Analysis of *Onchocerca volvulus* β-tubulin gene polymorphism in the Mbonge sub-division of Cameroon: Evidence of gene selection by ivermectin.

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Abstract

Ivermectin (IVM) still remains the only safe drug for the mass control of onchocerciasis, and the continued success of control programmes depends on its efficacy. However, the reliance on a single drug for decades might become problematic due to possible development of IVM resistance as has occurred in nematode parasites of livestock and the filarial heartworm of dogs. Drug resistance is a genetic phenomenon resulting from changes in the genetic profile of the parasite population that would be seen as a selection for particular alleles of genes. One gene shown to be linked to IVM selection in *Onchocerca volvulus* and also known to be associated with IVM resistance in veterinary nematodes is the beta (β) tubulin gene. Assessment of parasitological response profile of *O. volvulus* to IVM and genetic analysis of β-tubulin gene could reveal the association between *O. volvulus* worm genotype and IVM selection. Onchocercal nodules were surgically removed from onchocerciasis patients in two cohorts with different treatment histories: a group that had received repeated doses of IVM at least for the previous 3 years, and a control group with no history of IVM treatment. Reverse transcription (RT) PCR of β-tubulin transcripts revealed comparable expression levels in both IVM exposed and naïve worms. Restriction fragment length polymorphism of the β-tubulin gene revealed a selection of the G allele in IVM-exposed worms as against the T allele in the IVM naïve population. This evidence of IVM selection suggests that IVM resistance may be emerging in the Mbonge Sub-Division and thus requires monitoring.

Keywords: *O. volvulus*, Ivermectin, β-tubulin selection.

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Introduction

Human onchocerciasis commonly known as river blindness is one of the most devastating yet neglected tropical diseases. It is caused by the obligate human filarial nematode *Onchocerca volvulus* and transmitted by repeated bites of blackflies of the genus Simulium [1,2]. It is the second leading cause of infectious blindness after trachoma accounting for up to 270,000 cases in the world [3]. Worldwide, 99% of all infected people are living in Africa where 15.1 million people are infected and 120 million at risk of the disease [3,4]. In Cameroon, 2.81 million people are infected by the disease with 5.2 million people at risk of infection and an estimated nodule prevalence level of over 40% with a corresponding skin microfilaria prevalence level of about 60% [5]. A study in three drainage basins with contrasting hydrologic profiles in the rain forest areas co-endemic for onchocerciasis and loiasis in the Southern part of Cameroon reported that Meme drainage basin had the highest prevalence of nodule (39.1%) and micro-filarial (mf) (52.7%) [6].

Various governments, policy and decision makers at the forefront of the battle against onchocerciasis have continuously made efforts to control and eliminate the disease, presently relying largely on Community directed treatment with ivermectin (CDTI) [7]. Ivermectin (IVM) has limited macrofilaricidal efficacy thus, treatment of onchocerciasis with IVM has to be taken for about 15 years, corresponding to the life span of the adult worm [8]. However, the reliance on a single drug during decades might become problematic due to possible development of IVM resistance as has occurred in nematode parasites of livestock and the filarial heartworm of dogs [9].

Drug resistance is a genetic phenomenon resulting from changes in the genetic profile of the parasite population that would be seen as a selection for particular alleles of genes. Once the resistance phenotype has been unequivocally identified in the population, the genetic profile of the population is already altered. This genetic alteration can be expected not only in genes whose products are involved in the resistance mechanism, but may also be seen in genes not themselves involved in the resistance mechanism, but genetically linked to other genes that are involved in a resistance mechanism [10]. Gene selection is the first step in the development of drug resistance, it is therefore important to assess genetic changes in a population of parasites exposed to selection pressure [11].

Significant differences in the genetic polymorphism of β-tubulin gene between populations of *O. volvulus* from IVM exposed and IVM naïve people have been reported [9]. The entire β-tubulin gene has been screened for single nucleotide polymorphisms (SNPs) and a consistent relationship between four SNPs and the poor response of Ghanaian *O. volvulus* populations to IVM has been observed [11].

This work is aimed at assessing the β-tubulin gene for polymorphism associated with IVM resistance using *O. volvulus*

from IVM exposed and IVM naïve individuals in the Mbonge Sub-Division, South West Region of Cameroon. This will help in early detection or monitoring for IVM resistance in endemic communities.

Materials and Methods

Study design and selection of patients

This was a cross sectional population-based study designed to detect IVM selection by studying the genetic profile of worms in IVM exposed and IVM naïve populations. This study was carried out in Mbonge Sub-Division, an area hyperendemic for onchocerciasis, located in Meme Division, South West Region of Cameroon. Participants were placed in two groups: A group that had received repeated doses of IVM at least for the previous 3 years, and a control group with no history of IVM treatment. The protocols adopted for this study were reviewed and approved by the National Ethics Committee for Research in Human Health (CNERSH). Administrative authorisations were obtained from the Cameroon Ministry of Public Health and the South West Regional Delegation of Public Health. The objectives of the study were explained to all eligible individuals, and those who agreed to participate signed and kept a copy of the consent form.

Isolation of adult O. volvulus worms

Nodulectomy was done by a trained medical practitioner to excise a nodule from each patient into sterile 25 mL RPMI 1640 medium under aseptic conditions and transported to the laboratory. Digestion of the nodules to get worms was done according to the method of Schulz-Key (1977) [12]. Briefly, Nodular mass was cultured in fresh RPMI-1640 medium, supplemented with 0.25 mg/mL of gentamicin sulphate (Sigma) and collagenase (type 1) purified from Clostridium histolyticum (Sigma) at a final concentration of 0.5 mg/mL. The nodules were incubated at 37°C with constant gentle shaking over night before removing the individual worms from the digested nodule. The individual worms were separated by sex and submerged into a 1 mL incomplete culture medium (ICM) (RPMI-1640, supplemented with 25 mM HEPES, 2 g/L sodium bicarbonate, 2 mM L-glutamine, 150 units/mL penicillin, 150 µg/mL streptomycin and 0.5 µg/mL amphotericin B pH 7.4) using 24-well plates. The adult worms were allowed in ICM for 24 hours in 5% CO_2. These samples were then used for extraction of RNA or DNA.

DNA extraction

Ninety microliters of worm lysis buffer (0.1 M Tris-Cl pH 8.5, 0.1 M NaCl, 50 mM EDTA pH 8.0, 1% SDS), 4 µL of 20 mg/mL Proteinase K and 2 µL of 10 mg/ml RNase A were added to a frozen 10 µL aliquot of worms in a 1.5 mL microfuge tube. The worms were incubated at 65°C for 60 min. DNA was extracted using an equal volume of phenol/chloroform then precipitated with 10 µL 3 M NaOAC and 15 µL of ice cold 95% ethanol. The DNA pellet was air dried, resuspended in 50 µL dH_2O and stored at -20°C.

PCR amplification of genomic DNA fragments

PCR primers pairs flanking the regions of interest in *O. volvulus* β-tubulin gene were used for PCR amplification with the purified genomic DNA of individual adult worms as template (Table 1). PCR was performed using Red Taq Ready Mix PCR reaction mix (Sigma, Germany) in a total volume of 25 µL. PCR was carried out under standard conditions: initial denaturation: 95°C for 3 min., denaturation: 95°C for 1 min., annealing: (52ºC–55ºC (depending on the primer set) for 30 seconds, polymerisation: 68°C for 1 min. and final extension: 68°C for 10 min. The PCR products were separated on a 2% agarose gel and viewed under ultraviolet (UV) light using the molecular imager GelDocTM XR+ (BIORAD, USA).

Restriction fragment length polymorphism

The PCR products of various primer sets were digested immediately after amplification using three specific restriction enzymes (Table 1). The digestion products were incubated at 37 °C for 30 min. and then separated on an 8% 39:1 polyacrylamide gel (acrylamide: N, N-methylenebisacrylamide) in 1 × TBE at 115 V for 60 min. The gels were stained with ethidium bromide (0.5 g/ml) and visualized under UV light using the molecular imager GelDocTM XR+ (BIORAD, USA).

Ribonucleic Acid (RNA) extraction

Total mRNA was extracted from worms using the Recover AllTM Total Nucleic Acid Isolation kit (Ambion Life Technologies, USA) following manufacturer's protocol for 20 µL reaction. The extracted RNA was run on a 1% agarose gel to confirm the extraction. The isolated mRNA samples were then immediately used for cDNA synthesis.

Complementary Deoxyribonucleic Acid (cDNA) synthesis

The iScriptTM cDNA synthesis kit (BIORAD, USA) was used to synthesize cDNA from the extracted RNA following manufacturer's instruction for a 20 µL reaction mixture. Briefly, the reaction set up composed of 5 µL nuclease free water, 4 µL of 5X iScript reaction mix, 1 µL of iScript reverse transcriptase, and 10 µL of RNA was incubated at 25°C for 5 minutes, 42°C for 30 min, 85°C for 5 min. cDNA pools made up of eight IVM exposed samples and seven IVM naïve samples were prepared. The cDNA pools were quantified using SmartSpecTM plus Spectrophotometer (BIORAD, USA) and the expression profile of β-tubulin gene was checked between IVM exposed and IVM naïve worms using designed primers (Table 2).

To get the expression profiles of β-tubulin gene and OvGADPH

Table 1. *Features of primers used for the amplification of O. volvulus β -tubulin fragments containing the three polymorphisms investigated and restriction enzymes used to digest the amplified products.*

Primer set	Primer sequence (sense and antisense)	PCR product size [bp]	Annealing temperature [°C]	Restriction enzyme
SG-β-tub F and SG-β-tub R	5'-GCAACAATTGGGCTAAGGGAC-3' 5'-CGATCCGGATATTCCTCACGA-3'	300 (1557–1857)	52	AluI
SG-β-tub C/T F and SG-β-tub C/T R	5'-GTCTGCATCTCTTTCCAAGG-3' 5'-GCCCAATTGTTGCTAGCTCC-3'	486 (1083-1568)	55	NsiI
SG-β-tub T/G F and SG-β-tub T/G R	5'-TACACACCGTAGTCCTATGG-3' 5'- CCAGATCGACAAGGATTGC -3'	499 (970–1468)	53	AvaII

Table 2. *Features of primers used to investigate the expression profile of β-tubulin and OvGADPH genes.*

Gene	Primer set	Size (bp)	Annealing temperature [°C]
β-tubulin	F: 5'-GTCTGCATCTCTTTCCAAGG-3' R: 5'-GCCCAATTGTTGCTAGCTCC-3'	486 (1083-1568)	55
OvGADPH	F: 5'-GCAGTTGAAAAGGACACCGT-3' R: 5'-TGCACCTTCTACTCCCCATG-3'	224 (376-600)	56

Table 3. *Demographic data of participants.*

Patients parameters compared	IVM Exposed	IVM Naïve
Number (n) of subjects (n=36)	18	18
Female (Male)	11 (7)	7 (11)
Age range	8 to 57 years	6 to 55 years
Number of times subject has consecutively taken IVM	≥ 3 times	Never

Table 4. *Genotype and allele frequencies of β-tubulin gene 1183 T/G SNP in IVM exposed and IVM naïve worms.*

Status		Genotype			Allele	
		TT	TG	GG	T	G
IVM exposed (N=18)	N	7	11	0	25	11
	%	38.9	61.1	0.0	69.4	30.6
IVM naïve (N=18)	N	17	1	0	35	1
	%	94.4	5.6	0.0	97.2	2.8
Test statistics		χ^2=12.5, p=0.004			χ^2 = 10.0, p=0.0016	

(a house keeping gene), PCR was performed using RedTaq ReadyMix PCR reaction mix (Sigma, Germany) in a total volume of 25 µL. Standard PCR conditions were employed: 95°C for 3 minutes, 95°C for 1 min., 55 or 56°C (depending on the primer set) for 30 seconds, and 68°C for 1 min. Final extension was done at 68°C for 10 min. The PCR products were run on a 2.5% agarose gel and viewed under UV light using the molecular imager GelDocTM XR+ (BIORAD, USA).

Statistical analysis

Data was analysed using GraphPad Prism (version 5.0). All analytical tests were two tailed and P<0.05 was considered statistically significant at a confidence interval (CI) of 95%. The differences in proportion of the genotype and allele frequencies were assessed by Chi-square test.

Results

Demographic data of participants

A total of 18 IVM-exposed and 18 IVM-naïve subjects who live in the endemic region of Mbonge Sub-Division for at least the 3 previous years were recruited (Table 3).

Genotype and allele frequencies of β-tubulin gene

AvaII restriction analysis of β-tubulin gene revealed that the TT homozygous and TG heterozygous genotypes were present whereas the GG homozygote genotype was absent in both study populations (Figure 1).

The respective frequencies of the TT and TG genotypes among IVM exposed worms were 38.9% (n=7) and 61.1% (n=11). Among the IVM naïve worms, they were 94.4% (n=17) and 5.6% (n=1), respectively. The frequencies of the T and G allele among IVM expose worms were 69.4% and 30.6%, and among IVM naïve worms, they were 97.2% and 2.8%, respectively. The frequency of TT homozygote as compared to TG heterozygote was lower in IVM exposed group and higher in the IVM naïve group. Statistical analyses using Chi square test showed significant difference in genotype and allele frequency between the IVM exposed group and naïve group (all p-values <0.05) (Table 4).

Restriction fragment length polymorphism analysis of β-tubulin gene for 1308 C/T SNP using the NsiI restriction enzyme did not reveal any differences in banding pattern between the IVM-exposed and naïve populations (Figure 2). The same results were obtained for the insertion deletion (ID) polymorphism between 1557-1857 bp in the two populations with AluI restriction enzyme (Figure 3).

Expression levels of O. volvulus β-tubulin gene in IVM exposed and naïve worms

The concentration of cDNA pool of the IVM exposed and IVM naïve worms were successfully obtained using Smart SpecTM plus Spectrophotometer (BIORAD, USA). No difference in cDNA pool concentration was observed between the IVM-exposed worms (1.52 mg/mL) and cDNA pool of IVM naïve worms (1.53 mg/mL).

The expression levels of *O. volvulus* β-tubulin and OvGADPH genes were successfully obtained by RT-PCR using specific primers. PCR products were analysed on Ethidium Bromide-stained agarose gels and viewed under UV light using the molecular imager GelDocTM XR+ (BIORAD). Expression levels where the same in both IVM exposed and naïve worms (Figure 4). OvGADPH is a housekeeping gene and serve in normalization of expression levels.

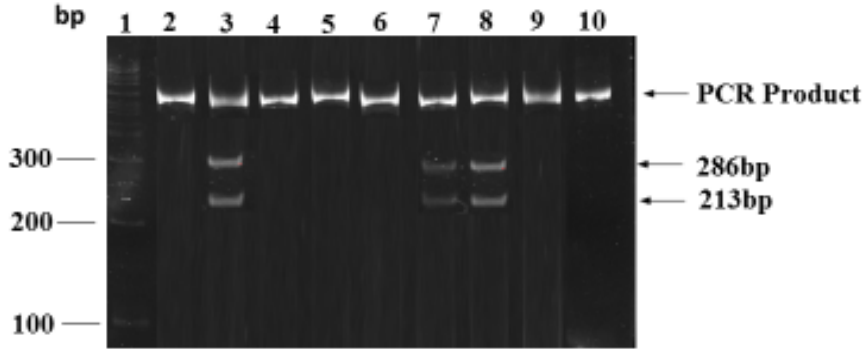

Figure 1. *AvaII restriction analysis of β-tubulin gene. PCR products amplified from SG-β-tub T/G primers were digested with AvaII and products were separated on 8% non-denaturing polyacrylamide gels, visualised under UV light. Lane 1: 100 bp ladder; Lanes 2, 4, 5, 6 and 9: TT genotype; Lanes 3, 7 and 9: TG genotype; Lane 10: Undigested amplified PCR product.*

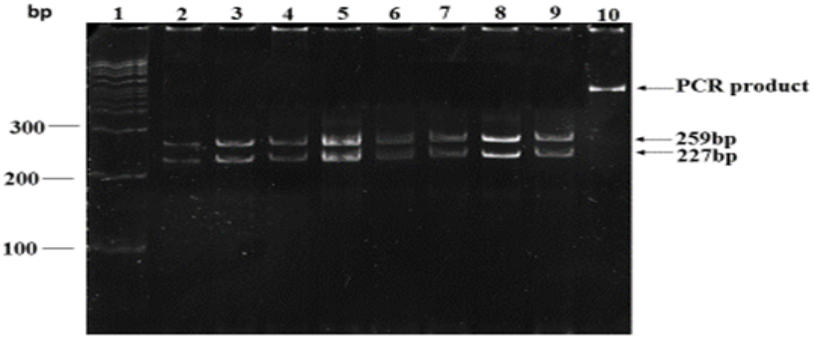

Figure 2. *NsiI restriction analysis of β-tubulin gene. The PCR products amplified from SG-β-tub C/T primers were immediately digested by NsiI endonuclease and products were separated on 8% non-denaturing polyacrylamide gels, visualised under UV light. Lane 1: 100 bp ladder; Lanes 2-9: Digested PCR product; Lane 10: Undigested amplified PCR product.*

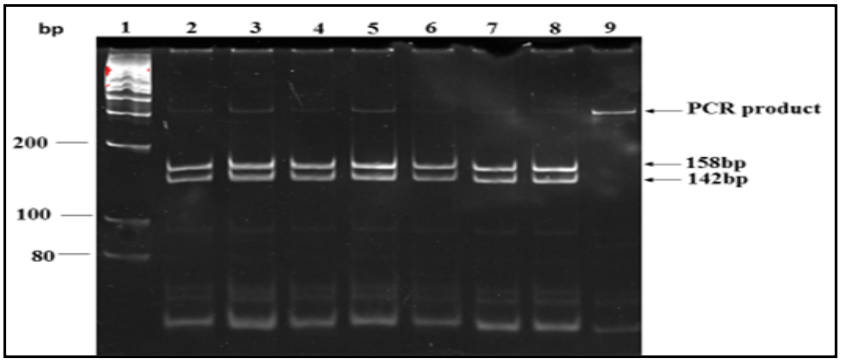

Figure 3. *AluI restriction analysis of β-tubulin gene. The PCR products amplified from SG-β-tub primers were digested with AluI endonuclease and products were separated on 8% non-denaturing polyacrylamide gels, visualised under UV light. Lane 1: 100 bp ladder; Lanes 2-9: Digested PCR product; Lane 10: Undigested amplified PCR product.*

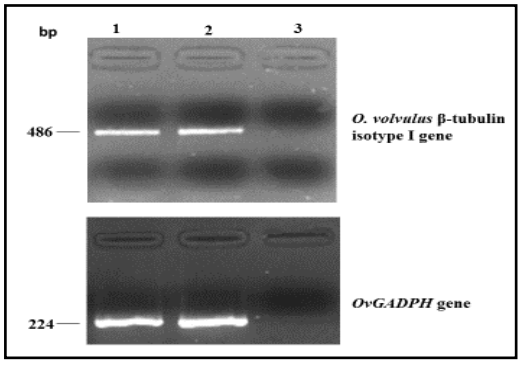

Figure 4. *Expression levels of O. volvulus β-tubulin and OvGADPH genes. RT-PCR was used to get the expression levels of O. volvulus β-tubulin and OvGADPH genes. The RT-PCR products were run on 2.5% agarose gel. Lane 1: IVM exposed sample; Lane 2: IVM naïve sample; Lane 3: Negative control.*

Discussion

The health, social and economic well-being of people in areas affected by onchocerciasis has drastically been improved with the aid of IVM through CDTI programmes and today known as Expanded Special Project for the Elimination of Neglected Tropical Diseases in Africa (ESPEN) [13]. However, there have been reports of suboptimal responses to IVM in terms of microfilarial loads after many rounds of treatment in some parts of the former APOC-controled areas where 14 or more rounds of IVM treatment have been distributed [14,15] thereby indicating some level of drug resistance. Gene selection is the first step in the development of drug resistance, it is therefore important to assess genetic changes in a population of parasites exposed to selection pressure [16].

Genotype and allele frequency distributions for the 1183 T/G SNP in the β-tubulin gene were compared between the IVM exposed and naïve worms using the Chi square test. The TT genotype and the T allele frequencies were lower in the IVM exposed worms than the IVM naïve worms.

The contrary situation was observed for the TG genotype frequency that was higher in the IVM-exposed worms than the IVM naïve worms (Table 4). These results are consistent with the findings of Nana-Djeunga et al. [8]. Where SNPs in β-tubulin selected in *O. volvulus* following repeated IVM treatment. These results indicate that IVM might be associated with a selection of the TG genotype and the G allele in preference to the susceptible genotypes by IVM (homozygote TT). Thus, worms with the selected genotype and allele continue to reproduce and contribute their gene pool to those of the next generation [16].

Genotype and allele frequencies of the 1308 C/T SNP and ID polymorphism between 1557-1857 bp in β-tubulin gene were also compared between the IVM exposed and naïve worms. No differences in genotype and allele frequencies distribution in the two study groups were observed (Figures 2 and 3). This result showed that neither the 1308 C/T SNP nor the ID polymorphism between 1557-1857 bp in the β-tubulin gene was associated with IVM selection and differs.

To find out if expression levels of the β-tubulin gene could play a role in drug resistance, RT-PCR was used to quantify mRNA levels of β-tubulin gene in IVM exposed and IVM naïve worms. OvGADPH, a housekeeping gene was used for normalisation [17]. In these two populations, the expression level of the β-tubulin gene was the same for both study groups (Figure 4) thereby revealing that the expression level of the β-tubulin gene may not directly play a role in drug resistance.

Conclusion

Genotype and allele frequencies of β-tubulin gene between IVM exposed and naïve groups were different at position 1183 with most of the IVM exposed worms having the G allele indicating that the G allele may be implicated in IVM selection whereas it was the same at position 1308 and between 1557-1857 bp. The expression level of β-tubulin gene was the same in the IVM exposed and IVM naïve worms. These results clearly serve as an evidence of β-tubulin gene selection by IVM in the Mbonge Sub division of Cameroon.

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