



Antimicrobial susceptibility of *Mannheimia haemolytica* and *Pasteurella multocida* isolated from ovine respiratory clinical cases in Spain and Portugal

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ABSTRACT

Antimicrobials are used to control respiratory disorders during the ovine rearing period. There is an urgent need to optimize the use of different antimicrobial families in livestock to tackle the general problem of antimicrobial resistance. The first step to addressing this problem is gaining insight into the antimicrobial susceptibility (pharmacodynamic) parameters of ovine pathogens. In this study, the key pharmacodynamic parameter (MIC) was determined for *Pasteurella multocida* and *Mannhaemia haemolytica* isolated from ovine respiratory clinical cases using accepted laboratory methods for bacterial isolation, identification and MIC determination. In the case of *Pasteurella multocida*, for sulfamethoxazole/trimethoprim, MIC_{range}, MIC₅₀ and MIC₉₀ values were 0.004–32 µg/mL, 0.063 µg/mL and 1 µg/mL; for tetracycline 0.016–256 µg/mL, 1 µg/mL, and 32 µg/mL; for enrofloxacin 0.002–32 µg/mL, 0.016 µg/mL and 0.5 µg/mL; for doxycycline 0.063–32 µg/mL, 1 µg/mL and 16 µg/mL. In the case of *Mannhaemia haemolytica* for sulfamethoxazole/trimethoprim, MIC_{range}, MIC₅₀ and MIC₉₀ values were 0.004–1024 µg/mL, 0.063 µg/mL and 1 µg/mL; for tetracycline 0.063–256 µg/mL, 8 µg/mL, and 64 µg/mL; for enrofloxacin 0.004–32 µg/mL, 0.032 µg/mL and 16 µg/mL; for doxycycline 0.063–256 µg/mL, 2 µg/mL and 16 µg/mL. The antimicrobial pattern showed good susceptibility for ovine respiratory pathogens to various licensed antibiotics including fluoroquinolones and sulfonamides. However, the antimicrobial susceptibility of antibiotics in the tetracycline family was variable. Doxycycline showed a better antimicrobial pattern than tetracycline. Finally, antimicrobial susceptibility monitoring programs are recommended to provide evidence-based guidance for antimicrobial therapy of bacterial diseases.

1. Introduction

In Spain and Portugal, the production of lambs is based on small ewe production farms. However, the market of fattening animals needs lambs from many farms to achieve a minimum size in feedlots (González et al., 2016), such that these facilities have sufficient volume to satisfy meat distribution channels. This industry organization has advantages in terms of achieving market goals but has serious drawbacks in terms of disease management because mixing animals of different origins is a well-known risk factor for disease outbreak in many veterinary species (Thrusfield, 2018).

Respiratory disease remains one of the most challenging problems in intensive ovine production systems. The term ovine respiratory disease complex (ORDC) describes a polymicrobial syndrome that results from a combination of infectious agents, environmental stressors,

population size, management strategies, age, and genetics. This syndrome causes reduced performance, and an increase in mortality rates and production costs in the ovine fattening industry worldwide (Black et al., 1997; Luzón and de las Heras, 1999; Gonzalez et al., 2001; Vilallonga, 2013). The aetiology of the ORDC has been in continuous progression due to pathogen evolution as well as in management and stressor changes in ovine feedlots (González et al., 2016). This ORDC is the consequence of impairment of the normal respiratory immune system due to pathogens that are able to damage these defences and establish infection on their own. Several pathogens have been involved in ORDC, making it a multifactorial complex. In any case, *Mannheimia haemolytica* (MH), *Pasteurella multocida* (PM), *Mycoplasma ovipneumoniae* and *Biberstena threalosi* are the most common etiologic agents involved (Gonzalez, 2015) but viruses like Parainfluenza 3, Pestivirus, Adenovirus 6 and Syncytial Respiratory Virus could predispose an

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animal to bacterial colonization in some cases (Brodgen et al., 1998; Martin and Cid, 2013; González et al., 2016). On the other hand, many non-infectious predisposing factors are involved in the ORDC, such as poor environmental conditions (e.g., deficient ventilation), density, stressors (weaning, feeding changes, transport and social stress), subacute acidosis, season of the year (summer in Spain and Portugal) and oxidative stress (pulmonary hypertension and damage to epithelia). Finally, coccidiosis could be one of the most important trigger factors (González et al., 2016) mainly during the first week at the feedlot.

The clinical manifestation of affected lambs can be septicemic, acute, subacute, chronic and subclinical (Gómez and Garijo, 2013; Gonzalez, 2015). Although ORDC is the most important cause of mortality in lambs older than 42 days of age (Lacasta et al., 2008), clinical signs of the syndrome may not be as evident highlighting the importance of the chronic or subclinical form. Thus, close to 33% of lambs raised in current feedlots have some degree of lesions in their lungs at the slaughterhouse (Gonzalez, 2015). Moreover, 75% of lambs were asymptomatic and had never been treated despite presenting some lung lesions at slaughter (Luzón and De las Heras, 1999).

The diagnosis, prophylaxis and treatment strategies for ovine respiratory disease complex should be adapted, in a case-by-case situation, depending on the relevance of the agents involved. As a general approach, the medical preventive programs should be based on applying measures to control diseases in a cost-effective way such as improving environmental conditions, decreasing density, mitigating stressors and controlling subacute acidosis and parasitism. Other measures include vaccinating against the major bacterial diseases and the use of antimicrobials to control bacterial diseases with a therapeutic or metaphylactic goal. Unfortunately, there are few registered vaccines and antimicrobials to apply in ovine animals. Thus, antimicrobials remain an essential tool to control ORDC under field conditions (Martin and Cid, 2013; Fernandez and Rey, 2013).

The major issues for practitioners when treating a large population of animals with antimicrobials are maximizing the likelihood of a favorable clinical outcome and minimizing the appearance and development of antimicrobial resistance. To optimize the use of these drugs, it is critical to have updated pharmacokinetic (PK) and pharmacodynamic (PD) data about antimicrobials (Mckellar et al., 2004). Unfortunately, there is a scarcity of public knowledge about the PK and PD of antimicrobials in ovine medicine. On the other hand, it has been strongly recommended that program be developed to monitor the usage of antimicrobial agents and the occurrence of antimicrobial resistance among food animals at the European level (Aarestrup et al., 2008; EMA AMEG, 2014, 2019). In Spain, a national antimicrobial resistance monitoring program has been underway since 2014 (PRAN, 2014). The bacterial species monitored, the antimicrobial agents tested, as well as the methodology used, are being homogenized to make data comparable between and across species in Spain (PRAN, 2019). In addition, this program also provides data on the consumption of antimicrobial agents in all veterinary species. Today, there is no public or updated information about antimicrobial susceptibility of ovine pathogens involved in ORDC in Spain and Portugal. Much of the information available is very old, was obtained by obsolete methodology (Diker et al., 1994) or comes from a geographical area unrelated to the European context (Marru et al., 2013).

The main objective of this work was to obtain pharmacodynamic information about *Pasteurella multocida* and *Mannhaemia haemolytica* in ovine animals following a methodology recommended by a national antimicrobial resistance control program in Spain. This information is necessary to obtain updated information to optimize the use of antimicrobials for this species following the general recommendations about prudent use of antimicrobials.

2. Material and methods

2.1. Animals and sampling

One-hundred twenty-eight respiratory clinical cases from 60 ovine feedlots were studied between February of 2015 and March of 2019 in Spain and Portugal. In these cases, a percentage of the feedlot showed respiratory symptoms (cough, fever, depression and dyspnoea) and the mortality rate significantly increased *versus* the baseline situation during the respiratory outbreak due mainly to respiratory causes. It was not sampled any animal that was treated with antimicrobials prior to sample collection. In each clinical case, a minimum and maximum number of 3 and 5 animals, with overt respiratory symptoms, were sampled, respectively. These animals were randomly selected if more than 5 animals showed respiratory symptoms at the same time. Unfortunately, it was not possible to have information about exact age, breed and days on feed of the animals because this data is not individually recorded in ovine fattening farms. Samples were drawn from pneumonic lesions in the lung during necropsy (64 samples) or from tracheobronchial lavages of live animals showing respiratory symptoms (63 samples). In the case of lung sample collection, a section of 5 x 5 cm of lung was collected from a lobule including healthy and pneumonic tissue. On the other hand, tracheobronchial lavage was carried out using a previous published method adapted to ovine (Hoffman, 2008). Briefly, 30–40 cm of a catheter of 2.7 mm of diameter was inserted through one of the nostrils into the trachea. Then, 20 cm³ of physiological serum were flushed and immediately aspirated with the same syringe. Afterwards, between 3 to 10 cm³ of tracheobronchial lavage were drawn. This sample was stored in a sterile container and sent immediately to the laboratory. In only one case, a nasal swab (one sample) was also collected from a sick lamb.

2.2. Bacterial isolation and identification

The surface of the lung was sterilized using surgical material at high temperature. Afterwards, an incision was performed with a scalpel and a sterile loop was used to sample inside the lung. In the case of tracheobronchial lavages, samples were uniformly mixed, and 10 µl were streaked on the blood agar plate surface with a sterile loop. Finally, swab samples were surfaced onto the blood agar. For all the samples, sterile loops were surfaced onto blood agar (tryptic soy agar containing 5% sheep red blood cells) (BA) plates (Oxoid PB 5039A) with an incubation at 35–37 °C in aerobic conditions for 24–48 h. Identification of isolates were carried out by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF, Bruker Daltonics, Bremen, Germany). Individual strains were stored at –70 °C in skim milk. For MIC testing, bacteria were thawed and cultured two times on the same agar media.

2.3. Antimicrobial compounds

Trimetropim (batch J1511036) and sulfadiazine (batch PX 1-SD-1510033) were kindly provided by Laboratorios Jaer SA whereas doxycycline hyclate (batch YD140201015) was provided by Laboratorios SP Veterinaria. Tetracycline hydrochloride (batch T8032) and enrofloxacin (batch17849) were purchased commercially from Sigma Aldrich. All the antimicrobials were reconstituted based on manufacturer's recommendations. Fresh stock solutions or those prepared from frozen samples (–70 °C) were used. For quality control, *Staphylococcus aureus* ATCC 29,213 from American Type Culture Collection (ATCC) control strain was included in each susceptible assay to ensure performance of the susceptibility assays; MIC values needed to be within acceptable ranges for each organism/drug.

2.4. MIC determination

MIC testing was carried out following the recommended CLSI

procedure for ten strains, five of PM and MH (VET08, 2018) that were randomly chosen between all the available ones. Briefly, Mueller-Hinton broth (MHB) containing a two-fold concentration of drug was added to the first column of a 96-well micro-dilution tray and serially diluted concentrations with MHB solution were prepared (from 0.001 to 1024 µg/mL for all the antimicrobials). A 0.5 McFarland density, established with a calibrated nephelometer (Biosan Medical-Biological Research & Technologies, Riga, Latvia), of *Pasteurella multocida* and *Mannheimia haemolytica* was further diluted to 5×10^5 cfu/ml, added to the microdilution tray containing drug and incubated for 18–24 h (35–37 °C) in aerobic conditions. To ensure the inoculum concentration, the counting of bacterial colonies per mL (cfu/mL) was carried out by serial dilution over the surface of a blood agar plate for 10 strains. The MIC was established as the lowest drug concentration inhibiting visible growth. This standard MIC procedure was repeated twice on separate days and the average value was accepted as the final one. In parallel, MIC for the same *Pasteurella multocida* and *Mannheimia haemolytica* strains were performed by test strip (Epsilon test or E-test) to evaluate the agreement between the two techniques. For MIC determination by test strip, tetracycline (TET) (Oxoid Limited, Hampshire, United Kingdom), doxycycline (DOX) (Liofilchem S.R.L., Roseto degli Abruzzi, Italy), trimethoprim/sulfamethoxazole (SXT) (Liofilchem S.R.L., Roseto degli Abruzzi, Italy), and enrofloxacin (ENR) (Liofilchem S.R.L., Roseto degli Abruzzi, Italy) strips were used. It was not feasible to use the E-test with sulfadiazine/trimethoprim because it is not commercially available. The inoculum was prepared as previously described. Briefly, a 0.5 McFarland density, established with a nephelometer (Biosan Medical-Biological Research & Technologies, Riga, Latvia), of *Pasteurella multocida* and *Mannheimia haemolytica* was spread on a Muller Hinton Agar plate (Oxoid Limited, Hampshire, United Kingdom) using a sterile cotton swab and the test strip was applied to the agar surface. After 18 h of incubation at 37 °C the MIC value was read from the scale in units of µg/mL where the ellipse edge intersects the side strip.

For the rest of the bacterial strains of PM and MH, minimum Inhibitory Concentrations (MIC) of SXT, TET, ENR and DOX were determined using the E-Test technique based on the results obtained using both techniques.

2.5. Data analysis

MIC distributions were determined for each species-antimicrobial combination. The MIC distributions were used to define MIC₅₀, MIC₉₀, and a tentative epidemiological breakpoint ECOFF for differentiation between susceptibility and strains with some gene of resistance. MIC₅₀ and MIC₉₀ were defined as MICs inhibiting 50% and 90% of the strains, respectively. ECOFFs were determined from MIC distributions for each species-drug combination, as recommended by EUCAST (Turnidge et al., 2006), with susceptible strains being the population with MIC at or below the ECOFF and strains with some gene of resistance having a MIC of > ECOFF from an epidemiological point of view.

The agreement between the microdilution and E-test technique to determine MIC was assessed using the Lin's concordance correlation coefficient as described by Watson and Petrie, 2010.

3. Results

Pasteurella multocida (n = 60) and *Mannheimia haemolytica* (n = 68) were isolated in most cases, although *Biberstenia threalosi* and *Escherichia coli* were found, but only in a few cases. These latter microorganisms were not included in the study due to the low number of available strains. As explained in the Material and Methods section, the MIC value was determined using microdilution and E-test techniques for 10 randomly selected strains whose MIC values were highly variable. The agreement between the two techniques was perfect (> 0.999) for enrofloxacin, and substantial (between 0.950 and 0.990) for

Table 1

Lin's concordance correlation coefficient between the MIC value obtained using microdilution techniques versus the E-test method.

Lin's concordance correlation coefficient	MIC Sulfadiazine/trimethoprim	MIC TET	MIC ENR	MIC DOX
E-test SXT	0.980	NA	NA	NA
E-test TET	NA	0.980	NA	NA
E-test ENR	NA	NA	0.999	NA
E-test DOX	NA	NA	NA	0.930

NA: Non-applicable.

tetracycline and sulfamethoxazole/trimethoprim. In the case of doxycycline, the agreement between the two techniques was moderate (0.930). Based on these results (Table 1), the E-test was used to determine the antimicrobial susceptibility for all the strains included in the study.

MIC data for PM and MH strains and the four antimicrobials are shown in Table 2. In the case of *Pasteurella multocida*, for sulfamethoxazole/trimethoprim, MIC_{range}, MIC₅₀ and MIC₉₀ values were 0.004–32 µg/mL, 0.063 µg/mL and 1 µg/mL; for tetracycline 0.016–256 µg/mL, 1 µg/mL, and 32 µg/mL; for enrofloxacin 0.002–32 µg/mL, 0.016 µg/mL and 0.5 µg/mL; and for doxycycline 0.063–32 µg/mL, 1 µg/mL and 16 µg/mL. On the other hand, in the case of *Mannheimia haemolytica* for sulfamethoxazole/trimethoprim, MIC_{range}, MIC₅₀ and MIC₉₀ values were 0.004–1024 µg/mL, 0.063 µg/mL and 1 µg/mL; for tetracycline 0.063–256 µg/mL, 8 µg/mL, and 64 µg/mL; for enrofloxacin 0.004–32 µg/mL, 0.032 µg/mL and 16 µg/mL; and for doxycycline 0.063–256 µg/mL, 2 µg/mL L and 16 µg/mL.

The distributions of MIC for SXT, TET, ENR and DOX are shown in Figs. 1, 2, 3 and 4, respectively, and tentative ECOFF values are superposed on the figures as a red line. The distributions of SXT and ENR are clearly unimodal for PM and MH. The percentage of strains with a MIC value above the ECOFF value at 99% of the confidence level (Figs. 1 and 3) of SXT and ENR were 16.7 and 15% for PM and 25 and 33.8% for MH, respectively. On the other hand, the distributions for TET and DOX were bimodal for both pathogens. The percentage of strains with a MIC value above the ECOFF value at 99% of the confidence level of TET and DOX were 40 and 11.7% for PM and 52.9 and 25% for MH, respectively (Figs. 2 and 4).

4. Discussion

The choice of an antimicrobial and the design of a rational dosing regimen depends on the knowledge of the microorganism that causes the disease (clinical experience or isolation), the action of the drug on the microorganism (pharmacodynamics), the action of the drug on the animal treated (toxicity) and the availability of the drug for the animal in question (pharmacokinetics). Other considerations include the appearance of antimicrobial resistance, animal welfare and the economic cost of the treatment (Fraile, 2013). In the current work, updated information was obtained about antimicrobial susceptibility (pharmacodynamic parameter) of ovine respiratory pathogens under field conditions in Spain and Portugal. Currently, little is known about antibacterial susceptibility distributions among the target pathogens of sheep and lambs in EU countries. The current study aims to address this gap by determining the minimal inhibitory concentrations (MICs) for two major respiratory tract pathogens recovered, prior to antibiotic treatment, from diseased lambs in Spain and Portugal. One of the critical points is the selection of antimicrobials to be tested. In this case, we focused on the antibiotics most frequently used through oral (premix or water) administration for lambs in Spain and Portugal. Thus, sulfadiazine and trimethoprim (SXT), tetracycline (TET), enrofloxacin (ENR) and doxycycline (DOX) were chosen following this criteria. From a strictly scientific point of view, it would have been advisable to include antimicrobials of the macrolide, fenicol and beta-lactamic

Table 2

MIC₅₀, MIC₉₀ and tentative epidemiological breakpoint (ECOFF) with a confidence level of 99% for *Pasteurella multocida* (n = 60 strains) and *Mannhaemia haemolytica* (n = 68 strains) isolated from lung or tracheobronchial lavage of lambs with respiratory symptoms.

Antimicrobial	Bacterial species	MIC ₅₀ (µg/mL)	MIC ₉₀ (µg/mL)	Tentative Epidemiological breakpoint (ECOFF) (µg/mL)
Sulfamethoxazole/trimethoprim (SXT)	<i>Pasteurella multocida</i>	0.063	1	0.5
	<i>Mannhaemia haemolytica</i>	0.063	1	0.25
Tetracycline (TET)	<i>Pasteurella multocida</i>	1	32	2
	<i>Mannhaemia haemolytica</i>	8	64	4
Enrofloxacin (ENR)	<i>Pasteurella multocida</i>	0.016	0.5	0.125
	<i>Mannhaemia haemolytica</i>	0.032	16	0.25
Doxycycline (DOX)	<i>Pasteurella multocida</i>	1	16	8
	<i>Mannhaemia haemolytica</i>	2	16	4

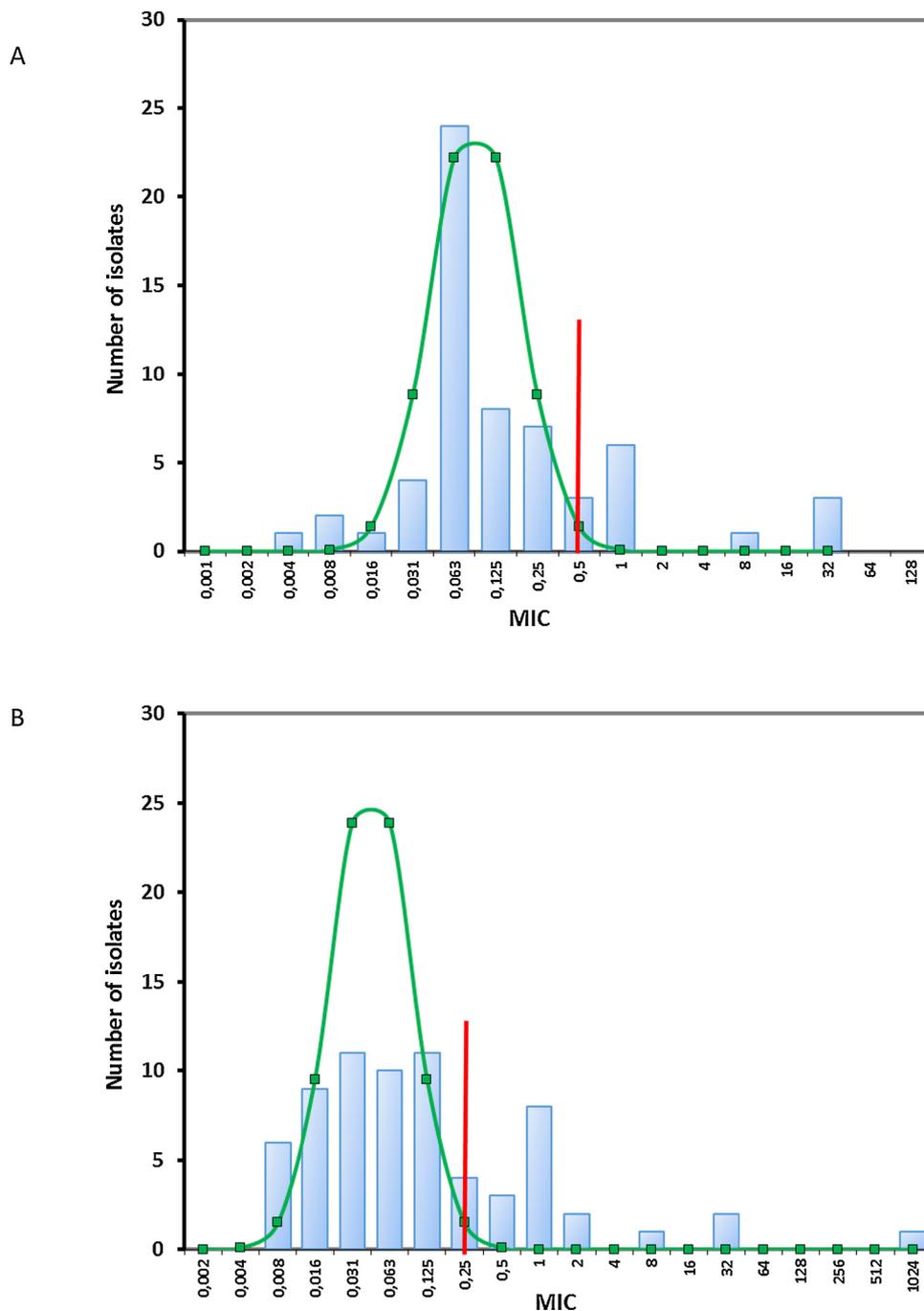


Fig. 1. MIC distribution of sulfamethoxazole/trimethoprim for *Pasteurella multocida* (A) and *Mannhaemia haemolytica* (B) isolated from lung or bronchoalveolar lavage of lambs with respiratory symptoms. Epidemiological breakpoint (ECOFF) is indicated by a red line (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

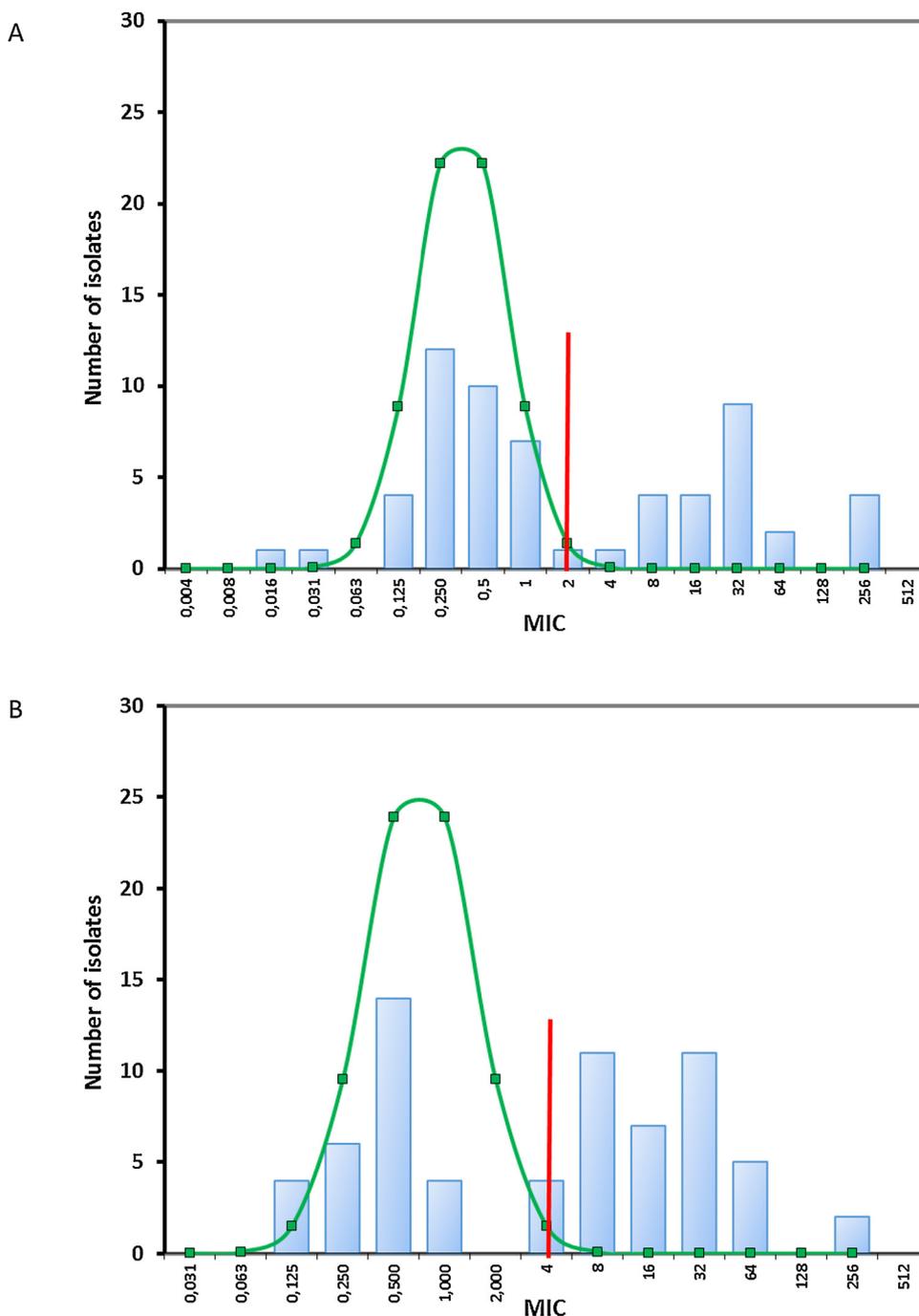


Fig. 2. MIC distribution of tetracycline for *Pasteurella multocida* (A) and *Mannhaemia haemolytica* (B) isolated from lung or bronchoalveolar lavage of lambs with respiratory symptoms. Epidemiological breakpoint (ECO) is indicated by a red line (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

families to monitor more precisely the antimicrobial susceptibility of these ovine pathogens but there is a shortage of veterinary medicinal products for use in lambs with these active ingredients. Thus, information gained would have been of little practical use.

The measurement of growth inhibition is carried out by minimum inhibitory concentration (MIC) determination. The MIC is the lowest antimicrobial concentration that inhibits *in vitro* the growth of the target bacteria in specific conditions of incubation *in vitro* (usually after 18 to 24 h in a culture medium at 37 °C and with a standard amount of inoculums) (Mckellar et al., 2004). In this study, the antimicrobial susceptibility was determined using artificial culture media as recommended by international guidelines on antimicrobial susceptibility

determination (CLSI, 2018). This methodology does not emulate the natural biophase in which bacteria grow *in vivo*, such as blood, or interstitial and intracellular fluid. This could lead to a deviation when using the information obtained *in vitro* to predict the clinical outcomes *in vivo*, but the results are more reproducible and comparable between laboratories and the prediction of clinical efficacy, taking into account this pharmacodynamic information, is acceptable from a practical point of view (Mckellar et al., 2004).

Our samples were collected from diagnostic specimens and we do not have the whole history of antimicrobial treatments received in those animals before arriving at the finishing farm. This could have biased the results towards a more resistant bacterial population that

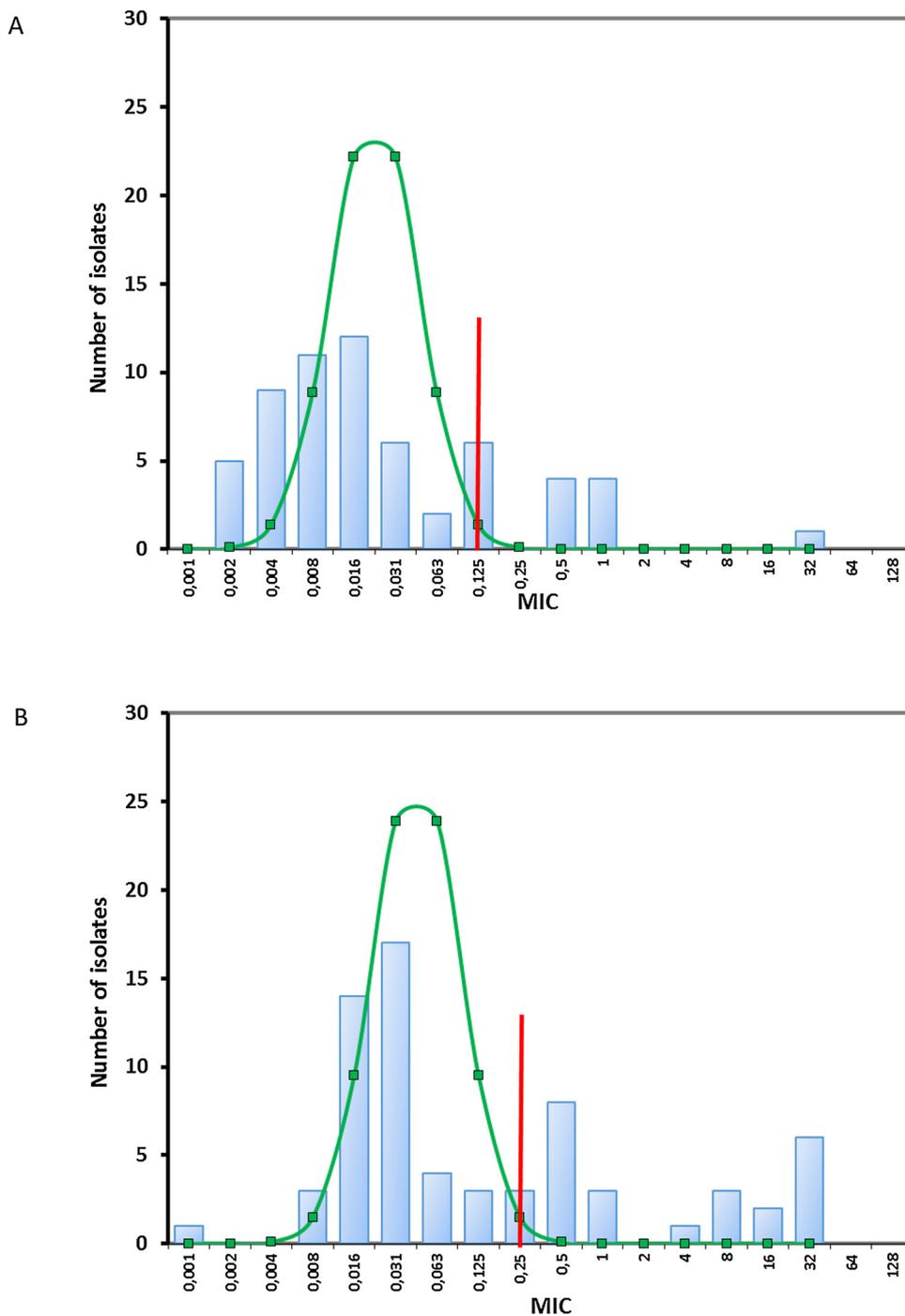


Fig. 3. MIC distribution of enrofloxacin for *Pasteurella multocida* (A) and *Mannheimia haemolytica* (B) isolated from lung or bronchoalveolar lavage of lambs with respiratory symptoms. Epidemiological breakpoint (ECO) is indicated by a red line (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

may not be representative for animals that have never received any antimicrobial therapy. The methods used to test the activity of antimicrobials against pathogens are mainly agar dilution, broth microdilution, E-test and diffusion disk (Kelly et al., 1999). Nowadays, broth microdilution is the standard method for this determination because the diffusion disk technique and E-test were found to be unreliable for some antimicrobials (e.g., colistin) due to poor diffusion properties in agar. Nevertheless, other methods may also be used if correctly validated as in the case of the E-test. This procedure consists of a continuous stable gradient of antimicrobial agent corresponding to 15 two-fold dilutions on a strip. We have carried out studies with the E-test to test its

agreement with broth microdilution (BM) for two ovine pathogens and four antimicrobials. Our results indicate that the agreement is good enough to use in place of BM for our research. The E-test technique is less labor-intensive and more cost-effective than BM for this particular case. The good agreement between the E-test and BM was previously demonstrated for cefditoren with *Streptococcus pneumoniae* (Kelly et al., 1999) and for telithromycin with pneumococci (Davies et al., 2000). Nevertheless, it must be studied on a case-by-case basis as a lack of agreement between the E-test and BM was recently published for colistin and enterobacteriaceae, probably due to its poor diffusion properties in agar (Turlej-Rogacka et al., 2018).

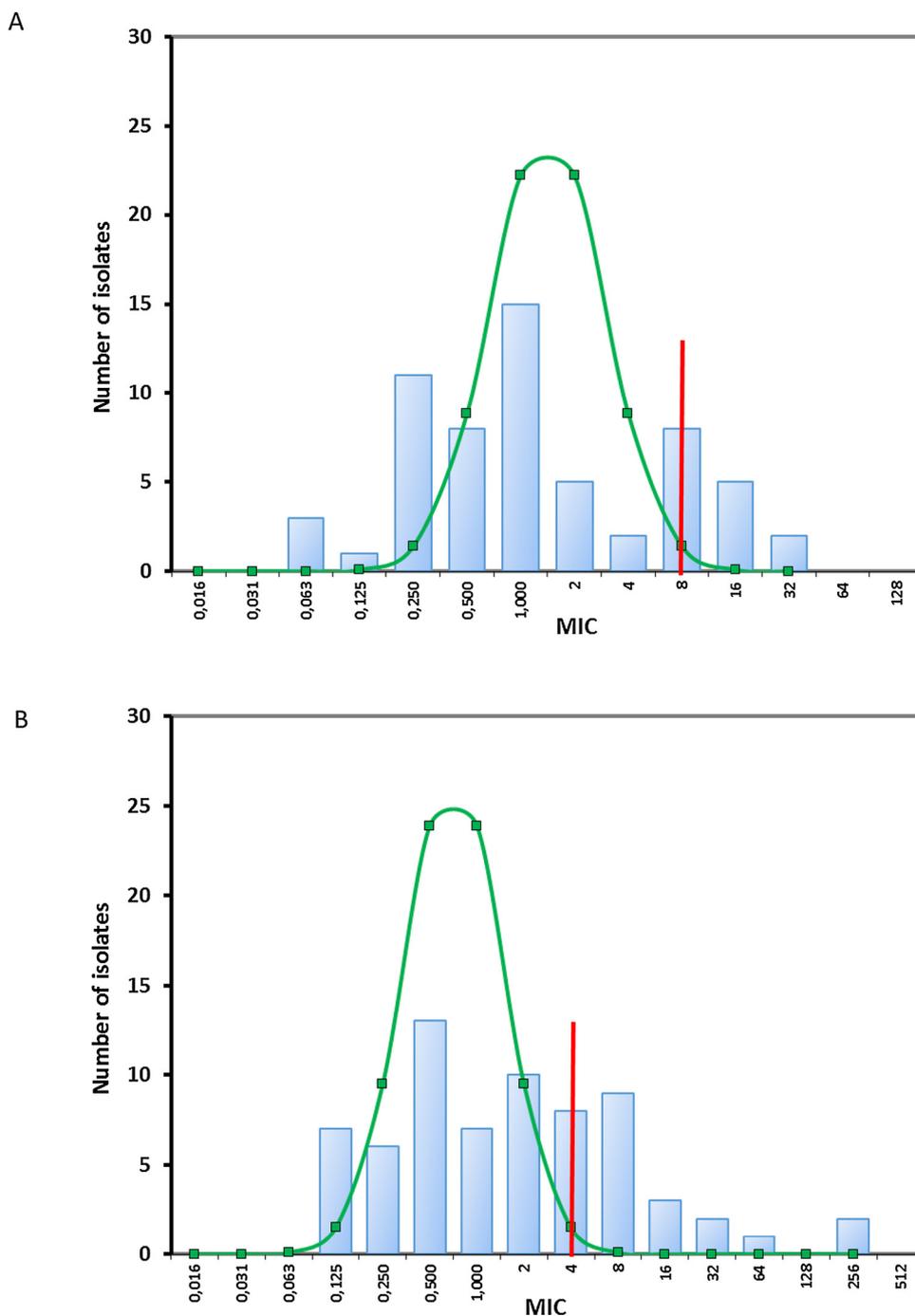


Fig. 4. MIC distribution of doxycycline for *Pasteurella multocida* (A) and *Mannhaemia haemolytica* (B) isolated from lung or bronchoalveolar lavage of lambs with respiratory symptoms. Epidemiological breakpoint (ECOFF) is indicated by a red line (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Empirical treatment is generally based on knowledge of susceptibility patterns of the different bacterial pathogens to antimicrobial agents used in the particular animal species. However, there is a shortage of information regarding the antimicrobial susceptibility among disease-causing bacteria from lambs. For the current work, clinical veterinary breakpoints are not available for lambs. Thus, isolates were not categorized as susceptible or resistant from a clinical point of view. It is, therefore, important to present the MIC frequency distributions to allow some interpretation from a practical point of view (Schwarz et al., 2001). This is feasible because the susceptible population, referred to as wild type by EUCAST, is also characterized by the absence of acquired resistance mechanisms and/or mutations leading to

resistance.

Unfortunately, it is not possible to compare the MIC data in our study with other studies in lambs because there are no recent studies using similar antimicrobial agents and guidelines. The information available is very old, was obtained by obsolete methodology (Diker et al., 1994), comes from a geographical area unrelated to the European context (Marru et al., 2013) and/or was obtained with pathogens isolated from mastitis cases (Lollai et al., 2016; Serrano-Rodríguez et al., 2017). On the other hand, the majority of bovine respiratory cases are associated with *Pasteurella multocida* (PM) and *Mannhaemia haemolytica* (MH) infections, which makes comparisons across species feasible. Our study demonstrates that, taken as a whole, antimicrobial

susceptibility of PM and MH in Spain and Portugal is relatively high for many licensed antibiotics for ovine respiratory disease, as was previously published for bovine animals (El Garch et al., 2016). Curiously, the antimicrobial susceptibility profile is better for PM than for MH not only in ovine but also in bovine animals (El Garch et al., 2016). On the other hand, the antimicrobial susceptibility is not similar across antimicrobial families for ovine animals. Thus, for enrofloxacin, doxycycline and sulfonamides, the percentage of PM and MH strains with a MIC value above ECOFF was relatively low (less than 25% in most of the cases) suggesting that many of them could obtain a positive clinical outcome after treatment with these antimicrobials. However, the situation is different for tetracycline for which the former percentage was higher (40% and 53% for PM and MH, respectively) than that previously described for the rest of the antimicrobials suggesting that the treatment with this drug could be associated with poor clinical outcomes in many cases. Finally, the clinical breakpoint described for tetracycline in PM and MH isolated from bovine (2 µg/mL) has the same value for PM (2 µg/mL) or is one dilution lower (4 µg/mL) for MH than the tentative ECOFF value described for ovine animals. On the other hand, the clinical breakpoint described for enrofloxacin in PM and MH isolated from bovine (0.25 µg/mL) is one dilution higher (0.125 µg/mL) or a similar value (0.25 µg/mL) than the ECOFF value described for PM and MH in ovine animals, respectively. Both results suggest that the clinical breakpoints for tetracycline and enrofloxacin could have the same value as those described for bovine animals. These data should be validated with accepted methods to establish clinical breakpoints (Turnidge and Martinez, 2017; Toutain et al., 2017). Unfortunately, a similar analysis cannot be performed for SXT and DOX due to the lack of clinical breakpoints.

5. Conclusions

The results of this study showed an antimicrobial pattern with good susceptibility of ovine respiratory pathogens to various licensed antibiotics including fluoroquinolones and sulfonamides. The antimicrobial susceptibility of antibiotics in the tetracycline family is variable. Doxycycline showed an antimicrobial pattern better than tetracycline. Tetracycline should only be used when the susceptibility test has shown efficacy. Finally, antimicrobial susceptibility monitoring programs of important veterinary pathogens are necessary to provide evidence-based guidance for antimicrobial therapy of bacterial diseases.

Declaration of Competing Interest

Authors do not have any conflicts of interests about this research work.

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References

- Aarestrup, F.M., Oliver Duran, C., Burch, D.G., 2008. Antimicrobial resistance in swine production. *Animal Health Reviews* 9 (2), 135–148. <https://www.ncbi.nlm.nih.gov/pubmed/18983723>.
- Black, H., Donachie, W., Duganzich, D., 1997. An outbreak of *Pasteurella multocida* pneumonia in lambs during a field trial of a vaccine against *Pasteurella haemolytica*. *New Zeal. Vet. J.* 45, 58–62. <https://www.researchgate.net/publication/7713221>.
- Brodgen, K.A., Lehmkühl, H.D., Cutlip, R.C., 1998. *Pasteurella haemolytica* complicated respiratory infections in sheep and goats. *Vet. Res.* 29 (3–4), 233–254. <https://www.ncbi.nlm.nih.gov/pubmed/9689740>.
- Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals. VET08. CLSI. <https://clsi.org/standards/products/veterinary-medicine/documents/vet01/>.
- Davies, T.A., Kelly, L.M., Jacobs, M.R., Appelbaum, P.C., 2000. Antipneumococcal activity of telithromycin by agar dilution, microdilution, E test, and disk diffusion methodologies. *J. Clin. Microbiol.* 38 (4), 1444–1448. <https://www.ncbi.nlm.nih.gov/pubmed/10747123>.

- Diker, K.S., Akan, M., Hazirolu, R., 1994. Antimicrobial susceptibility of *Pasteurella haemolytica* and *Pasteurella multocida* isolated from pneumonic ovine lungs. *Vet. Rec.* 134 (23), 597–598. <https://veterinaryrecord.bmj.com/content/134/23/597>.
- El Garch, F., de Jong, A., Simjee, S., Moyaert, H., Klein, U., Ludwig, C., Marion, H., Haag-Diergarten, S., Richard-Mazet, A., Thomas, V., Siegwart, E., 2016. Monitoring of antimicrobial susceptibility of respiratory tract pathogens isolated from diseased cattle and pigs across Europe, 2009–2012: VetPath results. *Vet. Microbiol.* 194, 11–22. <https://www.sciencedirect.com/science/article/pii/S0378113516300918>.
- Answers to the Requests for Scientific Advice on the Impact on Public Health and Animal Health of the use of Antibiotics in Animals. EMA AMEG. https://www.ema.europa.eu/en/documents/other/answers-requests-scientific-advice-impact-public-health-animal-health-use-antibiotics-animals_en.pdf.
- Answer to the Request From the European Commission for Updating the Scientific Advice on the Impact on Public Health and Animal Health of the Use of Antibiotics in Animals - Categorisation of Antimicrobials. EMA AMEG. https://www.ema.europa.eu/en/documents/other/answer-request-european-commission-updating-scientific-advice-impact-public-health-animal-health-use_en.pdf.
- Fernandez, S., Rey, J., 2013. Procesos Respiratorios producidos por *Mycoplasma*. Epidemiología y Control de los procesos respiratorios en pequeños rumiantes. Monografía. Revista Pequeños Rumiantes. Publicación de la Sociedad española de Ovinotecnia y Caprinotecnia 13 (3), 20–29. <https://dialnet.unirioja.es/servlet/articulo?codigo=4179120>.
- Fraille, L., 2013. Antimicrobial Therapy in Swine. A Practical Approach. Ed. Servet. Grupo Asis Biomedica S.L., pp. 25–43. <https://store.grupoasis.com/en/swine/235-antimicrobial-therapy-in-swine-practical-approach-9788494101496.html>.
- Gómez, M.T., Garijo, M.M., 2013. Bronconeumonías Verminosas. Epidemiología y control de los procesos respiratorios en pequeños rumiantes. Monografía. Revista pequeños rumiantes. Publicación de la Sociedad española de Ovinotecnia y Caprinotecnia. 2013 13 (3), 44–55. <https://dialnet.unirioja.es/servlet/articulo?codigo=4179125>.
- Gonzalez, J.M., 2015. Factores Que Condicionan La Supervivencia De Los Corderos Tipo Ternasco. Estudio Del Complejo Respiratorio Ovíno: Presentación, Formas Lesionales, Agentes Implicados Y Serotipificación De *Pasteurella haemolytica*. Tesis doctoral. Universidad de Zaragoza, Zaragoza (España). <https://dialnet.unirioja.es/servlet/tesis?codigo=203037>.
- Gonzalez, J.M., De Las Heras, M., Ferrer, L.M., Figueras, L., Garcia De Jalón, J.A., Lacasta, D., 2001. Las neumonías catarrales crónicas influyen negativamente en los índices productivos del cordero tipo ternasco. XXVI Jornadas Científicas SEOC 2001. <http://seoc.eu/es/2001-sevilla/>.
- González, J.M., Bello, J.M., Rodríguez, M., Navarro, T., Lacasta, D., Fernández, A., De las Heras, M., 2016. Lamb feedlot production in Spain: Most relevant health issues. *Small Rumin. Res.* 142 (83), 87. <https://www.sciencedirect.com/science/article/abs/pii/S092144881630044X>.
- Hoffman, A.M., 2008. Bronchoalveolar lavage: sampling technique and guidelines for cytologic preparation and interpretation. *Vet. Clin. North Am. Equine Pract.* 24 (2), 423–435. <https://www.sciencedirect.com/science/article/abs/pii/S0749073908000266?via%3Dihub>.
- Kelly, L.M., Jacobs, M.R., Appelbaum, P.C., 1999. Comparison of agar dilution, microdilution, E-test, and disk diffusion methods for testing activity of cefditoren against *Streptococcus pneumoniae*. *J. Clin. Microbiol.* 37 (10), 3296–3299. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC85550/>.
- Lacasta, D., Ferrer, L.M., Ramos, J.J., González, J.M., De las Heras, M., 2008. Influence of climatic factors on the development of pneumonia in lambs. *Small Rumin. Res.* 80 (28), 32. <https://www.sciencedirect.com/science/article/abs/pii/S0921448808001740>.
- Lollai, S.A., Ziccheddu, M., Duprè, I., Piras, D., 2016. Characterization of resistance to tetracyclines and aminoglycosides of sheep mastitis pathogens: study of the effect of gene content on resistance. *J. Appl. Microbiol.* 21 (4), 941–951. <https://www.sciencedirect.com/science/article/pii/S0022030217300413>.
- Luzón, J., De las Heras, M., 1999. Influencia de los procesos respiratorios en la mortalidad de corderos tipo ternasco y en los decomisos en el matadero. XXIV Congreso SEOC. Patología, comunicación 15. <http://seoc.eu/es/1999-soria/>.
- Marru, H.D., Anijajo, T.T., Hassen, A.A., 2013. A study on Ovine pneumonic pasteurellosis: isolation and identification of *Pasteurella* and their antibiogram susceptibility pattern in Haramaya District, Eastern Hararghe, Ethiopia. *BMC Vet. Res.* 9, 239. <https://www.ncbi.nlm.nih.gov/pubmed/24289236>.
- Martin, M.C., Cid, M.D., 2013. Pasterellosis Neumónica ovina y caprina. Epidemiología y Control de los procesos respiratorios en pequeños rumiantes. Monografía. Revista Pequeños Rumiantes. Publicación de la Sociedad española de Ovinotecnia y Caprinotecnia. 2013 13 (3), 6–16. <https://dialnet.unirioja.es/servlet/articulo?codigo=4179117>.
- Mckellar, Q.A., Sanchez Bruni, S.F., Jones, D.G., 2004. Pharmacokinetic/pharmacodynamic relationship of antimicrobial drugs used in veterinary medicine. *J. Vet. Pharmacol. Ther.* 27, 503–514. <https://www.researchgate.net/publication/8129387>.
- Plan estratégico y de acción para reducir el riesgo de selección y diseminación de la resistencia a los antibióticos, 2014. Plan Nacional Resistencia a Antibióticos (PRAN). Agencia Española De Medicamentos Y Productos Sanitarios. <https://www.aemps.gob.es/publicaciones/publica/plan-estrategicoantibioticos/v2/docs/plan-estrategico-antimicrobianos-AEMPS.pdf>.
- Plan estratégico y de acción para reducir el riesgo de selección y diseminación de la resistencia a los antibióticos, 2019. Plan Nacional Resistencia a Antibióticos (PRAN). Agencia Española De Medicamentos Y Productos Sanitarios. <https://www.aemps.gob.es/laAEMPS/planificacion-AEMPS/docs/Plan-estrategico-2019-2022.pdf>.
- Schwarz, S., Kehrenberg, C., Walsh, T.R., 2001. Use of antimicrobial agents in veterinary medicine and food animal production. *Int. J. Antimicrob. Agents* 17, 431–437. <https://www.sciencedirect.com/science/article/abs/pii/S0924857901002977>.

- Serrano-Rodríguez, J.M., Cárceles-García, C., Cárceles-Rodríguez, C.M., Gabarda, M.L., Serrano-Caballero, J.M., Fernández-Varón, E., 2017. Susceptibility and PK/PD relationships of *Staphylococcus aureus* strains from ovine and caprine with clinical mastitis against five veterinary fluoroquinolones. *Vet. Rec.* 180 (15), 376. <https://www.ncbi.nlm.nih.gov/pubmed/28213532>.
- Thrusfield, M., 2018. *Veterinary Epidemiology*. Veterinary Clinical Sciences. Royal School of Veterinary Sciences, fourth edition. University of Edinburgh. https://books.google.es/books?hl=es&lr=&id=3e5LDwAAQBAJ&oi=fnd&pg=PR18&dq=Veterinary+epidemiology.+Veterinary+Clinical+Sciences&ots=A_AKtY1aW&sig=gA-Cr4R1-HEGzKP4Gm-DVlli1Co#v=onepage&q=Veterinary%20epidemiology.%20Veterinary%20Clinical%20Sciences&f=false.
- Toutain, P.L., Bousquet-Mélou, A., Damborg, P., Ferran, A.A., Mevius, D., Pelligand, L., Veldman, K.T., Lees, P., 2017. En route towards european clinical breakpoints for veterinary antimicrobial susceptibility testing: a position paper explaining the VetCAST approach. *Front. Microbiol.* 8, 2344. <https://www.ncbi.nlm.nih.gov/pubmed/29326661>.
- Turlej-Rogacka, A., Xavier, B.B., Janssens, L., Lammens, C., Zarkotou, O., Pourmaras, S., Goossens, H., Malhotra-Kumar, S., 2018. Evaluation of colistin stability in agar and comparison of four methods for MIC testing of colistin. *Eur. J. Clin. Microbiol. Infect. Dis.* 37 (2), 345–353. <https://www.ncbi.nlm.nih.gov/pubmed/29177612>.
- Turnidge, J.D., Martinez, M.N., 2017. Proposed method for estimating clinical cut-off (CO_{CL}) values: an attempt to address challenges encountered when setting clinical breakpoints for veterinary antimicrobial agents. *Vet. J.* 228, 33–37. <https://www.sciencedirect.com/science/article/pii/S1090023317301867>.
- Turnidge, J., Kahlmeter, G., Kronvall, G., 2006. Statistical characterisation of bacterial wild-type MIC value distributions and the determination of epidemiological cut-off values. *Clin. Microbiol. Infect.* 12 (5), 418–425. <https://www.sciencedirect.com/science/article/pii/S1198743X14620048>.
- Vilallonga, D., 2013. Estudio de la etiología e impacto económico de los decomisos en un matadero de ovinos. Tesis doctoral. Universidad de Extremadura, Extremadura (España). http://dehesa.unex.es/bitstream/handle/10662/622/TDUEX_2013_Vilallonga_Vazquez.pdf?sequence=1&isAllowed=y.
- Watson, P.F., Petrie, A., 2010. Method agreement analysis: a review of correct methodology. *Theriogenology* 73 (9), 1167–1179. <https://www.ncbi.nlm.nih.gov/pubmed/20138353>.