International Journal of Scientific & Engineering Research, Volume 8, Issue 11, November-2017
ISSN 2229-5518

Species Composition of Phlebotomine Sandfly (Diptera:Psychodidae)Vectors of Leishmaniasis in Katsina State, Northern Nigeria

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Abstract—To determine the composition of Phlebotomine sandfly vectors of leishmaniasis in Katsina state northern, Nigeria, Multiplex Polymerase Chain Reaction of the mitochondrial DNA fragment was used to identify species of sandflies captured in the field. Trapping of sandflies was carried out biweekly using sticky paper traps that were set in six trapping points in the two stable foci of leishmaniasis in the study area between January-March (dry season) and June-August (rainy season) 2016. During the study, a total of 2,232 sandflies belonging to two sandfly genera (*Phlebotomus* and *Sergentomyia*) were collected out of which 573 (25.67%) were *Phlebotomus* species. Analysis of PCR product by electrophoresis indicates the presence of *P. duboscqi* in all the collection points. *Phlebotomus orientalis* known to transmit *Leishmani adonovani* in other parts of the world was also recorded in two of the collection points. This is the first time *P. orientalis* is been detected in Nigeria.

Keywords—Cytochrome b gene, Phlebotomine, Sandfly, Katsina State.

1 INTRODUCTION

Phlebotomine sandflies are insects belonging to order Diptera (true flies) and the family Psychodidae. About 800 species of sandflies have been identified which are placed in five genera viz: *Brumptomyia, Lutzomyia, Phlebotomus, Sergentomyia,* and *Warileya* (Kettle, 1993; Wall and Shearer, 2001). Although, majority of sandfly species play no part in the transmission of leishmaniasis in nature, about 10% of sandfly species have been incriminated as vectors of leishmaniasis in different parts of the world ((Killick-Kendrick, 1999; Bates, 2007; Kato *et al*., 2010). Among the many genera of psychodidae, only species of *Phlebotomus* and *Lutzomyia* are currently proven or suspected vectors of human leishmaniasis although, few studies have recently suggested the possible involvement of some species of the genus *Sergentomyia* in the transmission of Leishmaniasis in the Old World, (Mukherjee *et al*., 1997; Senghor *et al*., 2011).

Recent studies have shown emergence and resurgence of many vector and vector borne diseases in many parts of the world including Africa. These situations have been associated with ecological and climatic changes which have favored an increase in vector population densities. While some species of sandflies have disappeared due to human environmental disturbance, others have become more abundant due to migration and/or have adapted to the environments by changing their behavior (Ostfield *et al.,* 2004). These changes on the composition of sandflies population could cause increase in leishmaniasis transmission. Despite their undisputable importance in human medicine, inadequate and inconsistent attention has been paid to sandfly species and leishmaniasis in Nigeria. The few studies on the distribution of sandflies in northern Nigeria were based on morphological identification (Lewis, 1961; Asimeng 1990; Agwale *et al.,* 1995), which most times have limitations compared to molecular techniques. In addition, the presence of intraspecific variation and cryptic species frequently complicates classification based on morphological features (Bauzer *et al.,* 2007) thus the use of molecular technique of PCR analysis of molecular characteristic maker(cytochrome b gene) in the present study investigate the species composition of *Phlebotomus* fauna in leishmania endemic foci of Katsina state, Nigeria.

2 MATERIAS AND METHODS

2.1 Study Area

Katsina state lies at the extreme northern margin of Nigeria and shares boarder with Niger Republic. The state is located between latitude $11^0 08^1$ N and $13^0 22^1$ N and longitude $6^0 52^1$ E and $9^0 20^1$ E. It covers a total area of about 23,938 km^2 with a total population of about 5,801,584 people (Census, 2006). The state is boarded by Niger republic in the north, Kaduna state in the south, Zamfara state in the west and, Kano and Jigawa state to the east. The climate of Katsina state is hot and dry for most of the year with maximum day temperature of about 38 ^{0}C in the month of March and April and a minimum temperature of about 22^{0}C in the month of December and January. Total annual average rainfall ranges from 600 to 780mm with characteristic Guinea savannah vegetation in the southern half of the state and Sudan savannah vegetation in the northern part of the state.

2.2 Sampling sites /Points

Three sampling points (villages) each in two of the Local Government Areas (LGAs) known to be endemic for

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cutaneous leishmaniasis were selected using systematic random sampling technique. The sampling points in each of the LGAs were marked 'Sp 1 - 3' (Figure 1 and 2).

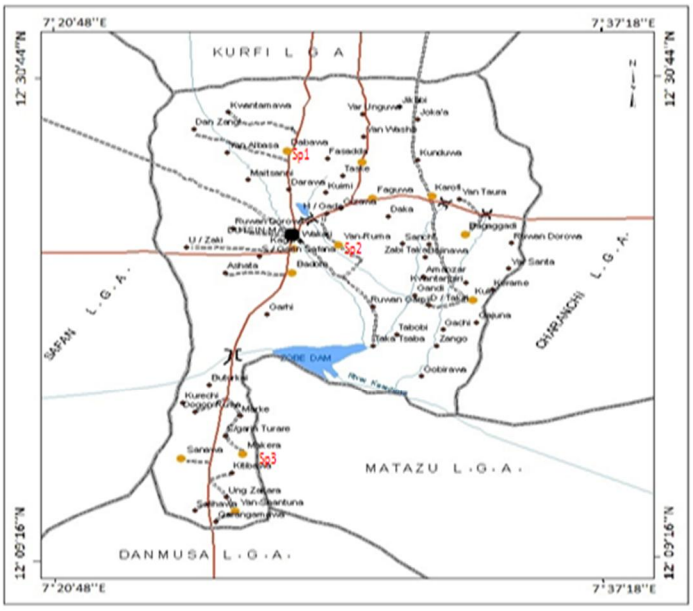

Figure 1. Map of Dutsin-ma Local Government area (Source: NASA/NOAA Sport Image 2015)

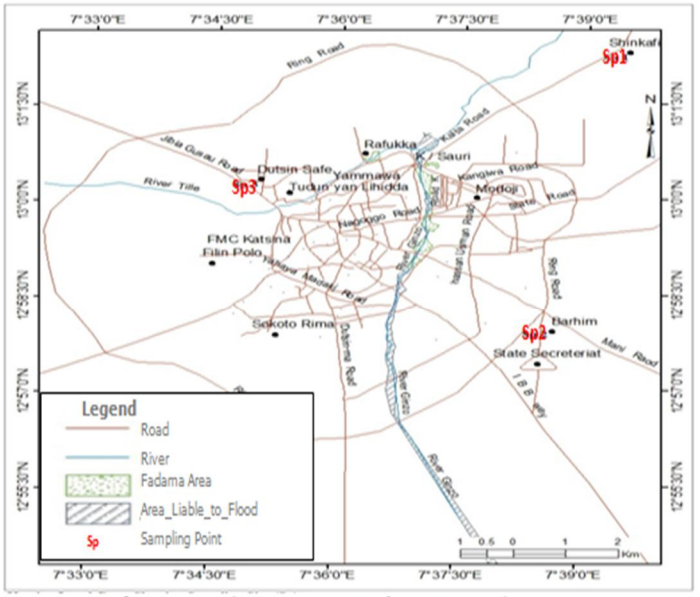

Figure2. Map of Katsina Local Government area (Source: NASA/NOAA Sport Image 2015

2.3 Collection of Sand flies and Morphological Identification

Sandflies were collected from outdoors twice-weekly from January-August 2016. For each collection time, 30 sticky paper traps were set near the flies' shelters such as holes in rocks, caves and ventilation shaft of termites' hills between 5:00–6:30 p.m. and collected between 6:00–9:00 a.m. the next morning. Flies captured were preserved in 70% ethanol in labeled sample bottles and transported to laboratory for analysis. In the laboratory, sandflies were washed once in 1% detergent then rinsed with tap water until the sample is free of soap and finally rinsed in a small Petri dish containing Phosphate buffered saline (PBS). Identification was made based on morphological characteristics using standard taxonomic keys of Kirk and Lewis (1951).

2.4 DNA Extraction

Extraction of DNA was carried out using phenol/chloroform method (Green and Sambrook, 2017). Sandflies were first crushed and grinded in tubes using pestle, homogenized and lysed in 400 µL DNA extraction buffer and 10µl of protenase K. Extracted DNA was left to dry out in open tubes for 3-10 min and the pellets were re-suspended in 20 µL of distilled water.

2.5 Molecular Identification of *Phlebotomus* species

Amplification ofCytochrome bgene (cyt b gene) was carried out in 6 pooled samples with each pool representing a sampling point. Portions of DNA extracted from pools of sand flies in a location were used as template. Species-specific primer sequences (Table 1) related to *P. dobuscqi*, *P. papatasi* and *P. orientalis* were designed based on the cyt b gene using online primer design tool (Primer3 and BLAST) from NCBI database, and used for PCR amplification. Identification of sand fly species was based on the multiplex PCR product of the mitochondrial DNA fragment as described by Saccagi *et al.* (2008).

The brief reaction set-up was as follows: DNA Template 2µl, Primer1 (forward) 1µl Primer2 (reverse) 1µl and dH_2O 16µl. These were added to the premix in a PCR tube and subjected to PCR amplification. The cycling conditions were Pre-denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C for 60 seconds, 30 cycles of extension at 72 °C for 30 seconds and a final extension at 72^0C for 5 minutes.

The products of the PCR were loaded onto 1.5% agarose gel marked with ethidium bromide and visualized by ultraviolet trans-illumination. The band size of each amplicon was estimated by comparison with a 100 base- pair (bp) molecular-

Weight ladder.

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2.6 Statistical analysis

Relative abundance was calculated as the percentage composition of an organism of particular kind relative to the total number of the organisms captured in the area. Test of association between phlebotomine sandflies and study area, distribution of male female flies; and *Sergentomyia* and *Phlebotomus* species were carried out using Chi-square analysis. Probability (P) ≤ 0.05 was considered significant.

3 RESULTS

A total of 2,232 phlebotomine sandflies were collected during the study period out of which 74.33% belong to the genus. *Sergentomyia* while the genus *Phlebotomus* formed 25.67%. The distribution of phlebotomine sand flies caught in the six villages of Dutsin-ma and Katsina LGAs is presented in Table 2. Data analysis shows a significant difference ($p<0.05$) in the distribution of *Phlebotomus* species and *Sergentomyia* species in both LGAs with both species recording higher abundance in Dutsin-ma LGA than in Katsina LGA.

Table 1. List and details of primers used in this study

Target	Primer pair	Name/Accession	Sequences (5'-3')
Cyt b	7	*P.* dobuscqi/KR 020580.1	CCGCCATCCCTTATCTAGGAA AGGGGTTACAAGAGGATTTGCT
	1	*P. papatasi*/HM992926.1	TCCGCCATCCCTTATCTAGGA GGACGAGCTCCGATTCATGT
	1	*P. orientalis*/F161203.1	TCCTCCCCATATTCAGCCAGA CAGGGCGAGCTCCAATTCAT

Designed from NCBI database (using Primer3 and BLAST)

Table 2. Distribution of Phlebotomine sand flies in of Dutsin-ma and Katsina LGAs of Katsina State

	Abundance		
	Genus		
	Phlebotomus	Sergentomyia	
LGA/Village	No. Collected (%)	No. Collected (%)	Total No. Collected (%)
Dutsin-ma /			
Dabawa	132 (23.04)	189 (11.39)	321 (14.38)
Kuki	190 (33.16)	632 (38.10)	822 (36.83)
Makera	36 (6.28)	86 (5.18)	122 (5.47)
Sub-total	358 (62.48)	907 (54.67)	1,265 (56.68)
Katsina /			
Barhim	17 (2.97)	98 (5.91)	115 (5.15)
Dutsin Safe	121 (21.12)	264 (15.91)	385 (17.25)
Shinkafi	77 (13.44)	390 (23.51)	467 (20.92)
Sub-total	215 (37.52)	752 (45.33)	967 (43.32)
Grand-total	573 (25.67)	1659 (74.33)	2232

$x^2 = 10.5709$, DF = 1, P = 0.001149

The distribution of male and female phlebotomine sandflies of *Phlebotomus* and *Sergentomyia* collected during the study period is presented in Table 3. Out of the total 573 *Phlebotomus* species collected, males formed 71.20% while the females formed 28.80%. Similarly, out of the total (1,659) *Sergentomyia* collected from both Dutsin-ma and Katsina LGAs, the composition of male and female flies was 74.92% and 25.08% respectively. Although higher population of males were recorded than females, statistical analysis shows no significant difference ($p>0.05$) in the distribution of male and female phlebotomine sandflies of both genera.

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Table 3. Sex Distribution of Phlebotomine Sand flies in Sampled Villages of Dutsin-ma and Katsina LGAs

	Genus					
	Phlebotomus			Sergentomyia		
LGA/Village	No. collected	Males (%)	Females (%)	No. collected	Males (%)	Females (%)
Dutsin-ma/						
Dabawa	132	98 (17.10)	34 (5.93)	189	112 (6.75)	77 (4.64)
Kuki	190	142(24.78)	48 (8.38)	632	504 (30.38)	128 (7.72)
Makera	36	26 (4.54)	10 (1.75)	86	71 (4.28)	15 (0.90)
Sub-total	358	266(74.30)	92 (25.70)	907	687 (75.74)	220 (24.26)
Katsina/						
Barhim	17	7 (1.22)	10 (1.75)	98	79 (4.76)	19 (1.15)
Dutsin Safe	121	89 (15.53)	32 (5.58)	264	188 (11.33)	76 (4.58)
Shinkafi	77	46 (8.03)	31 (5.41)	390	289 (17.42)	101 (6.09)
Sub-total	215	142(66.05)	73 (33.95)	752	556 (73.94)	196(26.06)
Grand-total	573	408 (71.20)	165 (28.80)	1659	1243 (74.92)	416 (25.08)

$\chi^2 = 2.8314$, DF = 1, P = 0.09243

A total of 1,555 phlebotomine sandflies were caught in Katsina and Dutsin-ma LGAs during the Wet season with a mean catch of 777.5±187.38 per LGA. In the Dry season, 677 flies were caught with an average/mean catch of 338.5±23.33 per LGA. Chi-square analysis shows a significantly higher ($p<0.05$) number of Phlebotomine flies collection during Wet season than Dry season in the study area. Members of the genus *Sergentomyia* formed the major proportion of sandflies collected in the two seasons.

Table 3. Seasonal Distribution of Phlebotomine Sand flies in Dutsin-ma and Katsina LGAs

Season/ LGA	No. of Phlebotomine Sandflies Collected	No. of Phlebotomus sp. Collected (%)	No. of Sergentomyia sp. Collected (%)
Wet			
Dutsin-ma	910	201 (22.09)	709 (77.91)
Katsina	645	143 (22.17)	502 (77.83)
Total	1,555	344	1,211
$\bar{x}$	777.5±187.38	172±41.01	605.5±146.37
Dry			
Dutsin-ma	355	157 (68.56)	198 (44.20)
Katsina	322	72 (31.44)	250 (55.80)
Total	677	229	448
$\bar{x}$	338.5±23.33	114.5±60.10	224±36.77

$\chi^2 = 33.8567$, DF = 1, P = 0.00001

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Multiplex PCR technique targeting the mitochondrial DNA fragment (cytochrome b gene region) in six pooled samples of *Phlebotomus* species indicated the presence of *P. duboscqi* in all the six pooled samples collected from the six villages. However, *P. orientalis* was also detected in two of the pooled samples from two villages of Katsina LGA. The result of gel Electrophoresis shows that *P. duboscqi* generated a bands size of 344 bp in all the samples tested, while *P. orientalis* generated bands size of 236 bp in lane 2 and 3 (Figure 3).

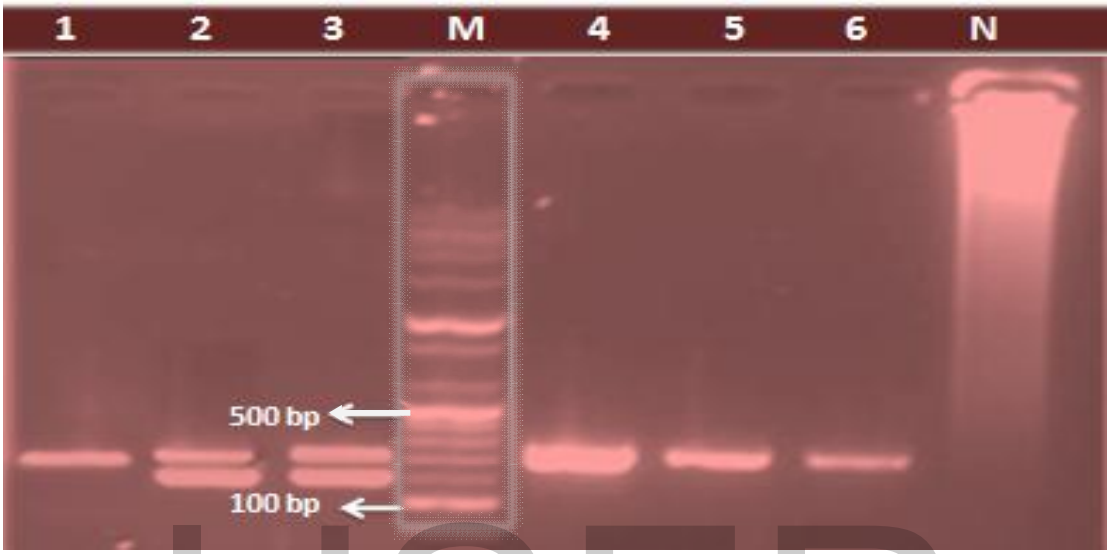

Figure 3. Multiplex PCR amplification of Cytochrome b gene using specie-specific primers of *P.duboscqi*, *P. orientalis* and *P. papatasi* for specie identification. { M: 100 bp size marker; lanes 1,4,5 and 6: *P. duboscqi* (344 bb); lanes 2 and 3: *P. duboscqi* (344 bp) and *P. orientalis* (236 bp)}

4 DISCUSSION

The study reveals the presence of phlebotomine sandflies in the rural villages of Dutsin-ma and Katsina LGAs. The sandflies fauna in the area is composed of two genera namely, *Phlebotomus* and *Sergentomyia*. The predominance of members of the genus *Sergentomyia* (74.33%) in the study area are in conformity with the records of Dedet *et al.* (1980), Desjeux *et al.* (1983), Asimeg (1988), Basimiki *et al.* (1992) and Adamu *et al.* (2012). The overwhelming number of male (73.97%) compared to female flies observed in this study is similar to an earlier report of Agwale *et al.* (1995) who recorded 69.57% males of Phlebotomines in Katsina state. Chaniotis *et al.* (1971) suggested that such disparity between male and female phlebotomine sandflies could be due to differential sex mortality wherein, some females die after completing few genotrophic cycles.

Seasonal variation in the abundance of sandflies in both Dutsin-ma and Katsina LGAs observed in this study during rainy season than dry season is to be expected. The pattern observed in this study has been observed in studies conducted in India (Tiwary *et al.,* 2013), Mali (Anderson *et al.,* 2011), Brazil (Sirlei *et al.,* 2016) and several other studies. It was reported that the extent to which sand fly population densities vary throughout the year depends on the local climate, with significant seasonal changes in temperature and precipitation resulting in fluctuations in sand fly numbers, which will be lowest during the coldest and/or driest seasons of the year (Lawyer and Perkins, 2004). According to Kasap and Alten (2005), sufficient moisture in the environment is required for egg survival. Therefore, the marked seasonal changes in the abundance of phlebotomine sandflies observed in the study area could be attributed to lower humidity during dry season than wet season thereby reducing the breeding population. This is important for epidemiological surveillance because of the risk of transmission of Leishmaniasis in the study area.

The detection of *Phlebotomus duboscqi* in the area is consistent with the reports of Lewis (1961), Asimeng (1985) and Agwale *et al.* (1995) who reported *Phlebotomus duboscqi* as the only probable vector of leishmaniasis in northern savannah zone of Nigeria. The detection of *P. orientalis* in the study area (Katsina LGA) for the first time is of significant public health implication. *Phlebotomus orientalis* which though has never been reported in Nigeria but known to occur in neighboring

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ISSN 2229-5518

countries like Chad (Sheik-Mahammed and Velema, 1999; WHO, 2010), Niger (Abonnenc *et al.,* 1964) Sudan (Elnaiem *et al.,* 1998) and Ethiopia (Hailu *et al.,* 1995) is the vector of *L. donovani,* the causative agent of visceral leishmaniasis. The disease is characterized by irregular bouts of fever, weight loss, enlargement of spleen and liver, anaemia and is usually fatal if left untreated in over 95% of cases. Visceral leishmaniasis occurs in over 65 countries with an annual incidence of 500,000 cases worldwide (Desjeux, 2001). In African, the disease was earlier reported from Central African Republic, Chad, Ethiopia, Gambia, Gabon, Kenya, Niger, Somalia, Sudan, and Uganda (Joseph *et al.,* 2014). Although pcr analysis of kDNA in pooled samples of *P. orientalis* showed no indication of *L. donovani,* it is commonly reported to be transmitted by this sandfly vector. The detection of *P. orientalis* in Katsina LGA could be associated to many factors among which include increased trans-boarder movement of human due to Boko Haram insurgency. Human movement has been identified as a key behavioral factor in many vector-borne systems (Stoddart *et al.,* 2009). It was predicted that should climate change result in suitable temperature, sandfly species could rapidly establish in countries currently on the edge of their range (Ready, 2010). Few years ago, Naucke *et al.,* (2013) noted that the range of geographical distribution of phlebotomine sandflies in the Mediterranean region of Europe has increased. Such an assertion may partly explain the probable spread of *P. orientalis* from Niger Republic which shares common border with Katsina State, Nigeria.

5 CONCLUSION

The distribution of *Phlebotomus duboscqi* in the study area confirmed the previous reports on presence of these flies in Katsina state, Nigeria. However, suspected vectors of *L. donovani* (*P. orientalis*) were also found among the *Phlebotomus* sandflies collected. The detection of *P. orientalis* in the study locations indicates the possible emergence of these vector in Nigeria as a consequence of Boko Haram insurgency which has forced movement of man and his livestock across the borders for fear of been killed..

Recommendations

There is a need for continues surveillance and monitoring of humans as well as sand fly vector populations in the affected communities to facilitate risk assessment and ensure adequate management and prevent outbreak of leishmaniasis in the area. Follow up survey on identification of *Phlebotomus orientalis* in the study should be conducted to further evaluate the claim in this study.

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International Journal of Scientific & Engineering Research, Volume 8, Issue 11, November-2017
ISSN 2229-5518

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