Comparative Immunology, Microbiology and Infectious Diseases 63 (2019) 148–153

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

First detection of *Mycoplasma wenyonii* in France: Identification, evaluation of the clinical impact and development of a new specific detection assay

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ARTICLE INFO

Keywords:
Mycoplasma wenyonii
Mycoplasma ovis
Hemoplasma
Cattle
Anemia
polC

ABSTRACT

Mycoplasma wenyonii, a hemoplasma infecting cattle, was never detected in France. In 2014, evocative inclusions were observed in erythrocytes from cattle presenting milk drops, anemia, and edema in Brittany (France). A survey was then initiated to investigate the epidemiological situation and correlate mycoplasma detection with clinical signs. For this purpose, a new PCR assay targeting *polC* gene was designed. Comparative results with published PCR assays place this new one as more specific, allowing a one-step diagnosis without further sequencing. A total of 181 cows were included in this study and 4.97% (n = 9) were positive, resulting in the first molecular identification of *M. wenyonii* in France. All positive animals presented anemia, edema and milk drop. When selecting animals presenting evocative clinical signs, the prevalence of *M. wenyonii* in Brittany was estimated to 25.6%. Further studies are needed to evaluate the importance of the infection, the implication of arthropods and the existence of asymptomatic carriers.

1. Introduction

After a period of confusion, *Haemobartonella* and *Eperythrozoon* species were reclassified as haemotropic mycoplasmas (hemoplasmas) based on molecular phylogenetic evidences [1,2]. Haemoplasmas species are commonly infecting erythrocytes of a wide range of animals. Two species are suspected to infect cattle: *Mycoplasma wenyonii*, formerly known as *Eperythrozoon wenyonii*, and *Candidatus* Mycoplasma haemobos. Data on *Candidatus* Mycoplasma haemobos are very scarce [3] and most reports on cattle haemoplasmas only relate to *M. wenyonii* [4,5].

M. wenyonii is an acknowledged pathogenic species infecting cattle worldwide. Blood smears reveal that this mycoplasma is found attached at the surface of red blood cells and/or free in the plasma [1]. Co-infections have been demonstrated with *Anaplasma marginale*, *A. phagocytophilum*, *Babesia* spp. and *Theileria* spp. [6]. In cattle, the majority of *M. wenyonii* infections remains subclinical [7,8], but signs such anemia, pyrexia, hind limb edema (hock), swollen teats, decreased milk production and weight loss have been observed [8–10]. Chronical infection has also been reported in a Charolais bull in the USA [7]. The use of oxytetracycline is an effective therapeutics in most of the cases. However, no antibiotic protocol has been proven to eliminate infection and infected animals may remain carriers for life [9].

Modalities of dissemination are unknown but horizontal transmission by blood-sucking arthropods has been basically accepted as the most common route for haemoplasma infection [6]. Abortion in association with *M. wenyonii* detection from dam blood suggested that vertical transmission to the conceptus may be an alternative route [11]. Some studies have reported that dam-to-fetus transmission is possible, but the importance of this route for infection is unknown too [12,13]. Vertical transmission seems possible for *Candidatus* Mycoplasma haemobos too [3]. Risk factors associated to infection have been poorly explored. Housed cattle seem more susceptible to infection that grazing cattle and cattle between 1 and 3 years old appear to be the more susceptible to infection [14].

In cattle presenting clinical signs, the usual diagnostic method relies on blood smears stained with Giemsa, but this technique shows low specificity and sensitivity (i.e. possible Howell-Jolli bodies artifact) [8].

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https://doi.org/10.1016/j.cimid.2019.01.010
Received 21 March 2018; Received in revised form 30 November 2018; Accepted 14 January 2019

More recently, methods developed for diagnosis included qualitative PCR [15], real-time PCR [16,17], direct PCR from whole blood sample without DNA extraction [14], and loop-mediated isothermal amplification (LAMP) assay [18]. Yet, these methods have on main drawback in that they were unsatisfactory to discriminate easily bovine hemoplasmas from other mycoplasmas of ruminant origin (i.e. *M. ovis*), which is an important objective [19]. To overcome this issue, we developed a highly specific PCR assay including a plasmid for positive control.

We report hereafter the first clinical cases due to *M. wenyonii* (MW) observed in a dairy herd Brittany, France. Suspected cases, based on visual examination of blood smears or imprecise PCR assays have been presented as oral presentation in cattle medicine congresses, but this is subsequently the first identification of MW in France. The clinical signs consisted in hyperthermia (up to 40 °C), anemia, and lower limbs and teat edema, and the disease affected up to 40% of the cows. Blood smears and molecular evidence using protocols as described by Tagawa et al. [4] and Sasaoka et al. [11], confirmed the diagnosis. Our second objective was to evaluate the feasibility and performances of an original one step detection based on gene *polC*.

2. Materials and methods

2.1. DNA samples

DNA was extracted from 200 µl of EDTA-blood samples collected from 2 cattle with clinical signs and positive smear evocating infection by *Mycoplasma wenyonii* (sample 141145 and 141146, collected in France [10] using a QIAamp DNA Blood Mini Kit (QIAGEN), eluted with 100 µl of buffer AE and stored at −20 °C until use. DNA extract from sample known as positive from previous investigation on *M. wenyonii* in Japan [16] was used as positive control for PCR detection. For defining the specificity of the PCR assay, genomic DNA from other mycoplasma species (*Mycoplasma ovis*, *M. fermentans*; *M. hominis*; *M. orale*; *Achleplasma laidlawi*; *M. capricolum* subsp. *capricolum*; *M. mycoides* subsp. *capri*; *M. leachi*; *M.putrefaciens*; *M. ovipneumoniae*; *M. bovigenitalium*; *M. conjunctivae*; *M. arginini*; *M. gallisepticum*; *M. canis*; *U. diversum*; *M. bovis*; *M. agalactiae*) were used and extracted from liquid culture as described elsewhere [20,21] except for *M. ovis* for which it was extracted from whole blood of a positive animal. The *Mycoplasma* species have been chosen either for their renown existence in ruminants (n = 13) or for their existence in common domestic animals or humankind (n = 5)

2.2. PCR detection assays

Four set of primers were used for PCR assays (Table 1). Primers for 16S_rRNA and primers for *rnpB* were designed according to Tagawa et al. [4] and Sasaoka et al. [11] respectively. Based on the alignment *pol*C sequences available in databases for MW and MO, forward and reverse primers were designed to amplify a 500 bp product. Using the Primer blast software, these primers were predicted *in silico* to specifically amplify MW. The same strategy was used to designed MO specific primers.

PCR assays were performed using an Eppendorf Mastercycler ep-Gradient thermocycler in 25 µL reaction mixtures containing 0.4 mM of each primer, 1X PCR reaction buffer (with MgSO4, New England Biolabs [NEB], Evry, France), 200 mM dNTPs and 2.5 U Taq DNA polymerase (NEB). Reaction mixtures were subjected to 2 min at 94 °C, 30 cycles (except for 16S rRNA: 35) of 30 s at 94 °C, 30 s at 58 °C (except for 16S rRNA: 62 °C), 30 s at 72 °C, and a final elongation step of 5 min at 72 °C. Annealing temperature was determined based on data obtained from a gradient of temperature. PCR products were analyzed by gel electrophoresis in 2% agarose for 16S RNA [4] and *rnpB* [11] assays or in 1% agarose for MW-*polC* and MO-*polC* assays (Molecular weight standard: Smart Ladder, Eurogentec, Liège, Belgium).

2.3. PCR sensitivity and design of replicative plasmid as positive controls

PCR products were purified (QIAquick PCR Purification Kit, QIAGEN, Courtaboeuf, France) and cloned into pGEMT-easy plasmid vector (Promega, Charbonnières les bains, France). Recombinant plasmid vectors were transformed in *Escherichia coli* strain DH5a (Invitrogen/Life-Technologies, Courtaboeuf, France) grown in LB medium supplemented with ampicillin, and purified with QIAprep Spin Miniprep kit (QIAGEN). DNA fragment insertion was monitored by PCR with the appropriate specific primers, followed by sequencing. Plasmid concentration was determined by Qubit Fluorometric Quantitation (ThermoFischer, Villebon sur Yvette, France). PCR assays were performed with serial dilution of the purified plasmid as templates to determine the minimal concentration for which amplification is detected. Each experiment was repeated three times to assess repeatability and we obtained every time the same results. For comparison, the PCR assay was run in parallel with serial dilution of positive samples.

2.4. DNA sequencing

Sequences of PCR product and plasmid were obtained by Sanger direct sequencing method using specific primers at the sequencing facility of UMR 5165 (CNRS, UPS, CHU Purpan, Toulouse, France). Sequence analysis was done with Chromas lite (available at http://www.technelysium.com.au/chromas_lite.html) and NCBI blast tools (https://blast.ncbi.nlm.nih.gov/).

2.5. Case detection study

A case detection study was implemented in 2016 with the help of veterinarians in French Brittany. Cases were included in the study on the basis of cattle presenting: anemia without hemoglobinuria, edema of the limbs and/or the udder, transient milk drop, excluding anaplasmosis and babesiosis, medical history was recorded. A total of 181 cows in 6 herds were included in the study: 34 suspected clinical cases

Table 1
Primers and PCR conditions.

Targeted gene	Primers	Ta[a]	Expected amplicon size		References
			M. wenyonii	*M. ovis*	
16S_rRNA	16S-F2 (5'-GGCCCATATTCCTRCGGGAAG-3') 16S-R2 (5'-ACRGGATTACTAGTGATTCCA-3')	62 °C	193 bp	176 and 193 bp	Tagawa et al. [4]
rnpB	rnpB-F (5'-AGTCTGAGATGACTRTAGTG-3') rnpB-R (5'-TRCTTGMGGGGTTTGCCTCG-3')	58 °C	189 bp	187 bp	Sasaoka et al. [11]
MW_polC	MW-PolCF1 (5'-GTAGTACCACCACCAGAGCAG-3') MW-PolCR1 (5'-ACTACCTCGTGCCCAAGTG-3')	58 °C	530 bp	–	This study
MO_polC	MO-PolCF1 (5'-GGACAGTGAGCGGAGATGG-3') MO-PolCR1 (5'-AAGAGTGGGGCTTGAATGGG-3')	58 °C	–	545 bp	This study

[a] Temperature of annealing.

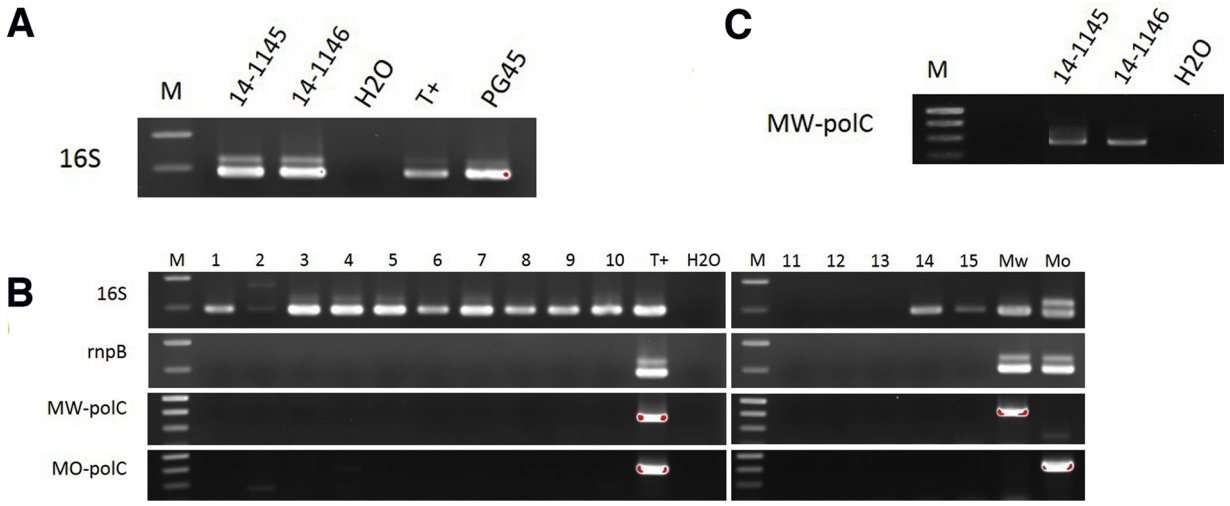


Fig. 1. *M. wenyonii* PCR assays. Princeps cases detection targeting 16S rRNA (A) or polC (C) genes, and assays specificity testing (B). T+: MW for 16S rRNA, *rnpB* and MW-*polC*; MO for MO-polC; M: Smart ladder Eurogentec; 1: *Mycoplasma fermentans*; 2: *Achleplasma laidlawi*; 3: *M. capricolum* subsp. *capricolum*; 4: *M. leachi*; 5: *M. putrefaciens*; 6: *M. ovipneumoniae*; 7: *M. mycoides* subsp. *capri*; 8: *M. bovigenitalium*; 9: *M. conjunctivae*; 10: *M. arginini*; 11: *M. canis*; 12: *U. diversum 310*$^{\#}$; 13: *U. diversum 246*$^{\#£}$; 14: *M. bovis*; 15: *M. agalactiae**; 16: *M. wenyonii**; (*: DNA extract of blood from positive animal, $^{\#}$: culture after PK lysis, $^{£}$: no culture growth).

Table 2
Specificity of the PCR assays targeting the detection of MW.

Species and reference of the strain or isolate	Host	PCR assay			
		16S rRNA	*rnpB*	MW-*polC*	MO-*polC*
M. wenyonii isolate 14-1146 this study	Ruminants	+	+	+	−*
M. ovis isolate 10-373, this study	Ruminants	+*	+	−*	+
M. fermentans type strain PG 12	Human	+	–	–	–
M. hominis type strain PG21	Human	+	–	–	–
M. orale type strain CH 19299	Human	+	–	−*	−*
A. laidlawii type strain PG8	Free	+	–	–	−*
M. capricolum subsp. *capricolum* type strain California kid	Ruminants	+	–	–	–
M. mycoides subsp. *Capri* type strain Y goat	Ruminants	+	–	–	–
M. leachii type strain PG50	Ruminants	+	–	–	–
M. putrefaciens type strain KST	Ruminants	+	–	–	–
M. ovipneumoniae type strain Y98	Ruminants	+	–	–	–
M. bovigenitalium type strain PG 11	Ruminants	+	–	–	–
M. conjunctivae type strain HRC581	Ruminants	+	–	–	–
M. arginini type strain G230	Ruminants	+	–	–	–
M. gallisepticum type strain PG31	Poultry	+	+w	–	–
M. canis type strain PG14	Ruminants	–	–	–	–
U. diversum type strain A417	Ruminants	–	–	–	–
M. bovis type strain PG45	Ruminants	+	–	–	–
M. agalactiae type strain PG2	Ruminants	+	–	–	–

+: amplicon at expected size; −: no amplification detected; −*: weak signal at a different size than expected; +w: positive weak signal at the expected size.

Table 3
Sensitivity of the PCR assays.

	Plasmid insert target	Detection threshold (genome/µL blood)
16S rRNA	MW	30
rnpB	MW	200
	MO	90
MW-*polC*	MW	1700
MO-*polC*	MO	240

MO: *Mycoplasma ovis*; MW: *Mycoplasma wenyonii*.

were collected and 147 samples from animals without clinical signs were also collected at the same time and from the same region. Animal blood samples were collected (5 mL, EDTA) for blood-smear primary detection using Diff Quick® coloration and, DNA extraction (QIAamp DNA Blood Mini Kit, Qiagen) for PCR detection using MW-*polC* amplification.

2.6. Nucleotide sequence accession numbers

Nucleotide sequences have been submitted to GenBank under accession numbers MH001426, MH001427, MH001428 and MH001429 respectively.

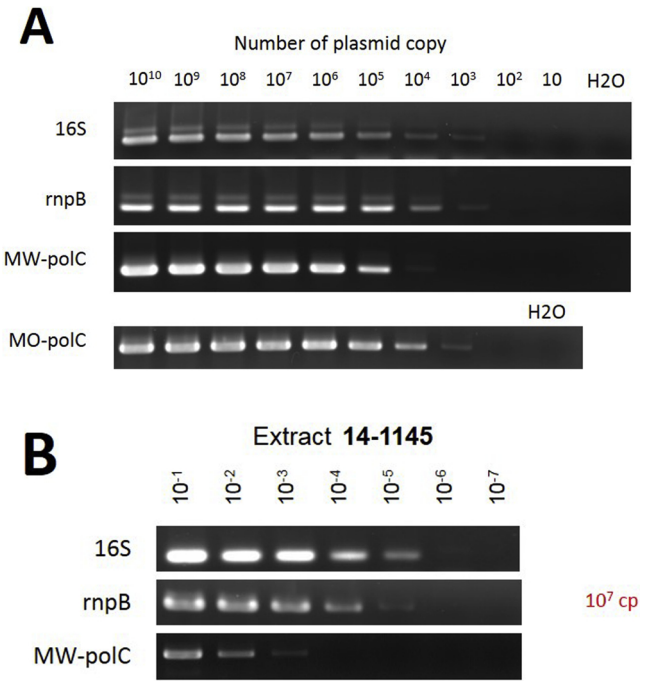


Fig. 2. Sensitivity testing of *M. wenyonii* PCR assays. (A) 16S rRNA, *rnpB,* MW-*polC and* MO-*polC* PCR with specific plasmid in serial dilution corresponding to 10 to 10^{10} gene copy as template (B) Quantification of gene copy in positive sample 14-1145 : 16S rRNA, *rnpB,* MW-*polC* PCR with serial dilution of DNA extract as template.

3. Results

3.1. First detection of hemoplasmas in French ruminants

Using the PCR assay developed by Tagawa et al. in 2008, the presence of MW was detected in 9 blood samples (i) collected in France. We selected only 2 after the sequencing process (due to the 100% level of similarity between the 9) from two cows suspected to be infected by MW, namely 14-1145 and 14-1146, (see Material and methods) and (ii) from an animal known as being infected by MW, as positive control. More specifically, with these samples, a PCR product of the expected size, 193-bp, was detected. Regardless of the stringency of the PCR conditions, the same PCR assay yielded a similar size product with DNA extracted from *M. bovis*, another ruminant mycoplasma species known to infect ruminant and initially included in our study as a negative control (Fig. 1A). The 193-bp PCR product of 14-1145, 14-1146 and *M. bovis* were sequenced. Data showed that 14-1145 and 14-1146 sequences are identical and closely matched sequences belonging to the

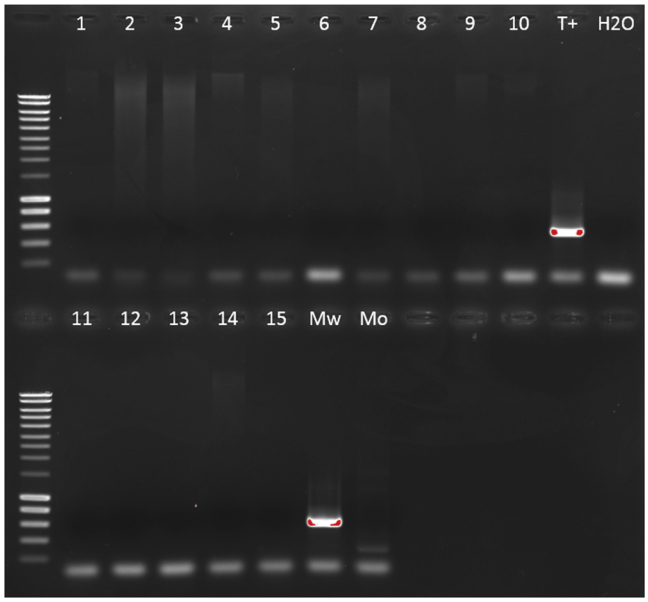


Fig. 4. *polC* gel *Mycoplasma wenyonii* specific.

16 s rRNA of MW and MO, with 0 to 2 SNPs, depending on the strains used for comparison. For *M. bovis*, the sequence was identical to the of *M. bovis* 16 s rRNA. All together these data suggested that the PCR developed by Tagawa et al. [4], while detecting hemoplasmas, does not discriminate between MO, MW and MB. When we tested the PCR assay developed by Sasaoka et al. [11], which one targets the *rnpB* gene, *M. bovis* DNA gave a negative result while 141145 and 141146 yield the expected size product which sequences match specifically the MW. Yet, MO was positive (Fig. 1B).

3.2. Development of a specific PCR for MW and MO

Overall, these results reflect the lack of a proper diagnostic assay that would allow unambiguously the detection of MW in one single step. To fill this gap, we designed a PCR assay targeting the *pol*C gene to amplify specifically a 500 bp product. This was further confirmed *in vitro* using genomic DNA extracted from MW, and MB with a 55 °C optimum temperature (Fig. 1C). The same strategy was used to designed MO specific primers. The specificity of these PCR assays, further designated as MW-*polC* or MO-*polC*, was then tested against 19 *Mycoplasma* species, of which 15 are known in ruminants (Table 2). Results shown in Table 2 and illustrated in Fig. 1 were compared to PCR assays previously developed. All species but two, *M. canis* and *U. diversum*, were detected in our hands with the assay targeting the 16S rRNA [4]. The PCR assay developed by Sasaoka et al. [11] was more specific; yet

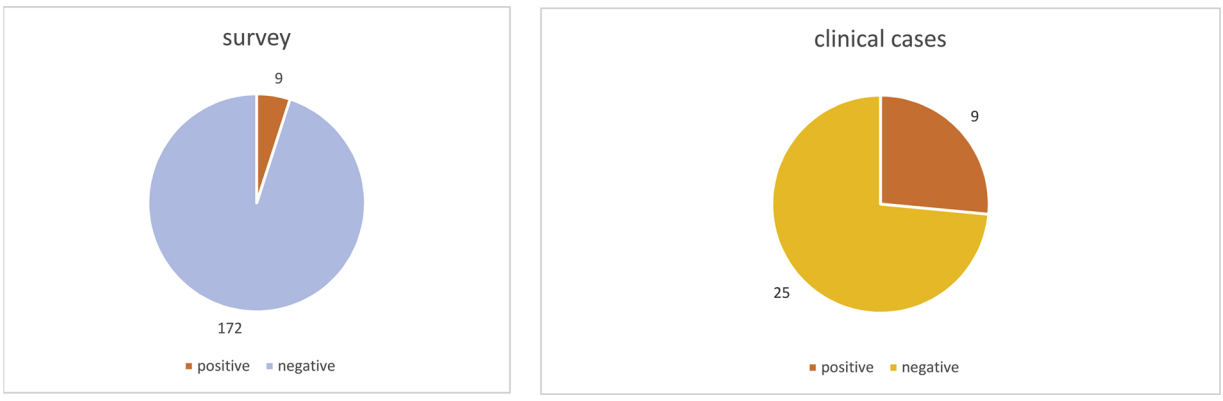
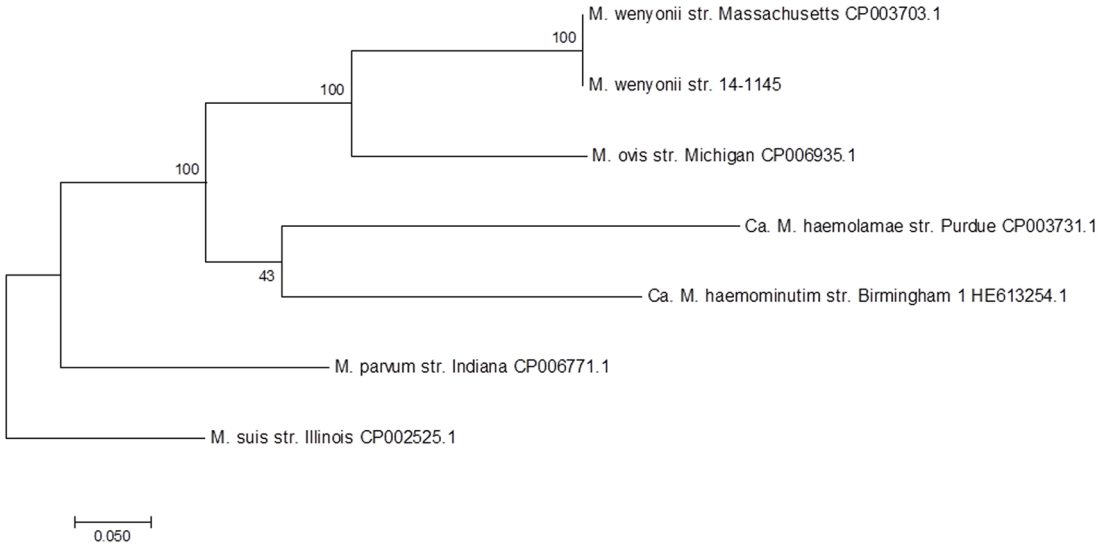


Fig. 3. Global survey results. Detection results in all animals or when selecting animal on clinical signs.



Phylogentic tree based on *polC* gene sequence

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model [1]. The tree with the highest log likelihood (-22940.9572) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 7 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 4202 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [2].

Fig. 5. *polC* phylogenetic tree [24,25].

amplification was obtained with MO. Overall, in our hands, comparison of the different PCR assays currently available points towards MW-*polC* as being the more specific for MW detection.

3.3. Development of molecular positive controls

Since MW and MO are not cultivable, the use of a field sample as positive control which availability is limited poses the question of the reproducibility and of the estimation of the sensitivity of the assay. To address these issues, we engineered a set of 4 replicative plasmids carrying sequences targeted by the PCR assays and corresponding to: the MW- or MO- 530 bp or 545 bp *polC* sequence respectively, the 189 bp MW *rnpB* sequence and the MW 16 s rRNA. Replicates of PCR assays using serial dilution of known plasmid concentration indicates that MW-*polC* is less sensitive than other PCR assays, with that targeting the two 16 s rRNA gene copies being as expected the most sensitive (Table 3 and Fig. 2A). Replicates of PCR assays using serial dilution of a field sample (Fig. 2B) permit to confirm these sensitivity differences and estimate the bacterial load of a clinically infected animal to 10^7 bacteria/ml of blood assessing that the sensitivity of MW-*polC* assay is sufficient enough to detect clinical cases.

3.4. Case detection study

Cases were recruited on basis of clinical signs evocating infection by *M. wenyonii* as detected by veterinarians in Brittany. Animals without clinical signs were also sampled at the same time. A total of 34 samples from adult dairy cattle presenting clinical signs were collected and 147 from animals without clinical signs. All were analyzed with MW-*polC* PCR assay. Of the 34 animals presenting signs evocating *M. wenyonii* infection, 9 were positive while the 147 samples from asymptomatic animals were all negative (Fig. 3).

4. Discussion

This study presents the molecular and clinical identification of a hemoplasma in cattle in France. To the authors´ knowledge, this is the first report of a molecular investigation of *M. wenyonii* in this country. Hemoplasma organisms (named before 2001 *Eperythrozoon*) have been identified in a variety of mammals (including cattle) and the infection by these organisms has been associated with extravascular hemolytic anemia, reproductive disorders, milk drop syndrome, edema of dependent parts, growth retardation… of varying intensity: subclinical infection seems common [22]. In Brittany (France), evocative inclusions in bovine erythrocytes have been observed for the first time in 2014, associated with clinical signs in adult cows [10].

Our study confirms the presence of *Mycoplasma wenyonii* in France. Surprisingly, in our survey 'Candidatus M. haemobos' was not detected unlike in other European countries [3,23], but this species is even less documented that MW. As expected, the number of infected animals by MW is much higher when selecting them on the basis of a clinical suspicion (9/34: 25.6%) than when sampling them at random in the same area (9/181: 4.9%). The selected significant clinical signs were: anemia without hemoglobinuria, edema of the limbs and/or the udder, transient milk drop. Of course clinical anaplasmosis and babesiosis were excluded by optical microscopy and PCR (data not shown). No coinfection was observed in our study but is likely to occur from time to time since their potential vectors share the same biotope [12,22].

Additionally, the purpose of our study was to address the specificity of previously published PCR assays, and to develop a specific molecular diagnosis a one-step assay, without any subsequent amplification. We demonstrated that no other assay is as discriminant as the one we developed: using MW-*polC* amplification (with the probes we developed) allows detecting only *Mycoplasma wenyonii,* and no other mycoplasma of ruminant origin (Figs. 4 and 5). Moreover, the amplicon size is Mw-specific and quite different from *M. ovis* amplicons, an important objective [19] we fulfilled. At first, the suitability of this assay in a real-time PCR was not the purpose of this study.

Sensitivity of the existing and new test has been evaluated. The detection threshold is as low as 1.7 10^6 copies of the genome per mL total blood, but it is less sensitive than PCR assays targeting the 16S rRNA gene copies which are, as expected, the most sensitive. The future

development of a real-time PCR will be the way to increase the sensitivity of the detection.

Sensitivity study was made possible thanks to the engineering of recombinant plasmids. These have several advantages over positive controls from the field infected animals whose reproducibility is poor and/or variable: they can be produce in large amount and offer reproducible and comparative data across labs.

Further studies are needed to evaluate the importance of MW infection. The systematic exploration of anemia in cattle could be the first step, as this symptom appears to be the more consistent in cattle of all ages. Studies on coinfections with *Theileria* spp., *Babesia* spp., *Anaplasma* spp. and *Bartonella* spp. are to be initiated too, because infectivity and disease could be associated with coinfection, apart from other factors to determine. Correlations of MW infection with productivity and growth might be evaluated subsequently.

As there is no clear consensus either on the potential vectors, or on transmission, arthropod trapping and investigation on asymptomatic or latent and chronic carriers are needed too.

The importance of vertical transmission is unknown too, and sampling calves before ingestion of colostral antibodies could help to specify the importance of this phenomenon.

The tools we developed can contribute to target these objectives.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

Authorship

LX. Nouvel and MC. Hygonenq carried out DNA extraction and PCR design and analysis. E. Martinelli and G. Catays carried out part of DNA extraction and PCR analysis. Ph. Lepage and E. Collin carried out animal sampling and case story collection. H.Inokuma provided positive controls and PCR protocols. C. Citti, LX. Nouvel and R. Maillard drafted the manuscript. LX. Nouvel, F. Schelcher, C. Citti and R. Maillard conceived and participated in the design of the study, which was coordinated by R. Maillard. All authors read and approved the final manuscript.

Declarations of interest

None.

Acknowledgment

This work was supported by the French Ministry for Agriculture and Food and by the National Institute for Agricultural Research (INRA).

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