease characterized by intense itching and skin depigmentation, lymphadenitis, elephantiasis and serious visual impairment leading to blindness in most cases (Jacob *et al.*, 2010). It was reported as the world's second leading cause of blindness (Ibeh *et al.*, 2006). It was estimated that about 140 million people are at risk of onchocerciasis globally, out of which 35 million people are infected, and a greater percentage of the infected people live in Africa (CDC, 2010; WHO, 2014; APOC, 2015). Nigeria has the highest world prevalence of onchocerciasis, with nine (9) different cytoforms of the blackflies presumed to be the vectors of the disease (Ibeh *et al.*, 2007). They include *S. damnosum* Nile form, *S. damnosum* Volta form, *S. sirbanum* Siba form, *S. sirbanum* Sudanese form, *S. soubrense* Beffa form, *S. squamosum* A, *S. squamosum* B, *S. squamosum* C, and *S. yahense* (Maikaje *et al.*, 2015).

Because fast-flowing streams and rivers are favourable breeding sites for blackflies, people living around such waters in endemic areas are most at risk of *O. volvulus* infection (Adeleke *et al.*, 2011). Physicochemical characteristics of the river and streams that serve as breeding sites are reported to correlate with the distribution of the different species of blackflies (Najeeb *et al.*, 2015). The abundance of blackflies in an area and the fear of Onchocerciasis are usually associated with the restriction of socioeconomic activities such as

farming, fishing, and fruit-gathering (Adler *et al.*, 2004). This results in a severe decline in the socio-economic development of that area as fertile riverbanks are abandoned for fear of the disease (Adeleke *et al.*, 2010). In men, Onchocerciasis is contracted through the bite of a black female fly infected with *O. volvulus* (Jacob *et al.*, 2010). WHO has identified onchocerciasis as one of the most neglected tropical diseases (WHO, 2012), and hence they do not receive the attention they need. Therefore, molecular characterization of female black flies to determine their infectivity is crucial in estimating the risk of Onchocerciasis in an area. The entomological surveys of blackflies are an essential component in determining if *O. volvulus* transmission has been interrupted and/or if focal elimination of the parasite has been achieved (Roderiguez-Perez *et al.*, 1999).

Different molecular techniques have been used to estimate the infectivity of adult female blackflies caught in the wild (Crosskey, 1990). The 0-150 PCR pool screen technique can detect a single infected fly in pools of about 100 flies (WHO). In Africa and Mexico, several studies have proven that the 0-150 pool screening PCR assay generated estimates of infective flies' prevalence in the vector population indistinguishable from those obtained by traditional dissection of the flies (Jacob *et al.*, 2010). The epidemiological and entomological survey was conducted in some local governments of Kaduna State to assess the impact of long-term treatment with Ivermectin (Tekle *et al.*, 2012) but did not include the Kaduna metropolis. Reports by Amuta (1982) showed the prevalence and distribution of onchocerciasis and the occurrence of the vectors in some parts of Kaduna state but not within the metropolis river. Metropolis River is a section of river Kaduna within the Kaduna metropolis, where the vegetation and terrain around the river show the type that supports the

breeding of blackflies. The present study aimed to evaluate the distribution of infected blackflies and the physicochemical characteristics that support *Onchocerca volvulus* infected blackflies' distribution along river Zururu Malalin Gabas Kaduna and their molecular characterization using 12SrRNA and 0-1500V.

MATERIALS AND METHODS

Sampling site

The study was conducted along River Zururu, a tributary that terminates into river Kaduna at Malalin gabas, from October 2015 to November 2016. Sampling points were selected for the study based on the anthropogenic activities prevalent around the river. The vegetation (grasses, shrubs and trees) and terrain known to support the breeding of blackflies were considered. The sampling site (Malalin gabas) is located between latitudes 10°

36' 7.31" N and longitude 7° 30' 8.09" E at an elevation of 597m along the northern axis of Kaduna (Figure 1).

Sampling and pool screening of flies

Adult black flies were caught randomly using hand scooping nets from the long and short grasses, shrubs and tree branches which serve as their resting sites along the river banks and preserved in 80% ethanol, which were later taken to the laboratory for morphological and entomological identification using the keys of external morphology (Takaoka, 2002), and also 150 identified blackflies were screened for *S. damnosum* and *O. volvulus* DNA using pool screening technique at the DNA laboratory Kaduna. The flies were aliquot into pools of fifty (50) flies in three replicates for DNA extraction.

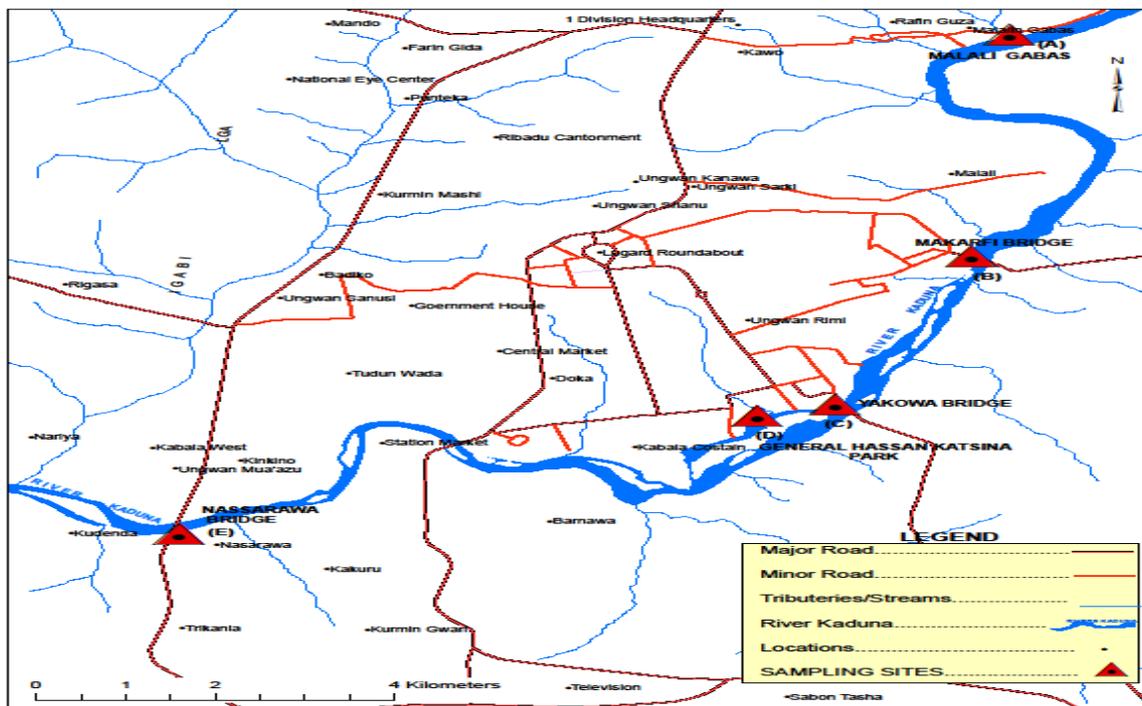


Figure 1: Map of River Kaduna within Kaduna Metropolis and the sampling station's environment. (Source: Department of Geography, NDA Kaduna)

DNA extraction and purification

Each pool of blackflies was placed into a 1.5µl eppendorf tube, rinsed thoroughly with distilled water and crushed using a blunted plastic pestle for DNA extraction. Four hundred µl of lysis buffer (100mm NaCl, 10mm Tris-HCl, pH 8.0, 1mmEDTA, 0.1% sodium dodecyl sulphate 100mg/ml) (Bioneer company) was added followed by 10µl of proteinase K in each tube and homogenized. This was incubated at 65°C for 1 hour after which it was vortexed 3 times at intervals of 20 minutes for another 1-hour incubation period. Four hundred µl of phenol-chloroform was added and vortexed for 15-20 seconds and spun for 10mins and other protocols were observed as reported by Post *et al.* (2011) and Gopal *et al.* (2012) for purified DNA extraction.

Polymerase chain reaction (PCR) for *S. damnosum* and *O. volvulus* gene amplification

The PCR was carried out according to the method of Alhassan *et al.* (2014) to characterize *Simulium* and *Onchocerca* species contained in the vector by amplifying the 12S rRNA and 0-150OV genes respectively (Tang *et al.*, 1996; Tang *et al.*, 2010). The primers and premix are the product of Bioneer Company, USA. The PCR was carried out using a reconstituted solution of 25µl premix as described by Post *et al.* (2011). The primer sequence used for the amplification of the 12S rRNA is forward 5'AATAGGGTATCTAATCCTAGTTT-3' and reverse 5'TATTGGTAAATTTTGTGCC-3' (Toe *et al.*, 1997), while the primer sequence used for the amplification of the 0-150OV are forward 5'GATTYTTCCGRCGAANARCGC-3' and reverse 5'GCRTRTAAATNYGNAAATTC-3' 0150-1633 (Rodriguez-perez *et al.*, 2015). PCR products were run on Agarose Gel Electrophoresis (Biorad, Italy).

Sequencing of *Simulium spp* and *Onchocerca spp* amplicons.

Sequence determination of the 12S rRNA gene was done following the routine protocol by DNA laboratory, Kaduna. PCR products of some positive samples of purified single DNA strands were sequenced with the Beckman Coulter CEQ 2000XL DNA analysis system (Macrogen, Seoul, South Korea). The sequence of the gene was aligned with the gene sequences recorded in the GenBank databases on the National Centre for Bioinformatics information (NCBI) using the basic local alignment search tool (BLAST) using the PCR machine by Labnet International incorporation Multigene II., BECKMAN COULTER

Amplification of the PCR products of the expected size was confirmed by electrophoresis (GIBCO BRL Electrophoresis Power Supply Life Technology). The sequence of the 12S rRNA gene was determined using a Dye terminator cycle sequencing with the quick-start kit, and the product was analysed with an ABI prism DNA sequence (ABI) (Pettersen *et al.*, 2009).

BLAST SEARCH for *Simulium spp* and *Onchocerca spp* amplicons using GenBank

The gene sequences of each product obtained were compared with known 12S rRNA gene sequences in the GenBank database by aligning with a blast search of the NCBI database to find the regions of similarity between the sequences (Zhao and Chu, 2014).

Physicochemical parameters

Physicochemical parameters of the water from the river where sampling was done were measured at the time of sampling where the blackflies were also collected and carried out once every month. Atmospheric temperature (AT), Water temperature (WT), Dissolved oxygen (DO), Biochemical oxygen demand (BOD), Electrical conductivity (EC), Total

dissolve solids (TDS), Alkalinity, Turbidity, and Velocity were all determined according to the American Public Health Association (APHA 2000) methods.

Statistical analysis

Data were analyzed using GraphPad Prism 7 (GraphPad Software, USA). Percentage and fold change was used to express the abundance of blackflies. A student t-test was used to compare the seasonal difference in the physicochemical parameters. The correlation between physicochemical parameters and the distribution of blackflies was estimated for physicochemical parameters that have a significant difference between the dry and rainy seasons using Pearson's correlation coefficient. $P < 0.05$ was considered statistically significant.

RESULTS

Seasonal abundance of blackflies and infection along river Zururu Kaduna

The total number of blackflies collected from the study site shows they are more abundant in the dry season with 1051 flies collected compared to the rainy season with 476 flies. The number of male and female flies collected were 706 and 345 flies respectively in the dry season while the male and female flies collected during the rainy season were 319 and 157 flies respectively (Table 1). Out of the 345 female flies collected during the dry season, 265 (76.8%) were examined and 75 (28.30%) were found to be infected with *O. volvulus*. All the female flies collected during the rainy season were examined and 40 (25.48%) were infected with *O. volvulus* (Tables 1 & 2).

Table 1: Seasonal abundance of blackflies along river Zururu Kaduna

Seasons	No. of flies collected	No. of female flies	Total
Dry season	706	345 (48.9%)	1051
Raining season	319	157 (49.2%)	476
Total	1025	502	1527

Table 2: Seasonal prevalence of *Onchocerca volvulus* infection in blackflies along river Zururu Kaduna

Seasons	No. of flies examined	No. of infected flies
Dry season	265	75 (28.30%)
Raining season	157	40 (25.48%)

Molecular characterization of S. damnosum and O. volvulus using 12SrRNA and 0-150OV primers

The 12SrRNA gene amplification of *Simulium* spp produced a band product at 196bp while that of *O. volvulus* DNA from *Simulium* spp produced a band product at 190bp (Figure 2A & 2B). Sequencing of the PCR products of the *Simulium* and the *Onchocerca* DNA produced nucleotide sequences of 170, 126 and 197 letters for the 12S rRNA. The alignment scores with a query length

and nucleotide sequence of 170 letters gave 93% alignment similarity for *S. damnosum*. The alignment scores with a query length and nucleotide sequence of 126 letters gave 90% alignment similarity for *S. maklarini* and *S. laterale* while the alignment scores with a query length and nucleotide sequence of 197 letters gave 78% and 77% alignment similarity for *S. middlemissae* range 1 and 2 respectively (Table 3).

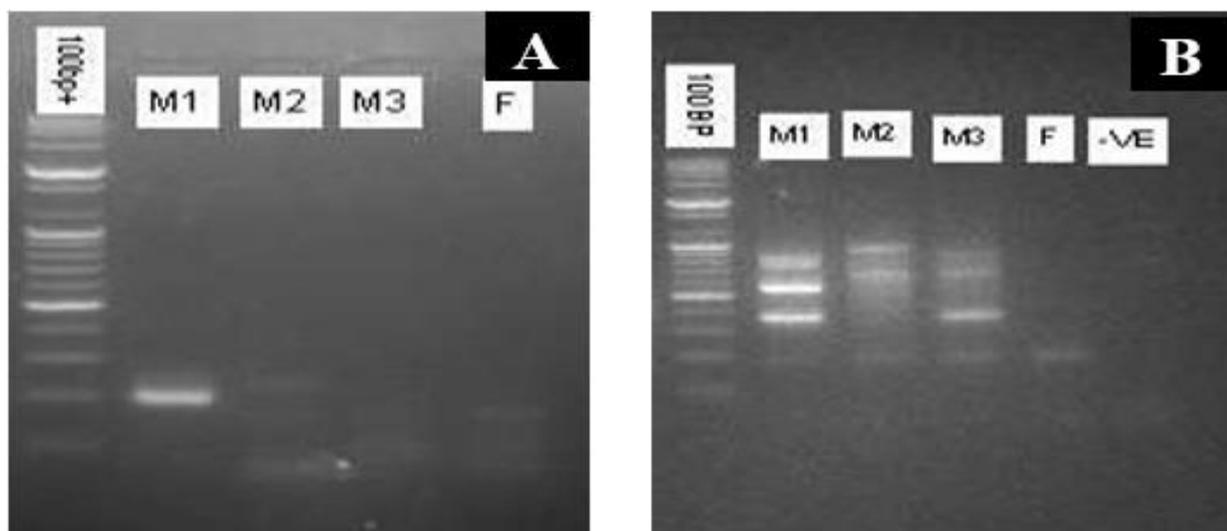


Figure 2: PCR band for 12SrRNA gene amplification of the *Simulium* spp. 196bp (A) and PCR band for 0-150OV gene amplification of *O. volvulus* from the *Simulium* spp 190bp (B). M1=*Simulium damnosum*, M2= *Simulium maklarini* and *Simulium laterale* M3= *Simulium middlemissae*

Physicochemical parameters

Student t-test revealed no significant change in atmospheric temperature (32.25 ± 2.40 and 30.23 ± 0.88), water temperature (27.18 ± 2.15 and 26.13 ± 1.66), pH (7.47 ± 0.15 and 7.25 ± 0.29), total dissolved solids (70.00 ± 10.95 and 81.67 ± 8.17) and conductivity (105.00 ± 0.88 and 123.33 ± 16.33) of the dry season compared to the raining season respectively at $P > 0.05$ (Figures 3,

4 & 5). Turbidity was significantly higher during the dry season (0.12 ± 0.05) compared to the rainy season (0.06 ± 0.05) (Figure 4) while alkalinity (55.83 ± 9.17 and 38.83 ± 10.61), biochemical oxygen demand (7.90 ± 0.55 and 4.45 ± 1.25), dissolved oxygen (11.22 ± 1.44 and 7.18 ± 1.77) and velocity (0.11 ± 0.03 and 0.07 ± 0.03) were significantly higher in the raining season compared to the dry season respectively at $P < 0.05$ (Figures 4 & 5).

Table 3: Summary of the BLAST searches using mitochondrial ribosomal RNA gene PCR Amplicons of the *Simulium* spp.

Pool	NS	QL	Query ID	BLAST hit/% identities	No. of Hits	Sequence ID	Accession
M1	170	170	230103	<i>Simulium damnosum</i> DNA sequence 14/15(93%)	1	gb/AF0104 10.1	AF010410.1
M2	126	126	201147	<i>Simulium maklarini</i> isolate M1 12SrRNA gene, partial sequence mitochondrial 18/20(90%) <i>Simulium laterale</i> isolate L1 12SrRNA gene, partial sequence mitochondrial. 18/20 90%)	2	gb /KT207435 .1 gb/KT2074 34.1	KT207435.1 KT207434.1
M3	197	197	222903	<i>Simulium middlemissae</i> 12SrRNA gene, partial sequence, mitochondrial gene for mitochondrial product. 127/163(78%)	10.2	gb/AF3334 67.1	AF333467.1

The No1 BLAST HIT corresponds to the alignment results with the highest identity with the mitochondrial genes, using 12SrRNA forward and reverse sequence of the mitochondrial genes 12SrRNA gene PCR Amplicon. M1, M2 and M3= Pools of 50 blackflies, QL= Query Length, NS= Nucleotide Sequence.

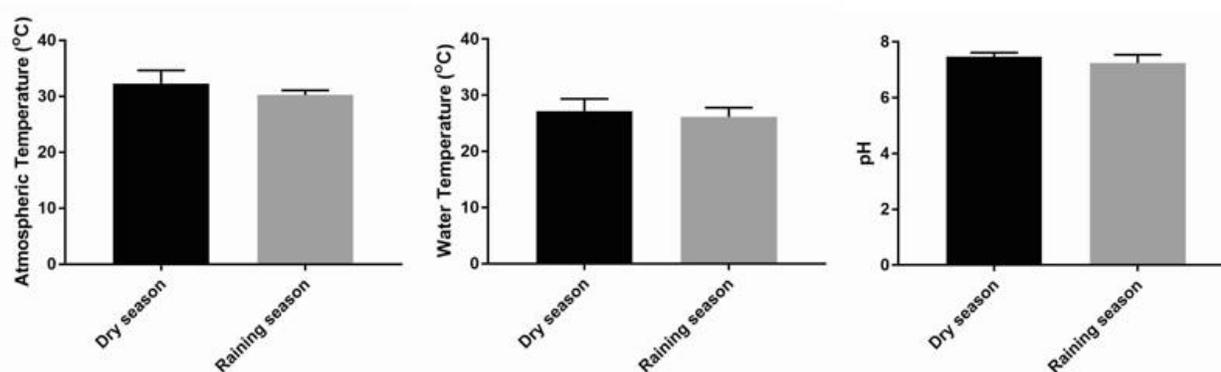


Figure 3: Atmospheric temperature, water temperature and pH of river Zururu Kaduna. Values are presented as mean±SD, *** indicates a significant difference at P<0.05.

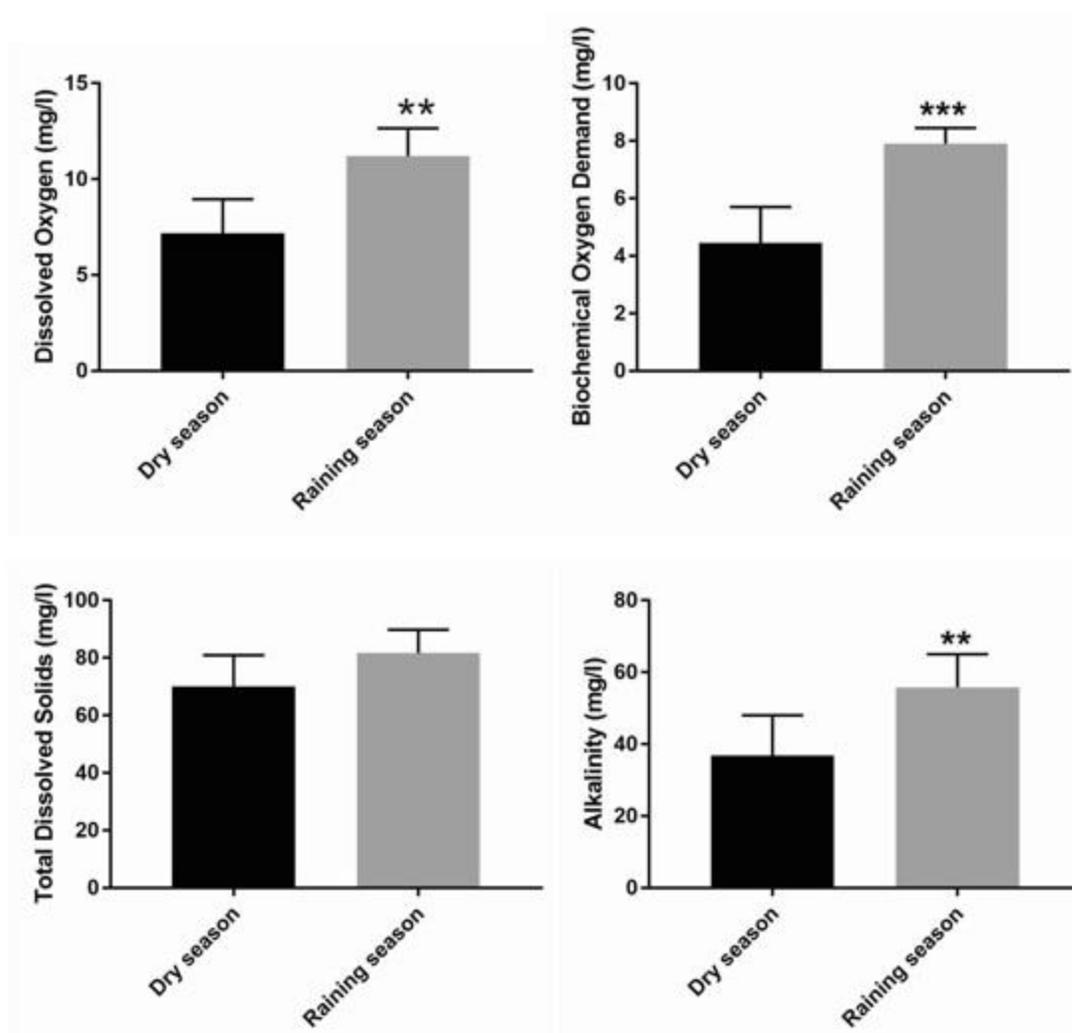


Figure 4: Dissolved oxygen, biochemical oxygen demand, total dissolved solids and alkalinity of river Zururu Kaduna. Values are presented as mean±SD, *** & ** indicates significant difference at P<0.05.

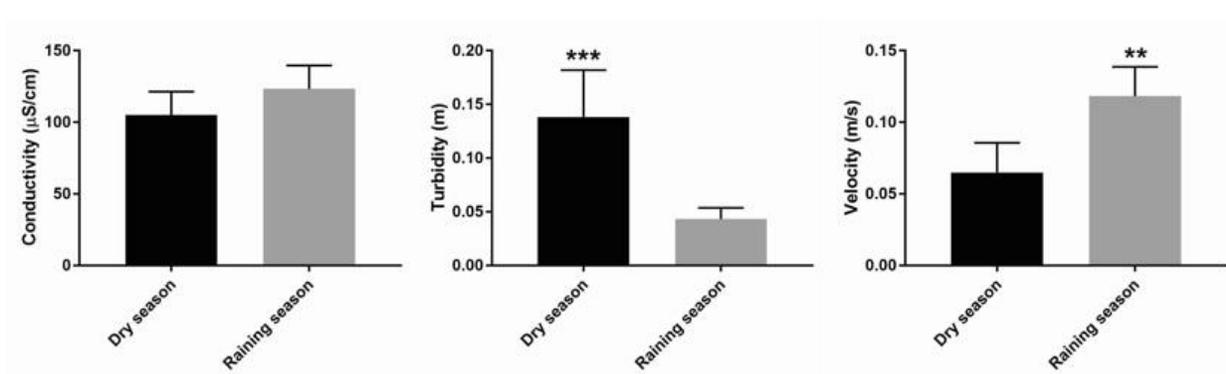


Figure 5: Conductivity, turbidity and velocity of river Zururu Kaduna. Values are presented as mean±SD, *** & ** indicates significant difference at P<0.05.

During the dry season, there was a significant positive correlation between dissolved oxygen

with the number of flies as well as between velocity and the number of flies at P<0.05 (Table

4). There was no significant correlation between biochemical oxygen demand with a number of flies, alkalinity with the number of flies, as well as turbidity with the number of flies in the dry season at $P>0.05$ (Table 4). During the rainy season, a significant negative correlation was observed between biochemical oxygen demand with the number of flies at $P<0.05$ (Table 4). No significant correlation was observed between dissolved oxygen with the number of flies,

alkalinity with the number of flies, turbidity with the number of flies and velocity with the number of flies at $P>0.05$ (Table 4). The result suggests that the dissolved oxygen level and velocity of river Zururu at Malalin gabas creates a good breeding site for blackflies while a decrease in biochemical oxygen demand during the rainy season creates a favourable environment for blackflies breeding.

Table 4: Physicochemical parameters and the distribution of blackflies at river Zururu Kaduna

Parameters	Dry season (r)	P value	Raining season (r)	P value
Dissolved oxygen (mg/l)	0.8704	0.0241*	0.3593	0.4843 ns
Biochemical oxygen demand (mg/l)	0.0000	0.9990 ns	-0.8239	0.0438 *
Alkalinity (mg/l)	0.7997	0.0562 ns	0.4989	0.3138 ns
Turbidity (m)	-0.06930	0.1269 ns	0.1448	0.7843 ns
Velocity (m/s)	0.9192	0.0095 *	0.1333	0.8012 ns

r = Pearson's correlation coefficient, ns= non-significant difference, * indicates significant difference at $P>0.05$.

DISCUSSION

This study showed blackflies are in abundance along river Zururu and there is an indication of infection with *O. volvulus*. This indicates that people living around the river or visitors coming around the river or those engaged in farming and fishing activities around the river may be exposed to bites and infection since there is a possibility of the blackflies harbouring the microfilaria. According to Weatheral (1995), the percentage of molecule infection in sample flies might indicate transmission is very active, especially with a lot of activities going on along the river bank. The number of infected blackflies found in this study indicates that this site is moderately infested with the blood-sucking flies and the number of worms recovered from the dissected female flies and the DNA result indicates a regular availability of a

human source of blood meal and a suitable breeding and resting sites. The prevalence of infected flies is an important epidemiological tool to help the decision-makers in the control of blackflies.

The molecular methods based on PCR have proven to be sensitive and specific. A 196-bp region of the 12SrRNA gene was amplified by PCR using the primers 12SrRNA for the detection of the blackfly species. The amplified PCR band has proven the blackflies to be species of the *S. damnosum* complex. The 0-1500V PCR amplification was also sensitive and specific and has detected and identified *O. volvulus* at 190bp length comparable to the reports (WHO, 2014). This has proven that the *Simulium* spp. are infected with *O. volvulus* worms. The reaction product produced an amplicon of 190bp for the

parasite *O. volvulus* and 196bp for the blackfly *Simulium*, in agreement with (Johansson, 2009). The ability of the 12SrRNA gene to amplify the DNA extracted from the flies generating a band size of 196bp is comparable to the report of (Lee *et al.*, 2012; Alhassan *et al.*, 2014). The 0-1500V gene band size of 190bp observed from the amplified DNA extracted from the *O. volvulus* infected *S. damnosum* is comparable to the reports (Simon *et al.*, 1994). To determine the identity of these blackflies, we amplified a portion of the blackfly mitochondrial 12SrRNA gene from the DNA samples by PCR sequencing. A nucleotide sequence was obtained which was used to carry out the BLAST search. The sequence is informative in distinguishing several blackfly species from American blackfly species, according to Acharya (2016). A comparison of the sequence data BLAST from station A obtained from the amplicons with the GenBank database showed that the sequences were similar to members of the genus *Simulium* of the following species with a similarity percentage as follows; *S. damnosum* (93%), *S. maklarini* (90%), *S. laterale* (90%) and *S. middlemissae* (78%). The *S. maklarini* is a species in the *S. melanopus* species group formerly misidentified as *S. laterale* (Hojas and Post, 2000). The *S. laterale* is also a species group identified by Sabah and Sawarak in Malaysia (Hojas and Post, 2000), while *S. middlemissae* is from French Polynesia (Morales-Hojas *et al.*, 2001). Results from bioinformatics carried out on the DNA sequences from station A has shown that the *Simulium* genes share similarity with the genes of the GenBank database as follows, 93%, 90%, 90% and 78%. This study has shown that molecular characterization using the PCR technique is more sensitive and can detect the infection once the parasite DNA is found during amplification. These were species that have been reported elsewhere but never in Nigeria or even in Kaduna. The explanation for the presence of these three related species might be because of earlier

misidentification or gene mutations or probably because no researcher has ever worked there to identify the species present in that area, especially the molecular characterization.

However, the *O. volvulus* parasite sequencing using the 0-1500V did not produce any sequence. The study station site (Malalin gabas) is an area where no known research study has been carried out before, and neither has there been any literature report to that effect. The blackfly there poses a great threat to the communities and the rural farmers in close proximity to the river. Results of the study presented on the physicochemical parameter at the station indicate suitable breeding sites for the blackflies, and the immature stages since the adult blackfly and immature stages were recovered from the study site (Plate 1). Aquatic insects are reported to be bio-indicators of water quality, and many physicochemical parameters of the water, such as water temperature, DO, Turbidity, Velocity, and pH, are known to affect the distribution of blackfly species and their abundance in the streams of Doi Pha Horn Pok National Park, Thailand. (Wichai *et al.*, 2015).

The mean water temperature of the dry season is in agreement with the reports of Alison *et al.* (2007). The water temperature of the dry and rainy season is found to be suitable for *Simulium* immature and adult resting and breeding sites. Temperature is critical in blackfly distribution because adequate temperature favours early life cycle completion (Tongjura *et al.*, 2015). Sam-Wobo *et al.* (2014) and Adeleke *et al.* (2011) reported most species' larva development depends on adequate temperature. Ezegbo-Nwobi and Eneanya (2013) reported that water temperature influences the breeding of the *S. damnosum* complex in Nigeria.

The mean pH values obtained in this study during the dry and rainy seasons were within the normal range for biological activities, which is comparable to those reported by Mafuyai *et al.*

(1996), Bassey (1998) and Ibeh *et al.* (2007) of *Simulium* breeding sites in Nigeria, which is the pH range for most biological activities and also found to be suitable for the breeding of *Simulium* as reported by Sam-Wobo *et al.* (2014).

The mean conductivity values recorded for the two seasons were higher; this is in contrast to those reported by Opara *et al.* (2005), as conductivity does influence the development and distribution of the larval population of blackflies and also influences their breeding in Nigeria. There is a noticeable decrease in dissolved oxygen (DO) during the dry season compared to the rainy season, which might be due to water evaporation, high temperature during the dry season and low humidity during the rainy season; these values are comparable to the value reported by Bernotiene, (2006) and also that of Rabha *et al.* (2013), which are suitable for the breeding site and resting site of blackflies. There were higher alkalinity values reported in this study than those reported by Allison *et al.* (2007). The alkalinity values were higher in the rainy season than the dry season, which is also suitable for the blackflies since they were recovered at this site. The Biochemical oxygen demand (BOD) values obtained in this study are higher in the rainy season than in the dry season. Therefore, these values are within the range suitable for *Simulium* breeding and resting, which is comparable to the reports of Ezugbo-Nwobi and Eneanya (2013) and Rabha *et al.* (2013).

Turbidity values are very low during the dry season compared to the rainy season, but these

values are still within the range suitable for *Simulium* breeding and resting sites, which is comparable to the reports of Rabha *et al.* (2013). This can explain the presence of *Simulium* in these water bodies, especially in the larval stages which are believed to be water quality indicators as reported by Rabha *et al.* (2013). The mean velocity of the river in the rainy season is higher than in the dry season. This is because, during the rainy season, there is an increase in the river's flow due to rainfall which tends to increase its velocity. This is comparable to the report of several authors on the importance of water velocity on the distribution of blackflies and larval density (Palmer and Craig, 2000, Opoku, 2006, Figueiro *et al.*, 2008, Adeleke *et al.*, 2011).

CONCLUSION

From the study results, it could be concluded that there is an infestation of the area with blackflies, which are also infected with *O. volvulus*, which may indicate a salient and moderate transmission going on along river Zururu (Malalin gabas). The molecular analysis results show that the DNA of *O. volvulus* can be detected from the whole pool without separating the head from the body. It also showed that some physicochemical parameters such as dissolved oxygen, biochemical oxygen demand and velocity are correlated with the number of blackflies, making them essential and suitable for the breeding and resting of blackflies.

COMPETING INTEREST

None

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