38. Antimicrobial susceptibility of *Brachyspira hyodysenteriae* isolated in Italy from 2005 to 2013.

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ABSTRACT

Swine dysentery (SD) is a severe colitis of pigs caused by *Brachyspira hyodysenteriae* (Harris, 1999). The control of SD is still mainly based on the use of few antimicrobials, such as pleuromutilins, macrolides and lincosamides.

However more than half of the Italian isolates of *B. hyodysenteriae*were shown to be resistant to pleuromutilins, with a significant increasing trend in the last 10 years (Rugna, 2015).

The aim of this study was to compare the susceptibility of *Brachyspira hyodysenteriae* versus tiamulin, valnemulin, doxycycline, aivlosin, lincomycin and tylosin.

The antimicrobial susceptibility of 206 *B.hyodysenteriae* isolates from Italian herds from 2005-2013 was evaluated by a broth microdilution test. The MIC values were interpreted using clinical breakpoints (Ronne and Szancer, 1990) and epidemiological cut-offs (Pringle, 2012). The vast majority of the isolates (above 80%, approximately) showed MIC values above the epidemiological cut offs, indicating a previous exposure to antimicrobials.

Moreover, most isolates (98%) were clinically resistant to tylosin, about half (56%) to lincomycin and roughly a third (34%) to tiamulin.

Furthermore, a significant increase of non-susceptible isolates (resistant or intermediate) was seen for both lincomycin and tiamulin from 2008 to 2013. A non-negligible proportion of isolates (54%) showed no susceptibility to tylosin, lincomycin and tiamulin, with a significant increase from 2010 to 2013 compared to 2008. Finally, a correlation was found between the non-wild type status of isolates to aivlosin and tylosin ($p=0.025$) and between non-wild type status of isolates to tylosin and lincomycin ($p=0.016$).

In conclusion, the decreased susceptibility of Italian isolates of *B. hyodysenteriae* is not restricted to pleuromutilins, but involves most of the antibiotics used for the control of SD. An increase of multiresistant isolates was recorded in the last eight years, posing a significant threat to pig industry.

INTRODUCTION

Swine dysentery (SD) is a severe muco-haemorrhagic colitis of pigs resulting from colonization of the large intestine by anaerobic intestinal spirochete *Brachyspira hyodysenteriae* (Harris, 1972). Where *B. hyodysenteriae* infection is endemic, SD is a major cause of severe economic losses for the pig industry (Rohde, 2004).Traditionally, control of SD was based on the use of antimicrobials in feed. Pleuromutilins, macrolides and lincosamides are essential for treatment of SD (Pringle, 2012). However, resistance to pleuromutilins has increased over the past twenty years (Molnar, 1996; Rohde, 2004). In Italy this phenomenon was described for the first time in 2004 (Bonilauri, 2004). More recently, more than half of the Italian isolates of *B. hyodysenteriae* were shown to be resistant to pleuromutilins, with a significant increasing trend in the last 10 years (Rugna, 2015). At the same time, multi-resistant strains of *B. hyodysenteriae*, showing a decreased susceptibility to pleuromutilin, macrolides and lincosamides, have been isolated across Europe (Hidalgo, 2011; Sperling, 2011).

The aim of this study was to compare the susceptibility of isolates of *B. hyodysenteriae* from Italian pig herds to pleuromutilins, doxycycline, aivlosin, lyncomycin and tylosin.

MATERIALS AND METHODS

Brachyspira hyodysenteriae isolates

B. hyodysenteriae Italian isolates (n=206) were taken from the strain collections of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche (IZSUM) and the Istituto Zooprofilattico della Lombardia e della Emilia Romagna, Reggio Emilia. The isolates originated from diagnostic submissions received in 2005- 2013 from Italian pig herds. The species attribution of the isolates was confirmed by the assessment of strong haemolysis, microscopy and species-specific PCR (La, 2003). The cultures were stored at -80°C.

Susceptibility testing by agar dilution

Thawed isolates were grown on fastidious anaerobe agar supplemented with 10% equine blood (FAA) (National Veterinary Institute, Uppsala, Sweden) for three days at 42°C prior to susceptibility testing. The purity of all isolates was assessed by phase contrast microscopy. The MICs of tiamulin, valnemulin, tylosin, aivlosin, doxycycline and lincomycin were determined by broth dilution in VetMICBrachy panels (SVA, Uppsala, Sweden) as described previously (Rohde,2004). Briefly, bacteria harvested from FAA were suspended in brain heart infusion (BHI) broth to an estimated concentration of 10^8 ufc/ml. From this suspension, 300 µl was transferred to 30 ml BHI broth, supplemented with 10% fetal calf serum, to obtain a final inoculum concentration of 10^6 ufc/ml. Each well was filled with 500 µl of the inoculums. The panels were incubated in square GENbox anaerobic jars with GENboxanaer generator sachets (bioMèrieux) for 4 days on a shaker at 37°C. The MIC was set as the lowest concentration of the antimicrobial agent that prevented visible growth. Strain B78T (*B. hyodysenteriae*, ATCC 27164) was used as a control.
The following concentrations of antimicrobials were tested:

Tiamulin: 0.063 µg/ml, 0,125 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml,8 µg/ml; Valnemulin: 0.031 µg/ml, 0.063 µg/ml, 0.125 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml; Doxycycline: 0.125 µg/ml, 0,25 µg/ml, 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml; Aivlosin: 0.25 µg/ml, 0,5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 32 µg/ml; Lincomycin: 0.5 µg/ml, 1 µg/ml, 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 32 µg/ml, 64 µg/ml; Tylosin: 2 µg/ml, 4 µg/ml, 8 µg/ml, 16 µg/ml, 32 µg/ml, 64 µg/ml, 128 µg/ml.

The MIC values were interpreted using clinical breakpoints (Ronne and Szancer, 1990) and epidemiological cut-offs (Pringle, 2012).

Statistical analysis

Statistical analysis was performed with the χ for trend, each year from 2008 to 2013, with a $p \leq 0.05$ level of significance. To underline the strength of association, Odds Ratios (OR) with 95% confidence intervals (95% CI) were calculated.

RESULTS

The vast majority of the isolates showed MIC values above the epidemiological cut-offs (91% for tiamulin, 99% for lincomycin and 98% for tylosin), indicating a previous exposure to antimicrobials (Table 1).

Table 1: proportion of isolates classified as wild/non wild, according to the epidemiological cut-offs indicated by Pringle (2012)

Antimicrobial agents	Wild (%)	Non wild (%)
Tiamulin	9.2	90.8
Valnemulin	18.9	81.1
Doxycycline	6.3	93.7
Aivlosin	3.1	96.9
Lincomycin	0.97	99.3
Tylosin	1.97	98.3

Moreover, most isolates (98%) were resistant to tylosin, about half (56%) to lincomycin and roughly a third (34%) to tiamulin (Table 2).

Table 2: proportion of isolates classified as resistant, intermediate and susceptible, according to the clinical breakpoints indicated by Ronne and Szancer (1990)

Antimicrobial agents	Resistant (%)	Intermediate (%)	Susceptible (%)
Tiamulin	34	21,8	44,2
Valnemulin	29,2	21,8	49
Lincomycin	55,5	34,5	10
Tylosin	98	1	1

A significant increase of non-susceptible isolates (resistant or intermediate) was seen for tiamulin (p=0.0328) compared to 2008, the ORs being 8.4, 4.65 and 4.44 in 2011, 2012 and 2013, respectively. A similar increasing trend was observed for lincomycin (p=0.0276). The same evaluation could not be done for tylosin, because no susceptible isolate had been detected for three consecutive years.

About half the isolates (54%) showed no susceptibility to tylosin, lincomycin and tiamulin, so they were classified as multi-resistant. A significant increase in the proportion of multi-resistant isolates was observed (p=0.0039) from 2011 (OR=8.4) to 2013 (OR=4.32) compared to 2008 (Figure 1).

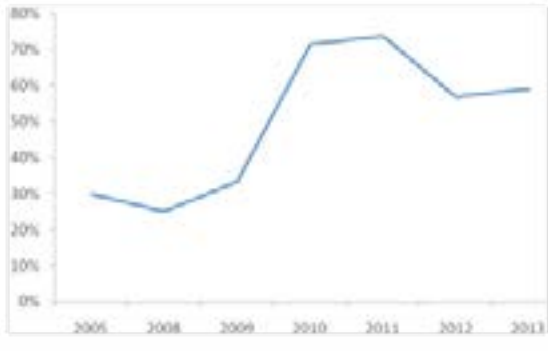

Figure 1: Line graph showing the proportion of multi-resistant *B. hyodysenteriae* isolates from 2005 to 2013.

Finally, a correlation was found between the non-wild type status of isolates to aivlosin and that of isolates to tylosin (p=0.025). The same correlation was found between non-wild type status of isolates to tylosin and that of isolates to lincomycin (p=0,016).

DISCUSSION

Epidemiological cut-offs give indications on the presence of acquired and mutational resistance mechanisms to a drug, and they are used to monitor changes of susceptibility in a bacterial population (Pringle, 2012). Most of the isolates included in the present study showed MIC values above this threshold, indicating a previous exposure to these antimicrobial agents. In some cases, such as in the case of aivlosin, the drug has only recently been approved for use in pigs in Italy and a previous exposure to this antimicrobial does not seem probable. However, a cross-resistance phenomenon can be postulated to explain this finding. In our study, a correlation was found between the non-wild type status of isolates to aivlosin and that of isolates to tylosin, an antimicrobial which has been extensively used in Italian pig production. This is in agreement with other authors, who indicated a possible cross-resistance mechanism between tylosin and aivlosin (Pringle, 2012).

Epidemiological cut-offs do not give indication on the susceptibility of *B. hyodysenteriae* isolates to therapy, this can instead be derived using the clinical breakpoints. However, these breakpoints have never officially been established for this bacterium. Therefore, the interpretation of MIC results is still based on the thresholds indicated by Ronne and Szancer in 1990, which do not include all the antimicrobials available today. According to the available clinical breakpoints, most of the isolates tested in this study showed poor susceptibility to the drugs approved for the control of SD. This reduced susceptibility is confirmed by the frequent reports of poor response to antimicrobial therapy on the field and it probably originated from the continuous treatment with antibiotics of herds affected by SD. It should be noted that this study is based on passive sampling, and these results are not to be considered representative of *B. hyodysenteriae* susceptibility prevalence in Italian pig herds. We can reasonably presume that the most severe cases, undergoing repeated treatment failures, were selected. However, in our study the decreased susceptibility involves most of the antibiotics used for the control of SD, with an increase of multi-resistant isolates in the last eight years. The presence and spread of these isolates can make the antibiotic therapy of SD ineffective and pose a significant threat to the pig industry.

To prevent a scenario in which no efficacious antimicrobial agents will be available to control SD, a more rational use of antibiotics and programmes for the eradication of *B. hyodysenteriae*, are strongly suggested.

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