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Original article

Antimicrobial susceptibility of *Brachyspira hyodysenteriae* isolated from 21 Polish farms

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Abstract

Swine dysentery (SD) is a common disease among pigs worldwide, which contributes to major production losses. Antimicrobial susceptibility testing of *B. hyodysenteriae*, the etiological agent of SD, is mainly performed by the agar dilution method. This method has certain limitations due to difficulties in interpretation of results. The aim of this study was the analysis of antimicrobial susceptibility of *Brachyspira hyodysenteriae* (*B. hyodysenteriae*) Polish field isolates by broth microdilution procedure. The study was performed on 21 isolates of *B. hyodysenteriae*, collected between January 2006 to December 2010 from cases of swine dysentery. VetMIC™ Brachyspira panels with antimicrobial agents (tiamulin, valnemulin, doxycycline, lincomycin, tylosin and ampicillin) were used for susceptibility testing of *B. hyodysenteriae*. The minimal inhibitory concentration (MIC) was determined by the broth dilution procedure. The lowest antimicrobial activity was demonstrated for tylosin and lincomycin, with inhibition of bacterial growth using concentrations >128 µg/ml and 32 µg/ml, respectively. In the case of doxycycline, the MIC values were ≤ 2.0 µg/ml. No decreased susceptibility to tiamulin was found among the Polish isolates and MIC values for this antibiotic did not exceed 1.0 µg/ml. The results of the present study confirmed that Polish *B. hyodysenteriae* isolates were susceptible to the main antibiotics (tiamulin and valnemulin) used in treatment of swine dysentery. Further studies are necessary to evaluate a possible slow decrease in susceptibility to tiamulin and valnemulin of *B. hyodysenteriae* strains in Poland.

Key words: MIC: *Brachyspira hyodysenteriae*, antimicrobial susceptibility, broth dilution

Introduction

Swine dysentery (SD) is a common disease among pigs worldwide, which contributes to major production losses. This severe mucohaemorrhagic diarrheal disease is caused by the anaerobic intestinal spirochete *Brachyspira hyodysenteriae*. On the basis of previous studies (Pejsak et al. 2007) almost 32% of Polish pig farms were found infected with the above-mentioned pathogen. In recent years, in most European countries, an increase of antimicrobial resistance to antibiotics routinely used for treatment of SD has been observed. Studies in this field were mainly performed in countries with high levels of pig production – Denmark, Spain, Italy, Germany, the United Kingdom and Czech Republic.

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The problem of antimicrobial resistance of many pathogens is caused by common and excessive use of antibiotics. The appearance of a resistance mechanism may lead to serious difficulties in treatment and control of bacterial infectious diseases in veterinary practice, related to prolonged, ineffective administration of antibiotics, which results in additional veterinary costs and interventions and problems with the elimination of the infectious agent from a herd.

Antimicrobial susceptibility testing of *B. hyodysenteriae* isolates is mainly performed by the agar dilution method. The most commonly used medium for this purpose is trypticase soy agar (TSA) supplemented with 5% ovine blood. However, this method has certain limitations, such as: difficulties in interpretation of results due to irregular growth of spirochaetes on the surface or more deeply in the agar plate, uneven distribution of antibiotic in the agar and short expiry date of TSA containing antibiotics. For practical purposes, it is highly important to evaluate the minimal inhibitory concentration (MIC) of field strains, defined as the lowest concentration of antimicrobial agent that completely prevents visible growth. Alternative methods for MIC determination (macrodilution and microdilution techniques) are characterized by higher precision and repeatability of results.

Therefore, the aim of the study was to evaluate the antimicrobial susceptibility of *B. hyodysenteriae* Polish isolates by broth microdilution procedure in order to monitor the situation in the field and to create a baseline of susceptibility results for Polish isolates.

Materials and Methods

Bacterial strains and growth conditions

Twenty-one field isolates of *B. hyodysenteriae* obtained from pigs from 66 different Polish farms between January 2006 to December 2010 were investigated. Fecal samples and/or large intestines (caecum and colon) were taken from weaners and fatteners suffering from diarrhoea, showing clinical symptoms and necropsy lesions suggesting swine dysentery. The material was also collected from farms where diversity in body weight was presented among weaners and fatteners. Identification of bacterium was performed by biochemical reactivity such as strong f-haemolysis, positive indole spot test (Binek and Szynkiewicz 1984, Belanger and Jaques 1991) and species-specific PCR based on amplification of the *tlyA*, encoding haemolysin gene of *B. hyodysenteriae* (Binek et al. 1995). Mixed cultures of *Brachyspira spp*. other than *B. hyodysenteriae* were not taken into account. The type strain: B78^T (ATCC 27164T) and the reference strain B204 (ATCC 31212) were included in the analysis as positive controls.

All the faeces and mucosal scrapings from the large intestines were streaked on TSA plates, supplemented with 5-10% fresh defibrinated ovine blood, L-cysteine (50 mg/l), spectinomycin (40 mgl), vancomycin (7 mg/l), yeast extract (500 mg/l) (Szynkiewicz and Binek 1986) and incubated in anaerobic jars (Merck, USA), in an atmosphere of 8-10% CO_2 and 90% H_2 (BD GasPak™ EZ Anaerobe Container System) for 3 days at 39.5°C. In order to obtain a pure culture of *B. hyodysenteriae*, after a first incubation a re-cultivation (overall 3 to 4 passages) on TSA medium was performed. For the further studies, field isolates were frozen at – 70°C. After thawing the isolates were passaged at least twice before susceptibility testing. The purity of the culture was checked by phase-contrast microscopic examination of aliquots.

Antimicrobials tested in broth dilution

To determine the susceptibility of the isolate, a VetMIC™ Brachyspira 48-well panel for susceptibility testing was designed in the Department of Antibiotics, National Veterinary Institute of the Swedish University of Agricultural Sciences, Uppsala, Sweden. The panels were coated with the six following antimicrobial agents: tiamulin, valnemulin (Novartis AG. Basel, Switzerland), doxycycline, lincomycin, tylosin and ampicillin (Sigma – Aldrich, Stockholm, Sweden), in arithmetically increasing concentrations. One well in each panel was free of drug and served as a bacterial growth control.

Broth dilution procedure

The broth dilution technique was performed according to basic dilution procedure, previously described by Kärlsson et al. (2002). Briefly, 3-day old bacterial culture was harvested from TSA agar plates and suspended in brain heart infusion broth (BHI, Difco), to a concentration of 10^8 CFU/ml, which corresponded to 2 full plastic loops (1 µl) according to the manufacturer's protocol (VetMIC™ Brachyspira). 300 µl of the suspension was then transferred to 30 ml of BHI broth, supplemented with 10% fetal calf serum, to obtain a final concentration of about 10^6 CFU/ml. Each well was filled with 500 µl of inoculated media. The plates were then covered with plastic lids and incubated in square GENbox anaerobic jars, with GENbox anaer Atmosphere generator sachets (bioMérieux, Lyon, France) for 4 days, on a shaker, at 37°C. To control anaerobic atmosphere in the jars, a dry anaerobic indicator strip

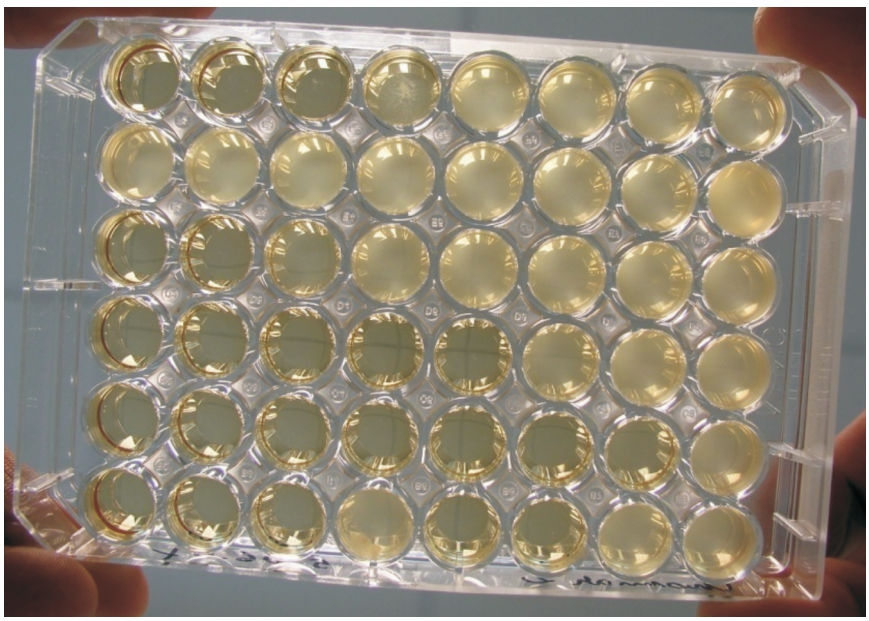


Fig. 1. VetMIC™ Brachyspira panel for susceptibility testing with six antimicrobial agents. Milky wells indicate growth of *B. hyodysenteriae* isolate; transparent wells contain clear broth and correspond to inhibition of bacterial growth.

Table 1. MIC breakpoints for antimicrobials mainly used in therapy of *B. hyodysenteriae* infections.

Antimicrobial	MIC breakpoints (µg/ml)			References
	Sensitive	Intermediate	Resistant	
Tiamulin	≤ 1	> 1≤ 4	> 4	Ronne and Szancer (1990)
Valnemulin	≤ 1	–	> 5	Novartis product information
Tylosin	≤ 1	> 1≤ 4	> 4	Ronne and Szancer (1990)
Doxycycline	0.125-0.25	1-4*		Pringle et al. (2007)
Lincomycin	≤ 4	> 4≤ 36	> 36	Ronne and Szancer (1990)
Ampicillin	≤ 8	16	> 32	CLSI (2007); EUCAST (2012) Guidelines

* decreased susceptibility

was used each time (BD, BBL™, USA). After incubation, the purity and homogeneity of each isolate was checked by phase-contrast microscopy and compared with the control well (Fig. 1). The interpretation of MIC values was based on criteria proposed by Rrnne and Szancer (1990), as well as on the recommendations of Kärlsson et al. (2003) and Pringle et al. (2007). The interpretations of MIC breakpoints for *B. hyodysenteriae* are presented in Table 1.

Results

The distribution of the MICs obtained using the broth dilution method of six antimicrobial agents used for 21 field isolates is presented in Fig. 2. The overall results from MIC determination of 6 antimicrobials for *B. hyodysenteriae* are presented in Table 2.

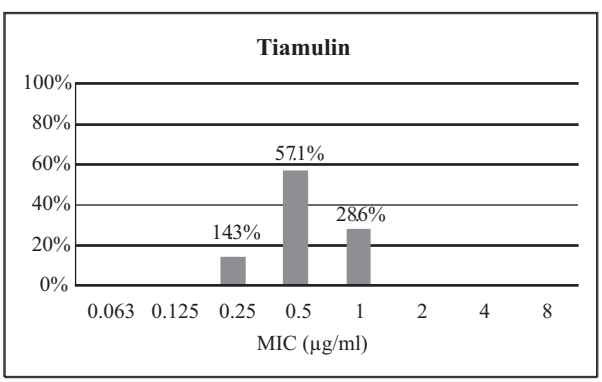
Using the breakpoints proposed by Rrnne and Szancer (1990) of ≤1.0 µg/ml for tiamulin, all the tested strains were fully susceptible. Over half of the total population (57.1%) showed a MIC value of 0.5 µg/ml, while one-third of isolates (28.6%) recorded values of 1.0 µg/ml. The activity of valnemulin was surprisingly lower than that for tiamulin, but based on the interpretation key (Table 1) 66.8% of isolates were considered susceptible with the breakpoint of 1.0 µg/ml and 33.2% were intermediate.

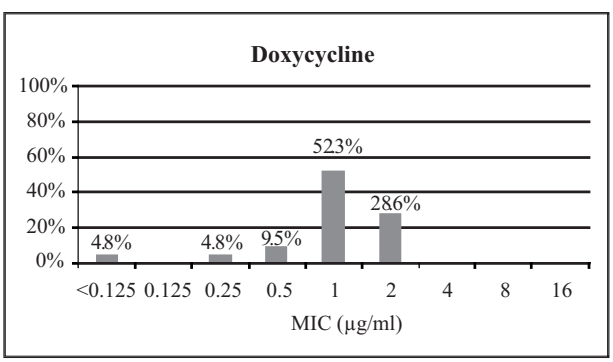
For doxycycline 80.9% of the isolates recorded MIC values of 1-2 µg/ml and these are considered of intermediate sensitivity (Pringle et al, 2007). Tylosin had the lowest activity, with the predominant number of isolates (85.6%) showing MICs higher than the range of concentrations used (>128 µg/ml). Regarding lincomycin, only 9.8% of isolates were considered susceptible (Ronne and Szancer 1990); however, the

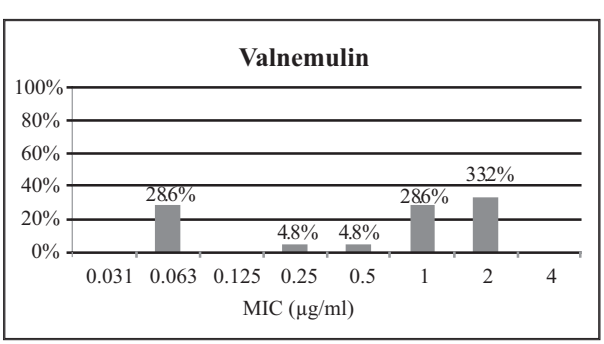
Table 2. Results of antimicrobial susceptibility testing of Polish field isolates of *B. hyodysenteriae* isolated between 2006-2010.

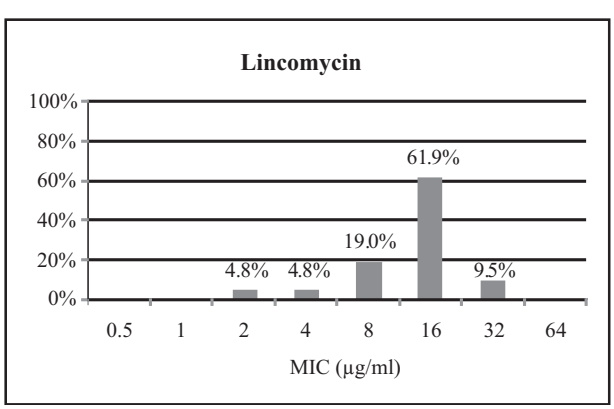
Antimicrobial	Range of tested concentrations (µg/ml)	MIC breakpoints (µg/ml)		
		Sensitive	Intermediate	Resistant
Tiamulin	0.063–8	21 (100%)	–	–
Valnemulin	0.031–4	14 (66.7%)	7 (33.3%)	–
Tylosin	2–128	–	–	21 (100%)
Doxycycline	0.125–16	2 (9.5%)	19 (90.5%)*	
Lincomycin	0.5–64	2 (9.5%)	19 (90.5%)	–
Ampicillin	0.5–32	21 (100%)	–	–

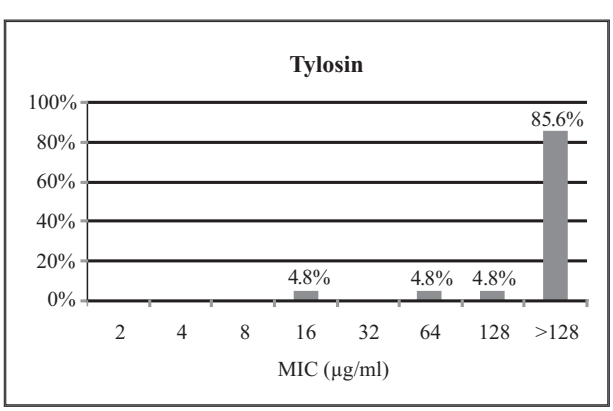
* decreased susceptibility











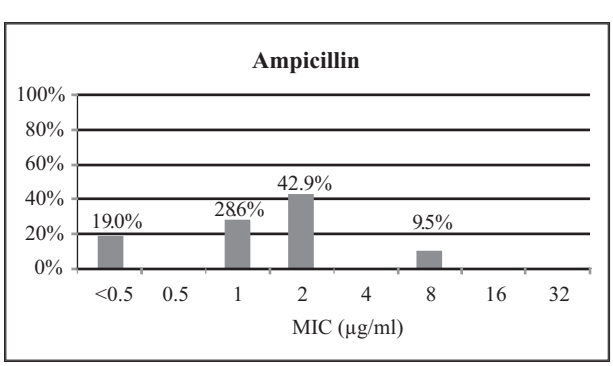


Fig. 2. Distribution of MIC of 6 antimicrobials for 21 Polish field isolates of *B. hyodysenteriae* collected between 2006 and 2010.

remainder were considered intermediately susceptible (Table 1). All of the isolates appeared sensitive to ampicillin according to the Clinical and Laboratory Standards Institute (CLSI) (2007) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) Guidelines (2012). However, these guidelines do not refer specifically to *B. hyodysenteriae* as they have not been established for this organism to date.

Discussion

The present study was the first monitoring trial performed in Poland to determine MICs for the antibiotics most commonly used in the treatment of SD. For this purpose 21 field isolates of *B. hyodysenteriae*, collected over a period of 5 years (2006-2010) from pigs from 66 farms distributed all over the country, were used.

The ring trial performed by Rasback et al. (2005) interchangeably confirmed that the disk diffusion method cannot be used for susceptibility testing of anaerobes. Therefore we decided to test our isolates according to the broth dilution procedure created by Kärlsson et al. (2002, 2003), based on general recommendations for anaerobic bacteria accepted by CLSI (2007) and EUCAST (2012). However, the interpretation of MIC values is still difficult and there are no approved standards for antimicrobial susceptibility testing of *B. hyodysenteriae*. The first steps toward standardization of the broth dilution procedure were made by Pringle et al. (2006) and these recommendations are still used (Hidalgo et al. 2009).

In this study poor susceptibility to tylosin was detected in most isolates (85.6% resistant strains). This is in agreement with previous findings confirming universal, worldwide resistance of *B. hyodysenteriae* isolates to tylosin (Binek et al. 1994, Molnar 1996, Gresham et al. 1998, Fossi et al. 1999, Kärlsson et al. 2001, 2003, Rohde et al. 2004, Uezato et al. 2004, Hidalgo et al. 2009). It is known that the point mutation that causes tylosin resistance at position 2058 in the 23S rRNA gene is also known to increase the MICs for the lincosamide antibiotics (Kärlsson et al. 1999), and this also may help to explain the trend for the increased MICs to lincomycin that was found in our study.

Results for lincomycin for individual isolates showed a similar trend, with a high number of isolates (61.9%) having reduced susceptibility to this drug (16 µg/ml). These data correspond to field observations, according to which for many years treatment of SD using this antibiotic was ineffective. Taking into consideration Rønne and Szancer criteria (1990), Hidalgo et al. (2009) reported high lincomycin resistance among Spanish field isolates, with MICs above 128 µg/ml. Polish studies confirmed resistance to lincomycin with MIC values reaching 32 µg/ml (9.5% of isolates).

Clinical breakpoints based on pharmacokinetic data and clinical efficacy proposed by Rønne and Szancer (1990) classify isolates with MIC values for tiamulin higher than 4 µg/ml as resistant. According to these criteria all Polish isolates were susceptible to this antibiotic. Later studies performed by Kärlsson et al. (2003) recommended a microbiological breakpoint of 0.5 µg/ml for monitoring the decreased susceptibility to tiamulin. On this basis almost 60% of the Polish field isolates of the bacteria should be considered as having reduced susceptibility to tiamulin. In this group 28.6% of the Polish strains had a MIC for this drug as high as 1 µg/ml. In contrast, according to Novartis Production Information (Table 1) classification for valnemulin, which belongs, with tiamulin, to the same group of pleuromutilins, indicated that field *B. hyodysenteriae* isolates were fully susceptible.

Analysis of results of German field strains (Rohde et al. 2004) from 102 isolates tested with broth dilution procedure indicates that only 75 (73.5%) were susceptible to tiamulin and 10 (9.8%) were resistant, with MIC values reaching a level of 8 µg/ml. For valnemulin 82 (80.4%) were susceptible and none was resistant, with the highest MIC of 4 µg/ml (Rohde et al. 2004). A decreased susceptibility over time to tiamulin and valnemulin among Czech isolates of *B. hyodysenteriae* was reported by Lobova et al. (2004). During a 3 year period the MIC_{50} and MIC_{90} of tiamulin increased from 0.062 and 0.25 µg/ml, to 1.0 and 4.0 µg/ml, respectively. Valnemulin MIC_{50} and MIC_{90} were initially below 0.031 µg/ml and within 3 years these levels reached 2.0 and 8.0 µg/ml, respectively.

In many countries pleuromutilin-resistant *B. hyodysenteriae* clones have created an increasing problem in the pig industry. Such a situation was recorded in the United Kingdom (Gresham et al. 1998), Hungary (Molnar 1996), Sweden (Kärlsson et al. 2003), Finland (Fossi et al. 1999) and Spain (Hidalgo et al. 2009), Japan (Uezato et al. 2004) and Australia (Kärlsson et al. 2002). Although *B. hyodysenteriae* isolates tested in Poland were susceptible to tiamulin, a tendency for decreasing susceptibility was identified. This finding was detected in earlier Polish studies, performed by Binek et al. (1994) and confirmed by our results.

The results of Pringle et al. (2007) for doxycycline susceptibility reveal two major groups of strains with different MICs, one highly susceptible – to *B. hyodysenteriae* strains originating from Sweden (MIC 0.125 – 0.25 µg/ml), and one with decreased susceptibility to German isolates (MIC 1 – 4 µg/ml). Minimal inhibitory concentrations proposed in this study place Polish isolates in a group with decreased susceptibility

to doxycycline (52.3% of isolates with MIC of 1 µg/ml and 28.6% with MIC of 2 µg/ml). For ampicillin all isolates were susceptible, which is similar to research performed by Hampson (2008), where the vast majority of isolates was susceptible and only 2 (3.3%) strains out of 60 were resistant, with MIC values above 32 µg/ml. These drugs are not used for the control of swine dysentery. The results of the present study confirm that Polish *B. hyodysenteriae* isolates are susceptible to the main antibiotics used in the treatment of swine dysentery. Due to the small number of isolates tested, further studies are necessary to evaluate a possible slow decrease of susceptibility to tiamulin and valnemulin of *B. hyodysenteriae* strains in Poland. It must be pointed out that long term studies may reveal isolates resistant to the above mentioned pleuromutilins. Therefore, monitoring of resistance should be continued in order to detect emerging trends in resistance and to control MIC values in the Polish swine population.

Acknowledgments

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