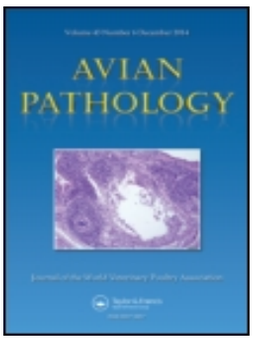

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Minimal inhibitory concentration of seven antimicrobials to *Mycoplasma gallisepticum* and *Mycoplasma synoviae* isolates from six European countries.

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Abstract

Mycoplasma gallisepticum and *Mycoplasma synoviae* are bacterial pathogens that cause disease in poultry, adversely affecting their health and welfare, and are a financial burden on producers. This MycoPath project is the first international antimicrobial susceptibility programme for mycoplasma pathogens isolated from poultry. Improved comparative analysis of minimal inhibitory concentration (MIC) results from participating countries was facilitated by using one laboratory determining all MICs. Chicken and turkey isolates were obtained from France, Germany, Great Britain, Hungary, Italy and Spain during 2014-2016. One isolate per farm was retained. The MIC of seven antimicrobial agents was determined using a broth microdilution method, with Friis Medium (*M. gallisepticum*) or Chanock's Medium (*M. synoviae*). Of the 222 isolates recovered, 82 were *M. gallisepticum* and 130 were *M. synoviae. M. gallisepticum* $MIC_{50/90}$ values were 0.12/0.5, 2/8, 0.5/4, 0.12/>64, 0.008/0.062, 0.008/32, 0.062/4 mg/L for doxycycline, enrofloxacin, oxytetracycline, spiramycin, tiamulin, tilmicosin and tylosin, respectively; and 0.5/1, 8/16, 0.5/1, 0.5/8, 0.25/0.5, 0.062/2 and 0.062/16 mg/L respectively for *M. synoviae.* A bimodal MIC distribution for the fluoroquinolone, enrofloxacin and the macrolides, spiramycin, tilmicosin and tylosin indicate that both species have sub-populations that are less susceptible *in vitro* to those antimicrobials. Some differences in susceptibilities were observed according to host species, *Mycoplasma* species, and country of origin. This study provides a baseline of novel data for future monitoring of antimicrobial resistance in poultry *Mycoplasma* species. Additionally this information will facilitate selection of the antimicrobial agents most likely to be effective, thus ensuring their minimal use with targeted and correct therapeutic treatments.

Key words: antimicrobial resistance, MIC, chicken, turkey, broth microdilution, *Mycoplasma gallisepticum, Mycoplasma synoviae*

Highlights:

- A European resistance monitoring survey for *Mg and Ms* from chickens and turkeys.

- Susceptibility of 82 *Mg* and 130 *Ms* strains to 7 licensed antibiotics was variable.

- Interpretation of mycoplasma MICs is problematic due to missing breakpoints.

Introduction

Mycoplasma gallisepticum and *Mycoplasma synoviae* are bacterial pathogens that cause disease in poultry (Ferguson-Noel, 2013). Their potential adverse health and economic impact on poultry production is so significant that these two *Mycoplasma* species are listed by the World Organization for Animal Health (OIE, 2018). *M. gallisepticum* is also included in the European Council Directive (European Council Directive 2009/158/EC) that facilitates trade between European countries. In 2018 the European poultry industry supplied 15,776,000 tons of poultry meat (AVEC, 2019) and 6,755,000 tons of eggs (European Commission, 2019).

M. gallisepticum causes chronic respiratory disease of domestic poultry, especially in the presence of management stresses and/or other respiratory pathogens. Disease is characterised by lachrymation, conjunctivitis, sneezing, cough, and by sinusitis, particularly in turkeys and game birds. Airsacculitis and pneumonia are considered the gross lesions related to *M. gallisepticum*; *M. gallisepticum* infection can result in loss of production and increased carcass condemnation in meat poultry, and a loss of egg production in layers. Transmission of *M. gallisepticum* infection occurs either vertically (*in ovo*) from an infected breeder flock to the progeny or horizontally by direct or indirect contact of susceptible birds with infected carriers or contaminated debris (Levisohn and Kleven, 2000). *M. synoviae* causes respiratory disease, synovitis, or may result in a silent infection. *M. gallisepticum* and *M. synoviae* strains vary in infectivity and virulence, and infections may sometimes be unapparent (Jordan, 1975; Kleven, 1998; OIE, 2018). In recent years, eggshell apex abnormalities have also been linked to *M. synoviae* infections (Feberwee *et al.,* 2009; Catania *et al.,* 2010). Catania *et al*. (2016) demonstrated a significant difference in daily egg mean weight and the number of eggs in *M. synoviae* experimentally infected birds.

The poultry industry has a number of approaches to maintaining healthy flocks (Mehdi *et al.,* 2018). Disease freedom and biosecurity methods are considered as the major

disease control approaches (Levisohn and Kleven, 2000; Ferguson-Noel *et al.,* 2020). Maintaining flocks free of pathogenic mycoplasmas, means that replacement stocks need to be obtained from mycoplasma-free sources and those birds are then raised in a single-age all-in all-out farm management system. Good biosecurity and an effective monitoring system are necessary aspects of this approach (Kleven, 2008), however disease control interventions are used as needed. Live vaccines are now commercially available for both *M. gallisepticum* and *M. synoviae*, but some studies report that they may not prevent infection (Feberwee *et al.,* 2006). "Even though the use of live vaccines is considered a good option in high prevalence areas, potential complications could arise in interpretation of seroconversion of the birds and ELISA positivity of the one-day old pullets (Moronato *et al.,* 2018)." This increases the complexity in interpreting laboratories results of the breeder flocks and there is also a potential risk of the vaccine reverting to a virulent form (Armour and Ferguson-Noel, 2015). Some producers, therefore, prefer to rely on biosecurity and as a consequence, antimicrobial agents are often needed for treatment and control of infections in particular for *M. synoviae.* It is mostly smallholder poultry producers and “hobby/backyard” poultry keepers that rely on antimicrobial agents to treat mycoplasma infections, while the major producers will sacrifice/cull all flocks to maintain *M. gallisepticum*-free status so that the associated trades can be protected. Infections with *M. synoviae* have possibly been perceived as less important; however some European countries have recognised the increased virulence of *M. synoviae* and are aiming to eliminate the infection (Landman, 2014; Michiels *et al.,* 2016).

In 2010 the Centre Européen d`Etudes pour la Santé Animale (CEESA) introduced a MycoPath programme, which assesses the antimicrobial susceptibility of four different veterinary *Mycoplasma* species isolated from cattle, pigs and poultry (de Jong *et al*., 2013; Klein *et al.,* 2017). The aim of the poultry programme was to create a pan-European collection of representative *M. gallisepticum* and *M. synoviae* isolates from clinical cases of diseased chickens and turkeys. The samples were only collected from poultry that had not recently been treated with antimicrobials, reducing the risk that any recent treatment would

not have residual antimicrobials, or temporary genetic changes impacting on the MIC levels obtained in this study. It is important to know if these pathogens are developing antimicrobial resistance, so that only effective antimicrobial agents are used for therapy, thus ensuring minimal use of antimicrobials by using targeted and correct treatments. The antimicrobials selected for this study are the most relevant antimicrobials which are licensed for commercial poultry use in Europe. In the AVEC annual report representing the European poultry meat sector it is stated that they are "committed to minimising the use of antibiotics in poultry production" but "zero use is neither ethical nor sustainable and poultry farmers and veterinarians need to have antibiotics to maintain the health and welfare of birds" (AVEC, 2019). Therefore, it is essential that the effectiveness of antimicrobial agents is monitored and maintained. However, recent antimicrobial susceptibility data of *M. gallisepticum* and *M. synoviae* isolates is very limited and national resistance monitoring surveys such as GE*RM*-Vet (2018) or Resapath (Anses, 2017) do not include *Mycoplasma* species isolated from poultry.

M. gallisepticum and *M. synoviae* isolates recovered from six European countries were tested against seven licensed antimicrobial agents, and their susceptibilities are reported here. Testing was carried out at a central laboratory using culture media suitable for optimal growth of these two *Mycoplasma* species. Although no standards for testing and interpretation of veterinary mycoplasma species are in place, standards for *Mycoplasma* species that cause significant human clinical disease have been published (CLSI, 2011; Waites *et al.,* 2012). The broth microdilution method used in this study essentially followed the guidelines of Hannan (2000) and CLSI (2011).

Materials and Methods

Collection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* isolates. *Mycoplasma* isolates came from France, Germany, Great Britain, Hungary, Italy and Spain. Samples were collected based on specific criteria and were sent to national laboratories for

culture, isolation and identification of *M. gallisepticum* and *M. synoviae.* The samples were collected during 2014 - 2016 and came from field cases reporting respiratory disease. Only one isolate per farm per clinical episode in a three month period was allowed, which minimizes the risk of epidemiologically related strains. Isolates had to be from geographically spread areas within each country and from chickens or turkeys that had no antimicrobial treatment in the previous 15 days. This procedure provides reassurance that the antimicrobial susceptibility data is representative for diseased birds without a history of antimicrobial therapy and prevents the collection of isolates biased in favour of resistance. In an attempt to achieve identical numbers of isolates from the participating countries, a fixed target number of 24 *M. gallisepticum* and 24 *M. synoviae* isolates were indicated for each country. The participating laboratories followed their standard *Mycoplasma* culture isolation and molecular identification procedures for obtaining pure cultures (Mattison *et al.,* 1995; Catania *et al.,* 2014; Kreizinger *et al.,* 2017). Isolates were stored at temperatures below -50°C, before transfer to the central laboratory (Don Whitley Scientific, Bingley, UK) on dry ice, or at ambient temperature as lyophilized cultures, together with a case report form for each isolate. Culture of the isolates at the central laboratory were checked for typical *M. gallisepticum* and *M. synoviae* growth characteristics and additional identity checks were performed by the central laboratory on a random selection of 12 isolates (5.4%). These selected *M. gallisepticum* and *M. synoviae* isolates were re-identified using a duplex PCR method, with primer pairs for the detection of each of these species (Buim *et al.,* 2009). The identity of all 12 isolates was confirmed.

Antimicrobial Testing. Antimicrobial susceptibility testing for all *M. gallisepticum* and *M. synoviae* isolates was carried out at the central laboratory. The isolates were checked for viability with *M. gallisepticum* in Friis Medium (Friis, 1975) and *M. synoviae* in Chanock's Medium (Chanock *et al.,* 1962), both with phenol red as an indicator and without the addition of antimicrobial agents. Each isolate was incubated in broth medium until a distinctive colour change was produced, then divided into aliquots and frozen at -70°C ± 10°C. The viable

count in one aliquot was determined by serial dilution and plating onto agar medium. During subsequent minimal inhibitory concentration (MIC) tests, aliquots were thawed and diluted to a cell density of 10^6 colony forming units (cfu) per ml, to produce a final inoculum density of nominally 5×10^5 cfu/ml in the MIC plates. *M. gallisepticum* NCTC 10115 (ATCC 19610) and *M. synoviae* NCTC 10124 (ATCC 25204) were used as quality control strains to monitor performance of the MIC test.

Minimal inhibitory concentration determinations were performed using a broth microdilution method. Seven antimicrobial agents, all EU-approved for medication in poultry, from four different antimicrobial classes; the fluoroquinolones, macrolides, pleuromutilins, and tetracyclines were tested. For each antimicrobial agent, a stock solution containing 1280 mg/L of the active ingredient was prepared using the appropriate solvents and diluents as specified in CLSI (2018) and dilutions were made in Friis Medium (*M. gallisepticum*) or Chanock's Medium (*M. synoviae*) to give a final test concentration range from 0.001 to 64 mg/L.

To determine the MIC for each isolate, 100 µL of the appropriate antimicrobial solution was distributed into the conical wells of polystyrene microtitre plates, before 100 µL of culture (thawed, pre-incubated for 1 hour and then diluted as described above) was added to each well to give a final cell concentration of approximately 5×10^5 cfu/mL. For each strain, a positive (growth) control well contained no antimicrobial with 100 µL of sterile medium in its place and a single well with 200 µL of sterile medium served as a negative uninoculated control. Immediately after inoculation, microtitre plates fitted with polystyrene lids were placed in a humidified atmosphere and incubated at 35°C ± 1 °C. Plates were examined every 24 hours. If no growth was evident in the positive control wells, plates were re-incubated for up to five days. For each isolate, MIC results were read as soon as adequate growth (unambiguous colour change) was visible in the positive control wells. All MIC plates were read against a white background to facilitate identification of colour changes in the medium from red (no growth) to orange/yellow (growth). The MIC of each antimicrobial was

recorded as the lowest concentration that completely inhibited growth. For the test to be considered valid, it was necessary for a definite colour change to be visible in the positive control well and for the negative control well to remain unchanged. The reproducibility of the test was demonstrated by ensuring that the MIC results of the quality control strains of this study fell within ± one doubling dilution around a central value. In cases where the MIC results obtained for an antimicrobial agent against one or more strains deviated markedly from the MICs obtained against the majority of strains, the MIC test was repeated on two further occasions. In such cases, the reported MIC value was obtained on at least two separate occasions.

The MIC ranges, MIC distributions, MIC_{50} and MIC_{90} values were determined for each antimicrobial and *Mycoplasma* species, and for each country. The MIC_{50} and MIC_{90} values are percentiles calculated from the complete set of MIC results for a given substance against a specified group of Mycoplasma strains. MIC_{50} is the lowest concentration of an antimicrobial agent at which growth is inhibited for 50% of tested strains. MIC_{90} is the lowest concentration of an antimicrobial agent at which growth is inhibited for 90% of tested strains.

Results

Collection of isolates. The number of isolates collected from either chickens or turkeys, varied between the participating countries for both *M. gallisepticum* and *M. synoviae* (Table 1). For *M. gallisepticum* 16 isolates (19.5%) were recovered from samples taken in 2014; 20 isolates (24.4%) from 2015, and 44 isolates (53.7%) from 2016. For *M. synoviae,* these figures were 5 (3.8%), 47 (36.2%) and 78 (60.0%), respectively. Demographic data were available for 90.1% of the isolates recovered. The vast majority of the samples in each country were from geographically spread farms from untreated flocks with mycoplasma-like clinical signs. The sample origin for chickens was 42.9% from layers, 15.0% from broilers and 42.1% from breeders; for turkeys these figures were 89.6% from fattening turkeys and

10.4% from breeders. Age of the chicken layers and turkey fatteners ranged from 98-686 days and 77-140 days old, respectively. The size of the chicken flocks was for layers 500-120,000, for broilers 2,500-40,000 and for breeders 19-27,000; for turkeys, the size of fattening turkey flocks varied from 500 to 42,000; breeder flocks from 18,000 to 36,000.

Minimal inhibitory concentration results. The distribution of chicken and turkey MIC results are detailed separately for the *M. gallisepticum* and *M. synoviae* isolates in Table 2, providing an overall comparison of the MIC distribution for both of these avian *Mycoplasma* species against the seven antimicrobials. Table 3 summarises the same results as MIC_{50}, MIC_{90} and MIC range separately for chicken and turkey isolates of *M. gallisepticum* and for *M. synoviae* as well as a combined MIC value for chicken and turkey isolates. The MIC ranges of the *M. gallisepticum* (n=4-6) and *M. synoviae* (n=5-10) control strains are also presented in Table 3. As a different and low number of isolates were collected from each country, direct comparison between countries is difficult, but three countries, Great Britain, Italy and Spain had more than ten *M. gallisepticum* isolates from chickens, so their MIC values are detailed and compared in Table 4. Similar comparisons are made for *M. synoviae* from chickens for France, Hungary, Italy and Spain (Table 5).

Both *M. gallisepticum* and *M. synoviae* showed a bimodal distribution for the fluoroquinolone enrofloxacin, although the two main MIC peaks were slightly less for *M. synoviae* (Table 2). For the two tetracyclines doxycyline and oxytetracycline MIC values showed a monomodal distribution, although for oxytetracycline the *M. gallisepticum* isolates were tending towards a bimodal distribution. In contrast, a bimodal distribution was observed for the three macrolides, spiramycin, tilmicosin and tylosin. For all macrolides the MIC values exhibited a broad range. The distribution of the MIC values for tiamulin showed two peaks for *M. gallisepticum*, while predominantly an even distribution for *M. synoviae* was observed.

With the exception of oxytetracycline at 0.5 mg/L and enrofloxacin at 2 mg/L the MIC_{50} values obtained for *M. gallisepticum* are low, at 0.12 mg/L or less (Table 3). However,

the MIC_{90} values are considerably higher, at 4, 4, 8, 32 and >64 mg/L for oxytetracycline, tylosin, enrofloxacin, tilmicosin and spiramycin, respectively. The bimodal populations and the high MIC values obtained with some isolates could suggest that *M. gallisepticum* has developed antimicrobial resistance against these antimicrobials. Doxycycline, enrofloxacin, oxytetracycline and tiamulin exhibited only slightly higher MIC_{90} as compared to MIC_{50}.

Comparison of the MIC values of *M. gallisepticum* chicken isolates from Great Britain (20 isolates), Italy (20 isolates) and Spain (14 isolates), results in potential trends (Table 4). Spain had markedly higher $MIC_{50/90}$ values for all seven antimicrobial agents; all MIC_{90} values for Italy were higher than those for Great Britain. Compared with Great Britain, Spain and Italy consistently had a bimodal population to all of the tested antimicrobial agents, with the second phase having higher MIC values. For *M. synoviae,* comparisons were made between France (13 isolates), Hungary (20 isolates), Italy (34 isolates) and Spain (20 isolates); differences among countries were less apparent (Table 5), but Hungary usually had lower $MIC_{50/90}$ values and lower maximum MIC values than the three other countries. *M. synoviae* isolates from Great Britain were not included for comparison because of the low number (n=7). The reason for these between-country differences in MIC values is not known, but one could speculate about the different levels of antimicrobial agent use for treatment of poultry, or it may be just differences in the poultry farming management where the isolates originated from.

The MIC comparison of turkey and chicken isolates is likely to be influenced by the low and different number of isolates from the participating countries (Tables 4 and 5). Using isolates from just one country, Italy could provide an acceptable comparison. Italian isolates included 20 *M. gallisepticum* from chickens and ten from turkeys, with 34 *M. synoviae* isolates from chickens and five from turkeys (Table 6). From the Italian samples some trends emerge. The *M. synoviae* isolates from both chickens and turkeys have higher MIC values for tiamulin than the *M. gallisepticum* isolates. For tilmicosin, although there is a bimodal population for all isolates and more isolates from chickens than turkeys, only the isolates

from chickens have MIC values above 16 mg/L. A higher number of *M. gallisepticum* turkey isolates (six out of ten) have MIC values for spiramycin at 64 mg/L and above compared to four out of 20 from chickens. The *M. gallisepticum* turkey isolates have no oxytetracycline MICs below 0.5 mg/L, whereas values for nine of the 20 chicken isolates are below 0.5 mg/L. The *M. synoviae* isolates have slightly higher enrofloxacin MICs (maximum 32 mg/L) than the *M. gallisepticum* isolates (maximum 8 mg/L).

Discussion

Although all participating countries were requested to collect equal numbers of *M. gallisepticum* and *M. synoviae* isolates, the number of isolates collected by each country varied. This is unlikely to be related to the size of the avian mycoplasma infections in the countries, but may be due to differences in practitioners requesting mycoplasma molecular tests in preference to culture and different time constraints of the practitioners. In addition, participating countries may use slightly different procedures for mycoplasma culture, isolation and identification which may have influenced the isolation rate.

At the outset it is important to emphasize that all of the data has great merit, however, care needs to be taken in interpretation of the results. The numbers of isolates per *Mycoplasma* species per country were small and were distributed between chickens and turkeys. Similarly, numbers between chickens and turkeys were in a few instances too low to draw definitive conclusions. Note that several factors may affect the rate of resistance including age of the birds, production system, different antimicrobial agent usage, source (diagnostic laboratory versus abattoir) or disease. Nevertheless some differences were observed and are worthy of including in the discussion.

Although only 17 *M. gallisepticum* isolates were from turkeys, some differences between isolates from chickens and turkeys were observed; mainly the MIC_{50} values of spiramycin and tilmicosin were higher in turkeys at 16 and 32 mg/L compared with 0.12 and 0.008 mg/L for the 65 chicken isolates (Table 3). From these results one may speculate that

farmers could use more macrolides in turkeys probably because the life cycle is longer in this species; or that the hosts exert different antimicrobial resistance selection pressures on the *Mycoplasma* species. It may also be due to isolates being obtained from different countries. When similar comparisons are made for *M. synoviae*, with 34 of those isolates from turkeys and 96 from chickens, the enrofloxacin and tylosin MIC_{50} values are higher for chickens at 8 and 0.25 mg/L, respectively, compared with 1 and 0.062 mg/L for turkeys. The MIC_{90} values of macrolides are also higher for *M. synoviae* chicken isolates: spiramycin, tylosin and tilmicosin at 16, 32 and 2 mg/L, respectively, compared with 2, 0.12 and 0.12 mg/L for turkey isolates. This is in contrast to the data observed for *M. gallisepticum.* For *M. gallisepticum* it could be speculated that this could be due to possible different treatment approaches. Usually turkeys are kept for meat, so they are kept for longer than chickens and no vaccines are available; antimicrobial treatment can be repeated during the production cycle. The difference in MIC results obtained for *M. synoviae* may be a result of treatment usually being applied in the layer sector to avoid egg production losses, or to reduce the presence of eggshell apex abnormalities (Catania *et al.,* 2010; Feberwee *et al.,* 2019). In broiler breeder production this approach may be justified to help contain the infection and reduce the risk of spreading infection in the broilers by vertical transmission. Early detection of infection and adequate knowledge of antimicrobial effectiveness, such as MIC data, can reduce the amount of antimicrobial agents used and still improve and increase broiler production (Fincato *et al.*, 2019).

Whilst the comparisons made between chicken and turkey isolates is informative in observing trends and potential risks of the avian mycoplasmas developing antimicrobial resistance, care is needed to not over interpret these *in vitro* tests in relation to the *in vivo* situation. Although all the testing was carried out in a central laboratory, essentially following the same procedures, comparisons between MIC values for *M. gallisepticum* and *M. synoviae* isolates may be affected by the use of the different growth medium, which was needed to provide the optimal growth conditions required for these two different *Mycoplasma*

species. In the Hannan (2000) recommendations for MIC testing against veterinary *Mycoplasma* species, the same control strains were used for *M. gallisepticum* and *M. synoviae* against enrofloxacin, oxytetracycline, tiamulin and tylosin, a comparison with this study controls is included in Table 3.

During the 1980's and 1990's several European studies describing the *in vitro* susceptibility of avian mycoplasmas were published, but recent European reports on MICs of *M. gallisepticum* are non-existent, and scarce for *M. synoviae* (Dufour-Gesbert *et al.,* 2006; Landman *et al.,* 2008; Kreizinger *et al.,* 2017; Catania *et al.,* 2019). Dufour-Gesbert *et al.* (2006) carried out MIC determinations on 36 *M. synoviae* isolates from French layers obtained between 2002 and 2003. They tested six of the same antimicrobial agents used in this study, not tilmicosin, and all of the MIC values were ≤1 mg/L, which is lower than some of the MIC values obtained for French isolates in this study. Landman *et al.* (2008) tested 17 *M. synoviae* Dutch isolates, 14 were from the respiratory tract and three from joints, two of which were from turkeys. For enrofloxacin, difloxacin, doxycycline, tylosin and tilmicosin they recorded MIC values at seven and 14 days. With the exception of two isolates where MIC values were 2 or 4 mg/L for enrofloxacin, all other results were below 1 mg/L at seven days. The 14 day results were higher, but that may be a minimal mycoplasmacidal concentration, rather than an MIC and could be due to antimicrobial activity decreasing during the longer incubation time. Kreizinger *et al.* (2017) tested 41 *M. synoviae* isolates from both chicken and turkey tracheas, mainly isolated between 2014 and 2016. Most isolates (26) were obtained from Hungary, the others came from Austria, Czech Republic, Slovenia, Ukraine, Russia and Serbia. These isolates were tested against a range of antimicrobial agents using a broth microdilution method. They reported some differences in MIC values between chicken and turkey isolates, with chickens having higher enrofloxacin MIC values. Overall similar MIC_{50} values for the same antimicrobial agents tested in this study were reported as well as elevated MIC_{90} values for most of the antimicrobial agents tested here except for tylosin, which had an MIC_{90} value of ≤0.25 mg/L compared with 16 mg/L in this study. In a recent

study Catania *et al.* (2019) investigated the antimicrobial susceptibility of 154 *M. synoviae* isolates from broiler chickens, layers and turkeys obtained between 2012 and 2017 in Italy. They tested them against seven of the same antimicrobial agents used in this study but with different mycoplasma culture media. The MIC ranges and MIC_{50} results were similar to those obtained in this study, but this current study had higher MIC_{90} values for spiramycin and tylosin both at 32 mg/L compared with 4 and 1 mg/L respectively, but lower for tilmicosin at 2 mg/L compared with >32 mg/L. Future MIC studies are needed to understand the observed differences.

Outside Europe, as early as 1994, Lin *et al*. (1994) reported high MIC_{50} values of oxytetracycline and spiramycin at >32 mg/L from Taiwanese *M. gallisepticum* isolates. Gharaibeh and Al-Rashdan (2011) showed increased MIC_{50} values for Jordanian *M. gallisepticum* isolates from 2007-2008 compared with isolates from 2004-2005. In comparison to 2004-2005 and 2007-2008, the values for tilmicosin, tylosin, enrofloxacin, doxycycline and oxytetracycline raised from ≤0.031 to 2, ≤0.031 to 0.125, ≤0.031 to 2, ≤0.031 to 0.062; 0.062 to 2 mg/L, respectively. It should be noted that the antimicrobial usage patterns in Jordan might be much different from the European countries. Gautier-Bouchardon (2018) reviewed the MIC values obtained for *M. gallisepticum* but comparative data for “old strains” and “new strains” is limited: the main finding is an increase in *M. gallisepticum* maximum MIC value of enrofloxacin from 1 to 10 mg/L and oxytetracycline from 0.5 to 4 mg/L.

A major concern is that the development of antimicrobial resistance may lead to antimicrobial treatment against avian mycoplasma infections being ineffective. Several authors have reported that *in vitro* passaging of isolates in antimicrobial agents at sub-lethal levels rapidly induces resistance in as little as ten passages. Zanella *et al*. (1998) demonstrated this with *M. gallisepticum* exposed to spiramycin, tylosin and enrofloxacin; Takahashi *et al*. (2002) to tylosin; Wu *et al*. (2005) to tylosin and tilmicosin. Similarly Gautier-Bouchardon *et al*. (2002) showed rapid development of antimicrobial resistance by both *M.*

gallisepticum and *M. synoviae* to enrofloxacin and tylosin. Interpretation of these higher antimicrobial susceptibility levels as antimicrobial resistance raises some issues as no defined epidemiological cut-off values or clinical breakpoints are set for these avian *Mycoplasma* species. However, Gerchman *et al*. (2011) linked the macrolide decreased susceptibility to mutations in the 23S rRNA gene in their study where 50% of the 50 *M. gallisepticum* isolates from Israel had resistance to tylosin and tilmicosin. Lysnyansky *et al*. (2015) also described mutations in the 23S rRNA gene of *M. synoviae* associated with high MIC values. Le Carrou *et al*. (2006) and Lysnyansky *et al*. (2013) associated high enrofloxacin MIC values in *M. synoviae* with amino acid substitutions in the *parC* gene. Data from another *Mycoplasma* species, *Mycoplasma bovis* has also demonstrated that high MIC values are linked with genetic mutations associated with antimicrobial resistance (reviewed in Lysnyansky and Ayling, 2016). Therefore it is likely that the high MIC values reported by previous workers are indicative of genuine antimicrobial resistance.

The *in vivo* effectiveness of antimicrobials has been the subject in many studies (Jordan and Horrocks 1996; Kempf *et al.,* 1997; Riazuddin *et al.,* 2017; Garmyn *et al.,* 2019). However Hinz and Rottmann (1990) investigated the re-isolation of *M. gallisepticum* following treatment with enrofloxacin, tylosin and tiamulin and they stated tylosin proved to be inadequate, whereas enrofloxacin was highly effective. Despite this Reinhardt *et al*. (2005) showed persistence of *M. gallisepticum* in chickens after treatment with enrofloxacin without development of resistance. Few cases document the use of antimicrobial agents to eradicate avian *Mycoplasma* infections from a flock; however Hong *et al*. (2015) describe the eradication of *M. synoviae* from a multi-age broiler breeder farm using intensive antimicrobial treatment which consisted of the continuous administration of tilmicosin, after two rounds of treatment with chlortetracycline, doxycycline and enrofloxacin.

The focus of this study has been on *M. gallisepticum* and *M. synoviae*, arguably the most pathogenic avian *Mycoplasma* species, certainly those that potentially have the most economic impact on poultry production. However many different *Mycoplasma* species have been isolated from poultry and most of those are thought to be commensal or possibly

opportunist pathogens. Therefore treatment of poultry with antimicrobial agents may inadvertently lead to antimicrobial resistance in these non-pathogenic *(*Beylefeld *et al.,* 2018) or potentially opportunist *Mycoplasma* species, as well as in other commensal or zoonotic bacteria such as *Salmonella* and *Campylobacter*.

Nhung *et al*. (2017) stated it is necessary to increase efforts to harmonize testing practices, to promote free access to data on antimicrobial resistance, which will improve treatment guidelines and monitor the evolution of antimicrobial resistance in poultry bacterial pathogens including the avian *Mycoplasma* species. Whilst all studies provide valuable information on the status of *in vitro* antimicrobial susceptibilities, this study has the advantage of using just one laboratory for the MIC determinations which provides consistent and comparative data for isolates from the different countries.

Conclusion

Use of a central laboratory to determine MICs against *M. gallisepticum* and *M. synoviae* in chickens and turkeys from several European countries gave useful comparative MIC data. This has allowed assessment of *in vitro* susceptibility of antimicrobial agents in treating the economically important mycoplasmas of poultry. It also provides a baseline for future comparisons to assess the development of antimicrobial resistance. An awareness of current MIC values facilitates initial selection of the most optimal antimicrobial treatment of these infections. This study demonstrates that some isolates of both *M. gallisepticum* and *M. synoviae* have high MIC values indicating that antimicrobial resistance is a risk, and further studies are required to determine their efficacy *in vivo*. Selection and use of antimicrobial agents to effectively treat avian mycoplasmoses requires knowledge of the organisms' antimicrobial resistance status. Indeed, the lack of clinical breakpoints emphasizes the need for establishing avian mycoplasma-specific clinical breakpoints to ensure a correct interpretation of the susceptibility results. The availability of interpretive criteria will assist

veterinarians in minimising antimicrobial usage and to promote targeted treatment options that will avoid development of more resistant strains.

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Conflict of Interest Statement

Some authors are connected with pharmaceutical companies, however the testing, interpretation of results and preparation of the manuscript have been carried out independently.

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Table 1. Summary of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* isolates from chickens and turkeys from each participating country during 2014-2016*.

Country	*Mycoplasma gallisepticum*			*Mycoplasma synoviae*		
	Chicken	Turkey	Total	Chicken	Turkey	Total
France	3	0	3	13	0	13
Germany**	0	5	5	4	6	10
Great Britain	20	2	22	7	0	7
Hungary***	8	0	8	20	21	51
Italy	20	10	30	34	5	39
Spain	14	0	14	20	0	20
Total	65	17	82	96	34	130

*Two *M. gallisepticum* isolates were recovered from samples collected in 2013.

**Four *M. synoviae* isolates were recovered from joint samples collected from turkeys.

***Four isolates were recovered from samples collected in the Czech Republic, three isolates were from Austrian samples and three isolates were from Romanian samples.

Table 2. Minimal inhibitory concentrations (mg/L) of seven antimicrobials for 82 *Mycoplasma gallisepticum* (Mg) and 130 *M. synoviae* (Ms) isolates from chicken and turkey obtained from European countries

Antimicrobial	Species	<0.001	0.001	0.002	0.004	0.008	0.016	0.031	0.062	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Enrofloxacin	Mg							3	19	7	5		3	6	19	18	2			
	Ms							1	2	2	9	18	20	5	4	26	41	2		
Doxycycline	Mg						1	1	21	30	12	13	4							
	Ms								11	17	34	40	21	7						
Oxytetracycline	Mg								4	16	15	16	10	5	10	4	1	1		
	Ms							3	6	9	29	42	33	5	2			1		
Spiramycin	Mg						1	9	20	16	2	1	2		2	2	3		5	19
	Ms									3	28	42	27	13	2	3	6	3	1	2
Tylosin	Mg				2	2	15	19	9	3	2	2	1	8	11	8				
	Ms					13	11	20	21	13	11	12		4	1	7	7	6	2	2
Tilmicosin	Mg	7		10	16	13	2	3	1			1				3	4	15	7	
	Ms				4	6	10	25	33	13	8	6	9	9	2	5				
Tiamulin	Mg			2	13	29	8	12	11	5	1	1								
	Ms				1		1		5	41	43	31	7	1						

Table 3. MIC_{50} and MIC_{90} (mg/L) for chicken and turkey *M. gallisepticum* and *M. synoviae* isolates obtained from European countries. The table includes the MIC range for the NCTC control strains.

Antimicrobial	Host species	*Mycoplasma gallisepticum* 65 chicken and 17 turkey isolates			*Mycoplasma synoviae* 96 chicken and 34 turkey isolates		
		MIC_{50}	**MIC_{90}**	**Range**	**MIC_{50}**	**MIC_{90}**	**Range**
Enrofloxacin	Chickens	2	8	0.031 – 16	8	16	0.062 – 32
	Turkeys	4	8	0.062 - 8	1	8	0.031 – 16
	All	2	8	0.031 – 16	8	16	0.031 – 32
	Control	N/A	N/A	0.016 - 0.031 (0.01)	N/A	N/A	0.12 – 0.5 (0.5)
Doxycycline	Chickens	0.12	0.5	0.16 – 1	0.5	1	0.062 – 2
	Turkeys	0.12	0.5	0.062 - 1	0.25	1	0.062 – 1
	All	0.12	0.5	0.16 – 1	0.5	1	0.062 – 2
	Control	N/A	N/A	0.062 – 0.25	N/A	N/A	0.062 – 0.25
Oxytetracycline	Chickens	0.25	4	0.062 – 32	0.5	1	0.031 – 32
	Turkeys	1	4	0.25 - 4	0.25	1	0.031 – 4
	All	0.5	4	0.062 – 32	0.5	1	0.031 – 32
	Control	N/A	N/A	0.12 – 0.25 (0.1)	N/A	N/A	0.12 – 0.25 (0.1)
Spiramycin	Chickens	0.12	>64	0.031 - >64	0.5	16	0.12 - >64
	Turkeys	16	>64	0.016 - >64	0.5	2	0.25 – 32
	All	0.12	>64	0.016 - >64	0.5	8	0.12 - >64
	Control	N/A	N/A	0.062 – 0.125	N/A	N/A	0.25 – 1
Tylosin	Chickens	0.031	4	0.004 - 8	0.25	32	0.008 - >64
	Turkeys	2	8	0.008 - 8	0.062	0.12	0.008 – 16
	All	0.062	4	0.004 – 8	0.062	16	0.008 - >64
	Control	N/A	N/A	0.031 – 0.031 (0.01)	N/A	N/A	0.031 – 0.12 (0.025)
Tilmicosin	Chickens	0.008	32	<0.001 – 64	0.062	2	0.004 – 8
	Turkeys	32	64	0.002 - 64	0.062	0.12	0.008 – 2
	All	0.008	32	<0.001 – 64	0.062	2	0.004 – 8
	Control	N/A	N/A	0.004 – 0.008	N/A	N/A	0.016 - 0.12
Tiamulin	Chickens	0.008	0.062	0.002 – 0.5	0.25	0.5	0.004 – 2
	Turkeys	0.031	0.062	0.002 - 0.12	0.12	0.5	0.016 – 1
	All	0.008	0.062	0.002 – 0.5	0.25	0.5	0.004 – 2
	Control	N/A	N/A	0.008 – 0.016 (0.0025)	N/A	N/A	0.062 – 0.5 (0.1)

N/A = not applicable

Data from Hannan, 2000 for control strain in brackets

Table 4. Comparison of $MIC_{50,}$ MIC_{90} and MIC range (mg/L) for *Mycoplasma gallisepticum* chicken isolates obtained from Great Britain (20 isolates), Italy (20 isolates) and Spain (14 isolates).

Antimicrobial	Country	MIC_{50}	MIC_{90}	Range
Enrofloxacin	Great Britain	0.062	0.12	0.031 – 0.12
	Italy	4	8	0.062 – 8
	Spain	4	16	0.062 - 16
Doxycycline	Great Britain	0.12	0.12	0.062 – 0.25
	Italy	0.12	0.25	0.016 – 0.5
	Spain	0.5	1	0.12 - 1
Oxytetracycline	Great Britain	0.25	0.5	0.062 – 0.5
	Italy	0.5	4	0.062 – 8
	Spain	4	8	0.12 - 8
Spiramycin	Great Britain	0.062	0.12	0.031 – 0.12
	Italy	0.12	64	0.031 - >64
	Spain	>64	>64	0.062 - >64
Tylosin	Great Britain	0.031	0.062	0.004 – 0.062
	Italy	0.12	8	0.031 – 8
	Spain	4	8	0.016 - 8
Tilmicosin	Great Britain	0.004	0.008	<0.001 – 0.008
	Italy	0.008	64	<0.001 – 64
	Spain	32	32	0.002 - 32
Tiamulin	Great Britain	0.008	0.016	0.004 – 0.031
	Italy	0.008	0.031	0.002 – 0.062
	Spain	0.031	0.12	0.004 – 0.12

Table 5. Comparison of $MIC_{50,}$ MIC_{90} and MIC range (mg/L) for *Mycoplasma synoviae* chicken isolates obtained from France (13 isolates), Hungary (20 isolates), Italy (34 isolates) and Spain (20 isolates).

Antimicrobial	Country	MIC_{50}	MIC_{90}	Range
Enrofloxacin	France	16	16	0.062 - 16
	Hungary	1	8	0.062 – 16
	Italy	8	16	0.12 – 32
	Spain	16	16	8 - 32
Doxycycline	France	0.5	1	0.12 – 2
	Hungary	0.12	0.5	0.062 – 1
	Italy	0.5	1	0.12 – 1
	Spain	1	2	0.25 - 2
Oxytetracycline	France	1	1	0.25 – 32
	Hungary	0.25	1	0.031 – 1
	Italy	0.5	0.5	0.12 – 2
	Spain	0.5	2	0.062 - 4
Spiramycin	France	0.5	8	0.12 – 64
	Hungary	0.5	2	0.25 – 4
	Italy	0.5	16	0.12 - >64
	Spain	1	16	0.12 - 4
Tylosin	France	0.12	0.5	0.031 - >64
	Hungary	0.016	0.25	0.008 – 2
	Italy	0.5	32	0.008 – 64
	Spain	2	64	0.062 - >64
Tilmicosin	France	0.031	1	0.016 – 8
	Hungary	0.031	0.25	0.004 – 2
	Italy	0.25	2	0.008 – 8
	Spain	0.25	8	0.031 - 1
Tiamulin	France	0.5	1	0.12 – 2
	Hungary	0.12	0.5	0.062 – 0.5
	Italy	0.25	0.5	0.12 – 1
	Spain	0.25	1	0.12 - 1

Table 6. Minimal inhibitory concentration distribution (mg/L) for *Mycoplasma gallisepticum* (Mg) and *M. synoviae* (Ms) isolates from chickens and turkeys obtained from Italy.

Antimicrobial	Species (No)	<0.001	0.001	0.002	0.004	0.008	0.016	0.031	0.062	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Enrofloxacin	Ms Turkey (5)										1			1	1	1	1			
	Ms Chicken (34)									1		6		2	2	9	13	1		
	Mg Turkey (10)										1				4	5				
	Mg Chicken (20)								3	1	1			2	7	6				
Doxycycline	Ms Turkey (5)										2	1	2							
	Ms Chicken									1	8	16	9							

	(34)																			
	Mg Turkey (10)								1	7	2									
	Mg Chicken (20)						1		6	7	4	2								
Oxytetracycline	Ms Turkey (5)											3	1		1					
	Ms Chicken (34)									1	3	14	15	1						
	Mg Turkey (10)											5	2	2	1					
	Mg Chicken (20)								2	5	2	2	5	1	2	1				
Spiramycin	Ms Turkey (5)										3						1	1		
	Ms Chicken (34)									1	13	10	2	1	1		3	1		2
	Mg Turkey (10)									3	1									6
	Mg Chicken (20)							2	7	4					1	1	1		3	1
Tylosin	Ms Turkey (5)								1	1						1	2			
	Ms Chicken (34)					1		3	2	3	3	9		1		4	3	4	1	
	Mg Turkey (10)						3		1					2	2	2				
	Mg Chicken (20)				1	1	3	6	1	1			1	1	2	3				
Tilmicosin	Ms Turkey (5)						1			1		1		2						
	Ms Chicken (34)				3	1												3	3	
	Mg Turkey (10)						3		1					2	2	2				
	Mg Chicken (20)	1		4	4	2	2									2	1	1	3	
Tiamulin	Ms Turkey (5)									2	1	1	1							
	Ms Chicken (34)									10	13	9	2							
	Mg Turkey (10)				1	3	1	2	2	1										
	Mg Chicken (20)			1	5	7	2	4	1											