Original Research Article

Molecular characterization of *Onchocerca volvulus* and *Simulium damnosum* and the physicochemical parameters supporting the fly distribution along the Gamji Park section of River Kaduna

Abstract

The blackfly (Simulium damnosum) is the primary vector of Onchocerca volvulus or river blindness parasite in most parts of Africa including Nigeria. This study aimed to evaluate the distribution and physicochemical parameters supporting Onchocerca volvulus infected blackflies along the Gamji Park section of River Kaduna and their molecular characterization using 0-150 OV and 12S rRNA. A grand total of 2689 blackflies were collected from the study site using hand scooping net and preserved in 80% ethanol for entomological studies. Out of these, 300 flies were randomly selected and divided into pools of 50 flies for DNA extraction and amplification. Purified DNA obtained was subjected to PCR analysis and amplified using the primers 12SrRNA for Simulium species and the 0-150OV for Onchocerca volvulus. The physicochemical parameters of the sampling station were also determined and correlated with number of flies caught .A non-significant negative correlation was observed between the dissolved oxygen, total dissolved solids, conductivity, velocity and number of blackflies caught during the dry (November to April) and rainy (May to October) seasons. Gene amplification generated a band size of 196bp for Simulium species and 190bp for Onchocerca volvulus. Sequencing and BLAST of the PCR products using the 12SrRNA confirmed the following identities: Simulium damnosum(COII) gene (94%), S. damnosum (ND4) gene (94%), S. damnosum Ni4 16s gene(100%), S. damnosum 16s rRNA gene(100%), S. damnosum strain Se-19 16s gene(100%), S. damnosum strain Se-12 16s gene (100%), S. damnosum country Uganda 16s gene (100%), S. damnosum UNI NADH gene(93%), and S. damnosum 16s rRNA gene (100%). In conclusion, the results suggested abundance of blackflies many of which were infected with Onchocerca volvulus in the area; indicating a silent and moderate transmission of Onchocerca volvulus going on along the river.

Keywords: Blackfly, *Onchocerca volvulus*, Gamji Park, River Kaduna, PCR, Sequencing, Physicochemical parameters.

INTRODUCTION

The Blackfly (Diptera: *Simuliidae*) is a small, humped, blood-sucking fly distributed worldwide usually along fast flowing streams and rivers where their early stages develop[1]. The flies are the primary vectors of the parasitic worm *Onchocerca volvulus* which causes onchocerciasis or river blindness disease [2].Onchocerciasis is a chronic disease characterized by intense itching and skin depigmentation, lymphadenitis, elephantiasis and serious visual impairment that may result to blindness [2]. Onchocerciasis was reported as the world's second cause of blindness in man [3]. About 140 million people are at risk of onchocerciasis globally, out of which 35 million people are infected with a greater percentage of these in Africa [4,5]. Nigeria has the highest world prevalence of onchocerciasis with nine different cytoforms of the blackflies [3]. These cyto forms include *S. damnosum* Nile form, *S. damnosum*

Volta form, *S.sirbanun* Siba form, *S. sirbanun* Sudanese form, *S.soubrense* Beffa form, *S. squamosum* A, *S.squamosum* B, *S. squamosum* C, and *S. yahense* [6].

Fast flowing streams and rivers provide favourable breeding sites for the black fly such that people living around such waters in endemic areas are most at risk of *O. volvulus* infection [1]. The physicochemical characteristics of the rivers and streams that serve as breeding sites are reported to correlate with the distribution of the different species of the blackfly [7]. The abundance of blackflies in an area and the fear of onchocerciasis are usually associated with the restriction of socio-economic activities such as farming, fishing and fruit gathering in that area [8]. This results to a serious decline in the socio-economic development of that area as fertile riverbanks are abandoned for fear of the disease [9]. In man, onchocerciasis is contracted through the bite of a female black fly infected with *O. volvulus* [10]. Onchocerciasis is one of the most neglected tropical diseases [11]. Therefore, the entomological survey of blackflies is an important component in determining the control of onchocerciasis in an area [10] while molecular characterization of female black flies to determine their infectivity is a crucial step in estimating the risk of onchocerciasis in the area.

The use of molecular techniques to evaluate the infectivity of adult female flies caught in the wild has previously been explored [12]. The 0-150OV PCR pool screening technique can detect a single infected fly in pools containing up to 100flies [13]. Several field studies in Africa and Mexico showed that the 0-150 pool screening PCR assay generated the estimates of the prevalence of infective flies in the vector population that were indistinguishable from those obtained by traditional dissection of the flies [10,13].

The Gamji (General Hassan Usman Katsina) Park, within Kaduna Metropolis, is traversed by a section of River Kaduna with vegetation and terrain that supports the breeding of blackflies [6]. Previous studies have shown that blackflies abound in the area [6,14]. However, information is scarce about the infectivity of the flies with *Onchocerca volvulus*. Entomological profile of blackflies and onchocerciasis transmission had been determined along the Gamji Park section of River Kaduna [6] but pool screening has been done. This study aimed to evaluate the distribution and physicochemical parameters supporting *Onchocerca volvulus* infected blackflies along the Gamji Park section of River Kaduna and their molecular characterization using 0-150OV and 12SrRNA.

MATERIAL AND METHODS

Sampling Station

The study was conducted at the Gamji (General Hassan Usman Katsina) Park section of River Kaduna located between latitudes 10°30'12.74"Nand longitude 7°27'9.01"East at an elevation of 580mwithin Kaduna metropolis (Figure 1). The area was purposively selected for the study based on the human activities such as swimming, fishing, bathing, washing of clothes and farming prevalent around that section of the river as well as the vegetation of trees, shrubs and grasses which have been reported to support the breeding of the blackflies [6] The study was conducted from November 2015 to October 2016 and divided into two seasons; dry season (November to April) and rainy season (May to October).

Pool Screening for fly infectivity

The blackflies were randomly caught from the study station using hand scooping nets from the vegetation serving as their resting sites along the river banks within the park. The flies were subsequently screened for *Onchocerca* parasite using the pool screening technique [13]. The collected flies were divided into pools of 50 flies in three replicates for the DNA extraction [15] and PCR amplification using the 0-150 OV and12S rRNA repeats.

Molecular Characterization

The pools of blackflies were thoroughly rinsed with distilled water and crushed using plastic pestle for DNA extraction. Thereafter, 400µl of lysis buffer (100mmNacl, 10mm Tris-Hcl, pH 8.0, 1mmEDTA,

0.1% sodium dodecyl sulphate 100mg/ml) was added followed by 10µl of proteinase K in each of the three tubes and homogenized. This was incubated at 65^{o}C for 1 hour after which it was vortexed 3 times at intervals of 20 mins for another 1hour incubation period. Four hundred microliters of phenol-chloroform was added and vortexed for 15-20 seconds and spun for 10mins. Other protocols for DNA extraction were observed as earlier reported [15,16].Polymerase chain reaction (PCR) was carried out [17] to characterize *Simulium* and *Onchocerca* species contained in the vector by amplifying the 0-150 OV and12S rRNA genes respectively [18]. The PCR was carried out using a reconstituted solution of 25µl premix [16]. The primer sequence used for 0-150 OV amplification are forward 5'GATTYTTCCGRCGAANARCGC-3' and reverse 5'GCRTRTAAATNYGNAAATTC-3' while the sequence used for 12S rRNA amplification are forward 5'AATAGGGTATCTAATCCTAGTTT-3' and reverse 5'TATTGGTAAATTTTGTGCC-3' [19-21]. Polymerase Chain Reaction was run on Agarose Gel Electrophoresis (Biorad, Italy) while all the premix and primers were purchased from Bioneer Company, USA.

Sequencing of *Simulium* and *Onchocerca* species amplicons

Sequence determination of 12S rRNA gene was done following the routine protocol by DNA laboratory, Kaduna Polymerase chain reaction products of some positive samples of purified single DNA strands were sequenced with 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 were determined using a Dye terminator cycle sequencing with quick start kit, and the product was analysed with an ABI prism DNA sequence (ABI) [20].

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

The gene sequences of each product obtained were compared with known 12S rRNA gene sequences in GenBank database by aligning with blast search of the NCBI database to find the regions of similarity between the sequences [22].

Physicochemical parameter

The water and atmospheric temperatures, biochemical oxygen demand, dissolved oxygen, electric conductivity, alkalinity, pH, turbidity, total dissolved solids and velocity of the water were measured. The atmospheric and water temperatures and pH were measured at five different locations and the average recorded. A pH/temperature meter (H1&424HANNA instrument) was used for the measurement. Dissolved oxygen (DO) was estimated as follows; Two (2 ml) of manganese chloride and 2 ml of alkaline iodide were added to two hundred and fifty milliliters of water sample followed by concentrated 2 ml H_2SO_4. The solution was shaken to dissolve the precipitate and 1% starch solution was added to indicate the presence or absence of dissolved oxygen. The mixture was titrated against 0.025N H_2SO_4 until it became colourless, and the volume of H_2SO_4 used was recorded. Biochemical oxygen demand (BOD) was calculated using the formula: $BOD = DO_1\text{-}DO_5/P$ where; DO_1= DO on day one, D_5= DO on day five and P= decimal volumetric fractions of sample used. Conductivity and total dissolved solids (TDS) were measured with a combined portable PHOx52 (combined conductivity and TDS analogue meter).[41] Turbidity was estimated by putting a secchi disc tied to a string into the river at a point until it becomes invisible. The string was pulled out until the disc becomes visible. The distance between the point of disappearance of the visibility of the disc and the point of its reappearance were measured in triplicate and the average value was recorded in meters. Alkalinity was estimated by taking 100ml of the sample water into a 250ml conical flask and 3 drops of methyl orange indicator was added and then titrated against 0.02N H2SO4 until the colour changes to pale pink. Average value of three readings was obtained, multiplied by 10 and expressed in mg/l .Water velocity was measured using a floating cork. The time

taken by the cork to pass two points of a distance of two metre, one metre before and one metre after an identified substrate was recorded and the velocity was calculated using the formula:Velocity =distance(in metres)/time(in seconds) expressed in m/s [41]

Statistical analysis

Data obtained during the study were analyzed with GraphPad Prism 7 (GraphPad software, USA). Blackfly abundance was expressed in percentage. Seasonal differences in the physicochemical [l parameters were compared using the student t-test. Pearson's correlation coefficient was used to determine the correlation between distribution of blackflies and physicochemical parameters for the dry and the rainy season .$P<0.05$ was considered statistically significant.

RESULTS

Infection rates and blackfly abundance at Gamji Park

The dry season has abundant blackflies (1405) compared to the rainy season (984). The number of female flies collected was 635 (45.2%) and 463 (47.1%) for the dry and rainy seasons respectively (Table 1). During the dry season, 476 blackflies were examined and 85 (17.9%) of these were infected with *Onchocerca volvulus*. During the rainy season, 453 blackflies were examined out of which 92 (20.3%) were infected with *Onchocerca volvulus* (Table 1).From the results of the studies more flies were caught during the dry season but the prevalence of infection was more in the dry season.

Chart 1 shows the fly collection and infection with Onchocerca volvulus during the study. A grand total of 2389 black flies were captured during the study and these were made up of 635 (45.2%) in the dry season and 984 (47.1%) in the rainy season. Among the flies collected, a total of 929 were examined for Onchocerca volvulus infection with 177 (19.1%) being infected. There was no significant ($p>0.05$) difference in infection between the dry (17.9%) and the rainy (20.3%) seasons.

Chart 1: Seasonal blackfly collection and examination of females for infection at Gamji Park, Kaduna, Nigeria.

Season	Total fly collection		Female fly examination for infection	
	No. collected	No. (%)of females	No. examined	No (%) infected
Dry season	1405	635 (45.2)	476	85 (17.9)
Rainy season	984	463 (47.1)	453	92 (20.3)
Grand total	2389	1098 (46.0)	929	177 (19.1)

Physicochemical parameters

No significant ($p>0.05$) change was observed between the dry and rainy seasons average for pH, atmospheric temperature, biochemical oxygen demand, conductivity, turbidity, water temperature and alkalinity (Figures 2, 3 & 4). The levels of dissolved oxygen and velocity were significantly higher ($p<0.05$) in the rainy season relative to the dry season while the levels of conductivity and total dissolved solids were significantly higher ($p<0.05$) during the dry season compared to the rainy season (Figures3 & 4).

Molecular characterization

The 12SrRNA gene amplification of *Simulium* species produced a band product at 196bp while that of *Onchocerca volvulus* DNA from *Simulium* species produced a band product at 190bp (Figs. 5A and 5B). Polymerase chain reaction of *Simulium damnosum* and the *Onchocerca volvulus* DNA produced nucleotide sequences of 267 and 184 letters for the 12S rRNA with no nucleotide sequence produced for the 0-150 OV.TheG2 and A3 showed the alignment scores with a query length and nucleotide sequence of 267 and 184 letters, giving 94%, 100% and 93% alignment similarity for nine descriptions for sequence producing significant alignments for *Simulium damnosum* genes (Table 4).Sequencing and BLAST using the 12SrRNA confirmed the following identities;. *damnosum* (COII) gene (94%), *S. damnosum* (ND4) gene (94%), *S. damnosum* Ni4 16s gene (100%), *S. damnosum* 16s rRNA gene (100%), *S. damnosum* strain Se-19 16s gene (100%), *S. damnosum* country Uganda 16s gene (100%), *S. damnosum* UNI NADH gene (93%), and *S. damnosum* 16s rRNA gene,100% (Table 4).

DISCUSSION

This study showed that the blackfly occurs in abundance in the Gamji park section of River Kaduna and there is an indication of infection with *O.volvulus*. The results suggest that people living around the area, visitors coming to the park and those engaged in farming and fishing activities around the river may be exposed to bites by the flies most of which harbor the microfilariae of *O. volvulus*. According to Ivoke [23], the percentage of molecule infection in sample flies might indicate that transmission is very active especially with a lot of activities going on along the river bank. The number of infected blackflies found in the present 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 results suggest a regular availability of human source of blood meal and suitable breeding and resting sites for the flies. 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 primer 12SrRNA for the detection of the blackfly species. The amplified PCR band has shown the blackflies to be species of the *Simulium damnosum* complex. The 0-150OV PCR amplification was also sensitive and specific and has detected and identified *Onchocerca volvulus* at 190bp length comparable to the reports of Katholi *et al*. [13]. This has suggested that the *Simulium* species collected and examined in the area were infected with *O.volvulus* worms. The reaction product of amplicons of 190bp for the parasite *O.volvulus* and the 196bp for the blackfly *Simulium* is in agreement with Hassan *et al*. [24]. The ability of the 12SrRNA gene to amplify the DNA extracted from the flies generating a band size of 196bp is comparable to previous reports [17,25]. The 0-150OV gene band size of 190bp observed from the amplified DNA extracted from the O. *volvulus* infected S. *damnosum* is comparable to previous reports [26-27].Amplification of a portion of the blackfly mitochondrial 12SrRNA gene from the DNA samples by PCR sequencing generated nucleotide sequences that were similar to nine strains of members of the genus *Simulium*. Consequently, the results suggest the presence of the blackflies actively feeding and infected with *Onchocerca volvulus*.

This study has shown the molecular characterization using PCR technique is sensitive and can detect the infection once the parasite DNA is found during amplification. However, the sequencing of the O *volvulus* parasite using the 0-150OV did not produce any sequence. Gamji Park is an area where research study has been carried out previously, although there hasn't been any literature report to that effect The presence of the infected black flies at this study site poses a great threat to the communities, visitors and the rural farmers in close proximity to the river.

Results of the study presented on the physicochemical parameter at the station indicate the availability of suitable breeding sites for the blackflies since the adult blackfly and immature stages were recovered from the study site. Previous studies have shown that aquatic insects serve as bio-indicators of water quality,

and many physicochemical parameters such as temperature, DO, turbidity, velocity, and pH of the water are known to affect the distribution of blackfly species and their abundance [28].

The mean water temperature of dry (27.47±1.94) and rainy (28.90 ±1.60) season agree with the reports of Allison *et al*. [29] and appear to be suitable for *Simulium* immature and adult resting and breeding sites. Previous studies in Nigeria suggest that water temperature is very important in blackfly breeding and distribution because larval development and survival depend on the availability of adequate temperature [1,30,31,32].

The mean pH values obtained in this study (7.70 ± 0.27) dry and (7.51± 0.14) rainy appear to be within the normal range for biological activities and comparable to previous reports in other parts of Nigeria [3,31,34].

The mean conductivity values recorded for the two seasons (93.17±17.93) rainy and (126.67±30.11) dry were high, with that of the dry season being higher than the rainy season. This is in contrast to those reported by Opara *et al*. [35], conductivity do influence the development and distribution of larval population of blackflies and also influence their breeding in Nigeria. There was a decrease in the mean dissolved oxygen (DO) values obtained during the dry season (6.85 ± 0.94) compared to the rainy season (13.73± 4.65) 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 those in previous reports [36,37]and suitable for the breeding and resting site of blackflies. The alkalinity of the water was higher (41.93±15.32) dry and (28.47± 10.39) than those reported elsewhere in Nigeria [29] but these appear to be suitable for the blackflies breeding in the study site. The Biochemical oxygen demand (BOD) values obtained in this study were higher in the rainy (3.76 ± 2.06) than the dry (2.79 ± 1.49) season, but these values were within the range suitable for *Simulium* breeding and resting as reported by other studies [32,37].

Water turbidity was low during rainy season (0.06 ± 0.03) compared to the dry season (0.15 ± 0.11) but these values are still within the range suitable for Simulium breeding and resting sites[37].Total dissolved solids (TDS) has a higher mean value during the dry season (86.67 ± 17.51) than the rainy season (61.58 ± 11.20). The mean velocity of the river in the rainy season (0.12 ± 0.03) was higher than in the dry season (0.08 ± 0.03) probably because during the rainy season there is an increase in the volume and flow of the river due to rainfall which tends to increase its velocity. This finding agrees with previous reports on the importance of water velocity in the distribution of blackflies and larval density [38,39,40].

CONCLUSION of the river water

In conclusion, therefore, the results of this study suggest that there is an infestation of the area with blackflies many of which were infected with *O. volvulus;* indicating a salient and moderate transmission of onchocerciasis going on at the Gamji Park section of Kaduna River. The results of the molecular analysis further suggest that the DNA of *O. volvulus* may be detected from the whole pool without separating the head from the body. The results further show that some physicochemical parameters such as dissolved oxygen, total dissolved solids, conductivity and velocity of the river water correlated with the number of black flies, making the environment important and suitable for the breeding and resting of blackflies in the area.

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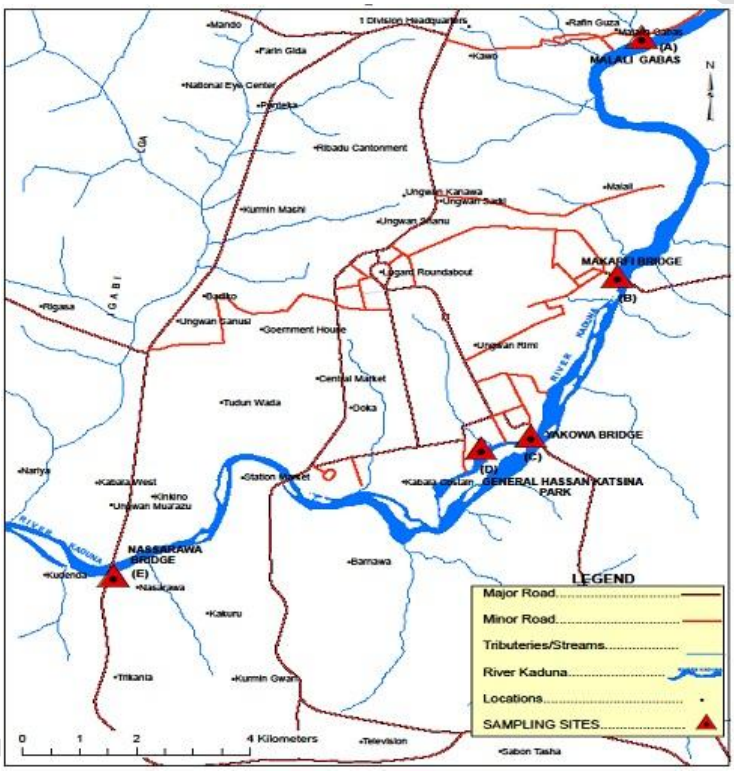

Figure 1: Map of River Kaduna within Kaduna Metropolis showing the sampling site (station D). (The map was created at Department of Geography, NDA Kaduna)

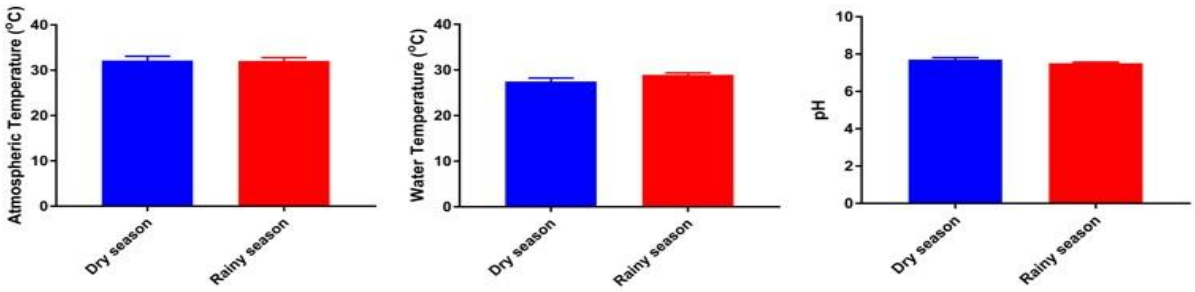

Figure 2: The pH, atmospheric and water temperatures of Gamji park Kaduna. Values are presented as mean±SEM, * indicates significant difference at P<0.05, SEM= standard error of mean.

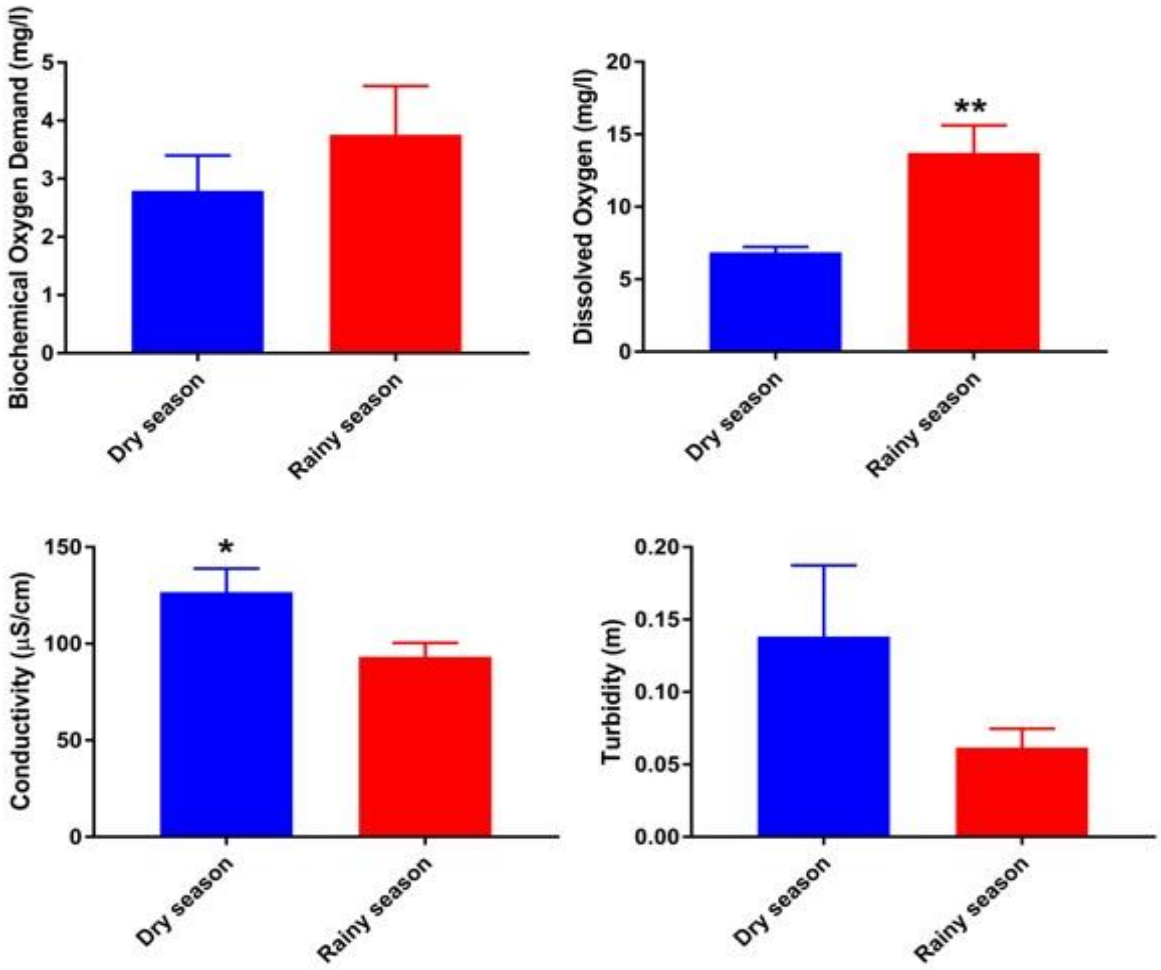

Figure 3: Biochemical oxygen demand, dissolved oxygen, conductivity and turbidity of Gamji park Kaduna. Values are presented as mean±SEM, ** indicates significant difference at P<0.05, SEM= standard error of mean.

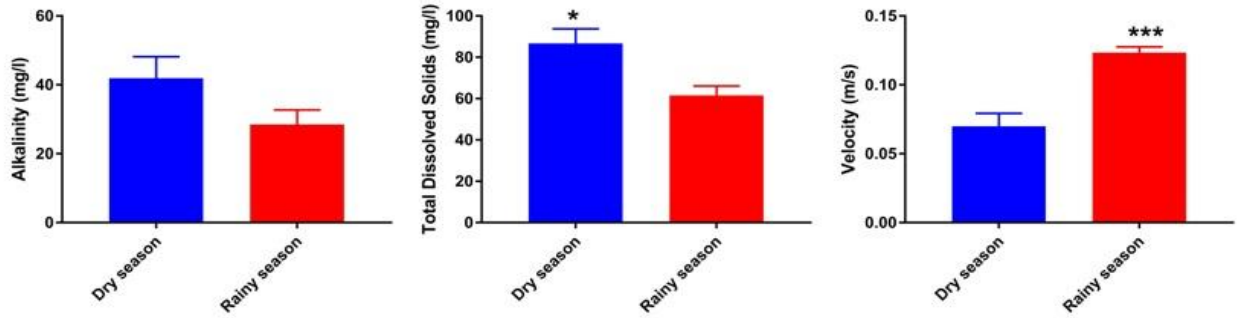

Figure **4: Alkalinity, total dissolved solids and velocity of Gamji park Kaduna. Values are presented as mean±SEM, *** indicates significant difference at P<0.05, SEM= standard error of mean.**

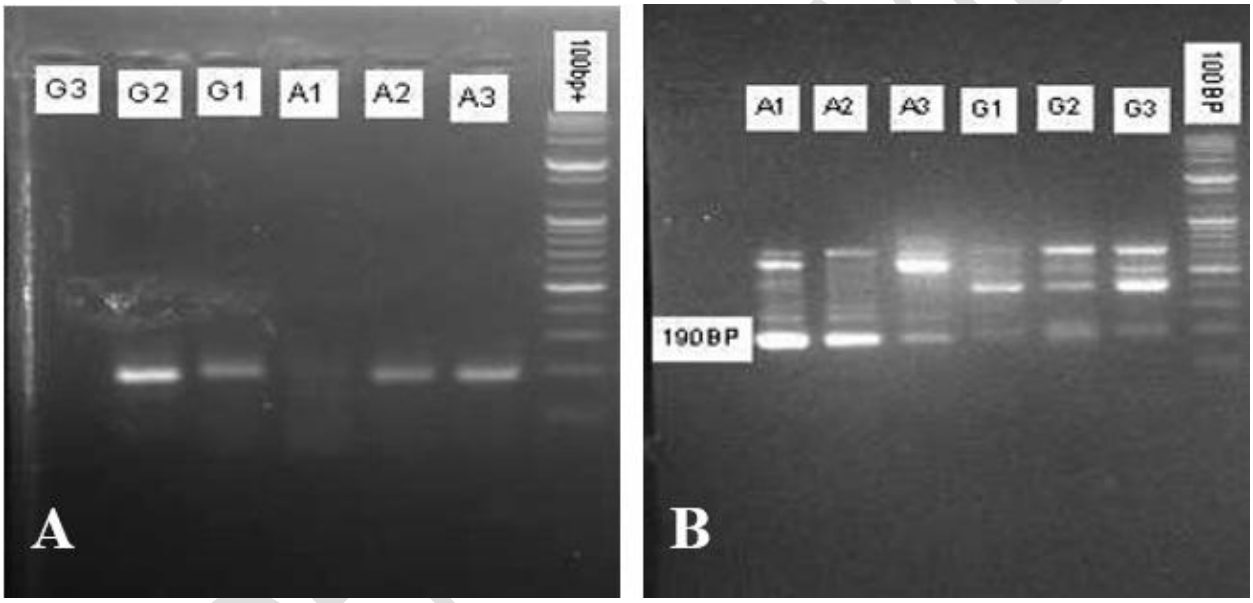

Figure 5: PCR band for 12SrRNA gene amplification of the *Simulium* spp. (196bp), lane G1-G3 and A1-A3 are *Simulium* spp(A) and PCR band for 0-150OVgene amplification of*Onchocerca volvulus* from the *Simulium* spp. (90bp), G1-G3 and A1-A3 are *O. volvulus* DNA from *Simulium spp*. (B)

Table 1: Seasonal blackfly abundance at Gamji Park Kaduna

Seasons	No. of flies collected	No. of female flies	Total
Dry season	1405	635 (45.2%)	2040
Rainy season	984	463 (47.1%)	1447
Total	2389	1098	3487

Table 2: Seasonal *Onchocerca volvulus* prevalence and infection rate at Gamji Park Kaduna

Seasons	No. of flies examined	No. of infected flies
Dry season	476	85 (17.9%)
Rainy season	453	92 (20.3%)

Table 3: Physicochemical parameters and the distribution of blackflies Gamji park Kaduna

Parameters	Dry season (r)	P value	Raining season (r)	P value
Dissolved oxygen (mg/l)	-0.6191	0.900 ns	-0.2873	0.5809 ns
Total dissolved solids (mg/l)	-0.1661	0.7532 ns	-0.2271	0.6653 ns
Conductivity (µS/cm)	-0.2380	0.6498 ns	-0.2365	0.6518 ns
Velocity (m/s)	0.7780	0.0684 ns	-0.1925	0.7148 ns

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

Table 4: Summary of the 15 BLAST hits searches using ribosomal RNA gene PCR Amplicons of the *Simulium* species

Pool	NS	QL	Query ID	Blast hit % Identities	Sequence	Accession
G2	267 letters 184 letters	267	168967	*S. damnosum* Cytochrome oxidase subunit II (COII) gene,	gb/DQ133093.1	DQ4133093.1
A3		184	153865	partial cds; mitochondrial 17/18 (94%)		
G2	267 letters 184 letters	267	168967	*S. damnosum* mitochondrion NADH dehydrogenase subunit 4	gb/U17728.1	U17728.1
A3		184	153865	(ND4) gene, partial cds 15/16 (94%)		
G2	267 letters 184 letters	267	168967	*S. damnosum* isolate Ni416s ribosomal RNA gene, partial	gb/AF416973.1	AF416973.1
A3		184	153865	sequence: mitochondrial 12/12(100%)		
G2	267 letters 184 letters	267	168967	*S. damnosum* 16s ribosomal RNA gene, partial sequence;	gb/AF127463.1	AF127463.1
A3		184	153865	mitochondrial gene for mitochondrial product. 12/12 (100%)		
G2	267 letters 184 letters	267	168967	*S. damnosum* strain Se-19 16s ribosomal RNA gene, Partial	gb/AF081908.1	AF081908.1
A3		184	153865	sequence; mitochondrial product 12/12 (100%)		
G2	267 letters 184 letters	267	168967	*S. damnosum* Se-12 16s ribosomal RNA partial sequence;	gb/AF081907.1	AF081907.1
A3		184	153865	mitochondrial gene for mitochondrial product 12/12 (100%)		
G2	267 letters 184 letters	267	168967	*S. damnosum* country Ugandan 16s ribosomal RNA gene, partial	gb/AF081904.1	AF081904.1
A3		184	153865	sequence; mitochondrial product. 12/12 (100%)		
G2	267 letters 184 letters	267	168967	*S. damnosum* UNI NADH dehydrogenase subunit 4 (NDA)	gb/U27114.1	U27114.1
A3		184	153865	gene mitochondrial gene encoding mitochondrial protein; partial cds 14/15 (93%)		
G2	267 letters 184 letters	267	168967	*S. damnosum* mitochondrial 16s ribosomal RNA gene, partial	gb/U17729.1	U17729.1
A3		184	153865	sequence 12/12 (100%)		

The No1 BLAST HIT corresponds to the alignment results with the highest identity with the mitochondrial genes, using 12S rRNA forward and reverse sequence of the mitochondrial genes 12S rRNA gene PCR Amplicon. G2 and A3= Pools of 50 blackflies, QL= Query length, NS= Nucleotide Sequence.