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Genetic heterogeneity in *Loa loa* parasites from southern Cameroon: A preliminary study

Tarig B Higazi[1], Amy D Klion[2], Michel Boussinesq[3] and Thomas R Unnasch*[1]

Address: [1]Division of Geographic Medicine, University of Alabama at Birmingham, Birmingham, AL USA, [2]Laboratory of Parasitic Diseases, National Institutes of Health, Bethesda, MD USA and [3]Institut de Recherche pour le Developpement, Departement Societes et Sante, Paris, France

Email: Tarig B Higazi - higazi@uab.edu; Amy D Klion - aklion@niaid.nih.gov; Michel Boussinesq - michel.boussinesq@wanadoo.fr; Thomas R Unnasch* - trunnasch@geomed.dom.uab.edu

* Corresponding author

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Abstract

Ivermectin (or Mectizan™) is widely used by onchocerciasis and lymphatic filariasis control programs worldwide. Generally, Mectizan™ is both safe and well tolerated. An exception to this general pattern is in some areas co-endemic for *Onchocerca volvulus* and *Loa loa*, where a number of severe adverse reactions to Mectizan™ have been noted in *L. loa* infected individuals. The vast majority of these severe adverse events have occurred in Southern Cameroon. This suggested the hypothesis that the parasites endemic to Southern Cameroon might form a distinct population that exhibited a phenotype of eliciting severe adverse reactions in *Loa*-infected individuals upon Mectizan™ exposure. To test this hypothesis, the DNA sequences of three potentially polymorphic loci were compared among *L. loa* parasites from Southern Cameroon and other endemic foci in Sub-Saharan Africa. Analysis of these data suggested that parasites from Southern Cameroon were at least as genetically diverse as those from other foci. Furthermore, no polymorphisms were noted that were unique to and shared among the parasite isolates from Southern Cameroon. Although a limited number of parasite isolates were tested, these results do not appear to support the hypothesis that *L. loa* parasites from Southern Cameroon represent a unique, genetically isolated population.

Findings

Ivermectin (Mectizan™) is a semi-synthetic lactone drug that exhibits broad anti-helminthic specificity [1]. Mectizan™ has become the drug of choice for mass chemotherapy campaigns to eliminate onchocerciasis as a public health problem [2]. In general, these mass chemotherapy projects have been associated with few complications. A notable exception occurs in some areas where the agent of onchocerciasis, *Onchocerca volvulus*, is sympatric with *Loa loa*, the causative agent of another filarial infection of humans. Over the past several years, severe adverse reactions to Mectizan™ treatment have been reported in individuals residing in onchocerciasis endemic areas that are also endemic for *L. loa* [3-5]. These adverse reactions are characterized by severe neurological complications, including incontinence, coma, and in some cases, death of the patient [3,5]. Interestingly, a large majority of the individuals suffering from such severe adverse reactions have resided in Southern Cameroon [6-8]. The development of severe reactions to Mectizan™ in *L. loa* infected individuals has had a negative impact on the design and implementation of Mectizan™ distribution campaigns to

control onchocerciasis in areas where *O. volvulus* and *L. loa* are co-endemic [9].

Parasitological examinations of patients who developed post-Mectizan™ encephalopathy showed a correlation between the risk of developing such reactions and the *L. loa* microfilarial load [10]. However, the finding that the vast majority of such severe reactions occurred in a localized geographic area also suggested that the parasites endemic to this area might represent a genetically distinct population that is particularly capable of inducing adverse reactions in the host upon being exposed to Mectizan™. If the parasites from Southern Cameroon represent a genetically distinct population, it would follow that genetic exchange with other parasite populations would be expected to be limited or non-existent. Such reproductively isolated populations often suffer from a genetic founder effect, resulting in a population in which the level of genetic heterogeneity is severely limited, and in which the distribution of alleles in the isolated population is often strikingly different from that seen in non-reproductively isolated populations. Furthermore, prolonged reproductive isolation of a population will result in the accumulation of population specific genetic polymorphisms. If this occurs, phylogenic analyses based upon data collected from isolates from within and outside of the isolated population should result in a phylogeny in which the isolates from within the isolated population are grouped together.

Very little is known concerning the degree of genetic diversity among different populations of *L. loa* and the role that such genetic polymorphisms may play in the adverse responses to Mectizan™ treatment. Simian and human strains of *L. loa*, which differ in the length of the microfilariae and their periodicity in the host have long been recognized [11,12]. Despite these differences, human and simian *L. loa* are believed to be the same species, as experimental studies have shown that parasites from these two primate hosts can hybridize [12]. Because parasites from the two hosts differ in periodicity and are transmitted by different species of the *Chrysops* genus, it has been proposed that the human and simian parasites "are at an early stage of radiative evolution" [11]. Whether hybridization of the simian and human strains is a co-factor in the development of post-Mectizan™ encephalopathy is unknown [13]. Some biochemical variation has also been reported among different populations of human *L. loa* [14], although these differences have not been explored at the genetic level.

Perhaps the most well documented case of the importance of genetic factors in the pathogenesis of a human filarial infection is in onchocerciasis. Human onchocerciasis exhibits two distinct clinical and epidemiologic disease patterns in the rain forest and savanna bioclimes of West Africa [15,16]. Studies have revealed a strong correlation between the classification based upon the epidemiological disease pattern and the pattern of hybridization to strain specific DNA sequences derived from a repeated sequence encoded in the nuclear genome of *O. volvulus* [17]. Subsequent studies have demonstrated that variation within this repeat population could be used as a tool for biogeographic studies [18,19]. In *Ascaris suum* [20], *Ostertagia osteragi* [21] and *Haemonchus contortus* [22] mitochondrially encoded sequences have proven to contain useful polymorphisms for population based studies. Similarly, the internal transcribed spacer (ITS) of the ribosomal RNA gene cluster has been used to develop markers capable of distinguishing different populations of *Haemonchus contortus* [23].

To test the hypothesis that *L. loa* endemic to Southern Cameroon represents a distinct population, four parasite isolates were obtained from Southern Cameroon. These parasites consisted of infective larvae obtained from four individual wild caught *L. loa* infected *Chrysops dimidiata* vector flies collected in the village of Ngat (3°25'N, 11°33'E), located approximately 50 km south of the capital of Yaoundé. Previous studies have reported cases of *L. loa* encephalopathy in this village [24]. Individual isolates were also obtained from blood collected from *L. loa* infected expatriate former residents of Nigeria, Gabon and The Democratic Republic of the Congo at the Laboratory of Parasitic Diseases at the National Institutes of Health in Bethesda, MD, USA. DNA was extracted from the parasite preparations, following previously described methods [25]. These DNA samples were then used as templates in PCR amplification reactions targeting three gene sequences: the mitochondrial 16S rRNA gene, the ITS2 domain of the nuclear rRNA gene cluster, and the 15r3 polyprotein gene in *L. loa*. The 15r3 gene has previously been shown to be polymorphic and useful in the development of a DNA based diagnostic assay for this parasite [26,27]. These gene sequences were chosen for study because, as discussed above, homologues (or related sequences) have previously been shown to be informative population based markers in studies of other parasitic nematodes. PCR amplicons resulting from these reactions were subjected to direct DNA sequence analysis to identify sequence polymorphisms.

All of the DNA templates were successfully amplified with the primer set derived from the mitochondrial 16S rRNA gene. However, no polymorphisms were noted in the amplification products analyzed (data not shown). Amplification products were also obtained from all of the samples targeting the 15r3 polyprotein gene. Several polymorphic sites were found in the 15R3 amplicons. Interestingly, sequences derived from DNA extracted from the

A

Sample	Identity						
1	Cameroon A	1					
2	Cameroon B	0.010	2				
3	Cameroon C	0.022	0.026	3			
4	Cameroon D	0.019	0.016	0.035	4		
5	Gabon	0.000	0.010	0.022	0.019	5	
6	Nigeria	0.000	0.010	0.022	0.019	0.00	6
7	Congo	0.000	0.010	0.022	0.019	0.00	0.00

B

Sample	Identity			
1	Cameroon A	1		
2	Cameroon B	0.011	2	
3	Gabon	0.015	0.015	3
4	Nigeria	0.037	0.030	0.034

C

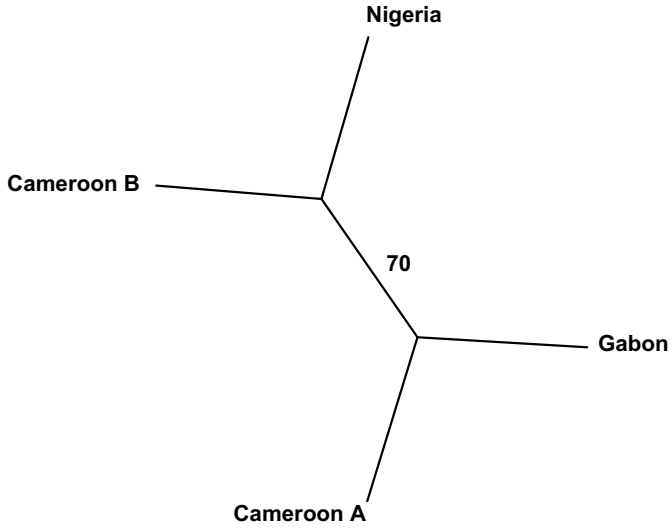

Figure 1
Analysis of polymorphisms in the 15r3 and ITS2 gene sequences of *L. loa*: Panel A: Pairwise differences in 15r3 amplicon sequences among *L. loa* isolates. The 15R3 gene fragment was amplified from *L. loa* genomic DNA (2.5 μl per reaction) using primers with the sequences 5' GGCACAAAACACTGCAGCAGTCCT 3' and 5' CAGCTGTCTCAAATCGAAGATTCT 3'. A total of 2.5 units of Taq polymerase (Roche Applied Biochemicals, Indianapolis, USA) was used in each 50 μl amplification reaction, together with the reaction buffer supplied by the manufacturer. Amplification conditions consisted of an initial denaturation of 5 minutes at 94°C, followed by 40 cycles consisting of 1 minute at 94°C, 1 minute at 49°C, and 2 minutes at 72°C. Reactions were completed by a final extension at 72°C for 7 minutes. The amplicon analyzed was 318 nucleotides long. Distances were calculated using the two parameter method [28]. Panel B: Pairwise differences in ITS2 amplicon sequences among *L. loa* isolates. The ITS2 gene fragment was amplified from *L. loa* genomic DNA (2.5 μl per reaction) using primers with the sequences 5' TAACAATGAAGATAAAGCGA 3' and 5' TTAGTTTCTTTTCCTCCGCT 3'. A total of 2.5 units of Taq polymerase (Roche Applied Biochemicals, Indianapolis, USA) was used in each 50 μl amplification reaction, together with the reaction buffer supplied by the manufacturer. Amplification conditions consisted of an initial denaturation of 5 minutes at 94°C, followed by 40 cycles consisting of 1 minute at 94°C, 1 minute at 50°C, and 2 minutes at 72°C. Reactions were completed by a final extension at 72°C for 7 minutes. The amplicon analyzed was 472 nucleotides long. Distances were calculated using the two parameter method [28]. Panel C: Phylogenetic tree developed from the ITS2 sequence data. The phylogeny was developed using parsimony methods, performing an exhaustive search of the data with the parsimony routines in the PAUP program package (v4.0, release 10) [29]. The robustness of the phylogeny was tested by running 1000 synthetic datasets with the bootstrap method in the PAUP program package. As indicated on the figure, the division of the four sequences into the two clades shown was supported 70% of the time in the bootstrap analysis.

blood of *L. loa* infected individuals from Gabon, Nigeria and The Democratic Republic of the Congo were identical (Figure 1, Panel A). One of the isolates from Southern Cameroon also contained a 15R3 sequence which was identical to that found in the parasite isolates from the other countries. However, the remaining three isolates from Southern Cameroon contained between 3 and 7 polymorphic sites when compared to the canonical sequence found in the other parasites (Figure 1, Panel A). These data suggest that the 15R3 gene sequences from parasites obtained from Southern Cameroon were at least as polymorphic as those from other *L. loa* foci in Sub-Saharan Africa. None of the polymorphisms were consistently found among the isolates from Southern Cameroon, suggesting that these polymorphisms probably did not arise from evolution in a genetically homogeneous, reproductively isolated population (data not shown).

Amplification of the ITS2 locus was successful from *L. loa* DNA samples from Nigeria, Gabon and two of four samples from Southern Cameroon. Again, several polymorphisms were noted among the four samples examined (Figure 1, Panel B). However, none of these polymorphisms were specific to and conserved in the two isolates from Southern Cameroon. Thus, an analysis of the sequence data using parsimony methods did not support a phylogeny that grouped the two isolates from Southern Cameroon (Figure 1, Panel C).

The data presented above are preliminary, and involve the analysis of a limited number of sequences from a small number of parasite isolates. Nevertheless, some conclusions are suggested by these results. First, it appears that genetic variation in parasites from the focus in Southern Cameroon is at least as frequent as in parasites from other locations. Second, no polymorphisms were noted which were unique to the parasites from Southern Cameroon and also shared among the isolates examined from this focus. Together, these observations argue against the hypothesis that the parasites found in Southern Cameroon represent a distinct, reproductively isolated population. Analysis of additional parasite samples and additional loci will be necessary to confirm this.

This study revealed a considerable amount of sequence variation in the two nuclearly encoded genes examined. If this level of sequence heterogeneity is representative of the rest of the nuclear genome, this suggests that the *L. loa* population from Southern Cameroon is fairly genetically heterogeneous. It is therefore possible that a proportion of the parasites from Southern Cameroon contain allelic variants of genes that result in enhanced virulence and/or pathogenicity in the face of Mectizan™ treatment. This might explain the fact that severe adverse reactions in response to Mectizan™ treatment are confined to a small proportion of the treated population. To test this hypothesis, it will be necessary to obtain parasites from individuals who exhibit the preliminary symptoms of a severe adverse reaction shortly following Mectizan™ distribution, when *L. loa* microfilariae are still likely to be present in the circulation.

Competing Interests
None declared.

Author's Contributions
TBH was responsible for carrying out the laboratory experiments described in the manuscript, including DNA isolation, PCR amplification and DNA sequence analysis. AK and MB were involved in the experimental planning and conducted the field work involved in obtaining the samples. TRU served as the Principal Investigator on the project. He was responsible for obtaining grant support for the project, for assisting in the experimental design, data analysis and for preparation of the manuscript.

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