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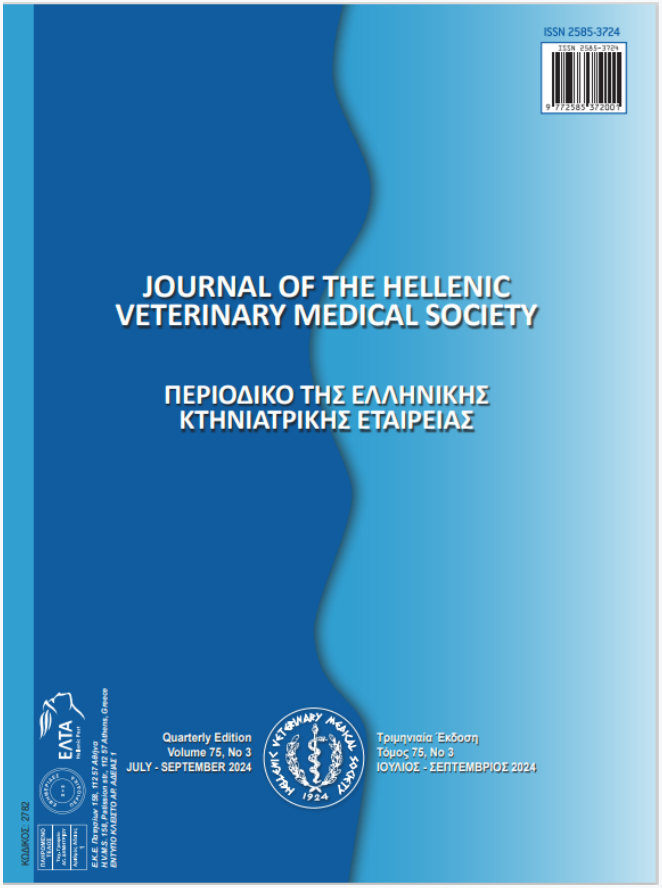

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Research article
Ερευνητικό άρθρο

Molecular detection of Potential Novel *Mansonella* species (Filarioidea: Onchocercidae) in equine and *Culicoides enderleini* from Africa

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ABSTRACT : A new genotype of a filarial worm from the *Mansonella* genus was determined in three donkeys from Algeria, as well as from four horses and two *Culicoides enderleini* biting midges from Senegal. Phylogenetic analysis based on the ITS1 and rRNA 5S genes show similar results clustering this filarial worm within the genus *Mansonella* with low identity with all known *Mansonella* species. Further research should be performed to characterise this *Mansonella* sp. OM-2015 and assess its prevalence in African countries.

***Keywords*:** Filaria ; *Mansonella* ; equine ; *Culicoides enderleini ;* biting midges ; PCR ; Africa ; Senegal ; Algeria.

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INTRODUCTION

Equine filariosis is an overlooked disease transmitted by dipteran vectors and caused by nematode species from the genus *Onchocerca, Setaria*, *Dirofilaria* and *Parafilaria*. Aside from their zoonotic potential, these parasites can cause various health issues in equine, leading to significant economic challenges (Radwan et al., 2016 ; Thurman et al. 1984). Adult nematodes parasitise the ligaments, aponeuroses, subcutaneous tissues and peritoneal cavities of equid. They produce microfilariae that migrate through the dermis and are ingested by blood-feeding arthropods. Once inside the insect, the larvae go through three stages of development, and during a new blood meal, the infectious larvae (L3) actively enter the host organism. (Abo-Aziza et al., 2022 ; El Namaky et al., 2017 ; Lia et al., 2016 ; Lima et al., 2020). Moreover, filarial worms have the ability to migrate to unusual locations such as the central nervous system and the anterior chamber of the eyeball, the most common form of migration in infected horses. This leads to severe pathological consequences (Bouchery et al., 2013 ; Hillyer et al., 2001 ; Junsiri et al., 2023 ; Yu et al., 2021).

Mansonella species are filarial nematodes in the family Onchocercidae that reside in subcutaneous tissues and intramuscular fascia. Their host range comprises a large variety of primates (including humans), carnivores, sciuridae, tupaiids and ungulates (Bain et al., 2015). The infective larvae (microfilariae) occur freely in the peripheral blood and are transmitted by biting midges of the genus *Culicoides* spp. (Diptera: Ceratopogonidae) and blackflies of the genus *Simulium* spp. (Diptera: Simuliidae) (Bain et al., 2015; Poole et al., 2019). Infection by members of the genus *Mansonella* is widespread in tropical Africa, Latin America, and the Caribbean Islands. Despite this, the incidence of mansonellosis is underestimated as it is considered of less pathogenic importance in comparison with onchocerciasis, lymphatic filariasis and loiasis (Bain et al., 2015; Medeiros et al., 2017). Occasionally, mansonellosis infections cause itching, joint pains, enlarged lymph glands, and vague abdominal symptoms but it often remains asymptomatic.

The species of *Mansonella* parasites, which principally infect animals, are also known to cause zoonoses in humans, such as *Mansonella rodhaini*, and some can use humans as their definitive hosts (Orihel and Eberhard, 1998). Three filarial species are responsible for causing human mansonellosis including *Mansonella perstans, Mansonella streptocerca*, and *Mansonella ozzardi* (Crainey et al., 2016; Downes and Jacobsen, 2010; Uni et al., 2004).

Culicoides (Diptera: Ceratopogonidae) are small haematophagous Dipterans that are important for their ability to transmit pathogens of microbial and parasitic origin. To date, 1347 species of biting midges have been described worldwide (Borkent and Dominiak, 2020) and *Culicoides enderleini* is very common throughout the Afrotropical region and is widely dominant in West Africa (Cornet and Brunhes, 1994).

The diagnosis of filarial infection through routine methods has some limitations, particularly when specific serological tests are not available. Molecular identification is currently the most effective method, which allows the characterization of filarial species and determination of their phylogenetic relationships with each other. Accurate identification and differentiation of filarial nematodes is critical for understanding their epidemiology, which can help in developing control methods against this pathogen (Abbas et al., 2016 ; Maharana et al., 2019 ; Ta-Tang et al., 2018). The current study presents an investigation into the molecular characteristics of lesser-known nematodes that are classified under the genus *Mansonella*. This investigation focuses on both equine and biting midges found in Algeria and Senegal.

MATERIALS AND METHODS

Study Areas, Collection of Insects and Blood Sampling

This survey was carried out on biting midges (*Culicoides* spp.) and equine blood samples in two African countries: Algeria and Senegal. Insects were collected using a CDC light trap (Sudia and Chamberlain, 1962), in the village of Dielmo (13°43′N,16°24′W) located southeast of Dakar (Senegal), very close to the border with Gambia. Since 2010, a non-malarial fever identification programme has been conducted (Mediannikov et al., 2013). We separated *Culicoides* specimens from other insects. Specimens were subsequently identified morphologically to the species level using an interactive identification key based on observation of wing patterns and body parts morphology (Cornet and Brunhes, 1994; Cornet and Chateau, 1970; Glick, 1990). Each identified specimen was then stored individually at -80°C for further DNA analysis.

Equine blood sampling was carried out in the following regions: El Tarf : (36°49′01″N, 8°24′47″E) in north-eastern Algeria and in four regions in Sene-

gal: Keur Momar Sarr (15°55'0″N, 15°58'0″) located in the north-west of Senegal, Dakar (14°41′37″ N, 17°26′38″W) located at the western tip of Africa, on the narrow Cape Verde peninsula, Sine Saloum (41°24'12.2»N, 2°10'26.5»E) in the north-western part of the Gambia basin, and Casamance (13°00C54.94"N, 15°19'3.68"W) in the south-western part of Senegal.

120 equines from Algeria and 199 from Senegal (including 93 from Keur Momar Sarr, 63 from Dakar, 40 from Sine Saloum and three from Casamance) were randomly selected (figure 1), blood samples were collected and conserved in labelled EDTA tubes and frozen at -20°C prior to molecular analysis.

Molecular diagnosis

All biological materials, including blood and *Culicoides* insects, were subjected to DNA extraction using the QIAamp Tissue Kit (Qiagen, Hilden, Germany) by Qiagen-BioRobot EZ1, according to the manufacturer's instructions. The extracted DNA was initially screened with qPCR using the set of primers and probe targeting the Inter Transgenic Spacer (ITS) that was able to amplify all species of *Mansonella* genus (Bassene et al. 2015). All positive *Culicoides* for ITS qPCR were systematically tested with a qPCR targeting *M. perstans*, which is endemic in Senegal (Bassene et al., 2015). We performed PCR reactions using a Thermal Cycler CFX96 Touch Detection System (Bio-Rad, Marnes-la-Coquette, France). Sample were considered positive when the Cycle threshold (Ct) value is lower than 35.

Additionally, all samples which were qPCR positive *Mansonella* spp. were also amplified with conventional PCR assays targeting ribosomal DNA ITS1 and 5S rRNA genes (table 1) (Jiménez et al., 2011 ; Xie et al., 1994). The conventional PCRs were performed in automated DNA thermal cycler (iCycler, BioRad, Munich, Germany). Each PCR reactions were carried out in a total volume of 50µl, consisting of 25µl of AmpliTaq Gold® 360 Master mix (Thermo Fisher Scientific, Applied Biosystems, Foster City, CA, USA), 18µl of ultra-purified water DNAse-RNAse free water (Eurogentec, Liège, Belgium), (1µl) of each primer and 5µl of DNA template.

The success of the PCR amplification was verified by 1,5 % of agarose gel electrophoresis and the PCR products were then purified using filter plate Millipore NucleoFast 96 PCR plate KIT following the manufacturer's recommendations (Machery-Nagel EURL, France).

Sequencing analyses were performed on the Applied Biosystems 3130xl Genetic Analyzer (Thermo Fisher Scientific, France), using the DNA sequençing BigDye Terminator Kit (Perkin-Elmer) according to the manufacturer's instructions. The obtained sequences were assembled on ChromasPro 1.7 software (Technelysium Pty. Ltd., Tewantin, Australia). Sequences obtained in this study and those obtained from GenBank were then

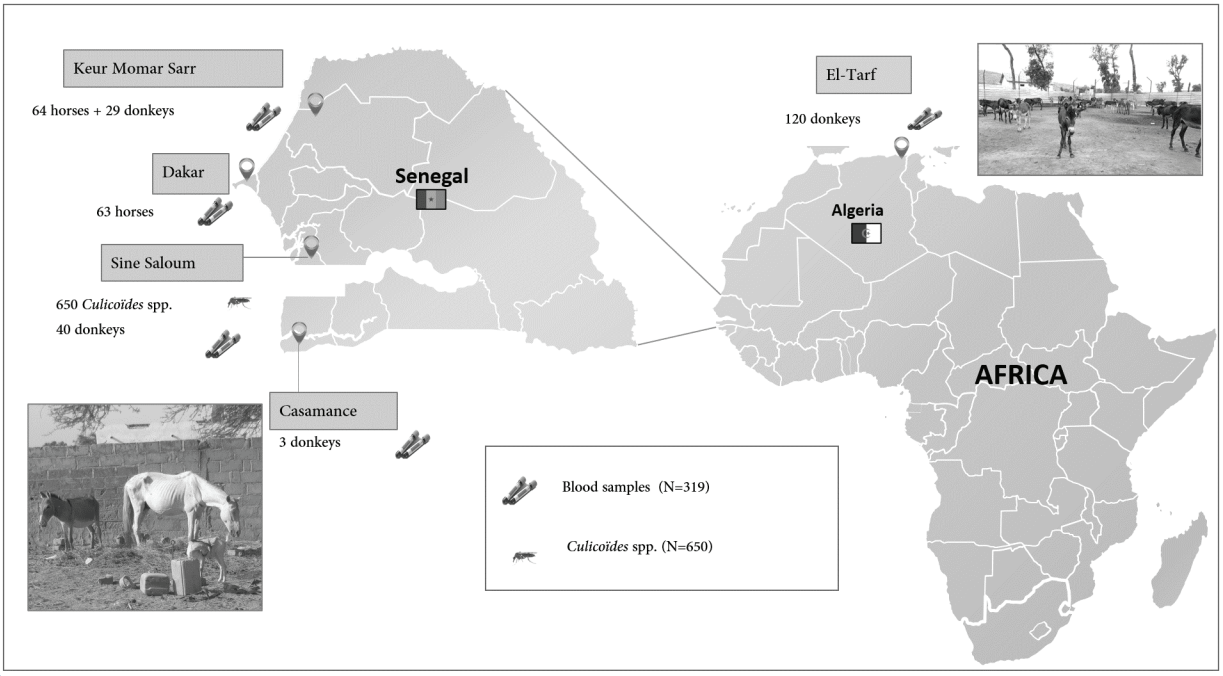

Figure 1: Survey locations with sample collection number

Table 1. PCR systems used in this study.

Targeted miroorganisms	Target gene	Primers & probes	Annealing temperature	Size of Amplicons (pb)	References
		qPCR			
***Mansonella* spp.**	ITS	F : 5'- CCTGCGGAAGGATCATTAAC-3' R : 5'- ATCGACGGTTTAGGCGATAA -3' 6-FAM-CGGTGATATTCGTTGGTGTCT-TAMRA	60°C/45s	237	(Bassene et al., 2015; Mourembouet al., 2015)
M. perstans	ITS	F : 5'- AGGATCATTAACGAGCTTCC-3' R : 5'- CGAATATCACCGTTAATTCAGT-3' 6-FAM-TTCACTTTTATTTAGCAACATGCA-TAMRA	60°C/45s	187	
		Standard PCR			
Filarioidea	5S	F : 5'-GTTAAGCAACGTTGGGCCTGG-3' R : 5'-TTGACAGATCGGACGAGATG-3'	55°C	421	(Xie et al., 1994; Jiménez et al., 2011)
M. perstans*, *Loa Loa* and *W. branchrofti	ITS1	F : 5'-GGTGAACCTGCGGAAGGATC-3' F : 5'-CTCAATGCGTCTGCAATTCGC-3'	58°C	485	

aligned using the ClustalW multiple sequence alignment program, which is included in the BioEdit software (Hall, 1999). phylogenetic inferences were obtained using Bayesian phylogenetic analysis with TOPALi 2.5 software (Biomathematics and Statistics Scotland, Edinburgh, UK) (Milne et al., 2004) within the integrated MrBayes program using the HKY85 + Γ substitution model. Numbers at the nodes are percentages of bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree**.**

RESULTS

A total of 650 *Culicoides* were examined. Morphological identification revealed the presence of four species including : 210 *C. enderleini*, 82 *C. imicola*, 182 *C. schultzei,* 108 *C. wansoni* and 68 *Culicoides* spp. Initial qPCR analysis confirmed that two *C. enderleini* from the village of Sourou were positive for *Mansonella* spp. However, qPCR specific for *M. perstans* was negative for both specimens. We managed to sequence amplicons of filarial 5S and ITS1 of the two *C. enderleini*. BLAST analysis revealed 87% similarity between the obtained 5S sequences and *M. ozzardii* (JF412305) deposited in GenBank. Similarly, with ITS1, the closest sequence to our amplicons is that of *M. ozzardi* (EU272180) with an identity percentage of 85%.

Mansonella spp. DNA was detected in 5.3% (17/319) of blood samples using ITS qPCR, we succeeded in amplifing and sequencing 7/17 (41.17%) of them targeting 5S rRNA spacer gene. These included three sequences which were obtained from Algerian donkeys, three from horses in Dakar and one from a donkey in Keur Momar Sarr. These sequences were between 86% and 88% identical to *M. ozzardii* (JF412305) (Figure 2).

We also obtained 6/17 sequences ITS1 rRNA gene from three donkeys in Algeria, two horses in Dakar and one donkey in Keur Momar Sarr. The BLAST result showed that the sequence of *M. ozzardi* (EU272180) is the closest (86%) to the sequences obtained in our study. The phylogenetic tree based on the ITS1 show the position of the *Mansonella* sp. identified in this study compared to other species of the genus *Mansonella*. (Figure 3).

All nucleotide sequences were deposited in GenBank under the following accession numbers: KR080175 for 5S and KR080176 for ITS1 for the sequences obtained in *C. enderleini.* This is also the case for the sequences described in the blood samples: MT786951, MT786954, MT786955, MT786953, MT786952 and MT786950 for the ITS1 sequences and MT795711, MT795716, MT795717, MT795712, MT795716, MT795713 and MT795714 for the 5S sequences.

DISCUSSION

Filariasis is one of the most critical parasitic sicknesses affecting equines worldwide, It is caused by different filarial worms from the families Onchocercidae and Setariidae.The best-known filarioid species that infect horses are from the genera *Onchocerca, Dirofilaria* and *Setaria*. These parasites can cause various problems, including fibrinous myocarditis and/or peritonitis, as well as tendinitis when they infect the muscles and ligaments (Abo-Aziza et al. 2022 ; Radwan et al., 2016). The current manuscript presents an investigation into the molecular characteristics of lesser-known nematodes that are classified under the genus *Mansonella*. This investigation focuses on both equids and biting midges *Culicoides enderleini* found in Senegal and Algeria

In this study, we detected the presence of a new genotype of *Mansonella* genus in equine from Senegal and Algeria, as well as in the *C. enderleini* biting midges from Senegal, using molecular assays. The primers used were based on conserved coding regions of the 5S rRNA and ITS1 which are characterized by their specificity in identifying and characterising filarial species (Jiménez et al., 2011; Xie et al., 1994). Phylogenetic analysis based on the concatenated 5S rRNA and ITS1 genes gave similar results: (i) sequences obtained from *Culicoides* and equine were almost identical and grouped together in a phylogenetic tree forming a cluster within *Mansonella* genus with good bootstrap support, meaning that these sequences represent the same species, and (ii) the amplified 5S gene and ITS1 sequences showed low identity with any recognized *Mansonella* species.

Unfortunately, we failed to obtain blood smears, thus, no morphological analyses and comparisons of *Mansonella* sp. OM-2015 were possible. Also, it is important to note that *Mansonella* adult stages are difficult to collect due to their deep localization inside body cavities and tissues. As a result, the diversity of this genus is not well investigated in animals (Gaillard et al., 2020). The most effective and accurate method for identifying these parasites from infected animals is through indirect approaches that target microfilarial DNA from blood or tissue fragments. (Chabaud and Bain, 1976).

Interestingly, *Mansonella* sp. OM-2015 was recently detected in equines from Egypt (Abo-Aziza et al., 2022), suggesting that it is widespread in Africa and that equines are the reservoir hosts. We strongly suspect that *Mansonella* sp. OM-2015 may be a new species. A thorough literature review showed that, except for the *M. perstans* species, no other *Mansonella* species have been reported in Senegal. *Mansonella* sp. genotype OM-2015 identified in our study differs significantly from *M. perstans*. To the best of our knowledge, our study reports for the first time the presence of filarial worm of the genus *Mansonella* in Algeria. We have also compared our sequences obtained from the 5S gene with the sequence of *M. streptocerca* published by Fischer et al. (1998) which is not available in Genbank and we found that the sequences are also different. In Africa, *M. streptocerca* has been found in primates (Mediannikov and Ranque, 2018). Moreover, In Gabon, potentially new species *Mansonella* sp. "DEUX" was discovered in febrile children (Mourembou et al., 2015 ; Sandri et al., 2021).

All African *Mansonella* reported from humans and great apes include *M. rodhainii*, *M. gorillae*, *M. vanhoofi*, *M. leopoldi*, and *M. lopeensis* (Bain et al., 2015). Genes from these species are unavailable in the Genbank, and their hosts (apes) have not been determined in Senegal. Also, there had been no report of any other valid species belonging to the *Mansonella* genus in Africa (Mourembou et al, 2015).

In addition, the investigations that we have carried out on the different *Culicoides* species have allowed us to detect *Mansonella* sp. OM-2015 for the first time in *Culicoides enderleini*. It should be recalled that various *Culicoides* species have been identified as potential vectors of *Mansonella* throughout the world including *Culicoides insinuatus* (Colombia); *C. guttatus* (Guyana, Suriname and Venezuela); *C. paraensis* (French Guiana, Argentina and Bolivia); *C. debilipalpis* (Bolivia and Argentina); *C. lahillei* (Argentina) *C. furens* (Haiti and St Vincent); *C. barbosai* (Haiti); *C. paraensis* (St Vincent) and *C. phlebotomus* (Trinidad) (Ta-Tang et al., 2018).

Morphological identification of microfilariae enables us to describe the features of *Mansonella* sp. OM-2015 and compare them to the aforementioned *Mansonella* spp. It is necessary to conduct an additional morphological study to determine whether *Mansonella* sp. OM-2015 is a *Mansonella* species that has not yet been molecularly characterized, a genetic variant or a new species.

CONCLUSION

We have identified a filaria species that could not be attributed to any molecularly characterized *Mansonella* species. It is likely that horses are the primary definitive hosts for this species, and *Culicoides enderleini* are the main vectors. In order to describe this potentially new species and expand our understanding of the vector and reservoir role of equids for these potentially zoonotic species, further morphological studies of these equine vector-borne nematodes are needed.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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