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ORIGINAL

A pilot study on the prevalence of loiasis in Equatorial Guinea using an accurate Nested PCR

Summary

Background: In the last years loiasis has emerged as a public health problem in areas where *Loa loa* is co-endemic with *Onchocerca volvulus, Wuchereria bancrofti* and other filarial parasites. The objective of this work was to carried out a preliminary field study on the prevalence of loiasis in Equatorial Guinea.
Methods: The study design was carried out in three villages situated in the continental region and the insular region from Equatorial Guinea. A total of 236 human blood samples were obtained from individuals living in the continental region (n=142) and on the island of Bioko (n=96). Blood samples were diagnosed by leucoconcentration and microscopy examination for microfilariae of *L. loa* and other filarial species. The molecular diagnosis was carried out by the *L. loa* specific nested PCR.
Results: The study results shown a 22.8% of loiasis prevalence by microscopy observation, whereas the nested PCR revealed a prevalence of 76.4% in the continental region. In the 94 samples obtained from individuals from the island of Bioko, loiasis was not detected either by microscopy analysis or by nested PCR.
Conclusions: The nested PCR used in this work showed itself to be an accurate technique that detects the presence of *L. loa* DNA and it could be a useful complementary tool in ascertaining more precise estimates of the prevalence of loiasis in Equatorial Guinea.
Key words: Diagnosis. Equatorial Guinea. Loa loa. Nested Polymerase Chain Reaction.

Resumen

Fundamentos: En los últimos años la loasis se ha convertido en un grave problema de Salud Pública en áreas donde *Loa loa* es co-endémica con *Onchocerca volvulus*, *Wuchereria bancrofti* y otras filarias. El objetivo de este trabajo fue llevar a cabo un estudio preliminar de la prevalencia de loasis en Guinea Ecuatorial.
Método: El diseño del estudio se llevo a cabo en tres poblaciones de la región continental y en la región insular de Guinea Ecuatorial. Se obtuvieron un total de 236 muestras de sangre procedentes de individuos de la región continental (n=142) y de la isla de Bioko (n=96). Las muestras fueron diagnosticadas por leucoconcentración y examen microscópico de *Loa loa* y otras especies de filarias. El diagnóstico molecular se llevó a cabo mediante una nested PCR específica para *Loa loa*.
Resultados: El resultado del estudio reveló una prevalencia de loasis del 22,8 % mediante diagnóstico microscópico y del 76,4% mediante nested PCR en la región continental. *Loa loa* no fue detectada ni por análisis microscópico ni por nested PCR en las 94 muestras obtenidas de individuos de la Isla de Bioko.
Conclusiones: La nested PCR utilizada en el trabajo y optimizada en nuestro laboratorio, es un método sensible que permite la detección de ADN de *Loa loa* y podría ser una herramienta complementaría útil en una evaluación más precisa de la prevalencia de loasis en Guinea Ecuatorial.
Palabras clave: Diagnóstico. Guinea Ecuatorial. *Loa loa.* Nested Polymerase Chain Reaction.

Introduction

Loiasis caused by *Loa loa* is endemic in West and Central Africa, where around 13 million people are infected[1]. It is well known that two thirds of *L. loa* infected individuals in endemic areas are without circulating microfilariae[2-4]. Also it has been confirmed that a significant proportion of amicrofilaraemic individuals in endemic areas carry adult worms. This phenomenon is known as "occult loiasis". Thus, microfilariae are typically detected fewer than 30% of the individuals by microscopy as this method is unable to detect occult infections[2]. On the other hand amicrofilaraemic individuals residents in endemic areas without clinical signs are designated as "putatively resistant". At this setting, the treatment of loiasis with diethylcarbamazine and ivermectin in patients with high microfilaraemia load (> 1000 mf/ml) is associated with serious adverse reactions, such as encephalopathy, glomerulopathy and severe hepatitis[5-9]. Therefore, accurate and specific diagnosis of human loiasis has a crucial importance in endemic areas where a majority of *L. loa* infected individuals are without circulating microfilariae. The ability to diagnose occult infection is an essential precondition for an accurate estimate of prevalence and to characterize endemic groups as microfilaraemic, amicrofilaraemic with "occult loiasis" and putative resistant individuals[10].

In addition, accurate identification of individuals with "occult loiasis", as they constitute a reservoir of infection, is compulsory to achieve a success implementation of control strategies[4]. In this way, a nested PCR, targeted on the repeat 3 region (15r3) of the gene coding for a *L. loa* 15 kDa polyprotein have been previously described and it proved to be specific (100%) and sensitive (95%) in relation to other sympatric filarial parasites[11]. Notably, the PCR detected "occult loiasis" in an endemic region of Gabon where the prevalence of loiasis is correlated with the intensity of transmission, especially in children[10,12].

The objective of this work was to carried out a preliminary field study on the prevalence of loiasis in Equatorial Guinea by the percent of loiasis diagnosed using microscopy with loiasis diagnosed using PCR.

Materials and Methods

Population

The study design was carried out in the continental region and the insular region from Equatorial Guinea. A total of 236 volunteers individuals aged >15 years and who had never received ivermectin treatment were considerated in the study. Individuals living in three villages situated in the continental region of Equatorial Guinea: Río Campo (Yengüe, YG, n=42), Mbini (Bicucbiny, BK, n=44) and Niefang (Miyobo, MB, n=54) and individuals attending the Hospital of Malabo, on the Island of Bioko, Equatorial Guinea (n=96) were included.

Samples

Human peripheral blood samples (n= 236) were collected at midday, between 10:00 and 16:00 hours, by venipuncture into vacutainers (1.3 ml) with EDTA (Starsdedt, UK).

Parasitological diagnosis

A concentration technique, Knott's technique[13], was carried out by diluting 1 ml of venous blood with EDTA, with 9 ml of 2% formaline in a conical tube. After mixing, tubes were centrifuged for 10 min at 500 rpm and the supernant discarded; the sediment was microscopically examined after staining with Giemsa.

Molecular diagnosis

Whole blood (200 μl) was used for DNA extraction. DNA was purified using the QIAamp DNA blood extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantification and puriy of the DNA was determined by spectrophotometry with a NanoDrop ND-1000 spectrophotometer (Nucliber, Madrid, Spain). Genomic DNA from *L. loa* was used as positive control.

DNA amplification was performed with the nested PCR based on the repeat 3 region (15r3) of the *L. loa* 15-kDa gene[11] with some modifications. All the PCRs were carried out in a final volume of 50 μl. The amount of DNA that was included in each reaction was 200 ng. The master mix for both PCRs consisted of 5 μl of 10X PCR buffer for Ampli-Taq Gold polymerase (1X) (Applied Biosystem, UK), 1 μl Ampli-Taq-Gold polymerase (5 U/μl) (Applied Biosystem), 0.2 mM of each deoxynucleoside triphosphate (dNTPs) (Amersham Pharmacia Biotech, Sweden), 2.25 mM of MgCl2 (Applied Biosystem, UK), 4 μl of BSA DNAse Free (0.8 μg/μl) (Amersham Pharmacia Biotech) and 0.001 mM of each set of primers 15r31-15r32 (initial PCR) and 15r33-15r34 (nested PCR)[11]. The nested PCR was carried out with 1 μl of DNA template from the first PCR reaction, diluted 4:1000 in DNase-free water. The working conditions were 1 cycle at 94°C for 9 min then 30 cycles (94°C 1 min, 65°C 1 min, 72°C 2 min) followed by 1 cycle at 72° C for 7 min for the two PCRs. The nested-PCR sample were electrophoresed through an 1% agarose D1 (Conda, Spain) in 1X TAE buffer, stained with ethidium bromide and visualizaed by trans-ilumination with ultra-violet light. Each of the samples was tested twice with each set of primers. Moreover, negative controls without DNA were included in each PCR. In order to avoid PCR contamination, sample preparation, reactions set-up and PCR amplifications were performed in separate rooms, with different lab coat and gloves.

Ethical Issues

This work received the ethical approval with a written consent of the Committe of the National Programme for the elimination of Onchocerciasis and other filariasis of Equatorial Guinea. All the participants were informed of the study and oral consent was obtained from all the individuals involved in the survey. The participants were calmed that they could take out from all or part of the consultation at any time. The investigators judged, on the basis of their experience, that written consent was not obtainable because the level of illiteracy in the population. The ethics committees accepted this restriction.

Results

A total of 236 blood samples from Equatorial Guinea were studied: 140 samples collected from the continental region (median age 42 years) and 96 samples from Malabo (insular region) (median age 30 years).

By microscopic examination, 32 out of 140 samples collected in the continental region were positive for *L. loa* microfilariae (22.8%), *M. perstans* was found in 47 out of 140 samples (33.5%) and 2 out of 140 samples were mixed infections (*L. loa*/*M. perstans*) (1.2%) (Table 1). On the other hand all the 96 samples from Malabo (Island of Bioko) were negative for *L. loa* microfilariae and 11 samples were positive to *M. perstans* (11.4%) (Table 2).

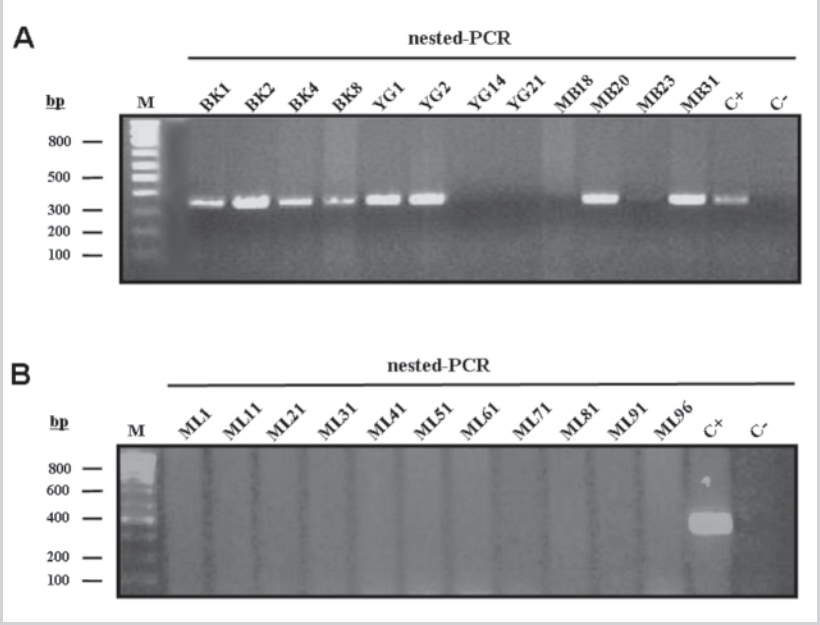

Figure 1. Representative nested PCR products of individuals samples. (A) Samples from the continental region, Equatorial Guinea: Yengüe (YG), Bicucbiny (BK) Miyobo (MB) and Malabo (ML) on the Island of Bioko. From the left to the right lanes: molecular weight marker (lane M), individuals samples (lane 2 to lane 14), positive control (lane 14) and negative control (lane 15). Specific band of 366 bp was observed in lane 2 to lane 7 and lanes 11, 12 and 13. (B) Samples from Malabo, on the Island of Bioko, Equatorial Guinea. From the left to the right lanes: molecular weight marker (lane M), individuals samples (lane 2 to lane 12), positive control (lane 13) and negative control (lane 14). In both cases amplified products were analyzed by 1% agarose gel electrophoresis.

By nested PCR, 107 out of 140 samples (76.4%) collected in the continental region were positive for *L. loa* DNA. These samples came from 34/107 *L. loa* microfilaraemic (31.7%) and 73/107 *L. loa* amicrofilaraemic (68.3%) individuals. There was impossible to verify the parasite eye passage in the 73 amicrofilaraemic people, although we considered that such individuals harbor *L. loa* adult worms.

Moreover 33 out of 140 samples (23.6%) were negative by nested PCR and 13 out of 33 negative samples (39.4%) were *M. perstans* positive by microscopic analysis (Figure 1a and Table 1).

In each continental village the global prevalence of loiasis by nested PCR was, respectively, 88% for Yengüe (YG, 37/42 samples), 79.6% for Miyobo (MB, 43/54 samples) and 61.3% for Bicucbiny (BK, 27/44 samples). In the same way, the prevalence of *L. loa* by nested PCR in amicrofilaraemic individuals with occult infection was 82.7% (24/29), 75.5% (34/45) and 46.8% (15/32) for Yengüe, Miyobo and Bicucbiny respectively (average prevalence of 68.3%) (Table 1).

Finally, nested PCR carried out in the 96 *L. loa* amicrofilaraemic samples from individuals of Malabo were also negative (Figure 1b and Table 2).

Discussion

The study results shown a 22.8% of loiasis prevalence by microscopy observation, whereas the nested PCR revealed a prevalence of 76.4% in the continental region. In the 96 samples obtained from individuals from the island of Bioko, loiasis was not detected either by microscopy analysis or by nested PCR.

These results of the *L. loa* prevalence study in Equatorial Guinea by microscopy diagnosis agree with the only two previous surveys carried out in the African country. In the first report, the *L. loa* prevalence estimated in 829 residents from eight villages located in Niefang district[14] was 12.2%. Moreover,

Table 1. Laboratory results obtained by microscopic diagnosis and nested-PCR in samples collected in three villages on the continental region of Equatorial Guinea

Sample Nº and origin	Blood microfilariae/ml *L. loa*	*M. perstans*	Nested PCR	Sample Nº and origin	Bloo Nested microfilariae/ml *L. Loa*	*M. perstans*	Nested PCR	Sample Nº and origin	Blood microfilariae/ml *L. Loa*	*M. perstans*	Nested PCR
BK1	0	70	+	YG8	0	10	+	MB18	0	6	-
BK2	50	600	+	YG9	0	0	+	MB20	0	6	+
BK4	0	90	+	YG14	0	0	-	MB21	20	0	+
BK7	0	20	-	YG15	0	0	+	MB22	0	8	+
BK8	570	0	+	YG16	0	0	+	MB23	0	0	+
BK12	0	4	+	YG18	0	0	+	MB24	0	0	+
BK14	0	20	+	YG20	0	120	+	MB27	0	0	+
BK15	0	2	+	YG21	0	0	-	MB28	0	0	+
BK16	0	2	+	YG22	0	20	+	MB29	0	0	+
BK17	0	40	+	YG28	180	0	+	MB30	0	0	-
BK19	0	0	-	YG29	0	0	+	MB31	30	0	+
BK21	0	30	-	YG32	2	0	+	MB35	0	0	+
BK25	0	10	-	YG34	0	60	+	MB36	0	0	+
BK27	0	40	+	YG35	0	8	+	MB37	0	0	-
BK29	0	60	-	YG38	0	10	+	MB39	0	0	+
BK31	10	0	+	YG40	0	0	+	MB40	0	0	+
BK34	0	0	-	YG41	0	0	+	MB42	0	8	+
BK38	0	0	-	YG42	0	0	+	MB43	0	4	+
BK39	0	0	+	YG45	2	0	+	MB44	0	0	+
BK40	0	0	+	YG46	6	0	+	MB47	0	0	+
BK41	140	0	+	YG47	0	4	-	MB49	10	0	+
BK43	0	0	-	YG48	0	0	+	MB50	0	0	+
BK45	30	0	+	YG49	30	0	+	MB51	0	0	+
BK46	600	0	+	YG51	0	0	-	MB53	0	0	-
BK47	0	0	-	YG52	0	30	+	MB54	0	0	+
BK50	0	10	+	YG53	2	0	+	MB55	0	0	+
BK52	0	0	-	YG55	0	70	+	MB58	0	0	+
BK53	6	0	+	YG56	0	50	+	MB60	0	0	+
BK54	0	0	+	YG57	0	600	+	MB62	0	0	+
BK55	0	0	-	YG58	90	0	+	MB63	0	6	-
BK56	0	40	-	YG60	10	0	+	MB66	0	0	+
BK58	0	4	+	YG61	60	0	+	MB68	0	0	-
BK61	2	0	+	YG62	110	0	+	MB70	0	0	+
BK62	0	0	-	YG63	160	0	+	MB72	0	10	+
BK63	10	0	+	YG64	4	0	+	MB73	0	0	+
BK64	0	0	-	YG65	50	150	+	MB74	0	0	+
BK67	4	0	+	YG66	0	50	+	MB84	0	30	-
BK68	0	70	-	YG67	0	300	-	MB85	0	40	-
BK69	7	0	+	YG68	0	30	+	MB87	0	70	-
BK70	0	2	+	MB1	150	0	+	MB88	0	100	+
BK72	0	4	+	MB2	0	0	+	MB89	0	50	+
BK74	0	0	-	MB6	10	0	+	MB90	0	20	+
BK75	8	0	+	MB8	70	0	+	MB91	0	80	-
BK76	0	0	-	MB11	20	0	+	MB92	100	0	+
YG1	0	10	+	MB12	0	8	+	MB93	0	0	-
YG2	0	2	+	MB15	0	0	+	MB94	50	0	+
YG5	0	0	+	MB16	0	0	+				

YG: Yengüe; BK: Bicucbiny; MB: Miyobo; +: positive result; -: negative result.

Table 2. Laboratory results obtained by microscopic diagnosis and nested-PCR in samples collected at the hospital of Malabo, on the island of Bioko, Equatorial Guinea.

Sample Nº and origin	Blood microfilariae/ml *L. loa*	*M. perstans*	Nested PCR	Sample Nº and origin	Blood microfilariae/ml *L. loa*	*M. perstans*	Nested PCR
ML1	0	0	-	ML49	0	0	-
ML2	0	0	-	ML50	0	0	-
ML3	0	0	-	ML51	0	0	-
ML4	0	0	-	ML52	0	40	-
ML5	0	0	-	ML53	0	0	-
ML6	0	0	-	ML54	0	0	-
ML7	0	0	-	ML55	0	0	-
ML8	0	0	-	ML56	0	0	-
ML9	0	0	-	ML57	0	0	-
ML10	0	0	-	ML58	0	30	-
ML11	0	0	-	ML59	0	0	-
ML12	0	0	-	ML60	0	0	-
ML13	0	60	-	ML61	0	0	-
ML14	0	0	-	ML62	0	0	-
ML15	0	0	-	ML63	0	0	-
ML16	0	0	-	ML64	0	0	-
ML17	0	0	-	ML65	0	0	-
ML18	0	90	-	ML66	0	0	-
ML19	0	60	-	ML67	0	0	-
ML20	0	0	-	ML68	0	0	-
ML21	0	0	-	ML69	0	0	-
ML22	0	0	-	ML70	0	0	-
ML23	0	0	-	ML71	0	20	-
ML24	0	0	-	ML72	0	0	-
ML25	0	10	-	ML73	0	40	-
ML26	0	0	-	ML74	0	60	-
ML27	0	0	-	ML75	0	0	-
ML28	0	0	-	ML76	0	40	-
ML29	0	0	-	ML77	0	0	-
ML30	0	0	-	ML78	0	160	-
ML31	0	0	-	ML79	0	0	-
ML32	0	0	-	ML80	0	0	-
ML33	0	0	-	ML81	0	0	-
ML34	0	0	-	ML82	0	0	-
ML35	0	0	-	ML83	0	100	-
ML36	0	10	-	ML84	0	0	-
ML37	0	0	-	ML85	0	20	-
ML38	0	0	-	ML86	0	0	-
ML39	0	0	-	ML87	0	0	-
ML40	0	0	-	ML88	0	0	-
ML41	0	0	-	ML89	0	0	-
ML42	0	0	-	ML90	0	0	-
ML43	0	0	-	ML91	0	0	-
ML44	0	0	-	ML92	0	0	-
ML45	0	0	-	ML93	0	0	-
ML46	0	0	-	ML94	0	0	-
ML47	0	0	-	ML95	0	0	-
ML48	0	0	-	ML96	0	0	-

ML: Malabo; +: positive result; -: negative result.

these figures changed when the altitude was considered; thus the higher *L. loa* prevalence (31.7%) and the lower *M. perstans* prevalence (57.3%) were found between 300-350 m of altitude. The *M. perstans* microfilaraemia prevalence determined by us was 33.5%, which was slightly lower than the one obtained by Vila Montlleo, *et al*. in 1990 (51.6%), and also the percentage of mixed infections was higher (14.8%) that the data shown in the present work (1.2%)[14]. The second study, an epidemiological survey on the prevalence of loiasis in Equatorial Guinea carried out by Sima *et al.* in 2003 (unpublished data), which microscopically examined 1166 blood samples estimated a global prevalence of 24% (281/1166), and specifically reported a prevalence of 32% (264/824) in the continental region. In contrast, the prevalence was the 4.97% (17/342) in the island (4.5% in the northern part and 6% in the southern part). The data presented in this work related with the samples collected in the Hospital of Malabo, situated in the northern part of Bioko, disagree with the previous unpublished study on the prevalence of loiasis on the Bioko island by Sima *et al.* in 2003 that revealed a 4,97% of prevalence, with a slightly higher prevalence in the southern part where *Chrysops* species are widely distributed, although it is worthy of mention that many patients are in close contact with the country mainland, patients visited or have lived in the continental region, and it remains unclear whether *L. loa* is actually transmitted[15].

However, in the two mentioned studies the *L. loa* specific nested PCR was not employed. In the present work, the nested PCR revealed a higher prevalence of the disease, estimated to be 76.4% (107/140), in the continental region. Notably, nested PCR detected *L. loa* in 100 % (34/107) and 68.3% (73/107) of microfilaraemic and amicrofilaraemic individuals, respectively. Thus, the nested PCR detected "occult loiasis" in amicrofilaraemic individuals. Our data are in absolutely accordance with the results previously obtained by Toure, *et al.*, who detected the parasite

in 84% (42/50) and 68% (106/157) of amicrofilaraemic individuals from Gabon[10,11,16-18].

According to the results obtained in this work by the nested PCR, the prevalence of loiasis in the three Equatorial Guinean villages from the continental region studied was 76.4%. Interestingly, these findings were not unexpected, as well as similar results were found in neighbouring countries, such as Cameroon and Gabon, which share the same ecological environmental characteristics with Equatorial Guinea and where the tabaniid vector has also been described[10,11,15,19]. There was impossible to verify the adult eye passage in the 73 amicrofilaraemic individuals so, we considered that these individuals with positive nested PCR harbour *L. loa* adult worms and thus have an occult infection. The 33 samples from individuals from the continental region, who were negative by both microscopic analysis and nested PCR, could represent non infected individuals or putative resistant individuals living in endemic areas, as previously described by Touré, *et al.*[10], although no immunological studies were done to confirm such hypothesis.

In summary, the nested PCR used in this work showed itself to be an accurate technique that detects the presence of *L. loa* DNA with high specificity and sensitivity, as previously described by Touré, *et al.*[11].

In the future, nested PCR could be a useful tool for a large-scale epidemiological surveys of individuals in the continental area as well as the Bioko island in ascertaining more precise estimates of the real prevalence of loiasis and in the development of effective control strategies in endemic areas such as Equatorial Guinea where it could be a useful complementary tool in Rapid Assessment Procedures for loiasis programs.

Conclusions

We conclude that *L. loa* specific nested PCR, followed by leucoconcentration and microscopic examination of thick blood films, are accurate tools for the detection and control of loiasis mainly in endemic areas, as Equatorial Guinea, where occult infection is present.

Acknowledgements

To Dr. Akue, Dept. of Medical Parasitology, Centre International de Recherches Medicales de Francesville, Gabon for kindly supplied genomic DNA from *L. loa*. To Rufino Nguema and Policarpo Ncogo for providing us with the epidemiological information of the samples from Equatorial Guinea. The authors would like dedicate this work in memory of Juanita Nsang.

Dr. González was sponsored by fellowships from the ISCIII and RICET-FIS. This study was supported by Grant MPY-1200/04 (Instituto de Salud Carlos III) and RETIC-RICET, RD06/0021/0019.

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