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Abstract

Chronic non-progressive pneumonia in small ruminants caused by *Mycoplasma (M.) ovipneumoniae* is mainly controlled by chemotherapy. In France, during the last decade, a rise in *M. ovipneumoniae* cases was recorded in both sheep and goats, suggesting a possible emergence. Whether this rise is associated with antimicrobial resistance, as observed in other ruminant *Mycoplasma* species, has yet to be examined.

The aim of the study was to characterize the diversity of *M. ovipneumoniae* strains circulating in France and assess their antimicrobial resistance, together with the underlying mechanisms, to help find an explanation for the increase in reported cases.

The genetic diversity of 56 strains isolated between 2007 and 2018 from sheep and goats was assessed using different subtyping methods. Their susceptibility to six antimicrobial classes was profiled by estimating Minimum Inhibitory Concentrations (MICs) using an optimised agar dilution method. Resistance mechanisms were explored by sequence analysis of rRNA targets.

A high genetic diversity of strains was evidenced, with consistent, marked animal-host clustering in the Hsp70 gene and whole genome sequence phylogeny. No clonal evolution could thus account for putative emergence. Apart from florfenicol, MICs were low except for a few isolates with increased

values for tetracyclines, macrolides and lincosamides. Hotspot mutations in the target ribosomal gene could explain increased tetracycline MICs. Other mechanisms are suspected for macrolide-lincosamide and florfenicol resistance.

The emergence of *M. ovipneumoniae* is thus not related to any increase in resistance or to a clonal spread. Explanations may lie in breeding practices.

Keywords: *Mycoplasma ovipneumoniae*, diversity, antimicrobial resistance, sheep and goat pneumonia.

Introduction

Since its first isolation in 1963, *Mycoplasma (M.) ovipneumoniae* has been described as the causative agent of chronic non-progressive pneumonia, also known as enzootic or atypical pneumonia in sheep (Cottew, 1979; Nicholas et al., 2008b). This disease has been reported worldwide, with a high prevalence in fattening lamb flocks. Some countries have recently seen an increase in cases (Nicholas et al., 2008b; Poumarat et al., 2014; Lindström et al., 2018; Manlove et al., 2019). It is generally associated with a variable morbidity but a low mortality with chronic cough, nasal discharge, and hyperthermia. It is nonetheless responsible for substantial economic losses due to lower ewe productivity and lamb growth (Besser et al., 2019; Manlove et al., 2019). Co-infection with other bacterial respiratory pathogens, including prominently *Mannheimia haemolytica*, or viruses, triggered by conditions of physiological stress or environment, is frequent, but its effects on the severity of the disease are not clear (Nicholas et al., 2008b; Besser et al., 2019). By contrast, clinical outcome seems to be correlated with increased *M. ovipneumoniae* concentration in the lungs (Nicholas et al., 2008b). *M. ovipneumoniae* in goats has been under-researched in comparison to sheep, although these animals are reported to present similar respiratory signs with potential severe forms (Nicholas, 2002; Gonçalves et al., 2010; Poumarat et al., 2014; Maksimović et al., 2017). This lesser recognition might be related to the presence of other *Mycoplasma* species more likely to be primarily diagnosed in the context of caprine pneumonia (Nicholas, 2002; Jaÿ and Tardy, 2019). Asymptomatic carriage of *M. ovipneumoniae* has been demonstrated in both sheep and goats (Nicholas, 2002; Maksimović et al., 2017). *M. ovipneumoniae* also causes pneumonia in wild ruminants, especially *Caprinae*, and so could threaten wildlife (Kamath et al., 2019).

Currently, despite greater awareness, surveillance data for *M. ovipneumoniae* are scant worldwide, possibly owing to limited diagnosis options. *M. ovipneumoniae* growth in the usual media is slower (24 to 72h with a peak at 48h) than that of most of other small ruminant *Mycoplasma* species and is associated with slight turbidity in liquid medium and colonies with atypical morphology (lacking a

62 “fried egg” appearance) on agar plates (Ackerman et al., 2019; Maksimović et al., 2020b). After
63 isolation, species identification relies on additional assays such as PCRs, antigenic detection or
64 MALDI-TOF (Poumarat et al., 1991; Nicholas et al., 2008a; Weiser et al., 2012; Spergser et al.,
65 2019). Direct quantitative molecular methods for detection (without preculture), such as qPCR or
66 isothermal amplification seem promising, but lack broad validation (Wang et al., 2020). Besides,
67 whereas in other *Mycoplasma* species, Antimicrobial Susceptibility Testing (AST) is becoming
68 increasingly part of diagnosis, antimicrobial resistance has been poorly studied in *M.*
69 *ovipneumoniae*, owing notably to difficulties in counting *M. ovipneumoniae* cells and so
70 standardizing the inoculum for AST (Ackerman et al., 2019). In veterinary *Mycoplasma*, AST relies
71 on determining MICs , but methodological guidelines are sparse (Hannan, 2000) and no clinical
72 interpretation criteria are yet available (Gautier-Bouchardon, 2018).
73 The intra-species genetic diversity of *M. ovipneumoniae* is considered high despite the limited
74 number of studies and tools available for worldwide comparison (Parham et al., 2006; Zhang et al.,
75 2013; Maksimović et al., 2017; Einarsdottir et al., 2018; Kamath et al., 2019). Genome-based
76 approaches are also limited owing to the small number of genomes currently available in public
77 databases (https://www.ncbi.nlm.nih.gov/genome/browse/#!/prokaryotes/2772/, last accessed
78 August 12, 2020). Like diagnosis, control tools for chronic non-progressive pneumonia are barely
79 progressing. Some attempts to develop a vaccine were promising but they faced several difficulties,
80 such as the need for high concentrations of antigen to induce an immune response comparable to
81 that of natural infection and a reduced efficacy in sheep with prior exposure to *M. ovipneumoniae*
82 (Einarsdottir et al., 2018). Hence good husbandry practices and antimicrobial treatment remain the
83 main ways to control *M. ovipneumoniae* (Besser et al., 2019).
84 France is the EU’s third largest producer of sheep and goat meat and milk
85 (https://www.europarl.europa.eu/RegData/etudes/BRIE/2017/608663/EPRS_BRI(2017)608663_EN
86 .pdf). The clinical monitoring of mycoplasmosis in France is done through a surveillance network,
87 Vigimyc, which centralizes the identification of *Mycoplasma* spp. recovered from clinical samples

by partner laboratories (Poumarat et al., 2014). Vigimyc is a "passive" surveillance network, the decision to test for *Mycoplasma* being solely on a veterinarian's initiative. There has been a recent increase in the number of isolates identified as *M. ovipneumoniae* through the network (Poumarat et al., 2014). It is still not known whether this rise is due to disease-controlling antimicrobial treatments becoming less effective.

The present work set out to characterize the diversity of *M. ovipneumoniae* strains circulating in domestic hosts in France and assess their antimicrobial susceptibility, together with the underlying mechanisms, to help explain the increase in reported cases.

Methods, techniques

M. ovipneumoniae isolates

Fifty-six *M. ovipneumoniae* isolates collected between 2007 and 2018 from sheep ($n = 28$) and goats ($n = 28$) in different regions of France were included in the study (Supplementary Table 1). Most ($n = 53$) originated from the epidemiological surveillance network Vigimyc. They were collected from animals with respiratory disorders in distinct herds, at different sampling dates. Three isolates were taken from sheep lungs, without lesions, from healthy herds sampled at slaughterhouses. When age was known (sheep $n = 20$ / goats $n = 23$), the sampled animals were mostly young (sheep $n = 17$ / goats $n = 15$). Isolates were identified using MF-dot and further confirmed by a species-specific PCR (Poumarat et al., 1991; Weiser et al., 2012). In addition, two previously sequenced strains, namely strain 14811 and the type strain Y98 (NCTC10151), available in the laboratory, were included in AST.

Culture conditions and antimicrobial susceptibility testing

All isolates were grown at 37 °C in an enriched CO_2 atmosphere (5%), in PPLO broth, modified as previously described (Poumarat et al., 1991) and supplemented with phenol red (0.002%).

AST was performed using the agar dilution method as previously described (Khalil et al., 2017) to determine the MICs, i.e. the lowest antimicrobial concentration at which no growth was observed.

Six drugs from different classes were tested: enrofloxacin (fluoroquinolones), oxytetracycline (tetracyclines), florfenicol (amphenicol), spectinomycin (aminosides) and members of the Macrolides-Lincosamides-Streptogramin-Ketolides (MLSK) group: tilmicosin (16 membered-ring macrolides) and lincomycin (lincosamides). They correspond to classes with either a market authorization in sheep or goats and therapeutic recommendations consistent with respiratory diseases or regularly used according to antimicrobial use survey in small ruminants in France (N. Jarrige, personal communication). Of note, florfenicol is licensed in sheep raised for meat production but banned in lactating animals.

The agar concentration in PPLO-plates was reduced to 0.7% (instead of the usual 1.2%) to improve the appearance of the colonies, i.e. round, non-confluent with regular borders (Cottew, 1979). Colonies were counted under a stereomicroscope, at 48–72 h after inoculation. Four to six antimicrobial-free control plates with an expected count of 30–300 CFU per 1 µL spot were also prepared to control potential inter-plate variability of inoculum size.

Firstly, only two antimicrobial concentrations corresponding to the intermediate and susceptible clinical breakpoints proposed by the CLSI for MIC interpretative criteria for *Pasteurellaceae* in cattle, were used (CLSI, 2015). These breakpoints are commonly used for ruminant *Mycoplasma* species with the same respiratory tropism as *Pasteurellaceae* (Poumarat et al., 2016; Khalil et al., 2017). These preliminary tests were conducted twice and enabled us to specify the antimicrobial concentration range to be tested to precisely define the MIC (Supplementary Table 1). MIC assays were then conducted with increasing two-fold dilutions of antimicrobial within the upper and lower limits previously determined. Assays were repeated two or three times. The final MIC values were determined taking account of both the preliminary tests and the final MIC assays. They were either the mode of three values or the repeated values obtained more than once. In several instances, repetitions did not enable us to define a mode, or values were different with equal occurrence; the MICs were then expressed as a range. In the absence of a designated control strain, we internally

calibrated one strain (F9821), compared with a *M. agalactiae* strain L15993 of which the MICs were duly determined previously (Poumarat et al., 2016).

Statistical analysis

For statistical analysis, the MIC values were converted into a continuous variable. These rank values were then used to compare different subpopulations of isolates (e.g. caprine versus ovine strains) using a Mann-Whitney test (significance level $p = 0.05$), as previously described (Poumarat et al., 2016).

DNA extraction and molecular analyses of *M. ovipneumoniae*

Genomic DNA was extracted from stationary broth cultures using a commercial kit (QIAamp DNA Mini Kit, Qiagen). The diversity of our *M. ovipneumoniae* isolate population was assessed both by Random Amplified Polymorphic (RAPD) DNA PCR and sequence analysis of the Hsp70 coding gene. RAPD was performed as previously described using only the Hum4 primer (5'-3': ACGGTACACT), which gave more reproducible patterns in our hands (Parham et al., 2006). The only modifications were a doubling of final dNTP concentration (200 µM) and a reduction of DNA final concentration (1 ng/µL). Amplification patterns were analysed using GelCompar II 6.6, and a dendrogram was drawn by the unweighted pair group method with arithmetic means (UGPMA, tolerance 2%).

The *hsp70* PCR was conducted as previously described (Zhang et al., 2013). The 1800 bp amplicons were sequenced at an external facility (Genewiz, Germany). For comparison purposes, sequences from Zhang et al. and Maksimović et al. were retrieved from the NCBI (GenBank accession numbers KC693973 to KC693989) and from the supplementary data of the paper, respectively. Four other sequences extracted from the genome of four strains available at NCBI were also included (see reference material in Supplementary Table 1). Sequences were aligned using ClustalW and trimmed to a common 537 nt region. A maximum likelihood phylogeny was estimated, built using Iq-tree (http://iqtree.cibiv.univie.ac.at/). The tree was finalized using Figtree and rooted using *M. ovipneumoniae* strain Y98 (NCTC10151).

PCR amplification and sequence analysis were also used to investigate potential hotspot mutations in ribosomal target genes, namely 23S rRNA and 16S rRNA, of strains with higher MIC values for macrolides-lincosamides and tetracyclines. Several PCR and sequencing primers were adapted from other work to cover frequently described mutation points. For 16S rRNA, U1 (593) (5'-GTTTGATCCTGGCTCAGGAYDAACG-3') and U8 (690) (5'-GAAAGGAGGTRWTCCAYCCSCAC-3') were used as PCR primers (amplification size 1500 bp) and U4 (538) (5'- GTAGTCCACGCCGTAAACG-3') for sequencing (Johansson et al., 1998). For 23S rRNA PCR, MOVI_23S_V1_F (5'- AGGGTGGCAACTGTTTATCAAAA-3') and MOVI_23S_V1_R (5'- ATTAAGGGTGGTATTTCAAGGTT-3') were adapted from Khalil et al. (amplification size 421 bp) and MOVI_23S_D2_F (5'-GTACCGTGAGGGAAAGGTGA-3') and MOVI_23S_D2_R (5'- CGCCATTCCACATTCAGTGC-3') were adapted from Prats-van der Ham et al. (amplification size 437 bp) (Khalil et al., 2017; Prats-van der Ham et al., 2017). Sequencing of 23S rRNA PCR products was performed respectively with MOVI_23S_V1_F and MOVI_23S_D2_F. Amplification protocols were run as originally published.

Whole-genome SNP phylogenetics using PhaME analysis workflow

Genomic DNA extraction of six cloned strains (see Table 1) was performed from 20 mL of stationary phase culture using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen). DNA samples were sequenced using Illumina MiSeq technology generating 2 × 150-bp pair-end reads (MiSeq, ICM Institute, Paris, France). A mean total of 4.32 million reads was generated for each of R1 and R2. Trimmed reads (using Trimmomatic-0.36) were used to construct a phylogenetic tree using PhaME workflow (Ahmed et al., 2015). Contigs from strains 14811, NZ90, SC01 and scaffolds from NM2010 were uploaded from the NCBI database (https://www.ncbi.nlm.nih.gov/genome/) and also included in the analysis. Raw reads, contigs or scaffolds were mapped to the reference genome of strain *M. ovipneumoniae* NCTC10151 (reference sequence NZ_LR215028.1) using the aligner bwa set in the control file. The maximum likelihood phylogeny was inferred using ModelFinder to find the best fit model, and Iqtree for tree

reconstruction with non-parametric bootstrap (500 replicates). The sequencing data has been deposited in the Sequencing Read Archive (SRA) under the Bioproject accession number PRJNA641331.

Results

Clinical surveillance results

Between years 2007 and 2019, 2512 *Mycoplasma* spp. isolates from small ruminants were received for species identification through our surveillance network Vigimyc (Poumarat et al., 2014). Of those isolates, 411 were identified as *M. ovipneumoniae*, mainly from respiratory samples (98%, 402/411), most often in association with *Pasteurellaceae* (87%, 297/341 with known bacteriology results).

Until 2011, fewer than 10 respiratory samples were *M. ovipneumoniae*-positive annually, while between 2012 and 2019, an annual mean of 48 *M. ovipneumoniae* isolates were identified with a maximum of $n = 73$ in 2019 (Figure 1). This progression suggests either (i) more frequent diagnosis due to better awareness or improved technical capacity for isolation, or (ii) a true increased incidence of the species. In sheep, between 2012 and 2019, the proportion of *M. ovipneumoniae*-positive respiratory isolates remained high and stable with an annual mean of 42% (SD 9%) and a maximum of 53% (49/91) reached in 2018. In goats, before 2011, a maximum of one *M. ovipneumoniae* isolate per year was identified, but from 2012 the mean annual number of isolates increased to 7-37, representing a maximum proportion of 41% (37/91) of isolates from respiratory samples in 2019, this proportion being almost equivalent to that of sheep (43% in 2019). Interestingly, the infected populations differed, young animals predominating in sheep samples when age was known (87% of 225 samples) but not in goats (57% of 113 samples).

This increase in the proportions of *M. ovipneumoniae* isolates, with a small lag between sheep and goats, suggests a possible rise in the species prevalence and a spillover from sheep to goats. This spread might also concern other animal species: in the 2007–2019 period, through Vigimyc, six

strains were isolated from respiratory samples in wild *Caprinae* (*n* = 3, *Rupicapra rupicapra*) and cattle (*n* = 3).

Given the similar proportions of *M. ovipneumoniae* in sheep and goat respiratory isolates in recent years of surveillance, we made a subset composed of half sheep and half goat isolates (28 isolates each). To assess evolution over time and influence of geographical origin, these isolates were recruited over the 2007–2018 period in various regions of France. Diversity analysis was performed on this subset to analyse the structure of the population and validate its suitability for AST, i.e. to rule out any clonal isolates.

Diversity of *M. ovipneumoniae* isolates

At the time of their collection, *M. ovipneumoniae* isolates were first identified using MF-dot and checked with the same method after subculture performed in this study. Antigenic patterns obtained in MF-dot were homogeneous, reacting solely with *M. ovipneumoniae* specific anti-sera, which rules out any *Mycoplasma* species mixtures. This identification was confirmed for all 56 isolates using a species-specific PCR, which also allowed us to assess the extracted DNA quality and its suitability for diversity tests. Two previously validated tests, RAPD and *Hsp70* polymorphism analysis (Parham et al., 2006; Zhang et al., 2013), were conducted on the whole panel, and a further genome-wise SNP phylogeny was performed on a sub-selection of strains for which the genomes were available.

In our hands, the RAPD assays proposed by Parham et al. resulted in better intra- and inter-assay repeatability using the Hum4 primer (preliminary assays not shown) (Parham et al., 2006). The profiles generated with our set of strains were composed of 1–12 fragments with a size ranging from 181 bp to 2838 bp, consistent with the results of Parham et al. (Parham et al., 2006). Most of the strains were tested only once. A UPGMA dendrogram was built using RAPD profiles and the Jaccard similarity coefficient (Supplementary Figure 1). The strains all showed different profiles with less than 90% similarity except for eight isolates clustered in four pairs. These results confirm the overall diversity of our panel of strains and its suitability for a MIC study. No influence of

sampling year, host species or geographical origin was evidenced. Two clusters (similarity >90%) were evidenced that corresponded to strains with potential epidemiological relatedness: these were two goat strains isolated the same year in the same region (F10330 and F9939 from herds 35 km away) or neighbouring regions (F10313 and F9865 from herds 150 km away). However, the other two groups of two strains with similarity >90% were less likely to be related. These were two goat strains isolated the same year but from two regions 300 km apart (F8988 and F9163) and one goat and one sheep strain (F10454, L15767, respectively) isolated in neighbouring regions at a 4-year interval. These discrepancies in a history of clustered strains underline the limits of RAPD PCR discrimination capacity as already observed in other studies (Maksimović et al., 2017). Moreover, despite our efforts, the RAPD intra-assay repeatability was poor.

Accordingly, the discriminatory power of another test based on polymorphisms in the gene coding for Hsp70 was evaluated. A total of 88 strains, including some from previous studies (Zhang et al., 2013; Maksimović et al., 2017), were compared by aligning a 537 nt sequence of the Hsp70 coding gene. A phylogenetic tree was inferred using the maximum likelihood method (Figure 2). A total of 48 sites were considered parsimony-informative, resulting in 77 patterns. In the consensus tree, goat isolates clearly gathered in a separate branch, while sheep isolates were split into several branches (Figure 2). This suggests that the Hsp70 coding gene could be a marker of the host specificity, as already proposed by Maksimović et al. (Maksimović et al., 2017). There were only four exceptions to this general grouping, with two ovine isolates retrieved within the caprine group (F11630 and Maksimovic_324) and two caprine isolates retrieved within the ovine group (F10330 and F8608). Several caprine strains appeared closely related.

Six strains, including two of the seemingly discrepant Hsp70 host-group strains, namely F10330 and F11630, further underwent a whole genome sequence analysis, and an SNP-based phylogeny was reconstructed using the NCTC 10151 genome as a reference (Supplementary Figure 2). The resulting maximum likelihood phylogenetic tree confirmed the clustering of strains as a function of

their animal host, whatever their geographical origin. However, within each group, the internal branch lengths were equivalent, suggesting a similar diversity for ovine and caprine strains.

The strain diversity analysis rules out the emergence of a clonal population and reveals sub-clustering depending on animal host. This should be considered in further AMR analyses.

MIC distribution of *M. ovipneumoniae* strains

M. ovipneumoniae MIC results are detailed in Supplementary Table 1, and distributions are represented in Figure 3. No significant evolution over time was evidenced whatever the drug considered, which is not surprising considering the short period of observation (2007–2018), so MICs were analysed globally. To discern a potential effect of the animal host species, which was shown to impact on the genetic clustering of strains, MIC distributions of goats and sheep were analysed independently (Figure 3).

For all the antimicrobials, a homogenous MIC distribution was evidenced with a dominant population below the intermediate clinical breakpoints for *Pasteurellaceae* (CLSI, 2015), except for florfenicol, with MIC distribution centred around the intermediate breakpoint (4 µg/mL). For oxytetracycline, tilmicosin and lincomycin, very few strains (1–5) showed increased MICs above the intermediate breakpoint. By contrast, the number of isolates with florfenicol MICs above the intermediate breakpoint (4 µg/mL) was high (n = 52), but their MICs increase was moderate with a maximum value of 8 µg/mL for nine isolates. Overall, our data suggest that *M. ovipneumoniae* isolates could be considered susceptible, with a few exceptions.

For florfenicol, tilmicosin, oxytetracycline and spectinomycin, sheep isolates had slightly higher MIC values than goat isolates, with a statistical significance of $p = 0.038$, $p = 0.005$, $p = 0.048$ and $p = 0.023$, respectively. These differences are consistent with the ban of florfenicol in lactating animals. However, this host-based difference in MICs needs to be cautiously interpreted as it never exceeded one dilution step.

One isolate, F10454, which was intermediate to florfenicol, and intermediate or resistant to tilmicosin and lincomycin, could be considered multi-resistant, *i.e.* non-susceptible to at least one agent in ≥3 antimicrobial categories excluding intrinsic resistance. Four isolates, namely F10435, F8588, L14811 and F10262, were non-susceptible to two classes, phenicols and macrolides. Cross resistance phenotypes with increased MICs to both macrolides and lincosamides, as in strain F10454, could be due to shared resistance mechanisms. However, this cross resistance to macrolides and lincosamides was not always the rule (see for instance isolates F10435 and F10262). These results prompted us to more closely investigate potential molecular mechanisms underlying resistance in isolates with increased MICs.

Molecular mechanisms of AMR

Resistance to tetracyclines and MLSK in animal *Mycoplasma* species is mainly associated with point mutations in ribosomal targets (Gautier-Bouchardon, 2018). To explore these potential mutation points in *M. ovipneumoniae* a set of isolates with high, intermediate or low MIC values were chosen for tetracyclines and MLSK, respectively (Table 1). For oxytetracycline, three groups of strains were explored: a reference population of five strains with MIC in the range ≤0.0625–0.125 µg/mL, a second group with four strains having increased MIC in the range 0.25–0.5 µg/mL and a third group with only two isolates having MICs exceeding or equal to 1 µg/mL (L16000, F11787). For MLSK and phenicols, 15 strains were tested, of which nine that had MICs in the range 0.5–8 µg/mL were considered as references, while six were considered as having increased MICs to one or several antimicrobial families.

Strain F10330, showing low MIC values, and the reference Y98 (NCTC10151) strain isolated in 1972 also with moderate MICs, were considered wild-type and their *rrs*, *rrl*, *rplD* and *rplV* genes were used as a reference. We considered *M. ovipneumoniae* strains to have only one *rrn* operon as it is the case for the type strain Y98 (NCTC10151), which is the only circularized genome available (reference sequence NZ_LR215028.1).

Positions 965, 966 and 967 (*E. coli* numbering) belonging to the Tet-1 binding pocket in the 16S rRNA (*rrs*) are identified in several *Mycoplasma* species as conferring a cumulative level of resistance after A->T, A->G or G->T substitutions (Amram et al., 2015; Khalil et al., 2017; Sulyok et al., 2017; Prats-van der Ham et al., 2018). In F10435 and F9820 harbouring the $A^{965}T$ substitution, MICs increased moderately, in the range 0.25–0.5 µg/mL, but this should be interpreted with caution as strain 14811 also had a 0.25 µg/mL MIC despite a wild type A^{965} genotype. In L16000 and F11787, the $A^{965}T$ transversion was associated with $A^{967}C$ mutation with a cumulative effect evidenced by MIC reaching respectively 1 µg/mL and 4 µg/mL. In F11787, a supplementary mutation outside Tet-1, namely $A^{1199}C$, could account for the higher MIC value. Outside Tet-1, no other mutation was evidenced except for $A^{1087}T$ in strain F8486 in association with a small MIC increase of 0.25 µg/mL.

Frequently described mutations in domains II and V of 23S rRNA gene (*rrl*) around positions 748 and 2058 were not evidenced in the test strains except for one transition $A^{2059}G$ in F10454 with an MIC ≥ 16µg/mL for both tilmicosine and lincomycine. In other isolates with increased MICs no mutations were evidenced in other positions previously associated with resistance in *Mycoplasma* spp. (534, 954, 1371, 1248, and 2447). However, it is of note that several positions of *M. ovipneumoniae rrl*, either corresponded to mutated nucleotides associated with MIC increases in *M. bovis* (A^{748} and T^{534}) or diverged from their counterpart, wild-type or not, in *M. bovis* G^{1371} and A^{752} when the transitions $C^{752}T$ and $C^{1371}T$ were associated with resistance in *M. bovis* (Lerner et al., 2014). Multiple alignments of the 23S rRNA within the *M. ovipneumoniae* isolates revealed no polymorphisms that were specific to isolates with increased macrolide MICs (≥ 8 µg/mL).

L4 and L22 proteins from *M. ovipneumoniae* are poorly conserved relative to those from *M. bovis* (identity of amino acids between Y98 and PG45 is 54% for L22 and 48% for L4), preventing useful comparison of point mutations. Amino acid sequences were thus explored within the *M. ovipneumoniae* species by comparing strains with low and high MICs (all strains sequenced by WGS in this study together with 14811 and Y98). Amino acid identity was higher for L4 (>97% in

aa) than for L22 (>94% in aa), which seems to be associated with a notably polymorphic region of L22 (position 117–123, internal numbering of aa) located outside the nucleotide binding site. No amino acid change seems clearly associated with increased MIC values. It should be noted that in position 77 of L4 (185 with *M. bovis* numbering), a change was present in F8588 in comparison to other strains ($Ser^{77}Leu$). In *M. bovis,* amino acid changes have been described in this position but their influence on MIC values seems unclear without association with *rrl* mutations (Gautier-Bouchardon, 2018). Being unobserved in other strains with similar MIC patterns, such as F10262, this amino acid change is thus unlikely to be associated with increased MIC.

In conclusion, mutations in hotspots (1–3 associated mutations with a cumulative effect) of the 16S rRNA could support the limited increase in tetracycline MIC values in *M. ovipneumoniae* strains. As for the 23S rRNA, the only strain with cross resistance to both macrolides and lincosamides harboured one hotspot mutation. In other strains with increased MIC for macrolides, no mutations in the 23S rRNA were evidenced, and the comparison of L4 and L22 did not evidence intra-species variations that accounted for increased MICs. However, the absence of low MLSK MIC values, even in older strains like Y98 collected in 1972, supports the hypothesis of a wild-type sequence of *rrl* associated with a potential low intrinsic resistance.

Discussion

Putative emergence and spillover of *M. ovipneumoniae*

In the last 12 years, a rise in *M. ovipneumoniae* detection has been observed throughout France through the Vigimyc surveillance network. No change in the diagnosis workflow, which could have improved detection, was made over that period, even though the first warning signs about *M. ovipneumoniae* (Poumarat et al., 2014) should have heightened awareness among veterinarians, who might then have sent more samples for diagnosis. This trend may also reflect a real increase in the incidence of *M. ovipneumoniae* in domestic species, as observed in other countries such as the UK, Sweden and the USA (Nicholas et al., 2008b; Lindström et al., 2018; Manlove et al., 2019).

369 The delayed surge observed in goats may result from a later outreach of *M. ovipneumoniae*
370 diagnosis in this neglected host or from a spillover from sheep, in conditions favouring its spread in
371 the goat reservoir. The spillover hypothesis is supported by the clustering of goat versus sheep
372 strains in our subtyping analyses, and by other examples of *M. ovipneumoniae* spillover from
373 domestic to wild species in France (to chamois as detected through Vigimyc surveillance, data not
374 shown) or in the USA where it was further associated with emergence (Kamath et al., 2019).

375 **Genetic diversity of *M. ovipneumoniae***

376 We then sought to determine whether the rise in *M. ovipneumoniae* diagnosis corresponded to a
377 clonal spread. The diversity of collected strains as assessed by RAPD-PCR was high but the low
378 repeatability of the technique prevented a reliable strain comparison. The genetic distance between
379 strains based on sequence analysis of the gene coding Hsp70 protein seemed more robust and
380 confirmed a high intra-species diversity and a strong influence of host species. The diversity of
381 strains together with the overall clustering according to the animal hosts are consistent with
382 previous findings (Parham et al., 2006; Zhang et al., 2013; Maksimović et al., 2017; Einarsdottir et
383 al., 2018; Kamath et al., 2019). Because of the few discrepancies in host-related clustering, we
384 selected a subset of strains to run whole genome comparisons. The WGS SNP phylogeny yielded an
385 unambiguous clustering of strains depending on their host species, whatever their geographical
386 origin. This suggests a strong co-evolution of the *M. ovipneumoniae* species with its animal host.
387 The six newly sequenced genomes will significantly expand the current set of 14 available genomes
388 for the species.

389 Interestingly, compared to the old Y98 type strain (Cottew, 1979), the evolutionary distance of our
390 strains was rather short and no major genomic evolution could be associated with putative
391 emergence over time. By contrast, in wild populations of *Caprinae*, where the disease has emerged,
392 a low diversity was evidenced as a result of spillover events followed by intra-species transmission
393 of geographical clones (Kamath et al., 2019). Hence the emergence in France is not linked to the
394 selection of a clonal population and might instead be found in other factors of putative emergence

such as a change in the animal host, e.g. through genetic selection of a breed or a change in breeding practices, such as antimicrobial use. Antimicrobial use is strongly driven by the animal species, age and production type, and differs substantially between young lambs or kids raised for fattening and lactating adults (with a potential withdrawal period). The availability of pre-mix forms and the cost of collective *vs.* individual treatment are also relevant. For example, tetracylines may be favoured over macrolides, in both species, because of a shorter withdrawal period for both milk and meat and the existence of pre-mix forms. However, it is not easy to document accurately the difference in use between goats and sheep, especially as for the latter only a few drugs currently have a marketing authorization. We undertook to analyse the antimicrobial susceptibility of goat *vs.* sheep strains.

Antimicrobial resistance

Several adaptations of the standard MIC protocols using the agar dilution method were made, where the reduction of agar concentration in the culture media proved successful for controlling the inoculum and colony morphology, despite the risk of punching the agar surface when using the multi-inoculator. This was overcome by several repetitions of the experiments.

Our study evidenced homogenous MICs distributions with low values mostly in the range ≤0.0625–8 µg/mL for all the antimicrobials tested. MICs remained dominantly below the intermediate breakpoint for *Pasteurellaceae* (CLSI, 2015), except for florfenicol, for which MICs were centred around this breakpoint. Whether this corresponds to an intrinsic or an acquired resistance could not be established owing to the absence of a wild-type population composed of old strains, except for the type strain Y98. Interestingly, *Pasteurellaceae* in both sheep and goats are highly susceptible to florfenicol (99%) (https://www.anses.fr/fr/system/files/LABO-Ra-Resapath2018.pdf) and so its use for treating mixed respiratory infection may favour *M. ovipneumoniae* in the chronic stage. Unfortunately, we did not get the chance to access specifically to the full data concerning presence of *Pasteurellaceae* (and their MICs) on the clinical samples out of which we isolated our *M. ovipneumoniae* strains. Our results are in overall agreement with the recent results of Maksimović

et al., i.e. strains with mostly low MIC values whatever the drug considered, although significant higher MIC values were obtained from goat strains with oxytetracycline, tylosin and enrofloxacin compared to sheep (Maksimović et al., 2020a). Some MIC discrepancies in the only common strain analysed in the two studies, namely Y98, were observed for aminosides, phenicols and macrolides. They may result either from methodological choices (agar *vs*. broth dilution method), highlighting the need to harmonise *Mycoplasm*a AST worldwide, or a difference in the strain variant stored in each laboratory.

Surprisingly, the host influence on MIC distribution remained limited, being statistically significant only for spectinomycin, oxytetracycline, tilmicosin and florfenicol but within a range of one dilution only. The host species difference evidenced by Maksimović et al. appeared more marked for enrofloxacin (MIC_{90} difference of three dilutions), oxytetracycline (seven dilutions) and tylosin (six dilutions), suggesting different patterns of use between the two countries (Maksimović et al., 2020a). In France, the situation of *M. ovipneumoniae* contrasts with that previously observed for the *M. agalactiae* species, one of the agents causing contagious agalactia syndrome in small ruminants (Poumarat et al., 2016), for which the animal host influence was marked for several drugs. Here, the limited effect prevented us from drawing any parallel with treatment practices.

The overall susceptibility of *M. ovipneumoniae* isolates contrasts with the status of *M. bovis*, a pathogen that has become resistant to several antimicrobial classes over the last 20 years (Gautier-Bouchardon, 2018) but is comparable to that of *M. agalactiae*, which has acquired moderate antimicrobial resistance over time, with a dominant low-MIC population (Poumarat et al., 2016). However, *M. agalactiae* strains reached higher values for tetracyclines in goats and for macrolides in both species (MIC_{90} of 8, 16 and 64 µg/mL respectively) than *M. ovipneumoniae*. *M. agalactiae* causes a variety of clinical signs including respiratory disorders, but also mastitis and arthritis, and this wider tropism might be one reason for different exposure of *Mycoplasma* species to antimicrobials, supporting differences in AMR when compared to *M. ovipneumoniae*.

Finally, MIC distributions of *M. ovipneumoniae* do not support any link between emergence and antimicrobial resistance, whatever the animal species considered.

Resistance mechanisms

For tetracyclines, point mutations detected in 16S rRNA of strains with moderately high MICs were consistent with observations in other *Mycoplasma* species (Gautier-Bouchardon, 2018). These include $A^{965}T$, $A^{967}T$ and $T^{1199}C$ as described in *M. bovis* (Amram et al., 2015; Khalil et al., 2017) as well as $A^{1087}T$ associated in *M. agalactiae* strains ($C^{1087}T$ on one allele) with an oxytetracycline MIC of 0.5 µg/mL, consistent with the effect observed here in the F8486 strain (Prats-van der Ham et al., 2018).

By contrast, for MLSK, only one strain of *M. ovipneumoniae* (F10454) with tilmicosin and lincomycin MICs ≥ 16 µg/mL harbours one mutation in domain V of the 23S rRNA, $A^{2059}G$, also observed in one allele of *M. hyopneumoniae* and *M. bovis* with tilmicosin MIC > 64 µg/mL (Lerner et al., 2014; Sulyok et al., 2017; Felde et al., 2018). In five other strains, the increase in MIC was high for tilmicosin (8–32 µg/mL) but moderate for lincomycin (2–4 µg/mL), a situation already observed in *M. agalactiae* in which it was linked to a mutation in the amino acid sequence of ribosomal proteins (Prats-van der Ham et al., 2017). In *M. ovipneumoniae*, ribosomal proteins and their amino acid sequences differ greatly from that of other *Mycoplasma* species, thus limiting potential comparison. However, intra-species alignment did not evidence any amino acid changes associated with this resistance to macrolides. This suggests the existence of other mechanisms accounting for the limited and progressive level of macrolide resistance, such as efflux pumps, which are rarely explored in *Mycoplasma* species. The mechanisms of florfenicol resistance in *Mycoplasma* have been under-explored, except for some substitutions in 23S rRNA proposed by Sulyok et al. for *M. bovis* (Sulyok et al., 2017). The absence of 23S rRNA mutations in all *M. ovipneumoniae* strains with an MIC of 8 µg/mL (except for F10454) also points to some other mechanism such as efflux or intrinsic resistance.

In conclusion, the occurrence of strains with higher MIC did not correspond to the onset of a marked AMR genotype that could emerge.

The absence of any markedly low MLSK MIC values, even in the Y98 reference strain isolated more than 40 years ago and thus expected to belong to the wild-type population, may point to a low basal intrinsic susceptibility. This can further be associated with the wild-type sequence of 23S rRNA, which included several nucleotides associated with increased MICs in other *Mycoplasma* species (A^{748}, T^{534}, G^{1371}, A^{752}). For instance, the $A^{534}T$ transversion in one *rrs* allele of *M. bovis* corresponds to an increase in tilmicosin MIC to 0.5 µg/mL (Sulyok et al., 2017).

Conclusion

In conclusion, the putative emergence of *M. ovipneumoniae* in respiratory mycoplasmosis in France is linked neither to a clonal spread nor to a marked increase in resistance associated with a particular AMR genotype. Continued monitoring of the spread and evolution of *M. ovipneumoniae* isolates in terms of diversity and susceptibility is needed in the years to come. Including wildlife isolates would be useful as these might play a role in the population dynamics as demonstrated in the USA (Kamath et al., 2019). This follow-up will rely on improved, harmonised diagnosis, including AMR with notably the potential determination of epidemiological cut-off values to interpret the MICs.

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Table 1: Polymorphisms in *rrs* (16S rRNA, in and outside the main tetracycline binding site), *rrl* (23S rRNA, domains II and V) genes in relation to MIC values for *M. ovipneumoniae* strains

		Tetracyclines			MLSK, Phenicols				
		MIC (µg/mL)	*rrs* [a]		MIC (µg/mL)			*rrl* [a]	
Isolate name	Animal host	Oxytetracycline	Tet-1	Outside Tet-1	Tilmicosine	Lincomycin	Florfenicol	domain II	domain V
F10330	**Goat**	**≤0.0625**	**wt**	**wt**	**4**	**1-2**	**4**	**wt**	**wt**
F11514	Goat	≤0.0625	/	/	4	2	4	nd	nd
F8525	**Goat**	**≤0.0625**	**/**	**/**	**4**	**2**	**2-4**	**/**	**/**
F8588	**Goat**	**≤0.0625**	**/**	**/**	**16**	**4**	**4-8**	**/**	**/**
L15948*	Goat	0.0625	nd	nd	2-4	1-2	4	/	/
F10943*	Goat	0.0625-0.125	nd	nd	2-4	2	2	/	/
F8486	Goat	0.25	/	$A_{1087}T$	2-4	2	4	nd	nd
F10454*	Goat	0.25	nd	nd	≥16	≥16	8	/	$A_{2059}G$
L16000*	Goat	1	$A_{965}T$; $A_{967}C$	/	4-8	2	4	nd	nd
F10297	Sheep	0.0625-0.125	nd	nd	2	2	4	/	/
Y98	Sheep	0.125	wt	wt	4	2-4	4	wt	wt
F11630	**Sheep**	**0.125**	**/**	**/**	**4**	**2**	**4-8**	**/**	**/**
F10803	Sheep	0.125	nd	nd	8-16	4	4	/	/
F10262	Sheep	0.125	nd	nd	16	4	4-8	/	/
14811	**Sheep**	**0.25**	**/**	**/**	**16**	**4**	**8**	**/**	**/**
F9139	Sheep	0.25	nd	nd	4	1	2	/	/
F9820	**Sheep**	**0.25**	**$A_{965}T$**	**/**	**4**	**2**	**4**	**/**	**/**
F10435	**Sheep**	**0.25-0.5**	**$A_{965}T$**	**/**	**16-32**	**2**	**8**	**/**	**/**
F11787*	Sheep	4	$A_{965}T$; $A_{967}C$	$T_{1199}C$	8	2	4	nd	nd

For *rrs*, polymorphisms were screened in between nucleotides 853-1538 for strains sequenced using Sanger method ('*',indicates two sequencing replicates) or along the entire allele for WGS sequenced strains (in bold). For *rrl*, mutations points were screened in between nucleotides 510-877 and 1820-2170 for strains sequenced using Sanger method or along the entire allele for WGS sequences strains. Within each animal host, isolates are sorted by increasing MICs for oxytetracycline and then tilmicosin.

[a] *Escherichia coli* numbering of nucleotides. The first letter indicates the wild type nucleotide (wt, strain F10330 and Y98 were used as susceptible reference genome), the number indicates the mutation position and the second letter indicates the substitution. The absence of mutation compared to the wild-type genotype is represented by "/"; nd, not done. Grey background emphasizes increased MIC values.

620 **Figure captions**

621 **Figure 1:** Annual number of respiratory isolates (black or white bars for goats and sheep,
622 respectively) and *M. ovipneumoniae* positive respiratory isolates (stripped black bars for goats and
623 dotted black bars for sheep) as collected through Vigimyc network between 2007 and 2019.

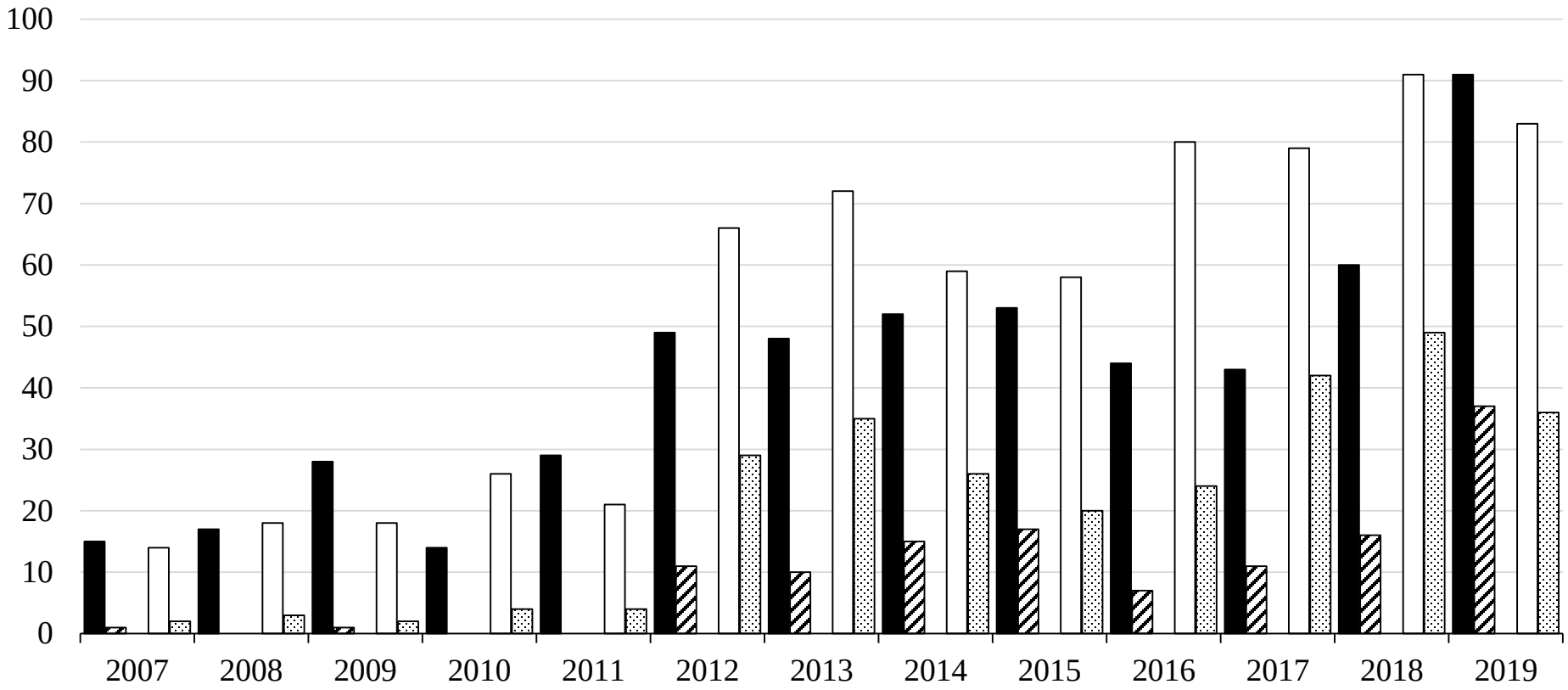
624 **Figure 2:** Maximum Likelihood phylogeny tree of 88 *M. ovipneumoniae* strains based on the
625 alignment of 537 bp-locus of the gene encoding Hsp70.

626 Values on branches are ultrafast bootstrap support (%) (1000 bootstraps). The scale bar indicates
627 the number of substitutions per site. A total of 49 parsimony informative sites were used that
628 generated 78 distinct patterns, using the TIM2+F+I+G4 model. The tree is rooted using the Y98
629 (NCTC10151) strain. The different strains are mentioned by their name followed by their country of
630 origin (ISO 3166 country codes) and department for French isolates, the year of isolation and the
631 host species. uk, unknown; CA, caprine; OV, ovine. The branch gathering all caprine isolates is
632 framed and isolates positioned in a branch not corresponding to their animal host are highlighted in
633 red.

634 **Figure 3:** Comparison of Minimal Inhibitory Concentration (MICs) distribution of six
635 antimicrobials for *M. ovipneumoniae* isolates from sheep (n=28) or goats (n=28).

636 X-axis MICs in µg/mL; Y-axis, number of strains, black and white bars represent goats and sheep
637 isolates, respectively. P <0.05 indicates a significant difference in MIC distributions between the
638 two populations of isolates. The arrows on X-axis indicate *Pasteurellaceae* intermediate clinical
639 breakpoint for cattle (CLSI, 2015).

640 For MICs expressed as a range, a 0.5 occurrence was attributed to the lower and upper limit of the
641 range (one dilution interval at most).



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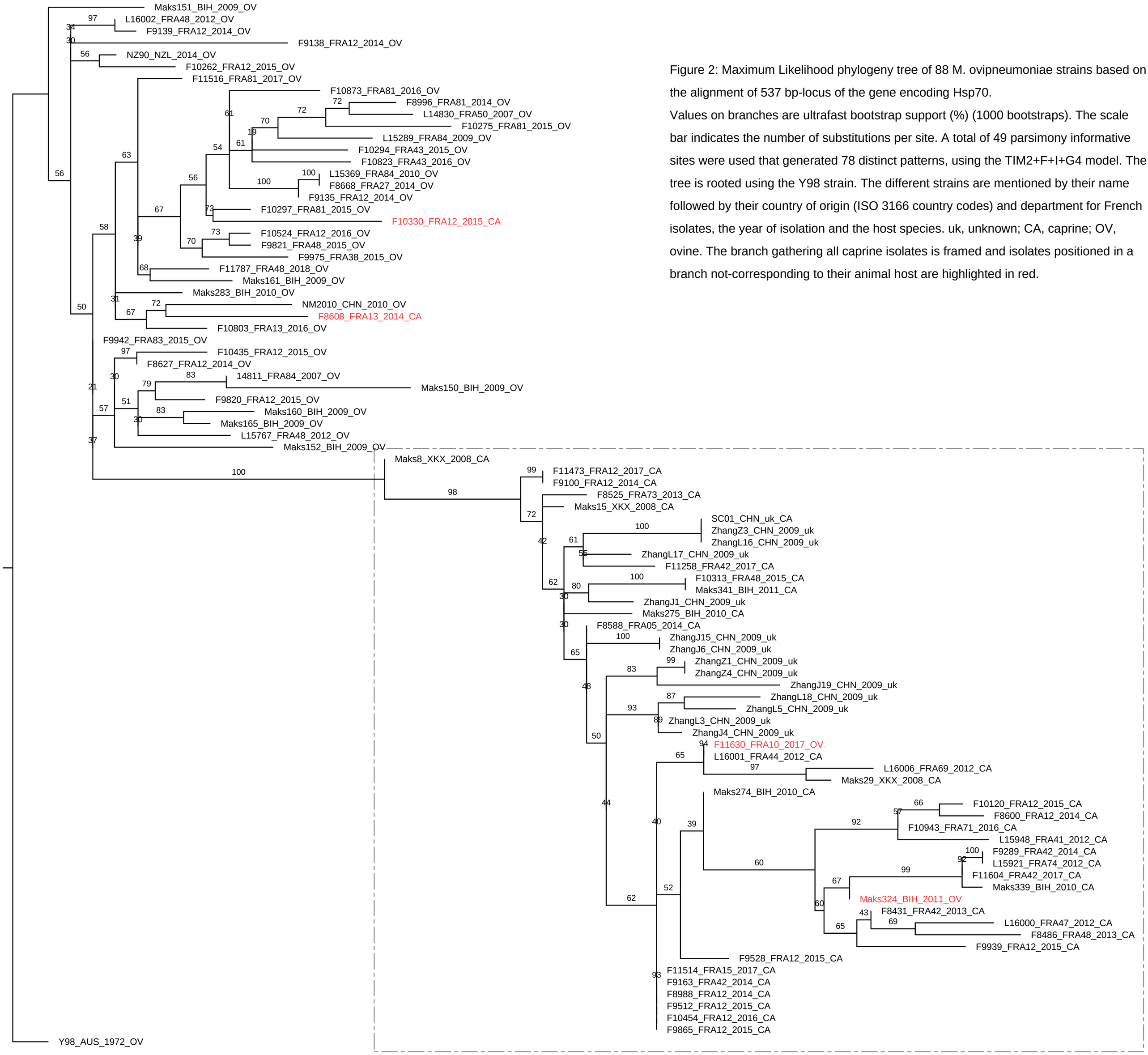

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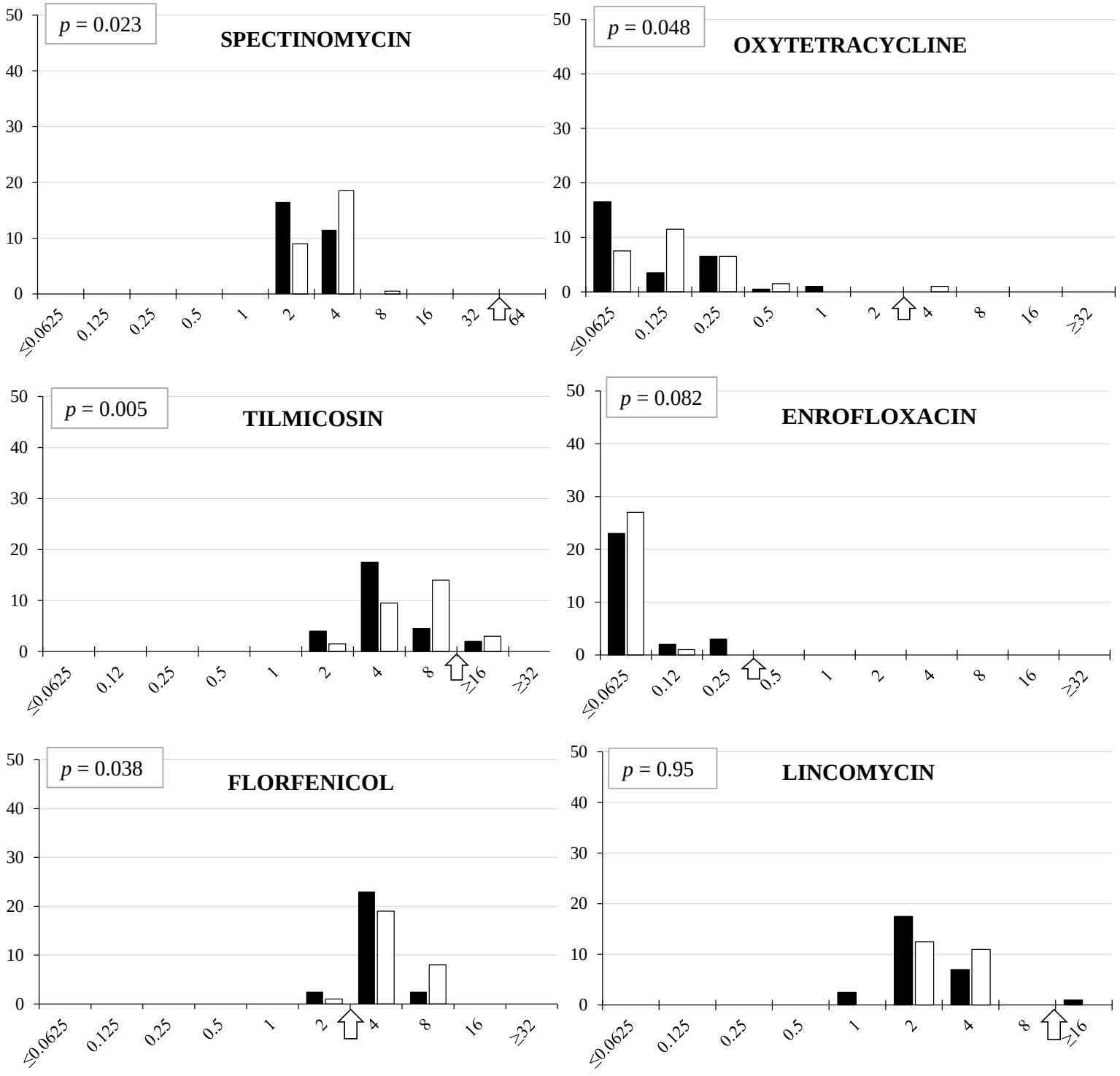

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