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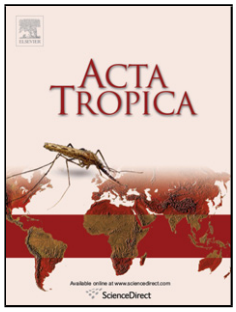

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Geographical Distribution and Species Identification of Human Filariasis and Onchocerciasis in Bioko Island, Equatorial Guinea

Short title: Updating Human Filariasis and Onchocerciasis in Bioko Island

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HIGHLIGHTS

- The filariae found were *O. volvulus, M. streptocerca, M. perstans* and *L. loa*.
- The filarial diagnosis performed by a RT-PCR improved specificity and sensitivity
- First gene annotations from *M. streptocerca* in GenBank

Abstract

Human filariae are vector-borne parasites and the causative agents of various diseases, including human onchocerciasis and lymphatic filariasis. Onchocerciasis causes a spectrum of cutaneous and ophthalmologic manifestations (including blindness) and has long been a major public health problem in Bioko Island (Equatorial Guinea). Bioko Island has been included in the WHO's Onchocerciasis Control Program since 1987. In Bioko Island, the specificity and sensitivity of clinical *Onchocerca volvulus* diagnosis is key. The objective of this work was to update onchocerciasis elimination progress in Bioko Island, after 18 years of mass ivermectin intervention, and the general filariasis situation through a rapid and accurate molecular method. A cross-sectional study was conducted in Bioko Island from mid-January to mid-February 2014. A total of 543 subjects were included in the study. Whole blood and one skin snip (from lumbar regions) were analysed with a real time PCR assay. Two other skin biopsies were analysed by an expert microscopist. All positive samples were confirmed by sequencing. Traditional microscopic examination of the skin biopsies failed to detect any microfilariae. However, 11 (2.03%) infections were detected using PCR assay, including one *O. volvulus,* two *Mansonella streptocerca,* seven *Mansonella perstans* and one *Loa loa*

infections. PCR assays in blood detected 52 filariae-positive individuals (9.6%) which harboured *M. perstans* or *L. loa*. The low prevalence of *O. volvulus* confirms the success of the Onchocerciasis Control Programme and suggests that Mass Drug Administration in Bioko Island can be interrupted in the near future. The very high prevalence of *M. perstans* found in skin snips assays raises doubts about the reliability of microscope-based diagnosis of *O. volvulus* infections.

Keywords: Filariasis, Onchocerciasis, ophthalmologic manifestations, Equatorial Guinea, Molecular diagnosis, microscopic diagnosis, ivermectin

1. Introduction

Human filariae are vector-borne and are responsible for various diseases, including human onchocerciasis (*Onchocerca volvulus*), lymphatic filariasis (*Brugia malayi*, *Brugia timori* and *Wuchereria bancrofti*), loiasis (*Loa loa*) and mansonelliasis (*Mansonella perstans*, *Mansonella ozzardi* and *Mansonella streptocerca*). The severity of disease caused by filarial infections in humans can vary dramatically and depends on the species as well as the level of parasitism. Some filarial species can cause severe diseases, while others can produce lighter symptoms or even may not produce symptoms or they go largely unnoticed (Marcondes, 2016; Simonsen et al., 2014).

In Bioko Island, part of the Republic of Equatorial Guinea in West Africa, *O volvulus, L. loa, M. perstans* and *M. streptocerca* have been described in humans (Cuello et al., 2009; El Haouri et al., 2001; Mas et al., 1995; Mas et al., 2006). Furthermore, there is an unpublished WHO report (Tchuem Tchuente et

al., 2008) describing the presence of *W. bancrofti* on the island but its real situation is unknown.

Onchocerca volvulus causes onchocerciasis or "river blindness" which is a chronic parasitic disease transmitted by black flies belonging to the genus *Simulium* (Dunn et al., 2015). It causes serious morbidities due to cutaneous and ophthalmologic manifestations, including blindness, intense itching and onchocercal skin disease (Dunn et al., 2015).

Loa loa causes loiasis, also known as African eye worm, which is transmitted by the bite of deerflies of the genus *Chrysops*. It usually consists of asymptomatic microfilaremia but some patients have episodic angioedema in the arms and legs and other cutaneous manifestations. In the human host, *L. loa* larvae migrate to the subcutaneous tissue where mature to adult worms, frequently passing through the eyes, mate and produce microfilariae (Padgett and Jacobsen, 2008). *Loa loa* infections have emerged as a public health problem in areas where it is co-endemic with *O. volvulus* because ivermectin mass drug administration (MDA) to eliminate onchocerciasis can produce fatal encephalopathic reactions among highly microfilaremic *Loa*-infected individuals (Fink et al., 2011; Gardon et al., 1997).

The different *Mansonella* species produce mansonellosis and are transmitted by the bite of bloodsucking insects: *Culicoides* spp. in all endemic areas, except in Amazonia where it is transmitted by *Simulium* spp. (Medeiros et al., 2015; Moraes et al., 1983). The disease is generally considered as innocuous, with few symptoms, fever, headache, articular pain and erythematous cutaneous plaques with pruritus. There are two species of the genus in West Africa. *M. perstans,* the most prevalent which is widespread in many parts of Sub-Saharan

Africa, where 33 countries reported cases including Equatorial Guinea (Vila Montlleo, 1990) and *M. streptocerca* distributed in West and Central Africa. The latter has the characteristic to be present in skin and can be easily mistaken with *O. volvulus* by unexperienced laboratory technicians when fresh unstained wet preparations of skin snips are examined or when its presence is not expected. In Uganda around 700 inhabitants were treated with ivermectin as a consequence of microscope-based misidentification (Fischer et al., 1997). The occurrence of *M. perstans* and *L. loa* parasites in the blood has also been seen to affect the accuracy of immunological assays routinely used for both lymphatic and onchocerciasis disease control monitoring (Bakajika et al., 2014; Luz et al., 2014; Wanji et al., 2015).

Onchocerca volvulus is the only filarial parasite that has been the target of control programmes in Bioko Island (Cuello et al., 2009 Mas et al., 1995; Mas et al., 2006). Bioko Island was included in Onchocerciasis Control Programme (OCP) activities in 1987 with the aim of eliminating the vector blackfly *Simulium* in river areas with known high rates of onchocercal blindness (Mas et al., 1995) and from 1998 in the African Programme for Onchocerciasis Control (APOC) with the objective of establishing sustainable Community-Directed Treatment with ivermectin (CDTI) (Alonso, 2009; Cupp et al., 2011). Mass drug administration with ivermectin on Bioko has been carried out for 18 years, and whilst this is expected to cause a reduction on the prevalence of infection (Dunn et al., 2015), it is uncertain how long mass drug administration would be needed on Bioko to eliminate the parasite, especially since the vector was eliminated from Bioko Island in 2005 as a result of APOC larviciding campaigns (Traore et al., 2009). A recent study described the epidemiological, clinical and

parasitological situation of onchocerciasis in Bioko Island using molecular and serological techniques and provides evidence that Bioko Island is moving fast towards elimination of the parasite after long term ivermectin distribution and the elimination of the vector in 2005, among other factors (Moya et al., 2016). Accurate filariasis identification is essential especially when other live microfilaria in skin can produce misidentification as *M. streptocerca* with *O. volvulus* in Equatorial Guinea (Mas et al., 2006) or atypical filarial localizations occurring in the human host make the microscopic diagnosis complicated (Tang et al., 2010). Thus, *O. volvulus* microfilariae, generally present in the skin, have been detected in blood samples (Duke et al., 1975; Fuglsang et al., 1974), and *M. ozzardi*, can be found in skin, although it is generally found in peripheral blood (Ewert et al., 1981; Moraes et al., 1983; Post et al., 2003). New strategies to accurately estimate the current epidemiological and entomological situation of Bioko Island are key to achieve the elimination of onchocerciasis and other filariasis. Understanding filarial parasite diversity in a region being targeted for control is thus important for effective onchocerciasis monitoring and control in Bioko and beyond (Marcondes, 2016).

The objective of this work is update the information concerning filariasis in Bioko Island using accurate molecular techniques, which are more sensitive and specific than standard methods, and able to detect human filarial parasites known to occur on the island.

2. Materials and Methods

2.1. Study area, study design and sample size.

Bioko Island is a part of the Republic of Equatorial Guinea. It is located in the Gulf of Guinea in Central Africa about 40 km southwest of the Cameroon coast. The island has a humid tropical environment and is divided into four administrative districts (Malabo, Luba, Baney and Riaba).

A cross-sectional study was conducted from mid-January to mid-February 2014. Sampling was carried out by multistage cluster survey. The sample size was computed using Epi-Info version 3.4.1 free software (10% hypothesized prevalence and 2% standard error). Firstly, twenty communities were randomly selected, proportionally to population distribution into urban or rural areas. Second sampling units were randomly selected households from an updated census from each community. In every selected household, all individuals aged 5 years or above who had permanently lived in Bioko Island during the last five years were recruited (Moya et al., 2016). A total of 543 subjects participated in the study, 224 men (41.25%) and 319 women (58.75%).

2.2. Samples collection.

The study complied with current national and international regulations and standards for biomedical research in human subjects. All participants, parents or guardians for children, gave written informed consent prior to the enrolment. The study was approved by the ethical advisory boards of Instituto de Salud Carlos III (ISCIII) in Spain and the Ministry of Health in Equatorial Guinea (CEI PI 21_2014).

Whole blood heparinised samples (2 ml) taken between 10 AM and 2 PM, and three skin snip biopsies from lumbar regions (two from right iliac crest and one from the left iliac crest) were collected from every participant. In total, 543 whole

blood and 1,629 skin snips samples were collected. Two out of three biopsies were immersed in saline solution prior to examination under microscopy for microfilariae in the field and 24 hours later in the Malabo Hospital Laboratory. The rest of biopsies and the whole blood samples were kept at 4ºC and shipped to the National Microbiology Center (ISCIII, Madrid, Spain), where further molecular analysis was performed.

2.3. Preparation of the DNA template.

The isolation of DNA from blood (200 µl) was performed using the QIAamp® DNA Blood Mini Kit (QIAGEN, Germany), according to the manufacturer's instructions. Skin snip biopsies DNA were extracted using the QIAamp® DNA Mini Kit. The biopsies were incubated at 56ºC overnight previous to DNA extraction following the manufacturer's instructions for DNA purification from tissues. DNA, independently of sample origin, was eluted with 200 µl distilled water and stored at 4ºC until use.

2.4. Filarial detection and identification by Real Time PCR (F-RT-PCR).

Detection of filarial species were performed using a Real Time PCR targeting the internal transcribed spacer one (ITS1) of the nuclear ribosomal gene of all filarial species modified from Tang *et al*. (2010). Positive samples were determined by post reaction analysis by melting temperature (Tm) curve of the amplified fragments (Tm=77.50°C ± 1.0°C) and the species identification was according to amplified product size performed by agarose or automatic gel electrophoresis and confirmed by sequencing. A size of 344 bp indicates

infection with *O. volvulus*, one of 312 bp infection by *M. perstans*, one of 301 bp by *W. bancrofti* and 286 bp infection by *L. loa*.

The PCR mixture consisted of 1x Quantimix Easy Probes (Biotools, B&M Labs, S.A., Spain), 0.2 µM of primer forward FIL2-F (5'-GGTGAACCTGCGGAAGGATC-3'), 0.2 µM of primer forward FIL 2-Loa (5'-GGTGAACCTGCRGMWGGATC-3'), 0.375 µM of reverse primer FIL2-R (5'-TGCTTATTAAGTCTACTTAA-3'), 2x EvaGreen® Dye (Biotium, Inc. Hayward, CA, U.S.A), 5µl of template DNA in a reaction volume of 20µl.

A Rotor-Gene 6000 (Corbett, Australia) was used to perform the amplification, beginning with 3 min at 95ºC, followed by 45 cycles of 10 s at 95ºC, 15 s at 50ºC and 15 s at 60ºC. Detection of positive samples was determined by melt program consisting of stepwise temperature increases of 0.5°C from 60°C to 95°C with fluorescence acquisition at each temperature transition.

2.5. Specific *Mansonella streptocerca* amplification.

In order to specifically detect *M. streptocerca* in the samples, a nested PCR targeting the conserved coding region of the 5S rDNA developed by Fischer *et al.*, (1998) was used.

2.6. Sequencing.

Amplified products were purified using Illustra DNA and Gel Band Purification Kit (General Electric Healthcare, England), then sequenced using Big Dye Terminator v3.1 Cycle Sequencing in an ABI PRISM® 3700 DNA Analyzer (Applied Biosystems, U.S.A.). All amplified products were sequenced in both directions, twice.

3. Results

Twenty communities were selected, more than half belonging to Malabo district where most of the inhabitants of the island live, but one was excluded as the samples were not properly conserved.

The analysis of the three skin snip biopsies from each individual were performed on site and re-examined in Malabo Hospital (2 skin snips) by microscopy and in Madrid (1 skin snip) by PCR. All samples (1,086) were negative when examined by microscopy, while PCR (F-RT-PCR) showed eleven positive cases which correspond to the 2.0% of the samples.

Species identification by amplified fragment size corresponds to seven *M. perstans*, one *L. loa*, one *O. volvulus* and two unknown filarial. The unique case of *O. volvulus* was detected in Riaba district, as well as the two unknown cases, together with five *M. perstans*. The other two *M. perstans* were found in Malabo district and the *L. loa* case in Baney district (Table 1).

The two unknown samples yielded a fragment around of 270 bp which did not correspond to any previously filarial size characterized. Both cases showed identical ITS-1 rDNA sequences and around 86 to 91% homology with *M. perstans*, *M. ozzardi* and *Mansonella mariae*. There was a strong suspicious of *M. streptocerca* infections which was confirmed by a specific PCR, using as target the 5S rDNA. The sequence obtained had a 100% homology compared with Fisher's *M. streptocerca* sequence published in his manuscript (Fischer et al., 1998). The sequences obtained of *M. streptocerca* 5S rDNA and ITS-1

rDNA from Bioko Island were deposited in the GenBank database with accession number KT224442 and KR868771 respectively.

The analysis in blood samples was performed exclusively by PCR in Madrid. Fifty two out of 543 whole blood samples were positive for some filaria (9.6%). Forty eight cases (8.8%) were characterised as *M. perstans* and four cases (0.7%) corresponded to *L. loa*. No other filariae were characterised in blood samples. The district with more positive cases was Riaba with 26 (37.1%), followed by the district of Baney (18 cases, 17.5%) and Malabo (8 cases, 2.5%), while no case was detected in Luba (Table 1).

In total, without discriminating between tissues, 57 individuals (10.5%) were infected with some species of filariae. The district with more cases was Riaba with 30 cases, which corresponded to 42.9% of the individuals analysed. In this district, the community 14 (Barrios Adyacentes) showed a 61.9% of infected population (Table 1).

Six out of eleven infected skin biopsies had the same corresponding infection in blood, the only *L. loa* case and five *M. perstans.*

4. Discussion

Although the microscopic examination of skin snips and Giemsa-stained blood samples is referred as the Gold Standard method for filarial diagnosis, it is well known that this technique is not the one of choice for cases with low level of microfilaremia and for mixed infections and, in general, the results depend on the microscopist's experience (Boatin and Richards, 2006). PCR offers an alternative to microscopy, as it has shown in many cases to have superior sensitivity and specificity (Tang et al., 2010).

Results in this cross-sectional study in Bioko Island showed that microscopy examinations of skin biopsies in all subjects were negative while molecular methods detected eleven positive cases (2%). It is already known that ivermectin mass treatment produces a reduction of Community Microfilarial Load (CMFL) and this has been demonstrated for Bioko Island in a previous study where CMFL dropped from 28.29 microfilariae/snip to 2.32 microfilariae/snip after eight years of treatment (Mas et al., 2006). The detection of 2% of infected skin biopsies in a context with very low microfilariae load confirms the higher sensitivity of the Real Time PCR used in this study vs microscopy.

Furthermore, accurate identification provided by the molecular methods, avoiding the microscopist's subjectivity, is essential for correct filarial identification. Ten out of eleven filariae found in skin were not *O. volvulus,* which, otherwise, could be easily mistaken if the microscopic observation were performed *in vivo.* This accuracy became much more important when, as a result of the success of onchocerciasis control activities, APOC paradigm has changed from control to elimination 'where feasible' (Traore et al., 2009) and for this new elimination goal the assessment of new methods with higher sensitivity and specificity than traditional methods is required (Olamiju et al., 2014). The only positive case of *O. volvulus* was found in a young woman from Riaba district who told that she had never been included into the APOC and she had never taken ivermectin. *Mansonella streptocerca*, previously described in Bioko Island (Mas et al., 2006) was only found in two people (0.4%), also in Riaba district.

Interestingly, all of our positive *O. volvulus* and *M. streptocerca* cases derived from the Riaba district. As *M. streptocerca* and *O. volvulus* are both susceptible to ivermectin (Fischer et al., 1997; Fischer et al., 1999), suggesting that Riaba district may not have received sufficient attention during the ivermectin MDA programme and thus future programmes should pay special attention to this area.

Mansonella streptocerca 5S rDNA fragment gene (KT224442) and the ITS-1 rDNA fragment gene (KR868771) are the first annotations for this species in the NCBI nucleotide database. This molecular identification was performed thanks to the partial nucleotide sequence of the 5S rDNA of *M. streptocerca* published in the manuscript of Fischer *et al*. (1998).

The presence of *M. perstans* DNA in skin is rare and not well documented, and it could be due to an improper sampling when biopsies are taken. However, two out of the seven *M. perstans* positive skin samples were negative in the corresponding blood sample. Although there are accounts of *M. ozzardi* occurring in the skin, the closely related *M. perstans* parasite is not generally regarded to occur in the skin and thus it was surprising that in this survey it was, in fact, the most commonly detected parasite found in both skin and blood sample assays (Medeiros et al., 2015; Simonsen et al., 2014). It is also surprising that we have detected 3 times more *M. perstans* positive skin samples than either *M. streptocerca* or O. *volvulus* positive samples. As *M. perstans* and *M. streptocerca* microfilariae are not easy to discriminate morphologically *in vivo* and *M. streptocerca* (which is easier to discriminate from *O. volvulus*) have been mis-identified as *O. volvulus* in the past (Fischer et al., 1997), there is thus a possibility that *M. perstans* occurring in the skin may have

been similarly mis-identified as *O. volvulus* in previous epidemiological studies in Bioko Island and in other localities in Africa where the two parasites co-occur. It is similarly possible that mis-identifications of *M. perstans* (which is more resistant to ivermectin and sometimes doxycycline treatments) as *O. volvulus* may have affected the accuracy of drug efficacy estimates made in the past (Awadzi et al., 2014; Hoerauf et al., 2001; Osei-Atweneboana et al., 2007).

In blood, the number of microfilariae cases is higher than in skin, 9.6% against 2%. The most represented species is *M. perstans* in 48 individuals (8.8%) spread in the entire districts but with higher prevalence in Riaba district. Historically, *M. perstans* infection was considered asymptomatic but in the last period it is more and more recognised as a pathogenic species (Fux et al., 2006). Several drugs, as diethylcarbamazine, praziquantel, ivermectin, and albendazole, have been used as treatment, but none has proven to be reliably and rapidly effective, for which reason this is not a good indicative of low ivermectin coverage for the APOC (Bregani et al., 2007).

Loa loa is also present but only in four individuals (0.7%), three of them in Baney district, at the Southeast of the island where the presence of *Chrysops spp*. (Diptera: Tabanidae) vector of loiasis is more prevalent (Cheke et al., 2003). Data of loiasis prevalence from the OCP of Equatorial Guinea obtained in 2003 indicate that *L. loa* was present in the 24% of inhabitants of the rural part of the continental region while in Bioko Island was only 5% (Jiménez et al., 2009). In this report the prevalence does not reach 1%. Loiasis has for long been regarded as a more or less benign fiariasis, and has been relatively poorly studied in most countries, including Equatorial Guinea. Interest in loiasis has increased in the last years due to the description of several cases of

encephalopathy in countries and patients with very high, coincident, *L. loa* microfilaraemia, who had been given ivermectin treatment for onchocerciasis (Molyneux et al., 2014). The low prevalence of *L. loa* in Bioko Island indicates that it is not a problem to perform MDA with ivermectin. Also, during those years where the program has been in operation, adverse effects cases have not been reported.

The Real Time PCR used in this study, F-RT-PCR, was based in a previously reported ITS-1 target filarial PCR assay which showed the capacity to detect and differentiate a wide range of filarial parasites as *O. volvulus*, *M. perstans*, *M. ozzardi*, *L. loa* and *W. bancrofti* between others (Tang et al., 2010). This assay was recommended by the National Reference Centre for Parasitology of Canada for the detection of *O. volvulus* (Ricciardi and Ndao, 2015) and it has also been successfully used in an epidemiological survey of *M. ozzardi* in Brazil (Medeiros et al., 2015) and also to confirm a *W. bancrofti* infection in Japan (Hagiya et al., 2014). Moreover, in this report, it is showed that the F-RT-PCR can be used to amplify *M. streptocerca* DNA from clinical samples, it is now apparent that the ITS-1 region can be successfully targeted for the detection and discrimination at least six types of human filarial parasites including all those that commonly occur in Africa. This novel assay is a one-step Real Time PCR assay and thus can provide sensitivity and accuracy to filariae diagnosis more than microscopy, especially in areas under some control programme; it is also faster than the original two-step nested PCR assay. The main drawback of F-RT-PCR is the identification of filarial species, which must be performed after a post analysis by gel electrophoresis or sequencing. Recently, other authors (Thiele et al., 2016) described a pan-filarial Real Time PCR to detect patent *O.*

volvulus infection in skin snips. These authors adapted our previously described method (Tang et al., 2010) to a Real Time PCR with melt curve analysis and they used to evaluate the presence of onchocerciasis in Uganda and Ethiopia. In their study they found 25.4% in Uganda and 4% in Ethiopia positive cases in skin snips but they just confirmed 24 out of 147 positive cases by sequencing asuming that all cases corresponded to *O. volvulus*, as no other filariae were expected in skin forgetting the previous misidentification in Uganda of *M. streptocerca* as *O. volvulus* (Fischer et al., 1997).

Conclusions

Four filarial species were found in the cross-sectional study performed in Bioko Island (Equatorial Guinea); *O. volvulus*, *M. streptocerca*, *M. perstans* and *L. loa*, all were detected in skin biopsy assays, while only *M. perstans* and *L. loa* were detected in whole blood assays. The filarial characterization was performed by a Real Time PCR since microscopy in skin was negative in all samples and in blood, microscopy was not performed due to time constraints, showing that the molecular diagnosis is a tool very important in the filariasis control programs. This report showed only a case of *O. volvulus* and two of *M. streptocerca,* which can be used as indicator of ivermectin coverage, all of them found in Riaba district suggesting that future Bioko onchocerciasis MDA should target Riaba. This study suggests that onchocerciasis is moving fast towards elimination after 18 years of ivermectin distribution and also suggests the possibility to interrupt ivermectin distribution in the near future, even more because the vector of onchocerciasis on the island was eliminated in 2005 and a re-introduction has not occurred yet.

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Table 1. List of communities with the names, corresponding number and number of cases and percentages by communities, species and tissues.

Community Number & Name	**Number of participants**	**District Name: Total of positive cases (percentage)**				**Blood**		**Skin**			
		Malabo (A)	**Baney (B)**	**Luba (C)**	**Riaba (D)**	***Loa loa***	***M. perstans***	***Loa loa***	***M. perstans***	***M. strptocerca***	***O. volvulus***
1 Santo Tomas de Aquino	**12**	0									
2 Zona Media B1 Baney	**23**		7 (21.2%)			2	5	1			
3 Manzana Casa Bola	**28**	1 (3.6%)					1				
4 Zona Alta A1 Baney	**36**		5 (13.9%)				5				
5 Alcalde 1 Malabo	**28**	0									
6 Cuartel de Colasesga	**31**	0									
7 Inasa Maule	**24**				11 (45.8%)	1	8			2 (2)*	
8 Manzana GETESA	**32**	2 (6.3%)					2				
9 Zona B Moka-Bioko	**21**			0							
10 IPECSA	**31**	0									
11 Bilelipa	**25**				6 (24.0%)		5		4 (1)*		
12 Zona Baja Baney	**34**		6 (17.6%)			1	5				
13 Santa Maria IV B	**40**	1 (2.5%)					1				

14 Barrios Adyacentes	**21**				13 (61.9%)		12		1		1 (1)*
15 Sampaka 1	**38**	1 (2.6%)					1				
16 Catedral Ela Nguema	**0**	ND									
17 Zona "D" C/1-25	**19**	1 (5.3%)					1				
18 Manzana Cachirulo	**21**	1 (4.8%)					1				
19 Santa Maria IV A	**43**	2 (4.7%)					1		2 (1)*		
20 Ruiché	**26**			0							
Total participants	**543**	323	103	47	70						
Number & percentage of positive cases by district all tissues and in blood		**9 (2.8%)** **8 (2.5%)**	**18 (17.5%)** **18 (17.5%)**	**0 (0.0%)** **0 (0.0%)**	**30 (42.9%)** **26 (37.1%)**						
Number & percentage of positive cases by species and tissue						4 (0.7%)	48 (8.8%)	1 (0.2%)	7 (1.3%)	2 (0.4%)	1 (0.2%)
Number & percentage of positive cases by districts, species and tissue			**57 (10.5%)**				**52 (9.6%)**			**11 2.0%)**	

(nº)* Positive cases detected just in skin without corresponding result in blood.