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SNP Typing of *Mycobacterium leprae* Clinical Strains in Côte d'Ivoire Reveals Genotype 4 Circulating

Amon Aby Christiane[1,2,*], **Coulibaly N'Golo David**[1], **Dehe Bahou Roger**[1,2], **Kouakou Henry**[3], **Kakou-N'Gazoa Solange**[1], **Djaman Allico Joseph**[1,2], **Bamba Vagamon**[3], **Dosso Mireille**[1]

[1]Molecular Biology Platform, Pasteur Institute of Côte d'Ivoire, BP 490 Abidjan, Abidjan, Côte d'Ivoire
[2]UFR Biosciences, Laboratory of biology and health, Felix Houphouet- Boigny University, Abidjan, Côte d'Ivoire
[3]Follereau Raoul Institute of Côte d'Ivoire, Adzope, Côte d'Ivoire
*Corresponding author: abychristiane@gmail.com

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Abstract Despite years of chemotherapy and the consequent reduction in the prevalence of leprosy in Côte d'Ivoire, there are still high levels of new detection cases. Thus the slow onset of leprosy and the reliance on physical examination for detection of the disease have limited the epidemiological follow-up necessary to understand and control transmission. The differentiation of isolates of *Mycobacterium leprae*, the causative organism of leprosy, has not yet been established in Côte d'Ivoire. Three SNP loci have been found informative and have been used to understand the evolution and global spread of *M. leprae*. Based on these reports and the availability of the whole genome sequence, a SNP analysis for strain typing has been realized. This preliminary study present the results of the *M. leprae* genotype study circulating in Côte d'Ivoire. For each clinically confirmed case of multibacillary leprosy (new cases or relapses), a sample of dermal juice was examined after Ziehl-Neelsen staining and a specific PCR targeting the *RLEP* repeat sequence. 31 isolates were used to search for single nucleotide polymorphism (SNP) present in the *M. leprae* genome at positions 14676, 1642875 and 2935685, then determined after sequencing of the mutation zones. The SNPs were determined with a yield of 44,92% (31/69). Genotype 4 was the only genotype founded from the 31 strains identified. This genotype corresponds to those previously reported as widespread in West African countries including Côte d'Ivoire.

Keywords: *M. leprae, single nucleotide polymorphism (SNP), genotyping, Côte d'Ivoire*

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1. Introduction

Leprosy or Hansen's disease is a chronic infectious disease caused by *Mycobacterium leprae*. The elimination of leprosy was achieved globally in 2002 [1]. However, the number of new cases reported worldwide has remained stable. According to official reports received in 2018, 250,000 new leprosy cases were reported in 131 countries, with 95% of them detected mainly in India, Brazil, Indonesia, and 20 other global priority countries [2]. These facts indicate that multidrug therapy (MDT), although effective in treating leprosy, is insufficient to prevent transmission [3]. Although the transmission of *M. leprae* is unknown, risk factors for the development of leprosy have been identified, including close contact with untreated multibacillary patients, nasal carriage (nose blowing, spitting, and aerosol), human susceptibility genes [4], and infection with soil-borne helminthes [5]. To understand the transmission routes of *M. leprae*, several genotyping methods have been used. These techniques used many targets based on nucleotide repeats polymorphism, such as variable numbers of TTC repeats polymorphism in the *rpoT* gene, the variable number of tandem repeats (VNTR) and single nucleotide polymorphisms (SNPs) [6]. VNTR polymorphisms are derived from bacterial replication and are therefore less stable than SNPs. SNPs are much more stable and less exposed to variation over short time intervals. Some authors showed that SNP typing retains its best application in long-distance transmission, such as national or global dissemination, or over historical time periods [7]. The *M. leprae* SNP genotyping system is characterized by four major single nucleotide polymorphism (SNP) types [1,2,3,4] and 16 SNP subtypes (A - P) [8].

In Côte d'Ivoire, with the use of multidrug therapy (MDT), the elimination goal was achieved at the national level with a recorded prevalence of 0.92 % per 10,000 habitants. However, according to the National leprosy elimination Program new cases infirmity degree II detected are gradually increasing (National Leprosy Eradication Program), reflecting continued transmission of the Hansen's bacillus in the country. Until now no information is available on circulating strains of *M. leprae* in Côte d'Ivoire. This preliminary study will globally determine the genetic variability of circulating strains based on SNPs markers.

2. Material and Methods

2.1. Ethical Statement

This study has been approved by the National Research Ethics Committee of Côte d'Ivoire as described in the study by Coulibaly [9].

2.2. Study Population and Sampling

The different samples used in this study were from the Institut Raoul Follereau of Côte d'Ivoire (IRFCI), a leprosy treatment center in the South of Côte d’Ivoire. Patients from whom the samples were collected came from 9 health districts spread over the Ivorian territory, including Man in the West, Daloa and Gagnoa in the Center-West, Adzopé in the South, Agnibilekro in the East, Daoukro in the Center, Lakota in the South-West and Bondoukou in the North-East (Figure 1). These samples were first examined by microscopy after Ziehl-Neelsen staining according to the protocol commonly used at IRFCI and recommended by the World Health Organization (WHO). Inclusion criteria were: multibacillary Leprosy positive for bacteriological examination according to WHO protocol, [10]. Molecular analyses were performed at the Institut Pasteur de Côte d'Ivoire (IPCI), the national reference laboratory for mycobacteria. The biological samples to be analyzed consisted of nasal swabs and dermal pulp fluid from each patient with a clinical diagnosis of leprosy. Dermal pulp fluid was collected from each right and left earlobe when possible. For microscopy, smears were taken from a nasal swab and dermal pulp fluid, whereas for molecular analyses, the nasal swab and dermal pulp fluid were recovered in a 2-ml microtube containing 500 µl (PBS).

2.3. DNA Extraction

The chemical extraction method used is the one described by Yoshikawa [11], using guanidine thiocyanate. Briefly, 300 µL of each sample was pretreated by boiling at 95°C for thirty minutes and then the DNA was extracted with the lysis buffer containing 5M guanidine thiocyanate, 50 mM Tris, pH = 8.0, 10mM EDTA, 5% 2-mercaptoethanol, 2% Triton X-100. After centrifugation, the lysate was transfered into a sterile 1.5 mL Eppendorf tube and DNA was precipitated with isopropanol and sodium acetate (3M). The DNA was then washed with absolute ethanol (96%) and finally resuspended in 150 µL of Tris-EDTA (TE). The DNA suspension was used immediately or stored at -20°C until needed.

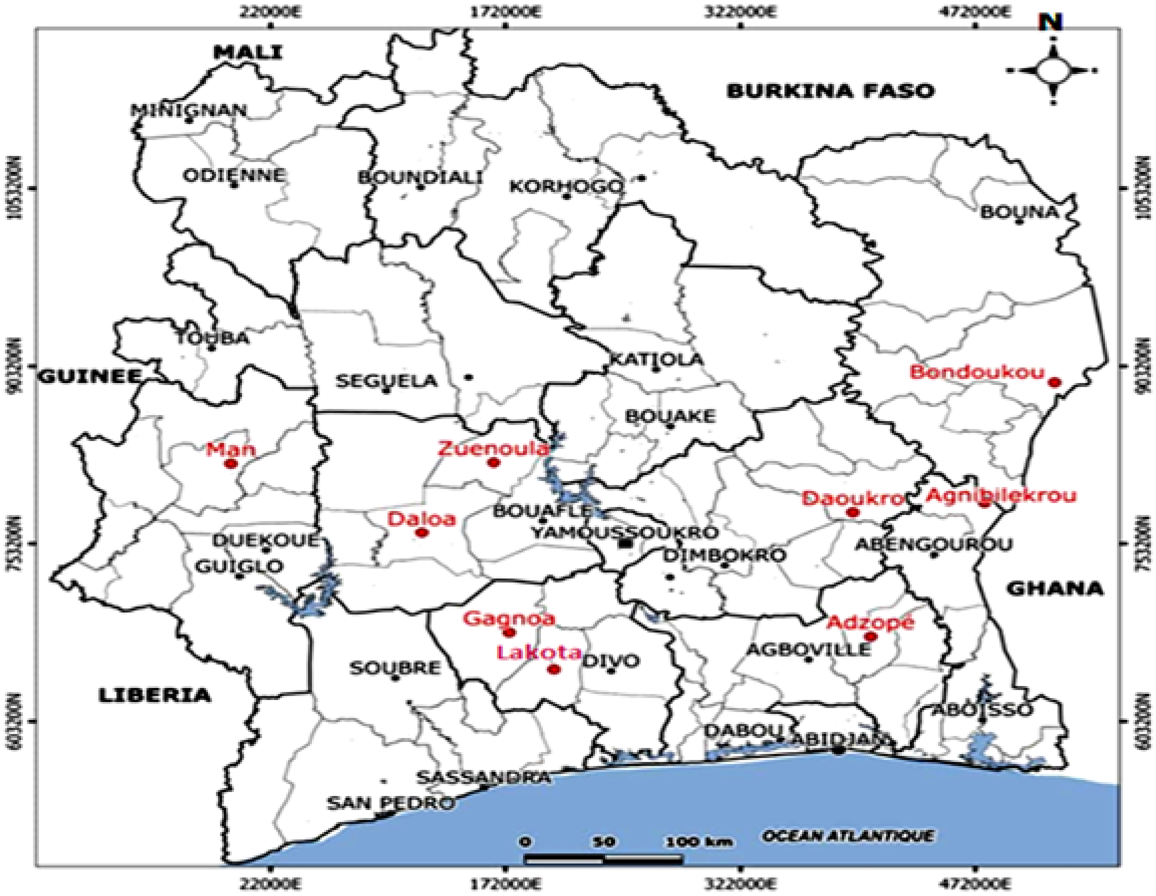

Figure 1. Distribution health districts of origin the patients

2.4. Confirmation of Clinical Cases of Leprosy

The clinical cases diagnosed were confirmed by detection of the *RLEP* repetitive elements in *M. leprae* genomic DNA. A conventional PCR method was used according to the protocol described above by Dehe [12]. Only positive samples for the *RLEP* gene were amplified using SNPs primers and then sequenced.

2.5. SNPs Genotyping and Sequencing

SNP PCR reaction was performed using Gotaq G2 polymerase Kit (PROMEGA, Madison, WI USA) in a final volume of 25 µL containing 5 µL of genomic DNA and 10 µM primers. SNP markers at positions 14676, 1642875 and 2935685(reference TN constraint numbering system) were evaluated according to the original method of Tio Coma [13].

The primers sequences used are presented in Table 1. Amplifications were realized using a GeneAmp® 9700 thermal cycler PCR system (Applied Biosystems, Singapore) with the following program for the 1st round: 10 min at 95°C, 1 min at 55°C and 2 min at 68°C for 45 cycles. In a 2nd round PCR, program was performed for 10 min at 94°C, 1 min at 48°C and 2 min at 68°C for 30 cycles according to the protocol of Reibel [14]. The PCR products were revealed in a 3% agarose gel (Sigma) SybrGreen incorporated. For the sequencing, the PCR products were recovered from the agarose gels, purified by the GFXPCR DNA and Gel Band purification cleaning kit (Cytiva), following the manufacturers' protocol. The sequencing was generated with an ABI 3500 24 capillaries Genetic Analyser sequencer (Applied Biosystems) and compared to *M. leprae* reference sequences accession number AY960580.1 for locus 1, AY960581.1 for locus 2 and AY960582.1 for locus 3available on GenBank (http://www.ncbi.nlm.nih.gov). For the detection of single nucleotid mutation, sequences were entered into BioEdit software version 7.0.9.0.

3. Results

3.1. Patient Geographic Repartition and Leprological Status

Three modalities of leprological status were reported for the 69 patients during the study: new cases, old cases and relapses. New cases constituted 49.27% of patients (Table 2).

3.2. Microscopy Diagnostic

Ziehl Neelsen staining was carried out on all previously processed samples. The Bascilloscopic Index (BI) of all patients was in the following range from 1+ to 5+ (Table 3).

Table 1. primers used for Nested PCR

	Primer A	Sequence (5'-3')	Primer B	sequence (5'-3')	Reference
1st PCR	SNP 14676-F1	CAGGTCTTGTGCGGATAA	SNP 14676-R1	AGGACACCTTCGACATGG	[16]
	SNP1 642875-F3	AATGGAATGCTGGTGAGAC	SNP1 642875-R3	CAATGCATGCTAGCCTTAATGA	
	SNP2 935685-F5	ATCTGGTCCGGGTAGGAATC	SNP2 935685-R5	CGTGCTGACTGTCATGAT	
2nd PCR	SNP 14676-F2	AATGGAATGCTGGTGAGAG	SNP 14676-R2	CCAATGCATGCTAGCCTTAATGA	
	SNP1 642875-F4	TGCTAGTTTAACCGAGTACTGCTA	SNP1 642875-R4	GTAGTAGTCTTCCAAGTTCTGGTG	
	SNP2 935685-F6	TACGGTGGTGTCGGTCTC	SNP2 935685-R6	ACCGGTGAGCGCACTAAG	

Table 2. Number of cases and patient status by geographic location

Origin	Number of cases	New cases	%	Old cases	%	Relapses	%
Adzopé	23	11		7		5	
Gagnoa	7	4		2		1	
Daloa	5	3		1		1	
Man	6	3		1		2	
Bondoukou	7	4		1		2	
Agnibilékro	6	1		3		2	
Daoukro	4	2		1		1	
Lakota	7	4		2		1	
Zuenoula	4	2		1		1	
Total	69	34	49,27%	19	27,53%	16	23,18%

Table 3. Microscopy results

		Patients	(n=69)
Caracteristics	Type	Number	%
BI	1+	25	36,23
	2+	16	23,18
	3+	11	15,94
	4+	10	14,5
	5+	7	10,15

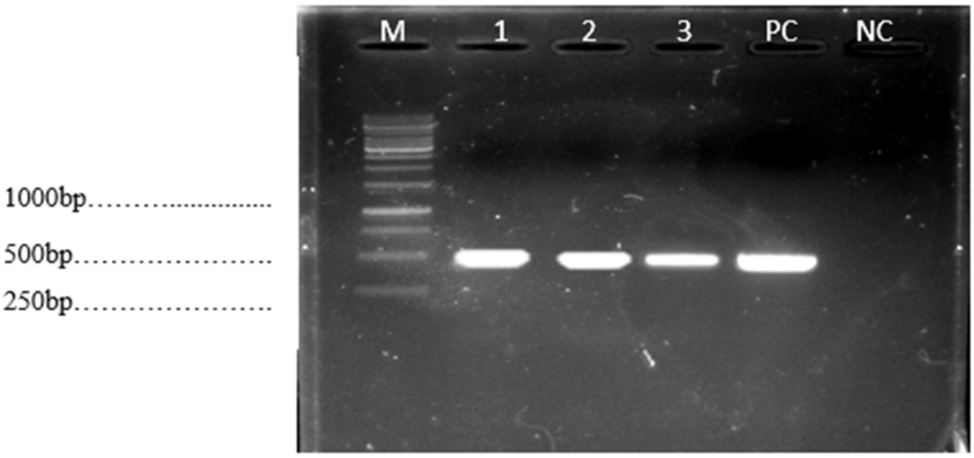

Figure 2. Revelation of *RLEP* PCR's products amplification on a 2% agarose gel (SybrGreen staining). M: molecular weight marker (1kb DNA Ladder Promega), line 1 to 3: samples, NC: negative amplification control. PC: positive amplification control

3.3. PCR Confirmation of Clinical Cases

The confirmation of clinical leprosy cases was performed by PCR; the detection of the *RLEP* target gave 69 positive out of 69 (100%) of the multibacillary samples. The Figure 2 shows the PCR products on a 2 % agarose gel.

3.4. SNP markers Amplification and Genotyping

The PCR reaction gave positive results at three SNP markers approaching 85% positivity (Table 4). Only 31 out of the 58 PCR positive samples targeting the three loci were used for sequencing. This choice was based on the quality of the fragments present in the agarose gel. The extracts with the highest DNA content were used. Figure 3 represents the PCR reaction gel.

Table 4. PCR results SNP markers

	PCR results			
PCR targets	**Positive**	**%**	**Negative**	**%**
Locus 1(SNP14676)	58	84.05	11	15.94
Locus2(SNP1642875)	59	85.50	10	14.49
Locus3(SNP2935685)	61	88.40	8	11.59

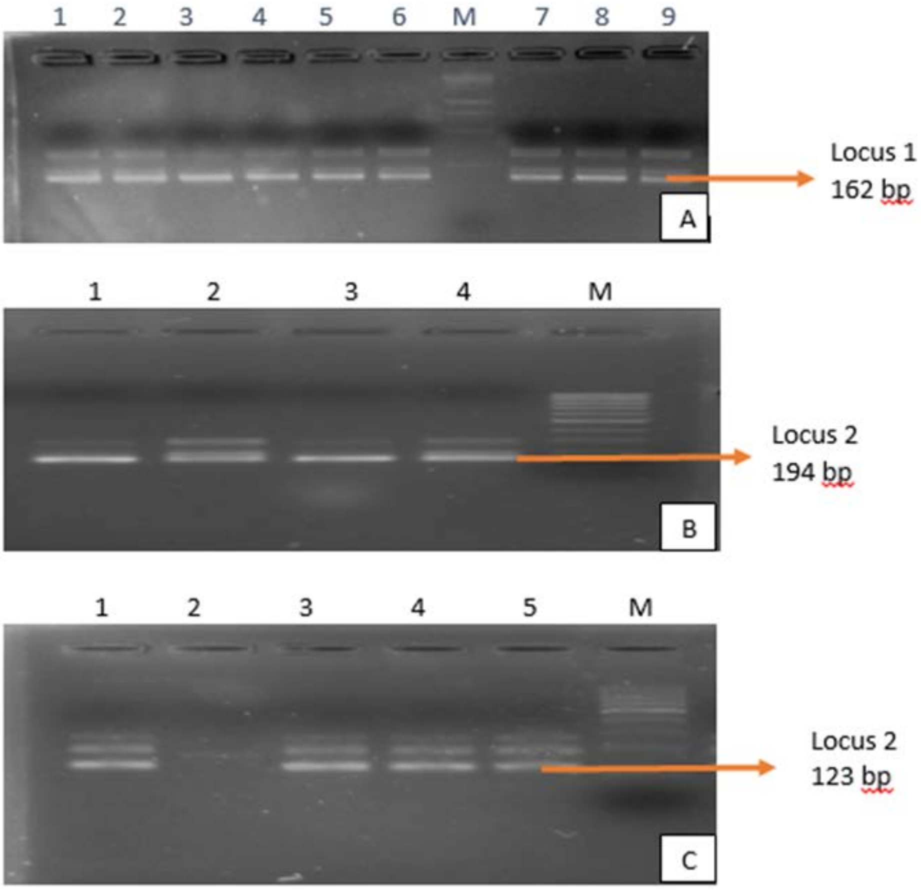

Figure 3. Electrophoretic amplification profiles of attended SNPs markers amplification: A: locus1 detection (SNP 14676); B: locus 2 detection (SNP 1642 875); C: locus 3 detection (SNP 2 935 685); M: Molecular weight marker (100bp); Numbers 1 to 9 represent samples

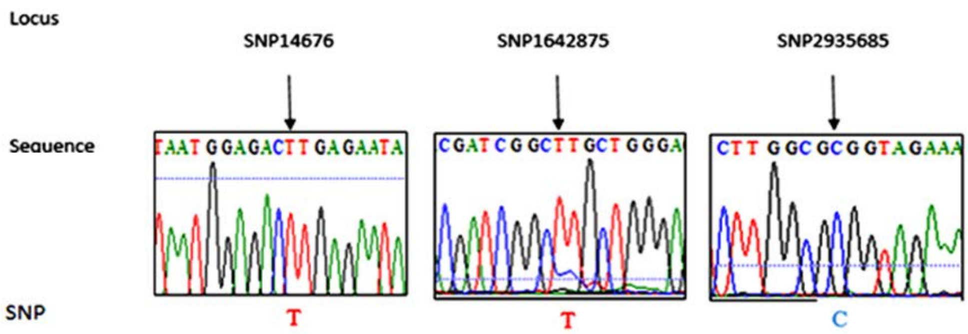

Figure 4. Sequence analysis of the three SNPs of *M. leprae* DNA

3.5. Sequencing and SNP Analysis

To determine the genotype of *M. leprae* in the samples, single nucleotide polymorphisms (SNPs) were analyzed at 3 loci [13]. Only simultaneously positive samples for the 3 loci were analyzed. Analysis of strains from Côte d’Ivoire revealed SNP type 4, characterized by T, T and C nucleotides at genomic positions 14676, 1642875 and 2935685, respectively (Figure 4).

Each locus was amplified by PCR and sequenced as described in the Materials and Methods. The vertical bars Indicate polymorphic base.

4. Discussions

Although Côte d'Ivoire has reached the threshold of leprosy elimination since 2001 [2], new cases of leprosy are still recorded with degrees II of disability. To understand the chain of transmission of the causative bacterium and determine the genetic variability of circulating strains in Côte d'Ivoire, SNP marker analysis could be performed on samples from multibacillary patients that provide sufficient bacillus-laden DNA. In this study, samples were obtained from patients diagnosed in Adzopé as well as from cases referred by the main affected health districts of the country. In Côte d'Ivoire, despite numerous studies on leprosy, the molecular epidemiology of *M. leprae* has not yet been elucidated. Recent studies have reported four genotypes defined by three single nucleotide polymorphisms (SNPs). SNPs, which are rare in *M .leprae,* have been reported to be useful in tracing the global spread of leprosy [15]. The present study explored the genetic diversity of *M. leprae* using SNP markers to reveal the distribution of leprosy in Côte d'Ivoire. However, after comparison of the sequences obtained in each locus with the GenBank database (BLAST), a high similarity of 98-100% was established between the reference sequence (TN) and the sequences of the Ivory Coast strains.

SNP types were examined on the basis of nucleotide pSNP types were examined on the basis of nucleotide polymorphisms at positions 14676, 164275 and 2935685 of *M. leprae* genomic DNA. Four types of SNPs type 1(CGA), type 2 (CTA), type 3 (CTC) and type 4 (TTC), have been reported previously [16].

The type 1 SNP of *M. leprae* was found to be widespread in Asia and East Africa, while type 3 was observed in European countries and the Americas. The type 2 SNP was found to be localized in strains from Ethiopia, North India and the type 4 SNP found in strains from West Africa [17].

This work showed that all clinical strains of this study belonged to type 4 SNPs. This genotype has been described in West African countries, confirming our results in Côte d'Ivoire. Moreover this genotype was found in a patient born in Haiti according to Reibel [14], and is present in several South American countries (Brazil, Bolivia, Uruguay, Venezuela and Mexico). This result could be explained by the fact that in West Africa, leprosy was introduced by the slave trade in the 18th century to the Caribbean islands, Brazil, and probably to other parts of South America. In this study, the genotype of *M.leprae* was identified from clinical samples of dermal juice, this result is close to those of Da Silva Rocha [18], who determined the genotype 4 of *M.leprae* from skin biopsy samples. Genotype 3 was identified in a French resident patient from Côte d'Ivoire, [14] which is different from the genotype observed in this study, and this genotype is normally observed in North Africa and America, likely reflecting the diversity of populations and origins of migratory flows.

In this study, genotype 4 was observed in relapsed and new cases. This could show a possible correlation between genotype and pathogenicity of the strain, as described in studies by Da Silva Rocha [18], where genotype 4 had a higher frequency in relapsed cases in Brazil. The prevalence of leprosy is also higher in the northern and eastern states of Brazil where genotype 4 predominates, this phenomenon may be due to social and health care differences between states [19].

5. Conclusion

The SNP types of all *M. leprae* from leprosy cases diagnosed in Côte d'Ivoire were consistent with what is known about their distribution in different regions of the world. They are genotype 4 of *M. leprae* which is found in West Africa.

A large-scale study in different endemic regions of Côte d'Ivoire would help to better assess the distribution of this genotype.

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Conflict of Interest

The authors declare that they do not have any conflict of interests.

List of Abbreviations

DNA: Deoxyribonucleic Acid
EDTA: Ethylene diamine tetraacetic acid
IB: Bacterological Index
bp: Basic pair
TE: Elution Buffer
µL: Microliter
SNP: Single Nucleotide Polymorphism
RLEP: Repetitive Element of *Mycobacterium Leprae*
M.leprae*: Mycobacterium leprae*
PBS: Phosphate-Buffer Saline
PCR: Polymerase Chain Reaction

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