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Purpose and overview of results of the Vigimyc Network for the epidemiological surveillance of mycoplasmoses in ruminants in France

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Mycoplasmas cause several diseases in ruminants including three that are registered as diseases of concern internationally by the World Organisation for Animal Health (OIE). The Vigimyc Network was established in France to monitor the status of these regulated diseases and of other economically harmful mycoplasmoses. Vigimyc maintains a collection of strains that is representative of the epidemiological picture nationally and this collection is regularly used to develop and validate diagnostic tests and for various studies aimed at improving knowledge of mycoplasmas, including their antimicrobial susceptibility and their pathogenicity.

Introduction

The class Mollicutes that gathers bacteria characterised by their small size and absence of a cell wall, is essentially represented in animals by species of the *Mycoplasma* genus. In ruminants, about forty species and sub-species of mycoplasmas have been described, including some that are pathogenic.

Three mycoplasmoses have economic consequences and an impact on trade that is sufficiently serious to warrant international control measures and classification of these diseases by the World Organisation for Animal Health (OIE). On the one hand, there are two «exotic» mycoplasmoses that pose a threat of re-emergence or emergence in France: contagious bovine pleuropneumonia (CBPP) and contagious caprine pleuropneumonia (CCPP) and, on the other hand, contagious agalactia (CA) that is widespread in southern Europe. CBPP is caused by *Mycoplasma (M.) mycoides* subsp. *mycoides* (*Mmm*). The disease originated in Europe and became a major worldwide panzootic in the 19th century. It is still highly prevalent in Africa and sporadic in Asia. In Europe, the implementation of an eradication programme in the 1980s and 1990s following widespread resurgence has led to no outbreaks having been recorded since 1999. CCPP is caused by *M. capricolum* subsp. *capripneumoniae* (Mccp). It was thought that this disease was limited to north-east Africa, but improved diagnostic methods have demonstrated that it is far more widely spread through Africa and Asia up to the borders of Europe, and that wildlife is possibly infected, particularly zoo animals. CA is a complex syndrome characterised by mastitis, arthritis, pneumonia and septicaemia. It is present worldwide with a strong impact in the Mediterranean area. It can be caused by several mycoplasmas: *M. mycoides subsp. capri (Mmc)*, *M. capricolum* subsp. *capricolum (Mcc)*, *M. putrefaciens* in goats and *M. agalactiae* in sheep and goats.

Although they are not listed by the OIE, *M. bovis* mycoplasmoses have become significant with the development of trade and herd mixing related to modern cattle farming. They manifest in a highly proteiform manner with mastitis, arthritis, otitis and pneumopathies. Bronchopneumonia in young cattle poses problems internationally and mastitis or even otitis are becoming frequent and an economic burden in some countries, particularly in North America.

In addition to these major diseases, other mycoplasmoses are beginning to cause concern. *M. ovipneumoniae* in small ruminants is considered to be an important factor in respiratory disease in some countries. *M. leachii*, reported sporadically in Europe, has been found to be highly pathogenic in cattle in China and in Australia (arthritis, abortions, mastitis). *M. canis* and *M. alkalescens* are also thought to be pathogenic in cattle. Moreover, many other saprophytic mycoplasma species are found in ruminants and are sometimes abundant. Isolation of a mycoplasma is therefore of no clinical relevance unless the specific species or sub-species is identified.

In order to monitor these diseases, we created an epidemiological surveillance network for mycoplasmoses in ruminants in France in 2003, the Vigimyc Network. This article describes the organisation of this network, presents an overview of results obtained over the last 5 years, and the scientific benefits resulting from Vigimyc.

Objectives and organisation of the Vigimyc Network

Vigimyc was originally designed as a diagnostic support service to stimulate diagnosis of mycoplasmoses, but has subsequently evolved and now covers a significant part of the country, and can therefore be considered a surveillance network, despite certain methodological limitations.

Vigimyc has the following objectives:

- to identify mycoplasma species isolated in ruminants;
- to determine the epidemiological picture and follow-up on mycoplasmoses in ruminants across France, particularly those that are listed by the OIE;
- to detect any emergence of new mycoplasma species or variants;
- to share scientific and technical data regarding mycoplasmas;
- to build up and make use of a collection of nationally representative strains.

Vigimyc is administered by ANSES-Lyon Laboratory and supervised by a steering committee made up of representatives from all the stakeholders in the network: participating laboratories, public authorities, practicing veterinarians, farmers and scientists.

Vigimyc is a «passive» surveillance network since the decision to test for mycoplasmas is solely the initiative of the practicing veterinarian. Mycoplasma detection by subculture from clinical specimens is carried out by departmental veterinary diagnostic

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laboratories with some technical guidance from the network which is regularly updated during inter-laboratory testing. When mycoplasmas are isolated, the primary culture is sent to us for identification, along with a standard registration sheet containing background data on the sample. Identification is carried out using dot immunobinding on membrane filtration (Poumarat *et al.,* 1991). Each primary culture is tested using a battery of hyperimmune sera representative of the main mycoplasma species found in ruminants and a specific monoclonal antibody against the agent causing CBPP. If the result is ambiguous or negative, additional testing is carried out by specific PCR protocols or PCR and/or sequencing of various "housekeeping genes". The result is then forwarded to the requester laboratory. All epidemiological data and identification results are centralised in a database and a summary is provided annually to the participants and members of the steering committee.

Current trends concerning mycoplasmoses in ruminants observed through Vigimyc over the last five years

Overall analysis

Key figures are presented in Tables 1 and 2. Over the period 2009-2013, 46 veterinary diagnostic laboratories participated in the network. A total of 1938 primary mycoplasma cultures from 1526 outbreaks were sent to ANSES Lyon, resulting in identification of 2105 isolates, taking into account mixes of species. These isolates were from 77 different French *départements*, i.e. 80% of the country, and 44% involved cattle, 38% goats, 12% sheep and 6% wildlife, primarily ibex.

In cattle, most isolates were from respiratory diseases in young animals and M. bovis was the most commonly identified mycoplasma. In goats, the isolates were mainly from contagious agalactia cases, with *Mmc, Mcc* and *M. putrefaciens* the most commonly isolated mycoplasmas. In sheep, isolates came primarily from respiratory disorders in lambs, with increasing isolation of *M. ovipneumoniae*. In mountain ungulates, isolates were either from pneumopathy lesions with an *M. agalactiae* characterisation, or from nasal or ear swabs, indicating high carrier levels of *M. feriruminatoris* in healthy animals.

Two non-pathogenic mycoplasma species, *M. bovirhinis* in cattle and *M. arginini* in all ruminants, were frequently isolated, alone or in combination, and are of no diagnostic significance.

There are no major changes since the last overview for 2003-2008 (Chazel *et al.,* 2010), with the exception of increased isolation of *M. ovipneumoniae* in sheep.

Picture for mycoplasmoses listed by the OIE

The specific agent for contagious bovine pleuropneumonia (CBPP), which was tested for systematically, was found in no animal species, whether cattle, its usual hosts, or small ruminants that can be occasional hosts.

There are three types of contagious agalactia (CA) caused by M. *agalactiae* in France: one in sheep, limited to the milk-producing area in the western Pyrenees and increasing sharply since 2006; one in goats that occurs sporadically across the country (11 outbreaks in eight départements between 2009-2013); and one in wildlife found in the Alps following an episode of mortality related to bronchopneumonia in ibex and chamois populations.

Caprine CA caused by *Mmc, Mcc* or *M. putrefaciens* has been found to be highly prevalent. It is mostly caused by *Mmc*, but the annual rate of *Mcc* isolation fluctuates significantly (higher than Mmc in 2013). *M. putrefaciens* is less frequent and is mainly related to mastitis. *Mmc* is also found sporadically in cattle and sheep. Some severe clinical forms of CA are very similar to contagious caprine pleuropneumonia (CCPP). Because it is difficult to grow *Mccp* on commercially available media, these outbreaks could go undetected. As a result, corresponding information has been distributed widely via Vigimyc so that any outbreak of serious pneumopathy along with high morbidity and mortality in goats would be reported to ANSES. The Agency would then be able to carry out a specific PCR test for CCPP directly on pleural fluid or the lung tissue without prior enrichment. In this way, two suspected cases were registered between 2009 and 2013 but were found to be related to *Mmc*.

Picture for *M. bovis* mycoplasmoses

M. bovis is the most commonly isolated mycoplasma in cattle in France but it is mainly found during pneumopathies, with an overall prevalence estimated at 15% on the basis of a one-off survey in 2013 among Vigimyc laboratories. The other clinical forms, mastitis, arthritis and otitis are far more infrequent. Only four outbreaks of mastitis across four départements were reported between 2009 and 2013. This very low to non-existent incidence does not appear to be an under-estimation bias since a study involving systematic testing on bulk tank milk in the Rhône-Alpes region arrived at the same conclusion (Arcangioli *et al.,* 2011). Arthritis is often associated with respiratory diseases while sporadic otitis outbreaks are starting to be identified by Vigimyc (eight outbreaks in three *départements* between 2009 and 2013).

Other mycoplasmoses

Until recently, *M. ovipneumoniae* was rarely isolated, even though very frequent co-infection with *M. arginini,* a fast-growing mycoplasma, could mask this infection. However, since 2010 and even though procedures have not changed, a much higher number of cases has been found in respiratory disease in small ruminants. Two hypotheses have been put forward to explain this progression: either evolution of the strains of interest, or a change in the type of samples being studied. Samples currently come primarily from lambs grouped for finishing, with the concentration of animals promoting high infection pressure.

Two species, *M. canis* and *M. alkalescens*, have emerged in cattle and are developing strongly in the United Kingdom and in some other European countries. They are thought to be involved in respiratory disease and for *M. alkalescens*, also in arthritis and mastitis. A retrospective study on older collections at ANSES has shown that these mycoplasmas have been present in France for a long time, with the oldest isolations dating from 1965 and 1993. Moreover, no real progression has been found in France for a decade.

No isolate with a profile indicative of *M. leachii* was detected in cattle, sheep or goats.

Scientific use of biological material generated by Vigimyc

Although the collection of mycoplasma strains assembled as part of Vigimyc cannot claim to be a biological resource centre, it does constitute a notable representative selection of the epidemiological picture in France over time, and reflects the biological diversity of mycoplasmas in ruminants. It enables method development and validation not only for diagnosis, surveillance and molecular epidemiology, but also up-stream, for analysis of changes and virulence factors in mycoplasmas.

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Development and validation of detection and identification methods for mycoplasmas

Since strains are constantly evolving and diagnostic techniques constantly improving, stakeholders working within the frame of Vigimyc need to regularly validate detection and identification methods for mycoplasmas. Indeed, degrees of specificity and versatility of techniques are sometimes called into question when they are assessed using large numbers of diverse samples, such as those provided by Vigimyc (Le Grand *et al.,* 2004; Marenda *et al.,* 2005). These difficulties are related to the high level of genomic plasticity in mycoplasmas (Marenda, 2014) which leads to sometimes significant diversity within (sub) species, despite sometimes close phylogenetic relationships between (sub)species. For example, denaturing gradient gel electrophoresis (DGGE) of sequences of 16S rRNA previously amplified by PCR, a technique that is widely used in the UK as the new universal method to identify mycoplasmas, has been found to be insufficiently selective for certain (sub)species that are very closely related phylogenetically in the more complex French epidemiological context (Tardy *et al.,* 2008). In contrast, MALDI-TOF-type mass spectrometry, tested more recently, appears to be very promising (Pereyre *et al.,* 2013). This technique can identify microorganisms by comparing their predominant protein profiles with an array of reference spectra. The Vigimyc collection is an excellent tool for regularly verifying the completeness and accuracy of this array.

Regular efforts are also made to develop new diagnostic techniques that are suited to the national epidemiological picture. For instance, a real-time PCR method, able to detect and identify the four etiological agents of CA simultaneously, has been developed and made available as a commercial kit as part of a partnership with a private company (Becker *et al.,* 2012). Furthermore, a PCR technique used for health watch has been designed to unequivocally distinguish between *Mcc*, frequently isolated in caprine CA, *M. leachii,* and above all *Mccp*, the agent underlying CCPP, through direct detection in clinical samples (Maigre *et al.,* 2008).

Molecular subtyping of strains

Molecular subtyping can be very useful in epidemiology and in disease control. The collection of strains stemming from Vigimyc is very useful in this way to compare strains from different times, hosts, diseases and regions. For example, subtyping the various isolates of *M. agalactiae* from our collection, specifically by Multiple Locus Variable number tandem repeat Analysis (MLVA) and macro-restriction followed by Pulsed Field Gel Electrophoresis (PFGE), has improved understanding of *M. agalactiae* CA at the national level. In particular, it was established that the various waves of ovine CA in the milk-producing region of the Pyrenees, including the most recent wave, were all the resurgence of a single clone located in this high livestock density region for at least the past 30 years (Nouvel *et al.,* 2012). In contrast, the strains of *M. agalactiae* isolated from sporadic caprine outbreaks are highly diversified, indicating a diffuse long-term enzootic in the country. More recently, strains of *M. agalactiae* isolated from Alpine ibex were found to be: i) very similar to one another, but ii) different from strains historically responsible for domestic CA in goats in the same valleys in Savoie, and iii) atypical compared to all currently known domestic strains, indicating an enzootic that is probably long-standing and specific to wild ungulates (Tardy *et al.,* 2012). In *Mmc* caprine CA, asymptomatic carriage and shedding appear to be frequent, with the outer ear in goats forming a favoured site where several strains or even species of mycoplasmas coexist (Mercier *et al.,* 2007). A series of surveys performed in partnership with ANSES Niort Laboratory provided an estimate of the prevalence of carrier level and allowed to collect strains that were not accessible *via* Vigimyc. In herds with no known history of mycoplasmosis, on average 8% of animals were *Mmc* carriers in the outer ear, and 5% of bulk tank milk was positive for *Mmc* (Tardy *et al.,* 2007). Subtyping of the various strains of *Mmc* by PFGE and micro-restriction followed by Southern Blot analysis of the insertion sequence profile showed, i) very high polymorphism in ear strains, ii) coexistence of several clones in healthy animals or in herds with no associated clinical signs, and, in contrast, iii) circulation of a single clone during a disease episode. Nonetheless, no difference was found between the carrier strains and clinical outbreak strains from Vigimyc, whether genetically or in terms of experimental virulence potential (Tardy *et al.,* 2010). As such, *Mmc* mycoplasmoses in goats appear to be latent enzootic infections with sporadic emergence of pathogenic strains. In these circumstances, applying a purely health-based prophylactic programme would seem bound to fail (Tardy *et al.,* 2007).

Given its current operational framework, Vigimyc is very effective at monitoring strain evolution from an antigenic and/or genetic point of view. Detailed characterisation of atypical strains is essential in order to maintain health watch that takes into account the genomic diversity of the various strains and their evolution, as well as emergence of new species or variants. Recently, a strain isolated from a clinical sample of caprine arthritis reacted with the specific monoclonal antibody targeting *Mmm*, the causative agent of CBPP. After molecular assessment, it was found to belong to the *Mmc* species. This cross-reaction was alarming bearing in mind that goats could be an occasional reservoir for CBPP, and could have cast doubt on the reliability of the serological screening test recommended for CBPP which is based on a competitive ELISA assay using the target epitope of this monoclonal antibody. A study of the variability of the epitope coding region in all the strains of *Mmc* enabled us to demonstrate that the probability of false positive clones is very rare and random (Tardy *et al.,* 2011) and therefore does not cast doubt on the reliability of screening, nor on the Vigimyc surveillance strategy in France.

Antimicrobial susceptibility

Unlike many other pathogenic bacteria in ruminants, mycoplasmas are not included in various surveillance networks for antimicrobial resistance since evaluating their antibacterial susceptibility requires specific techniques that are not available routinely in partner laboratories. However, reports from the field regularly indicate treatment failures with progression to chronic disease. In view of this, we used the strains available via Vigimyc to evaluate the current level of susceptibility of mycoplasma strains.

The first species tested was *M. bovis* which is often involved in infectious enzootic bronchopneumonia of calves, a multifactorial disease requiring large quantities of antibiotics and for which no thorough evaluation had been performed in France for 20 years. Using our strain collection, partly collected through Vigimyc, we were able to compare minimum inhibitory concentrations of various antibiotics used in veterinary medicine and likely to be active against mycoplasmas in 27 older isolates (1978-1979) and 46 recent isolates (2010-2012) of *M. bovis* from 73 separate outbreaks of infectious enzootic bronchopneumonia across France (Gautier-Bouchardon *et al.,*

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2014). A statistically representative loss of sensitivity was found for 8 antibiotics among 100% of contemporary strains. As a result, if we consider critical values accepted for pathogenic bacteria in the respiratory area in cattle, all contemporary mycoplasma strains would be classified as “resistant” to macrolides, tetracyclines, spectinomycin and florfenicol, and “intermediate” for fluoroquinolones. Bearing in mind that mycoplasmas are naturally resistant to all antibiotics that act on the cell wall (beta-lactams and glycopeptides), the therapeutic armamentarium against *M. bovis* mycoplasmoses would be extremely limited. Evaluation of the baseline level of resistance of other mycoplasma species to the various antibiotics used today is underway.

Use of strain diversity to develop knowledge on the Mycoplasma genus

The collection of strains obtained through Vigimyc provides biological resource for a number of research projects on the evolution of mycoplasmas, borders between species, virulence of strains, etc. In return, the information obtained is used to adjust our microbiological surveillance of mycoplasmoses. An example is the EVOLMYCO project (ANR-07-GMGE-001). It has provided the scientific community with 20 additional ruminant mycoplasma genome sequences, among which 8 correspond to strains from the Vigimyc collection (Dordet-Frisoni *et al.,* 2013; Dupuy *et al.,* 2013; Manso-Silvan *et al.,* 2013; Tardy *et al.,* 2012). Initial results from comparative genomics are forcing us to rethink current knowledge in mycoplasmology, and show that very few families of genes clearly distinguish strains based on their pathogenicity or their host. In addition, significant levels of horizontal gene transfer (HGT) between species that are not closely related but share the same ecological niche have been suggested *in silico* (Sirand-Pugnet *et al.,* 2007), calling into question the idea that mycoplasmas evolved primarily through downsizing, with massive gene losses. Our collection of strains has enabled us to look for potential vectors of HGT. Plasmids are minor contributors to HGT (Breton *et al.,* 2012), but integrative and conjugative elements (ICEs) appear far more promising and their inter-strain transfers have recently been reproduced *in vitro* (Dordet Frisoni *et al.,* 2013). Today, mycoplasmas appear to be genetic mosaics (Marenda, 2014). We have shown that the *M. leachii* species is in fact a genomic chimera between the *capricolum* and *mycoides* species and represents an excellent example of the genetic continuum between strains, beyond species borders (Tardy *et al.,* 2009). This new concept could call into question the very notion of species and thereby the taxonomy currently used to diagnose animal mycoplasmoses. In these conditions, diagnosis will probably move more toward an overall approach to mycoplasma diseases, with detection of trans-taxon virulence markers.

Table 1. Key figures for the Vigimyc Network for the 2009-2013 period: Number of treated outbreaks, studied animal species, types of animals and frequency of the various diseases of interest

	Hosts			
	Cattle	Goats	Sheep	Wildlifee
Volume of analyses and origin of strains				
Number of départements providing samples	62	55	34	1
Number of analysed isolates	856	725	237	120
Number of outbreaks	735	511	192	89
Distribution of samples based on host animal age (%)				
Adult animals	7	71	18	80
Young animals	76	18	57	5
Animal of unknown age	17	11	25	15
Distribution of samples based on type of disease (%) (Disease present alone or in combination with other clinical signs)				
Respiratory disease	89	24	68	32
Mastitis	2	36	2	0
Arthritis	2	15	2	0
Otitis	1	0	0	0
Septicaemia	0	2	0	0
Abortion	0	0	3	0
Eye disease	0	0	3	4
Unknown disease	5	19	20	21
Health follow-up	0	0	0	43
No disease	1	4	2	0

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Conclusion

The Vigimyc Network is unique in continental Europe, with only the UK having a comparable system. After a decade of operation, Vigimyc has largely fulfilled its initial objective of reviewing the epidemiological situation of regulated mycoplasmoses and mycoplasmoses with an economic impact in ruminants nationally. With its current organisation, characterised by an overall approach to all mycoplasmas and mycoplasmoses, it is perfectly suited to future changes in diagnosis and surveillance. Its strong point in the last few years has been above all the efforts to make the most of the strain collection generated by Vigimyc. This is probably the area in which Vigimyc will strengthen its activities by transferring its diagnostic role to partner laboratories, a change that has become possible thanks to recent technical innovations.

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Table 2: Distribution of the 2105 isolates (including mixes of species) identified between 2009 and 2013 based on animal species

	Host animal								
	Cattle (n=1029)		Goats (n=703)		Sheep (n=275)		Ibex (n=98)		Total
(sub)-species of mycoplasma	n	%	n	%	n	%	n	%	n
Pathogenic									
M. agalactiae	0		29		2		16	16.3	47
M. bovis	488	47.4	0		1		0		489
M. capricolum subsp. capricolum	0		192	27.3	0		1		193
M. capricolum subsp. capripneumoniae	0		0		0		0		0
M. leachii	0		0		0		0		0
M. mycoides subsp. capri	5		288	41	6		0		299
M. mycoides subsp. mycoides	0		0		0		0		0
M. putrefaciens	0		85		0		1		86
Unclear pathogenic potential									
M. alkalescens	34		0		0		0		34
M. canadense	13		0		0		0		13
M. canis	5		0		0		0		5
M. conjunctivae	0		0		6		0		6
M. feriruminatoris subsp. nov.	0		0		0		72	73.5	72
M. ovipneumoniae	0		24		78	28.4	0		102
Opportunistic									
Acholeplasma laidlawii	3		1		0		0		4
M. arginini	121		75		180	65.5	6		382
M. auris	0		3		0		2		5
M. bovigenitalium	10		1		2		0		13
M. bovirhinis	350	34	0		0		0		350
M. edwardii	0		1		0		0		1
M. yeatsii	0		4		0		0		4

n=number of isolates; %=proportion of isolates by host animal (the proportion is given only for the two most common (sub)-species).

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