



Antimicrobial susceptibility testing of Spanish field isolates of *Brachyspira hyodysenteriae*

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ABSTRACT

This study is the first conducted in Spain to evaluate antimicrobial susceptibility of field isolates of *Brachyspira hyodysenteriae*. One hundred and eight isolates of the bacterium, recovered from different Spanish swine farms between 2000 and 2007, were investigated. The minimum inhibitory concentrations (MIC) of erythromycin, tylosin, tiamulin, valnemulin, clindamycin and lincomycin were determined using a broth microdilution technique. Most of the isolates showed poor susceptibility to erythromycin (MIC₉₀ > 256 µg/ml), tylosin (MIC₉₀ > 256 µg/ml), clindamycin (MIC₉₀ > 4 µg/ml) and lincomycin (MIC₉₀ = 128 µg/ml). Reduced susceptibility to tiamulin and valnemulin was observed with a MIC > 2 µg/ml in 17.6% and 7.41% of the *B. hyodysenteriae* isolates, respectively. Moreover, a survival analysis permitted the detection of an increasing trend in the MIC values for almost all the antimicrobials used in the treatment of swine dysentery when comparing recent isolates (from 2006 to 2007) with those recovered in earlier years (between 2000 and 2004).

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1. Introduction

Swine dysentery (SD) is a severe muco-haemorrhagic colitis arising from colonization of the large intestine of pigs by *Brachyspira hyodysenteriae* (formerly *Serpulina hyodysenteriae*), a strongly β-haemolytic spirochaete (Hampson et al., 2006). The condition is seen mainly in grower or finisher pigs and less commonly in weaners, and it is characterized by obvious, wet, porridge-like diarrhoea that leads to dehydration, weight loss and, in extreme cases, death. The faeces are grey to chocolate-brown in colour and may contain plugs of mucus or flecks of fresh blood (Hampson et al., 2006). Although this condition is quite variable in its severity, SD is considered to be one of the most significant production-limiting porcine infections (Hampson et al., 1997), and it causes considerable financial losses arising from mortality, decreased growth rates, poor feed conversion, and treatment expenses (Hampson et al., 2006).

Due to the lack of commercial vaccines, the control and treatment of SD involves the use of antimicrobials, with tiamulin, valnemulin, tylosin and lincomycin as the drugs most commonly used for this purpose in the European Union (EU) (Hampson et al., 2006). However, the control of SD has been complicated nowadays by the emergence of strains of *B. hyodysenteriae* with reduced susceptibility to one or more of these antimicrobials recently reported in several countries (Molnar, 1996; Karlsson et al., 2001, 2003; Lobova et al., 2004; Rohde et al., 2004). As a consequence, careful use of the limited range of effective drugs cur-

rently available is now recommended (Karlsson et al., 2002a), and the monitoring of resistance in clinical isolates of *B. hyodysenteriae* has become highly desirable (Karlsson et al., 2002a; Rohde et al., 2004).

The development of pig production in Spain has been spectacular. It reached 3.3 million Tm in 2006, which is the second highest output in the EU (data from the Ministry of Agriculture and Livestock). A recent study concluded that SD is a major cause of diarrhoea among pigs of all ages that are raised on commercial farms in Spain (Carvajal et al., 2006). *B. hyodysenteriae* was identified in 32% of the farms and 12% of the faecal specimens collected from commercial pig farms with clinical signs of diarrhoea. In spite of their clinical importance, there are no previous data regarding the susceptibility of *B. hyodysenteriae* field isolates from Spain to antimicrobial agents.

The research reported here reports on the *in vitro* susceptibility of Spanish field isolates of *B. hyodysenteriae* to several of the drugs commonly used in the treatment and control of SD. A second aim was to determine whether the activity profile of any of these antimicrobial agents diminished over time.

2. Materials and methods

2.1. Bacterial strains and growth conditions

One hundred and eight field isolates of *B. hyodysenteriae*, obtained from faecal samples of pigs suffering from diarrhoea and submitted to the Laboratory of Infectious diseases in the Veterinary

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Faculty at the University of Leon between January 2000 and November 2007 for diagnostic examination, were investigated. One single *B. hyodysenteriae* isolate was tested per farm. The sampled farms were distributed all over the country.

For primary isolation, faecal samples were cultured on tryptose soy agar (TSA) medium supplemented with 5% ovine blood and antibiotics, as previously described by Jenkinson and Wingar (1981). Plates were incubated in an anaerobic atmosphere (10% hydrogen, 10% carbon dioxide and 80% nitrogen) at 39 °C. The bacteria were identified as *B. hyodysenteriae* according to their strong β -haemolysis and using a species-specific PCR based on the 23S rRNA gene (Leser et al., 1997). PCR, specific for *Brachyspira pilosicoli* (Muniappa et al., 1997), was performed on all the isolates to exclude the concomitance of this *Brachyspira* specie. Thereafter, plates positive for *B. hyodysenteriae* were subcultured until the pure state was reached on the TSA plates supplemented with 5% ovine blood (TSA-blood) in an anaerobic atmosphere, as mentioned above. The purity of all isolates was checked by phase-contrast microscopy. These isolates were stored in liquid nitrogen at the Department of Animal Health of the University of Leon, Spain.

The reference and type strains, B204 (ATCC 31212) and B78^T (ATCC 27164^T), were used as controls.

2.2. Antimicrobial agents and antibiotic panel

A susceptibility testing panel was designed using the following six antimicrobial agents: tiamulin hydrogen fumarate, valnemulin hydrochloride (Novartis Animal Health), tylosin tartrate, erythromycin (Sigma-Aldrich), clindamycin hydrochloride (Upjohn AB), and lincomycin hydrochloride (Pharmacia Animal Health).

Stock solutions of each of the antimicrobials were prepared with appropriate solvents according to the manufacturers' recommendations and stored at 4 °C. Five microliters of twofold serial dilutions in sterile Milli-Q water (Millipore) of each of the antimicrobials tested (for the range of final concentrations, see Fig. 1) were poured into 48-well tissue culture trays (IWAKI). Two wells were left empty and served as positive and negative growth controls. Plates were prepared immediately before using in a safety cabinet to prevent contamination.

2.3. Broth dilution procedure

Broth dilution was performed as described by Karlsson et al. (2002a, 2003). Briefly, bacteria harvested from TSA-blood plates were suspended in brain-heart infusion (BHI) broth to an estimated concentration (absorbance measuring) of $1-5 \times 10^8$ CFU/ml. Three hundred microliters of the bacterial suspension were diluted in 30 ml of BHI supplemented with 10% foetal calf serum and 0.5 ml of the final suspension was dispensed per well. The panels were incubated in anaerobic jars (GENbox, BioMerieux with AnaeroGen sachets, Oxoid) for 3–5 days on a rotary agitator at 38 °C. The minimal inhibitory concentration (MIC) was determined as the lowest concentration of antimicrobial agent that prevented visible growth. All the isolates were tested in duplicate and one dilution step difference was allowed for each antimicrobial agent between the two panels. When this difference existed, the highest MIC value was chosen for each drug. The reference and type strains, B204 (ATCC 31212) and B78^T (ATCC 27164^T), were also tested in duplicate at the start and at the end of the study as quality controls of the antibiotic panels. Aliquots (10 μ l) of the positive growth control were checked by phase-contrast microscopy to confirm pure growth.

2.4. Data processing and analysis

The strains and data yielded by the *B. hyodysenteriae* isolates were divided into two groups, according to the year of isolation,

in order to study trends in antimicrobial susceptibility over time. The first group was composed of 50 strains recovered between 2000 and 2004, whereas 58 isolates from 2006 and 2007 composed the second group. The lowest concentrations that completely inhibited the growth of 50% and 90% of the isolates, MIC₅₀ and MIC₉₀, respectively, were calculated for each of the antimicrobials. All data were stored and analysed using SPSS for Windows®.

A survival analysis was employed for comparing the resistance during the study period, as previously described (Stegeman et al., 2006). The inhibition of bacterial growth was the event, and the concentration of antibiotic to the event was used instead of time to the event. This type of analysis allows for the detection of changes over the entire range of concentrations. Growth or growth inhibition of *B. hyodysenteriae*, at each antimicrobial concentration tested, was recorded, and the data were censored when there was no inhibition at the highest concentration level. Moreover, 2 log transformations of the antimicrobial twofold serial dilutions were performed and adjusted to whole numbers starting from zero for a clearer graphical representation. Survival curves were compared using the Log Rank test at $\alpha = 0.05$.

3. Results

3.1. Antimicrobial susceptibility testing

The MICs of the six antimicrobial agents studied for the *B. hyodysenteriae* reference and type strains, B204 (ATCC 31212) and B78^T (ATCC 27164^T), obtained in the present and previous studies are shown in Table 1.

No differences higher than one dilution step for each antimicrobial agent were found between the two panels tested for any of the field or reference strains.

The distribution of the MICs of the six antimicrobial agents for the Spanish field isolates of *B. hyodysenteriae* is presented in Fig. 1. A clear unimodal population distribution was obtained for both the macrolides tested. The MICs for erythromycin were higher than the range of concentrations used ($>256 \mu\text{g/ml}$) for 96.3% of the isolates (104 out of 108). Similar results were recorded for tylosin, with MIC values equal to or greater than $256 \mu\text{g/ml}$ for 83.3% of the isolates (90 out of 108). In contrast, MICs for tiamulin exhibited a trend towards a bimodal distribution. One peak stood at $0.125 \mu\text{g/ml}$ (18.5% of the isolates), with a second at values above $2 \mu\text{g/ml}$ (17.6% of the isolates). For valnemulin, about one-third of the total population (29.6%) showed a MIC below $0.016 \mu\text{g/ml}$, while another third (34.25%) was in the range from 0.125 to $0.5 \mu\text{g/ml}$. In the case of clindamycin, a cluster of isolates (96.3%) with MICs in the region of $4 \mu\text{g/ml}$ was evident, whereas lincomycin showed a considerable population (74.1% of the isolates) with MICs of around $16 \mu\text{g/ml}$.

3.2. Changes in antimicrobial susceptibility over time

The distribution of antimicrobial resistance in the *B. hyodysenteriae* isolates recovered from clinical submissions in Spain from 2000 to 2004 and 2006 to 2007 is summarized in Table 2. The values of MIC₅₀ and MIC₉₀ for erythromycin, tylosin and tiamulin were identical in both time periods. However, the MIC₅₀ of lincomycin and clindamycin increased by one dilution step as time elapsed, although the MIC₉₀ did not change. Both the MIC₅₀ and MIC₉₀ values increased for valnemulin in the 2006–2007 period.

To investigate trends in the activity of the antimicrobial agents over time, a survival analysis was performed. The survival curves of the six antimicrobials for both periods are shown in Fig. 2. Survival curves for erythromycin, tylosin and clindamycin showed that a large proportion of the isolates were able to survive at the highest

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