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Susceptibility of *Brachyspira hyodysenteriae* Isolates to Doxycycline using Agar Dilution and *Epsilometer T*est

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Abstract

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The agar dilution method and the *Epsilometer* test were used to evaluate antibacterial susceptibility of 49 *Brachyspira hyodysenteriae* field isolates to doxycycline. A comparison of results obtained by the two methods revealed agreement for 84% of *B. hyodysenteriae* isolates; with 16% of isolates the Minimal Inhibitory Concentration values differing by two and more dilutions. Out of the total of *B. hyodysenteriae* isolates tested, 36 (73%) were classified as sensitive, 13 (27%) as intermediary, and none of the strains were found doxycycline-resistant. The demonstrated susceptibility of *B. hyodysenteriae* isolates to doxycycline suggests the possibility of using it for combination therapy with pleuromutilins of swine dysentery. The *Epsilometer* test has proved a practical alternative to dilution methods of *B. hyodysenteriae* susceptibility testing.

Etest, swine dysentery, Minimum Inhibitory Concentration, antibiotics

The number of farms infected with a clinical form of swine dysentery in the Czech Republic has grown significantly in recent years (Čížek et al. 2003). The causal agent of the disease is *Brachyspira hyodysenteriae*, an intestinal spirochete, causing serious mucosal haemorrhagic scouring, especially in fattening pigs (Harris et al. 1972). Economic loss in swine herds attacked with dysentery can be reduced by treating clinically ill pigs and prescribing preventive medication of feed to categories of swine that are at risk. In recent years, there have been increased reports of reduced clinical efficacy of first choice antibiotics in the Czech Republic (Čížek et al. 2002) as well as abroad (Molnár 1996; Karlsson et al. 2002). For this reason the appropriate choice of antibiotics for therapy is very important and should be made on the basis of laboratory test results whenever possible. The laboratory methods available are currently being standardized (Karlsson et al. 2002). There is also a need for new simple testing alternatives to complement the existing methods, which are rather demanding in terms of both time and material, such as the agar dilution method (ADM) and the broth dilution method (BDM). One such method could be the *Epsilometer* test (Etest), which has been used for susceptibility testing of anaerobic bacteria (Rosenblatt and Gustafson 1995) in human medicine. This trial focused on testing susceptibility of *B. hyodysenteriae* strains to doxycycline (tetracycline group of antimicrobial agents) using the Etest and the agar dilution method. The results achieved by the two methods were compared and the appropriateness of the Etest for *B. hyodysenteriae* susceptibility testing was assessed.

Materials and Methods

Bacterial cultures

The trial used 49 field isolates of *B. hyodysenteriae* isolated from clinical cases of dysentery in swine herds in the Czech Republic from 1999 to 2001. The strain *B. hyodysenteriae* ATCC 27164^T was used as a reference culture.

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B. hyodysenteriae isolates were verified using strong beta haemolysis, biochemical testing (Fellström and Gunnarsson 1995) and PCR (Elder et al. 1994). Brachyspira isolates were stored in a cryopreservation medium at -80 °C. Before test for resistance, the isolates were cultivated on Wilkins-Chalgren anaerobic agar (Oxoid) and strain purity was microscopically verified after cultivation in an anaerostat for 3 days at 37 °C. Microbiological checks of the cultures were made using culture smears stained with 1% crystal violet solution.

The agar dilution method

Doxycycline MIC (Minimal Inhibitory Concentration) values were determined using the agar dilution method applied on Wilkins-Chalgren anaerobic agar supplemented with 5% of defibrinated sheep blood. The 3-day culture was suspended in tryptose soya broth (BBL) up to 1° turbidity level on the McFarland turbidity scale. A suspension was prepared as a 1/100 dilution in sterile deionised water for final concentration 10^6 CFU/ml according the NCCLS methods for anaerobic bacteria (M11 A5, 2001). Subsequently, 20 µl of suspension of each isolate were inoculated to the surface of the agar containing 0.06; 0.125; 0.25; 0.5; 1; 2; 4; 8; 16; 32 µg/ml of doxycycline hyclate (Rhône Mérieux) in a rosette-like pattern and simultaneously to doxycycline hyclate-free agar. The inoculated plates were incubated anaerobically in the gas generating kit (BR38, Oxoid) for 3-4 days at 37 °C. The lowest concentration of the antibiotic preventing hemolytic activity and growth of the strain tested was recorded as MIC. The reference strain was *Brachyspira hyodysenteriae* -B78 – ATCC 27164^T.

The Etest

Simultaneously with the ADM, the Etest (AB Biodisk) was used in accordance with the manufacturer's instructions. Suspensions of cultures prepared in the same way as with the ADM method were diluted with saline solution to produce a final concentration corresponding to 0,5-1° on the McFarland turbidity standard. Wilkins-Chalgren anaerobic agar was inoculated with 2 ml of this bacterial suspension covering the whole agar surface. Immediately after removing the surplus suspension by using sterile pipette and leaving it to set about ten minutes, individual doxycycline strips (with concentration gradient 256-0.016 µg/ml) were applied to dry individual plates. The plates were placed in an anaerostat and incubated anaerobically for 3 days at 37 °C. With the field isolates, a reference strain of *B. hyodysenteriae* ATCC 27164^T was tested as a control. In the comparison of MIC values produced by the two methods, the differences of two or more dilutions were regarded as disagreement.

The MIC values obtained by the two methods were documented in Microsoft Excel and compared using 3 interpretation categories of susceptibility of anaerobic bacteria to tetracycline according to NCCLS M11-A5 (2001).

Results

MIC values for doxycycline obtained by the ADM and the Etest are stated in Table 1. Of the total of 49 *B. hyodysenteriae* field isolates tested by the ADM 36 (73%) were sensitive, 13 (27%) were intermediary and none were found to be doxycycline-resistant. The Etest MIC values differed for 8 isolates (16%). From these eight different isolates was sensitivity of four isolates proved by the ADM and the Etest, but four isolates were due to the differences in their MIC values influenced in the interpretation categories of susceptibility (two isolates were sensitive in the ADM and intermediary in the Etest and two isolates were sensitive in the Etest and intermediary in the ADM).

Table 1
Comparison MIC values of ADM and Etest susceptibility of 49 *B. hyodysenteriae* isolates to doxycycline

MIC value classification according to NCCLS (µg/ml)	Isolates proved by the ADM (%)	Isolates proved by the Etest	
		No. of agreeing isolates (%)	No. of disagreeing isolates (%)
Sensitive (≤ 4)	36 (73)	30 (83)	6 (17)
Intermediary (4-8)	13 (27)	11 (85)	2 (15)
Resistant (≥ 16)	0	0	0
Total No.	49	41 (84)	8 (16)

MIC values for doxycycline obtained by the ADM and the Etest by a reference strain of *B. hyodysenteriae* ATCC 27164^T are stated in Table 2. MIC values corresponded to NCCLS M11-A5 (2001) methodologies for anaerobic bacteria but the interpretation categories for *B. hyodysenteriae* were not still determined.

MIC_{50} values (1.0 μg/ml) and MIC_{90} values (4.0 μg/ml) for doxycycline obtained by the ADM and the Etest were not different.

Table 2
MICs of doxycycline for *B. hyodysenteriae* B78 (ATCC 27164^T)

Type strain	MIC DOX/ADM μg/ml	MIC DOX/Etest μg/ml
B. hyodysenteriae ATCC 27164^T	1	1.5

Discussion

There have been a growing number of cases of diminished efficacy of pleuromutilins - diterpens (valnemulin, tiamulin) in the treatment of swine dysentery in the Czech Republic and abroad (Lobová et al. 2004). Laboratory tests have shown that this is due to an increase in the MIC values for the first choice antibiotics pleuromutilins in the treatment of *B. hyodysenteriae* isolates (Karlsson et al. 2002, 2003; Smola and Čížek 2003). To determine susceptibility of *B. hyodysenteriae* to doxycycline, the ADM was used. In most laboratories, quantitative susceptibility testing of brachyspiras is commonly done by diluting the antibacterial substance in agar media (Kitai et al. 1987; Ronne and Szancer 1990; Smith et al. 1991; Trott et al. 1996; Duhamel et al. 1998; Hommez et al. 1998; Fossi et al. 1999) or by the broth dilution method (Hayashi et al. 1988; Buller and Hampson 1994; Karlsson et al. 2002). Apart from applying the agar dilution method, which has been used by our laboratory on a daily basis for several years, a semi-quantitative method, namely the *Epsilometer* test for *B. hyodysenteriae* susceptibility to doxycycline, was analyzed. *B. hyodysenteriae* strains were cultivated using Wilkins-Chalgren anaerobic agar, recommended in some trials for anaerobic cultivation based on NCCLS 2001 methodologies (Rokosz et al. 2001). The MIC values obtained by the two methods agreed in 84% of the isolates (41 isolates), while 16% of the isolates had differring MIC values; most notably the isolates with low doxycycline MIC values. Regarding these notable isolates, the inconsistencies were mainly due to difficult MIC value reading, ascribable to the blurred edges of inhibitory zones. The gradient of doxycycline concentrations in the Etest was also less steep compared with the doxycycline concentrations used with the agar dilution method. Despite these inaccuracies, the Etest can still be recommended for standard performance as a suitable alternative to agar dilution method.

Similar conclusions were obtained at in an assessment of Etest suitability for determination of MIC values of antibiotics for *Campylobacter jejuni* strains isolated from humans. The ADM/Etest agreement was 85.5% (Oncul et al. 2003), which is in line with the results we obtained. Rosenblatt and Gustafson (1995) evaluated the Etest by determining susceptibility of anaerobic bacteria to 14 antibiotic substances and found it a suitable alternative to the agar dilution method. Similarly Midolo et al. (1997) regards the use of the Etest for strains of *Helicobacter pylori*, which are demanding in terms of cultivation, as a simple-to-perform method. Matsumoto et al. (1999) found the Etest to be a simple method, correlating well with the agar dilution method in terms of accuracy.

Before this practical-to-use test can be applied in a routine practice in diagnostic laboratories, inter-laboratory comparative studies will have to be performed in order to standardize the method. The inaccessibility of pleuromutilines in the Etest remains a factor limiting the method to some extent. Evaluation of MIC values for doxycycline has shown that 73% of *B. hyodysenteriae* isolates are susceptible to doxycycline while none were doxycycline-resistant. These results suggest a high number (36 isolates) of doxycycline-susceptible isolates. Should there be *B. hyodysenteriae* strains with reduced susceptibility to pleuromutilines, doxycycline can be considered for combination therapy. An increased

effect of tiamuline in combination with chlortetracycline has already been proved for mycoplasms and other bacterial porcine pathogens (Stipkovits 1992).

Hodnocení citlivosti izolátů *Brachyspira hyodysenteriae* k doxycyklinu agarovou diluční metodou a *Epsilometr* testem

Agar diluční metodou a *Epsilometr* testem byla hodnocena *in vitro* antibakteriální citlivost u 49 terénních kmenů *Brachyspira hyodysenteriae* k doxycyklinu. Porovnáním výsledků obou metod byla zjištěna shoda u 84% kmenů *B. hyodysenteriae* a u 16% kmenů se hodnoty MIC lišily o více než dvě ředění. Z celkového počtu testovaných izolátů *B. hyodysenteriae* bylo 36 (73%) klasifikovaných jako citlivé, 13 (27%) jako intermediární a žádný kmen nebyl k doxycyklinu rezistentní. Prokázaná citlivost izolátů *B. hyodysenteriae* k doxycyklinu naznačuje jeho možné využití k terapii dyzenterie prasat. Pro jednoduchost provedení se *Epsilometr* test osvědčil jako alternativa k dilučním metodám při testování citlivosti *B. hyodysenteriae*.

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