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## High Prevalence of *Mansonella perstans* Filariasis in Rural Senegal

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**Abstract.** Large parts of African and American countries are colonized by *Mansonella*, a very common but poorly described filarial nematode. Bloodsucking flies of the genus *Culicoides* are suspected to be the vector of *Mansonella perstans*, but no study in Senegal has confirmed that *Culicoides* can transmit the parasite. Designed specific real-time quantitative polymerase chain reaction (qPCR) can be used to identify microfilaria in stained blood smears. This study was performed in July and December 2010 in the southeastern Senegal, which is known to be endemic for *M. perstans*. We analyzed 297 blood smears from febrile and afebrile resident people by qPCR. The global prevalence of *M. perstans* was approximately 14.5% in both febrile and afebrile individuals. The age group of > 30 years had the highest prevalence (22.0%). No *Culicoides* among 1,159 studied specimens was positive for *M. perstans* and its vector in Senegal still requires identification.

### INTRODUCTION

*Mansonella perstans* is a human filarial nematode that is highly prevalent in some areas across sub-Saharan Africa and South America.<sup>1,2</sup> The prevalence of this nematode is often very high in endemic areas, even in children, and increases with age. Four species of filariasis-causing nematodes belonging to the genus *Mansonella* are responsible for human mansonellosis, *M. perstans*, *M. streptocerca*, *M. ozzardi*, and *M. rodhaini*,<sup>3,4</sup> three of which have been described in Africa. *M. perstans* is considered to be the most frequent in Africa and is endemic in a large portion of sub-Saharan Africa, from Senegal to Uganda and south to Zimbabwe, as well as in South America.<sup>1</sup> Overall, more than 100 million people may be infected, and approximately 600 million people lived in the 33 countries are at high risk for *M. perstans* infection in Africa alone.<sup>1</sup>

Adult nematodes are 35–80 mm long, live in the body cavities including pericardium, mesentery, and perirenal and retroperitoneal connective tissues of humans.<sup>5</sup> The microfilariae are nonperiodic and circulate in the blood in equal amounts throughout the day and night. They can also often be found in salivary gland abscesses,<sup>6</sup> an intraocular position,<sup>7</sup> and the conjunctiva.<sup>8</sup> A vaginal localization was also reported by Bamba in 2012 in Burkina Fasso.<sup>9</sup> The longevity of adult *M. perstans* worms in humans is unknown, but the microfilariae persist for approximately 4 months.<sup>10</sup> *M. perstans* is currently considered to be of little pathogenicity and almost always asymptomatic. This might be partially explained by a modulated immune response because of repeated exposure to the parasite, as was previously shown for loiasis.<sup>11</sup> No studies showing statistically proved association of *M. perstans* infection with clinical symptoms have been performed to date, *M. perstans* could be the cause of hypereosinophilia in infected expatriates.<sup>12</sup> The reservoir of *M. perstans* is humans, and transmission appears to occur through the bite of bloodsucking *Culicoides* flies, which are abundant in tropical ecosystems. Research on *M. perstans* vectors has allowed Sharp<sup>13</sup>

to identify *Culicoides grahami* as a potential vector. Because studies performed in Cameroon have described *C. grahami*, *C. austeni*, and *C. ornatipennis* as potential vectors,<sup>14–16</sup> additional studies were performed in different countries to search for vectors of this parasite in endemic areas, including Rhodesia,<sup>17</sup> Congo,<sup>18</sup> and Nigeria.<sup>19</sup> In east Africa, Khamala and Kettle<sup>20</sup> studied the taxonomy of *Culicoides*, identifying 61 species that had no local role in the transmission of *M. perstans*. Despite the prevalence reported by Pfister in 1954<sup>21</sup> and Hocquet and others in 1964,<sup>22</sup> no preliminary study has been conducted to identify the vector of *M. perstans* in Senegal, west Africa.

The goal of this retrospective study was to evaluate the prevalence of this neglected parasite in a rural area of Senegal and to lay a foundation for future studies on the documentation of symptoms related to *M. perstans* infection.

### MATERIALS AND METHODS

**Study site.** The Bandafassi study area is located in the region of Kedougou, eastern Senegal, near the border between Senegal, Mali, and Guinea. It belongs to the Sudano-Guinean savannah ecological zone. The region is disadvantaged in comparison with average rural areas in Senegal because it is far away from the capital, Dakar (700 km), and it is the country's most poorly equipped region in terms of health infrastructure and programs. The local roads are bad, often impracticable during the rainy season, which lasts half of the year. The entire population is rural, with farming, including cereal crops (sorghum, maize, and rice), peanuts, and cotton, as the main activity. The water is drawn from collective wells and there is no electricity. The majority of dwellings are huts covered with thatched roofs and most of the compounds have no toilet facilities. There is one public health post within the area, located in the village of Banadafassi, managed by a public nurse. The closest hospital is at Tambacounda (250 km from the area).

Before the beginning of these studies, all participants, including parents or legal guardians of all children, provided written, individual informed consent. The national ethics committee of Senegal approved the project (program of identification of emergent pathogens—No. 00.87 MSP/DS/CNERS, June 2, 2010; malaria in school children—0000-91/MSP/DS/CNERS, June 3, 2010).

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**Blood specimen collection and examination.** We conducted interviews and sampling in July and December 2010 in rural Senegalese villages in the Kedougou region. The villages were included in a longitudinal prospective study to investigate malaria in school children in Senegal, including epidemiological risk, burden of disease, and control strategies, and a program for the identification of emergent pathogens. Medical examination and blood smear were performed for each child (school children) who had a fever > 37.5°C. For the second program (MALEMAF), 200 µL capillary blood was collected and combined with anticoagulant for molecular examination.<sup>23</sup> For each study, a specific designed questionnaire was completed. To determine the prevalence in healthy children, a transversal sampling was performed. Blood smears were stained with Giemsa and analyzed by microscopy to identify malarial trophozoites and gametocytes and the presence of microfilaria.<sup>5,24</sup>

On each slide, 200 oil-immersion fields (~0.5 µL blood) were examined, and the filarial:leukocyte ratio was measured.<sup>25,26</sup>

**Arthropod collection.** Insects were collected using a modified CDC light trap (BioQuip Products, Compton, CA), as previously reported.<sup>27</sup> In brief, the tulle net pockets were replaced with pockets of Terylene cloth, which traps tiny insects. The arthropods were collected in nine villages of Kedougou in April 2013 and April 2014. The traps were placed in different habitats, resulting in important variations in their qualitative and quantitative performance. In each habitat, we placed two traps, one inside a room occupied by a sleeper to capture endophilic *Culicoides* spp. and another outside to catch exophilic *Culicoides* spp.

**Morphological identification.** Using a stereomicroscope, *Culicoides* spp. were separated from other insects and sorted into the following groups based on wing morphology: *schultzei*, *imicola*, *magnus*, *milnei*, and other *Culicoides*.<sup>28,29</sup> Species identification was achieved by microscopic analyses of wing patterns and observation of different body parts (head, legs, and wing).<sup>28–30</sup> After identification, the insects were stored individually at –80°C for further analysis.

**Molecular identification.** For molecular identification, blood smears were scratched, and DNA was extracted using a modified 2.0% cetyl-trimethyl ammonium bromide (CTAB) (Sigma-Aldrich, Steinheim, Germany) method, as described previously.<sup>31</sup> Before extraction, samples were digested with proteinase K at +56°C for 1 hour. The final elution volume of DNA was 200 µL. The quality of the samples was controlled using quantitative polymerase chain reaction (qPCR) targeting the human β-actin gene, as described previously.<sup>32</sup>

DNA from *Culicoides* spp. was extracted as follows: body parts of midges were ground in 180 µL 2.0% CTAB and 20 µL proteinase K (activity equal to 600 mAU/mL solution or 40 mAU/mg of protein). The ground material was lysed by heating in a water bath at 56°C for 1 hour. Two hundred microliters of chloroform was added to the lysed material and the supernatant was recovered after centrifugation at 12,000 rpm for 5 minutes. The nucleic acids were precipitated with 200 µL isopropanol after 15 minutes of centrifugation at 12,000 rpm. The pellet was washed with 200 µL of 70.0% ethanol and centrifuged under the above condition for 5 minutes to remove the residual salt. Finally, the pellet was dried in a Speed Vac (Thermo Scientific, Courtaboeuf, France) for 3–4 minutes, and the DNA was dissolved in 100 µL elution buffer Tris-EDTA (TE) and stored at 4°C until further use.

We designed two new sets of primers and Taqman<sup>®</sup> probes (Life Technologies, St. Aubin, France) based on an alignment of the internal transcribed spacer 1 (ITS1, 18S-5.8S) of *Mansonella* spp., primarily *M. perstans* (5 sequences) and *M. ozzardi* (3 sequences), as well as *Wuchereria bancrofti* ITS1. Previously, only standard PCR developed by Keiser and others in 2008 and based on the internal transcribed spacer was proposed for identification of *M. perstans*.<sup>33</sup> The first set was designed to be specific for the genus *Mansonella* and consists of a forward primer, 5'-CCTGCGGAAGGATCATTAAC-3', a reverse primer, 5'-ATCGACGGTTTAGGCGATAA-3', and a probe, 6-FAM-CGGTGATATTCGTTGGTGTCT-TAMRA. The second *in silico* set is specific for *M. perstans*, with a forward primer, 5'-AGGATCATTAAACGAGCTTCC-3', a reverse primer, 5'-CGAATATCACCGTTAA TTCAGT-3', and a probe, 6-FAM-TTCACTTTTATTTAGCAA CATGCA-TAMRA. After qPCR validation, all samples were tested first with qPCR specific for *Mansonella* spp., and the positive samples were tested for *M. perstans*.

Molecular analysis of *Mansonella*-positive samples was performed using the primers which amplify 485 bp portion of ITS. The PCR consisted of DNA purification at 50°C for 2 minutes and an initial heating phase at 95°C for 15 seconds, followed by 40 cycles of denaturation (95°C for 10 seconds) and annealing (60°C for 45 seconds). A negative control was used to detect possible contamination and a positive control was used to ensure that the amplification conditions were appropriate. All amplifications in real-time PCR (qPCR) were performed in a total reaction volume of 20 µL containing 10 µL master mix Takyon<sup>®</sup> (Eurogentec France, Angers, France), 1 µL of each primer, 1 µL probe, 2 µL distilled water, and 5.0 µL DNA template. We performed all PCR reactions using the thermal cycler CFX96 Touch detection system (Bio-Rad, Marnes-la-Coquette, France).

The specificity of the PCR qPCR was tested with 100 blood samples collected from febrile French residents hospitalized at La Timone Hospital (Marseille, France) who had not visited an endemic zone for *Mansonella*.

**PCR validation.** To validate the PCR, qPCR we scratched and tested 54 blood smears from Senegal: five samples with microscopically identified *M. perstans* filariae (only these five slides were reported to be *Mansonella* positive during the routine examination in the malaria in schoolchildren research program) and 49 microscopically negative specimens collected from the malaria in-school program performed in Kedougou. The examination of the blood smears was performed by microscopy (×100 with immersion oil). The DNA extracted from the blood samples of 100 febrile French residents without blood *Mansonella* microfilariae and without a history of travel in endemic areas all tested negative. A comparison of the microscopy and PCR gave following results: five samples were positive by microscopy and PCR, no sample was positive by microscopy and negative by PCR, three samples were positive by PCR and negative by microscopy, and 46 samples were negative by both tests.

In brief, the sensitivity of the proposed qPCR compared with the microscopic examination was 100%, as we could detect all positive samples. Moreover, three samples for which we detected the presence of *Mansonella* genes by qPCR did not reveal microfilariae during microscopic examination, suggesting the higher sensitivity of the qPCR technique. The sensitivity of blood smear microscopy for the diagnostics of

mansonellosis was only 63% compared with qPCR. The specificity of the proposed method is also very high: none of the febrile patients from non-endemic regions produced positive results. In total, both techniques were concordant.

We sequenced the 485 bp-portion of ITS from 10 randomly selected patients, including three samples negative by microscopy. All sequences were identical among each other and to *M. perstans* ITS sequences deposited in GenBank (KJ631373 and others).

**Statistical analysis.** The data were analyzed using Epi Info software, version 7.0.8.0 (Centers for Disease Control and Prevention, Atlanta, GA). Statistical significance was defined as  $P < 0.05$ .

RESULTS

**Entomological examination.** A total of 1,159 *Culicoides* spp. were captured (509 in 2013 and 650 in 2014), the majority of which were females. We caught the most midges in the villages of Laminia and Banding, representing 66.2% (767/1,159) of the captures. After microscopic identification, we identified 10 species of *Culicoides*. *C. enderleini* and *C. oxystoma* were the most prevalent, representing approximately 34.1% (395/1,159)

and 15.0% (174/1,159), respectively, of the captured species. *C. enderleini* was well distributed in the study area, whereas *C. oxystoma* was localized in Laminia and Samekouta, the two villages closest to each other (Figure 1). The details of the *Culicoides* spp. captured during the study are presented in Table 1.

Using PCR, we found that none of the 1,129 *Culicoides* tested positive for *Mansonella*. All positive and negative controls gave proper results.

**Analysis of blood specimens.** A total of 297 blood smears were analyzed by PCR: 201 from febrile people (febrile people series) who requested care in health posts in the study areas and 96 from blindly selected healthy children (afebrile population series). The sex ratio (male/female) was 50%, and the age group was from 7 months to 45 years. We identified *M. perstans* in 29 specimens from the febrile people series; this represents a prevalence of 14.4% (29/201). In the afebrile population series, we identified 14 positive representing 15.0% (14/96). The global prevalence of *M. perstans* in this area was about 14.5% (43/297).

*M. perstans* is widely distributed in this area, with only a few villages having a low prevalence. The highest prevalence was observed in Nathia, Bundukundi, and Baraboye. Overall, although *M. perstans* was found to be highly prevalent among

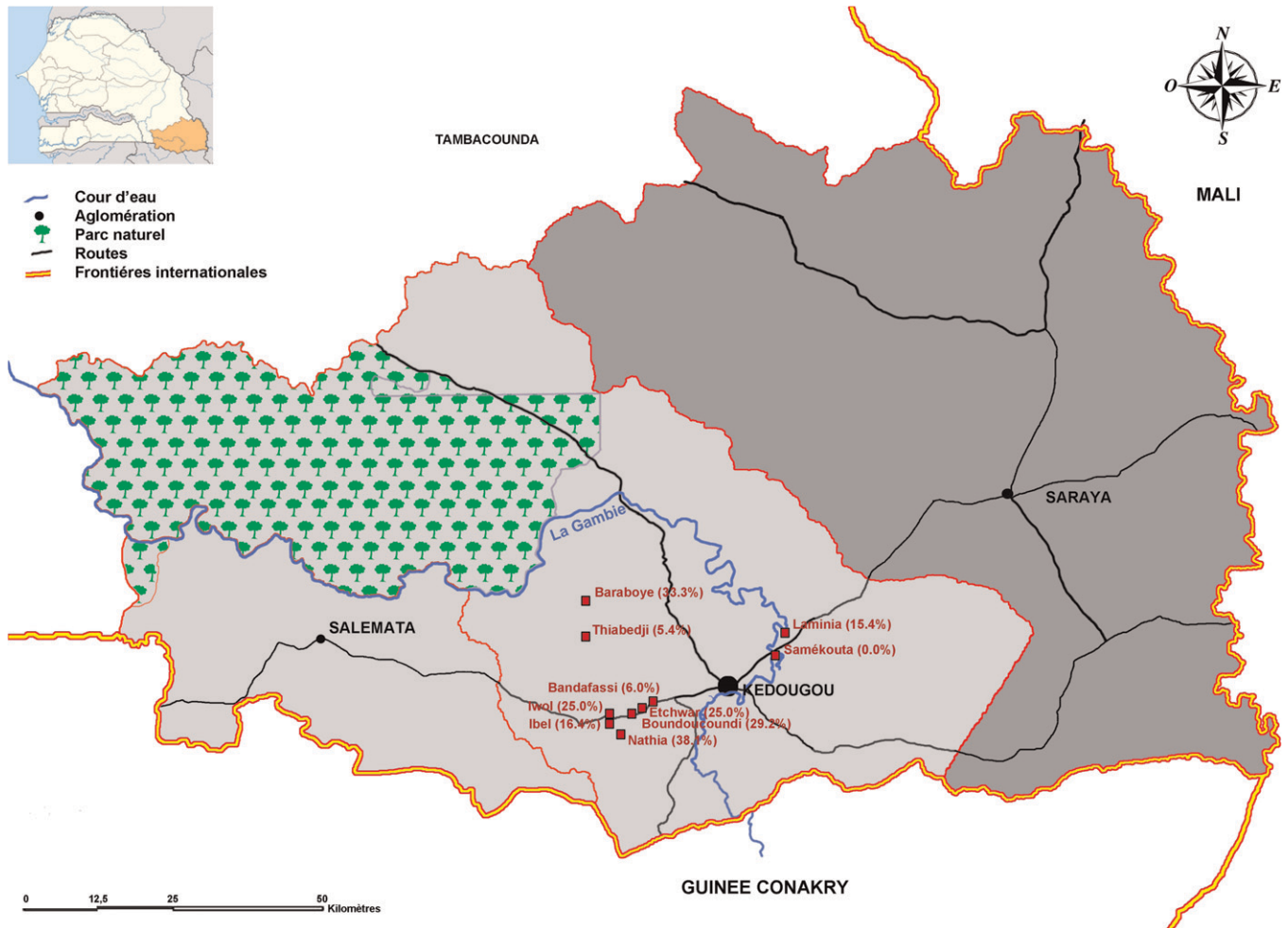


FIGURE 1. Study site in Senegal, with the prevalence of *Mansonella perstans* among the population in parentheses when available.



TABLE 1  
Biting midges diversity in the Kedougou region

	Bandafassi	Banding	Bundukundi	Ibel	Indar	Thiabedji	Nathia	Samékouta	Laminia	Total
<i>Culicoides distinctipennis</i>	0	0	0	2	0	0	0	0	0	2
<i>C. enderleini</i>	12	116	1	35	2	140	20	12	57	395
<i>C. fulvithorax</i>	1	1	4	7	1	0	1	0	0	15
<i>C. imicola</i>	2	26	40	14	7	0	5	12	13	119
<i>C. kingi</i>	0	4	2	10	0	0	11	4	33	64
<i>C. leucostictus</i>	0	1	0	0	0	0	0	0	0	1
<i>C. oxystoma</i>	0	0	0	0	0	0	0	4	170	174
<i>C. schultzei</i> spp.	0	0	0	0	0	0	0	0	1	1
<i>C. similis</i>	0	13	0	0	0	0	0	0	0	13
<i>Culicoides</i> sp. (group <i>distinctipennis</i> )	0	0	0	0	0	0	13	0	0	13
<i>Culicoides</i> sp. (group <i>similis</i> )	0	0	6	0	0	0	3	0	2	11
<i>Culicoides</i> spp.	1	115	2	3	1	0	6	3	3	134
<i>C. subschultzei</i>	0	71	0	0	0	0	1	4	83	159
<i>Alluaudomya</i> spp.	0	14	0	0	0	0	0	0	0	14
<i>Forcipomyia</i> spp.	0	8	0	0	0	0	0	0	0	8
NI	0	0	0	0	0	0	0	0	36	36
Total	16	369	55	71	11	140	60	39	398	1,159

NI = none identified.

people older than 29 years (33.3%). No cases were detected in the group of the patients younger than 12 months.

## DISCUSSION

The results of the comparison of techniques for detecting *M. perstans* showed that qPCR could be successfully used to identify microfilaria in the samples used, revealing excellent sensitivity and specificity. Indeed, the sensitivity of qPCR was even higher than that of microscopy. For the three cases in which qPCR rendered a positive result for a microscopically "negative" slide, we suggest that the filariae were actually either missed (or misinterpreted) during examination or destroyed during slide preparation or that *Mansonella* DNA was present inside phagocytes. It is known that the microfilariaemia maybe quite low in up to 70% of infected persons.<sup>34</sup> It is also well known that molecular methods are much more sensitive for the detection of parasitaemia (plasmodia)<sup>35</sup> and bacteremia (borreliae)<sup>36</sup> compared with microscopic examination. This also appears to be the case for mansonellosis. Unfortunately, the entire sample on the slides was used for DNA extraction and thus cannot be reexamined. Despite its cost, qPCR was found to be very interesting for a retrospective study of *M. perstans* in this locality. On the basis of all

these elements, we used qPCR for this study of filariasis in rural Senegal.

This retrospective study carried out using samples from the Kedougou area showed that *M. perstans* is widespread in this region. This wide distribution of microfilaria could be explained by the abundance of its vector and a good vectorial capacity. However, the vector of this microfilaria has not yet been identified in Senegal. Previous studies in other countries cited biting midges as vectors of *M. perstans*,<sup>37–39</sup> but our study did not identify *M. perstans* in 1,129 *Culicoides* spp. collected in the same region where the human blood samples were studied. Most villages were located beside either a waterway or a depression that filled with water during the rainy season, thus constituting larval lodgings for the vector.<sup>40,41</sup> *M. perstans* filariasis was found to affect all segments of the population, from school-age children to adults. The fact that the prevalence is higher among adults gave the impression that the immune response of the body against filariasis was absent as individuals infected in the second year (study results) should have ample time to develop antibodies against this parasite. However, as noted in other studies,<sup>1</sup> adults were infected as frequently as children. The fact that the prevalence is higher among adults (Table 2) indicates that they were more exposed than children, or this may be explained by a cumulative effect. Except for the village of

TABLE 2  
*Mansonella perstans* prevalence rates for age groups

	< 12 months	1–3 years	4–6 years	7–15 years	16–29 years	30–45 years	NA	Total
Bandafassi	0.0% (0/5)	6.2% (1/16)	0.0% (0/14)	4.5% (1/22)	0.0% (0/2)	50.0% (1/2)	10.0% (1/10)	6.0% (4/71)
Baraboye	–	–	–	33.3% (1/3)	–	–	–	33.3% (1/3)
Boundoucoum	0.0% (0/2)	0.0% (0/1)	0.0% (0/6)	54.0% (7/13)	–	0.0% (0/1)	0.0% (0/1)	29.2% (7/24)
Etchwar	–	0.0% (0/1)	0.0% (0/1)	50.0% (1/2)	–	–	–	25.0% (1/4)
Ibel	0.0% (0/1)	22.2% (2/9)	16.7% (2/12)	10.0% (3/31)	0.0% (0/1)	0.0% (0/1)	33.3% (4/12)	16.4% (11/67)
Iwol	–	–	33.3% (1/3)	0.0% (0/1)	–	–	–	25.0% (1/4)
Laminia	–	–	–	15.4% (2/13)	–	–	–	15.4% (2/13)
Nathia	0.0% (0/1)	100.0% (1/1)	0.0% (0/1)	41.2% (7/17)	0.0% (0/1)	–	–	38.1% (8/21)
Samecoute	–	0.0% (0/1)	–	0.0% (0/20)	–	–	–	0.0% (0/21)
Thiabedji	0.0% (0/1)	0.0% (0/7)	0.0% (0/9)	14.3% (2/14)	–	–	0.0% (0/6)	5.4% (2/37)
Other villages	0.0% (0/2)	40.0% (2/5)	0.0% (0/4)	20.0% (1/5)	20.0% (1/5)	50.0% (1/2)	11.1% (1/9)	19.0% (6/32)
Total	0.0% (0/12)	15.0% (6/41)	6.0% (3/50)	18.0% (25/141)	11.1% (1/9)	33.3% (2/6)	16.0% (6/38)	14.5% (43/297)

– = no data; NA = any information about the age.

Samecouth, where this parasite was absent, all other villages showed infection, suggesting that *M. perstans* is endemic in this area. Further studies could identify if the absence of mansonellosis in Samecouth identified in this study is real and propose possible explanations of this phenomenon. The Iwol village, at an elevation 440 m higher than Ibel, had a prevalence of 25%. This could be explained by the number of samples obtained in Iwol (4).

Despite of the number (1,159) of *Culicoides* spp. tested, we did not identify *M. perstans* in studied arthropods. Many investigated species of *Culicoides* are considered to be anthrophilic (*C. imicola*, *C. enderleini*, *C. oxystoma*, and *C. kingi*) in Senegal, and even to be implicated in the transmission of mansonellosis in Africa (*C. fulvithorax*).<sup>19</sup> All midges were collected in April, the month when the maximum frequency of microfilaria in human blood smears was identified (data not shown). This limited collection, even performed in the possible transmission season, limits this study. Systematic screening of *Culicoides* midges for *M. perstans* throughout a year would have been preferred. The fact that all *Culicoides* tested by PCR were negative may suggest the role of another vector of *M. perstans* in this area such as *Simulidae*, *Tabanidae*, or *Culicidae*.

Although *M. perstans* is highly prevalent in some areas in Senegal, it is not associated with febrile conditions. Proposed real-time PCR specific for *M. perstans* allows the identification of these filariae in the stained blood smears. Further investigations are needed to determine the clinical impact of *M. perstans* among the population and to identify the vectors of this highly prevalent nematode.

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