Brachyspira hyodysenteriae: detection, identification and antibiotic susceptibility

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ABSTRACT: 101 samples of faeces, colonic mucosa and rectal swabs taken from 100 pigs (29 commercial herds) were cultivated on Trypticase Soy Agar with 5% of sheep blood, spectinomycin (200 mg/l), vancomycin (50 mg/l), rifampicin (12.5 mg/l) and colistin (12.5 mg/l). Plates were incubated in an anaerobic container at 37°C for 5–7 days. 25 samples (10 faeces, 15 scrapings of colonic mucosa) were examined by darkfield microscopy for the presence of spirochaetes. In 80 samples (21 faeces, 31 rectal swabs, 28 scrapings of colonic mucosa) from diarrhoeic pigs 44 isolates of spirochaetes were identified by PCR method as *Brachyspira hyodysenteriae*. In 21 samples (20 rectal swabs, 1 scraping of colonic mucosa) taken from apparently healthy pigs in herds with swine dysentery were isolated weakly haemolytic spirochaetes: *B. intermedia* in 5 samples and phenotypic group III brachyspirae in 4 samples. Minimal inhibitory concentration (MIC) of tylosin, tiamulin and valnemulin was determined by the agar dilution method. To tylosin 16 out of 17 strains of *B. hyodysenteriae* were resistant (MIC 64 and 256 μg/l), one strain was susceptible (MIC l μg/ml). To tiamulin 17 out of 19 strains were susceptible (MIC from 0.016 to 0.25 μg/ml), one strain was intermediately susceptible (MIC 2 μg/ml) and one resistant (MIC 32 μg/ml). To valnemulin 17 out of 19 strains were susceptible (MIC from 0.016 to 0.064 μg/ml), one strain was intermediately susceptible (MIC 2 μg/ml) and one was resistant (MIC 8 μg/ml). Valnemulin resistant strain was also resistant to tiamulin.

Keywords: pigs; dysenteries; isolation; identification; *Brachyspira hyodysenteriae*; weakly haemolytic spirochaetes; PCR; MIC; tiamulin; tylosin; valnemulin

Brachyspira hyodysenteriae (*B. hyodysenteriae*) is the etiological agent of swine dysentery (SD), a severe mucohemorrhagic diarrheal disease of weanling to finishing pigs. SD is usually observed in 15–70 kg pigs, but the disease may also occur in adults and occasionally in suckling piglets (Harris *et al.*, 1999). The disease is primarily transmitted by ingestion of fecal material either from clinically affected pigs or from clinically normal pigs that carry *B. hyodysenteriae*. SD causes a tremendous financial loss due to mortality, decreased rate of growth, poor feed conversions and expenses for chemotherapy.

Although SD was first described in 1921 by Whiting *et al.*, the etiology remained unknown for 50 years. In 1971 Taylor and Alexander and Harris *et al.* (1972) described a pathogenic anaerobic spirochaete as the etiological agent of swine dysentery. The organism was called *Treponema hyodysenteriae*. Later it was shown that the organism belonged to a distinct genus. Stanton (1992) renamed it *Serpulina* and Ochiai *et al.* (1997) *Brachyspira*.

B. hyodysenteriae is a Gram-negative, motile, oxygen tolerant, anaerobic, loosely coiled spirochaete, haemolytic on blood agar. Strong complete haemolysis is a well established characteristic differentiating the highly pathogenic *B. hyodysenteriae* from all other less pathogenic or nonpathogenic intestinal spirochaetes.

A direct microscopic examination of faeces is of little benefit since the species cannot be readily distinguished morphologically and it can lead to a false diagnosis of swine dysentery. Therefore for a definitive diagnosis of SD, the isolation and identification of isolates to the species level is important due to the presence of less pathogenic or nonpathogenic intestinal spirochaetes (*B. intermedia*, *B. innocens* and *Brachyspira* group III) or the presence of pathogenic *B. pilosicoli*, etiological agent of porcine intestinal spirochaetosis (Trott *et al.*, 1996). The differentiation of spirochaete isolates is made biochemically or by PCR method (Fellstrom *et al.*, 1995).

The knowledge of minimal inhibitory concentrations (MICs) of the antimicrobial agents against *B. hyody-*

senteriae gives an indication of the degree of susceptibility of the microorganism to the agents. At present tylosin, tiamulin and valnemulin are the most commonly used antimicrobial agents for the prevention and for the treatment of SD.

The aim of this study was to verify the detection of *B. hyodysenteriae* using a selective culture medium, differentiation of the isolates by PCR method and to assess *in vitro* susceptibility and estimation of MICs of *B. hyodysenteriae* isolates in some swine herds from the central part of Bohemia.

MATERIAL AND METHODS

Pigs of 29 commercial herds with swine dysentery coming from the central region of Bohemia were used in this study. For the isolation of *Brachyspira* species 101 samples of rectal swabs, colonic mucosa and faeces were taken from 100 pigs. 80 samples (21 faeces, 31 rectal swabs, 28 scrapings of colonic mucosa) were from diarrhoeic pigs. 21 samples (20 rectal swabs and one scraping of colonic mucosa) were taken from clinically healthy pigs in herds with swine dysentery for the control of the efficiency of dysentery treatment or for identification of infected animals before transfer to another herd.

The samples were transported to the laboratory at 6–10ºC with minimal delay: (a) rectal swabs in Amies (charcoal) transport medium, (b) fresh faeces in a bottle filled up to the stopper, (c) tied up parts of the large intestines with pathological changes, (d) fresh carcasses.

All samples were cultured on Trypticase Soy Agar plates (TSA, Oxoid) containing spectinomycin (200 mg per l), vancomycin (50 mg/l), rifampicin (12.5 mg/l) and colistin (12.5 mg/l) (Leser *et al.*, 1997). Antibiotics were bought from Sigma Aldrich. The media were pre-reduced for 24 hours before inoculation. After inoculation the plates were incubated at 37ºC in an anaerobic container with anaerobic Gas Generating Kit (Anaerogen, Oxoid) for 5–7 days. 25 samples were examined by darkfield microscopy. Isolated strains of spirochaetes were identified by PCR method (Leser *et al.*, 1997) after isolation of DNA by boiling. Suspicious haemolytic bacterial colonies were dispersed by vortexing in 0.4 ml phosphate buffered saline (PBS) and centrifuged for 5 min. at 15 000 × g in a microcentrifuge. The pellet was suspended in 100 µl distilled water and boiled in a water-bath for 10 min. The lysed cells were subjected to PCR. The PCR assay was based on amplification of segments of 23S ribosomal DNA from *B. hyodysenteriae* and other weakly beta-haemolytic *Brachyspira* strains. The sequences used as primers were for *B. hyodysenteriae* forward 5´CGC TAA GTG ATG TAC TTG 3´ and reverse 5´AGC CTC AAC AAC CTT AAA GA 3´, for B. pilosicoli forward 5´ AGG TGA TGG TTA TCC TC 3´ and reverse 5´ AAC CTT AGG AAT TAT TTC TAA 3´, for *B. intermedia* forward 5´ CCG TTG AAG GTT TAC CGT G 3´ and reverse 5´ CGC CTG ACA ATG TCC GG 3´. For PCR, 1 µl of extracted DNA was amplified in a thermal cycler with heated lid (Biometra). The reaction volume was 25 µl in 0.2 ml microtubes. Final reaction conditions were 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 50 mmol/l KCl, 0.2 mmol/l of each deoxynucleotide triphosphate (Promega), 0.5 µmol/l of each primer and 0.63 IU of Tag polymerase (Promega). The DNA was initially denatured at 94ºC for 60 s, and subsequently amplified through 35 cycles of denaturation at 92ºC for 40 s, annealing for 40 s at 45ºC for *B. hyodysenteriae* and *B. pilosicoli* and at 60°C for *B. intermedia* and extension at 75ºC for 60 s. Final extension was at 75ºC for 5 min. The PCR products were analysed by electrophoresis on 2% agarose gel (Gibco BRL) in lx TBE (0.045 mol/l tris borate, 0.001 mol/l EDTA pH 8.0) and stained with ethidium bromide. Reference isolates of *B. hyodysenteriae* ATCC 27164, *B. pilosicoli* ATCC 51139 and *B. intermedia* ATCC 51140 were used as positive controls.

The susceptibilities of the spirochaete strains to tiamulin, valnemulin and tylosin were determined by the agar dilution method (Ronne and Szancer, 1990; Messier *et al.*, 1990). Appropriate amounts of stock antibiotic solutions were added to molten TSA containing 5% sheep blood. Final concentrations of tiamulin and valnemulin ranged from 0.016 to 256 µg/ml, of tylosin from 0.016 to 512 µg/ml. Antibiotic-free control plates were prepared using the same medium. Plates were dried for approx. 15–30 min on a laminar flow bench before inoculation. The isolates of *B. hyodysenteriae* were resuspended in sterile saline to MacFarland standard turbidity 1 using a spectrophotometer. Each plate with an antimicrobial agent was spot inoculated with 10 µl of suspension. All isolates were tested in duplicate. Plates were placed in anaerobic jars and incubated under anaerobic conditions as described above for 3–5 days at 37ºC and then examined. The lowest concentration of each antimicrobial agent at which no growth nor distinct haemolysis was visible in the inoculation spot was taken as MIC. This was compared with the haemolytic effect seen on control plates. Very weak and small haemolysis spots, faint

bases of growth and single colonies were disregarded (Hommez *et al.*, 1998). Interpretation of the MICs of tiamulin and tylosin was done according to interpretative criteria proposed by Ronne and Szancer (1990) and of valnemulin according to criteria obtained from Novartis Company (Novartis Animal Health Sector, Prague, Czech Republic, personal communication, 2001). The breakpoint for susceptible *Brachyspira* strains to tiamulin, tylosin and valnemulin is MIC ≤ 1 μg/ml, for intermediately susceptible strains > 1 and ≤ 4 μg/ml and for resistant strains > 4 μg/ml.

RESULTS AND DISCUSSION

TSA agar with 5% sheep blood, colistin, rifampicin, spectinomycin and vancomycin worked well for the growth of *Brachyspira* strains. Fifty-six spirochaete cultures were isolated on this medium from 101 examined samples of pig faeces, rectal swabs and colonic mucosa. Forty-seven *Brachyspira* cultures were isolated from samples taken from 80 diarrhoeic pigs (Table 1), nine from samples taken from 21 apparently (clinically) healthy pigs (Table 2). All antimicrobial agents used in TSA for primoculture have MICs greater than 100 μg per ml against *B. hyodysenteriae*, but they inhibited the faecal flora. However, the inhibition of faecal flora was not always complete.

After incubation of the primocultures Gram stained smears were prepared from the zones of haemolysis with a film of growth but without visible colonies. When spirochaetes with typical morphology were present, further subcultures were made on TSA with 5% sheep blood for further identification. Isolated strains were identified by PCR method, which offers a more rapid and sensitive approach to identification of the porcine spirochaetes (Atyeo *et al.*, 1998) because differentiation of the spirochaete species based on the intensity of haemolysis on blood agar and on the results of few biochemical and enzymatic tests are not completely specific by themselves (Leser *et al.*, 1997). From 80 diarrhoeic pigs 44 cultures were identified as *B. hyodysenteriae*, two as *B. intermedia* and one as *B. pilosicoli* (Table 1). In one faecal sample *B. hyodysenteriae*, *B. pilosicoli* and *B. intermedia* occurred jointly. Five strains of *B. intermedia* and four strains of *Brachyspira* group III were isolated from rectal swabs and colonic mucosa of 21 apparently healthy pigs. Neither *B. hyodysenteriae* nor *B. pilosicoli* was detected in these samples (Table 2).

Table 1. Detection of *Brachyspira* sp. in diarrhoeic pigs by bacteriological cultivation using a selective culture medium

Type of samples	Faeces	Rectal swabs	Colonic mucosa
Number (%) of examined samples	21 (100)	31 (100)	28 (100)
Positive findings			
B. hyodysenteriae	17 (80.9)	12 (38.7)	15 (53.6)
B. intermedia	1 (4.8)	1 (3.2)	0 (0)
B. pilosicoli	1 (4.8)	0 (0)	0 (0)
Brachyspira group III	0 (0)	0 (0)	0 (0)

Table 2. Detection of *Brachyspira* sp. in clinically healthy pigs by bacteriological cultivation using a selective culture medium

Type of samples	Rectal swabs	Colonic mucosa
Number (%) of examined samples	20 (100)	1 (100)
Positive findings		
B. hyodysenteriae	0 (0)	0 (0)
B. intermedia	4 (20)	1 (100)
B. pilosicoli	0 (0)	0 (0)
Brachyspira group III	4 (20)	0 (0)

Table 3 shows the comparison of the results of darkfield microscopy and selective cultural examination of 25 samples (10 samples of faeces and 15 samples of colonic mucosa). In 85.7% of *B. hyodysenteriae* culture positive samples the direct stains did not reveal the presence of spirochaete in three samples (14.3%). Stanton and Jensen (1993) also found that 12.5% of samples positive by selective culture were negative by direct microscopy in experimental swine dysentery in postweaning piglets. This result is not unexpected since culture methods using TSA with blood and antimicrobial agents are more sensitive than direct microscopy for detection of *B. hyodysenteriae* (Stanton and Jensen, 1993). But interestingly, four out of 25 samples (16%) (one sample of faeces, three samples of colonic mucosa) positive by direct microscopy were negative for *B. hyodysenteriae*, but one sample was positive for *B. intermedia* by selective culture. Olson and Fales (1983) and Stanton and Jensen (1993) similarly reported that 24–35% of rectal swabs contained visible spirochaetes but did not give any positive cultures. These authors suggested that the optimal methods for detecting *B. hyodysenteriae* in faeces should include both direct microscopy and selective culture. Direct micro-

Table 3. Comparison of the results of bacteriological examination and direct microscopy

Sample		Test result			
type of sample	number of sample	direct microscopy	selective culture		
			BH	BP	BI
Faeces	1	+	+	–	–
	2	+	+	–	–
	3	+	+	–	–
	4	+	+	–	–
	5	+	+	–	–
	6	+	+	–	–
	7	+	–	–	–
	8	–	+	–	–
	9	–	+	–	–
Colonic mucosa	10	–	+	+	+
	11	+	+	–	–
	12	+	+	–	–
	13	+	+	–	–
	14	+	+	–	–
	15	+	+	–	–
	16	+	+	–	–
	17	+	+	–	–
	18	+	+	–	–
	19	+	+	–	–
	20	+	+	–	–
	21	+	+	–	–
	22	+	+	–	–
	23	+	–	–	+
	24	+	–	–	–
	25	+	–	–	–
Number of positive findings		22	21	1	2

BH = *Brachyspira hyodysenteriae*
BP = *Brachyspira pilosicoli*
BI = *Brachyspira intermedia*

scopic examination can be used as a presumptive evidence of *B. hyodysenteriae* at least until the cultural examination is completed. Other morphologic types of spirochaete are present in the colon of normal and diarrhoeic pigs and can be confused with *B. hyodysenteriae* although the large size of spirochaete is a typical morphological characteristic for *B. hyodysenteriae*. Initial culture is therefore needed to obtain isolates for strain typing and for antimicrobial sensitivity testing.

The distributions of MICs of tiamulin, tylosin and valnemulin for *B. hyodysenteriae* strains are presented in Table 4. Tiamulin and valnemulin were the most effective antimicrobial agents *in vitro* against *B. hyodysenteriae*. 90% of the isolates were inhibited by 0.25 μg/ml of tiamulin and by 0.64 μg/ml of valnemulin (Table 5). The range of susceptibility to tiamulin and valnemulin was wide, from 0.016 to 32 μg/ml of tiamulin and from 0.016 to 32 μg/ml of valnemulin (Table 4). *In vitro* efficiency of tylosin was low, the MIC_{90} was 256 μg/ml (Table 5). The range of MIC of tylosin was from 1 to 256 μg/ml (Table 4).

The "tailing" in MIC distribution of *B. hyodysenteriae* with tiamulin was described by Hommez *et al.* (1998). The authors found one third of 36 strains of *B. hyodysenteriae* at least tenfold less susceptible than the type strain B78 with MIC < 0.03 μg/ml.

Table 4. Minimal inhibitory concentration (MICs) of tiamulin, tylosin and valnemulin against *B. hyodysenteriae*

Antibiotic	Number of tested strain	Number of strains with MICs (μg/ml)												
		0.016	0.032	0.064	0.125	0.25	1	2	4	8	32	64	128	256
TIA	19	2	4	4	3	4	–	1	–	–	1	–	–	–
TYL	17	–	–	–	–	–	1	–	–	–	–	3	–	13
VAL	19	12	3	2	–	–	–	1	–	1	–	–	–	–

TIA = tiamulin; TYL = tylosin; VAL = valnemulin

The interpretation of the MIC of tiamulin and tylosin in terms of susceptibility and resistance was done according to interpretative criteria proposed by Ronne and Szancer (1990). These are based on the serum, colon content and colon tissue concentrations normally obtained with these antibiotics. According to this interpretation all strains (89.5%) tested in this study are susceptible to tiamulin except one intermediately susceptible strain with MIC 2 μg/ml and one resistant strain with MIC 32 μg/ml. The MIC_{90} of tiamulin in the present study was <1 μg/ml. These results are comparable with the findings of other studies on antimicrobial effectiveness against *B. hyodysenteriae*. For example to tiamulin were susceptible 87.7% of *Brachyspira* strains tested in Austria (Dünser and Schweighart, 1998), 94.4% strains isolated in Belgium (Hommez *et al.*, 1998), 100% strains from Canada (Messier *et al.*, 1990) and from Denmark (Ronne and Szancer, 1990). The MIC_{90} of these isolates was < 1 μg per ml, analogically to our findings. Higher MIC_{90} for tiamulin (8 μg per ml) was reported by Buller and Hampson (1994) in Australia.

Table 5. Minimal inhibitory concentrations (MICs) of 50 and 90% susceptible *B. hyodysenteriae* strains

Antibiotic	MIC (μg/ml)		
	MIC_{50}	MIC_{90}	Tested range
TIA	0.064	0.25	0.016–256
TYL	256	256	0.016–512
VAL	0.016	0.064	0.016–256

TIA = tiamulin
TYL = tylosin
VAL = valnemulin
MIC_{50} = growth of 50% of tested strains is inhibited
MIC_{90} = growth of 90% of tested strains is inhibited

To tylosin only one out of 17 strains of *B. hyodysenteriae* was susceptible (MIC 1 μg/ml). 94.1% of strains were resistant. Similar results were reported for this antibiotic in Belgium (Hommez *et al.*, 1998), where 97.3% of *B. hyodysenteriae* strains were resistant. 100% resistance was found in Denmark (Ronne and Szancer, 1990) and in Australia (Smith *et al.*, 1991; Buller and Hampson, 1994).

17 strains (89.5%) of *B. hyodysenteriae* in the present study were inhibited by 0.064 μg/ml of valnemulin, one strain by 2 μg/ml and one by 8 μg/ml. The MIC breakpoint for valnemulin in *Brachyspira* strains has not been published. According to a personal communication from Novartis Company (Novartis Animal Health Sector, Prague, Czech Republic, 2001) the breakpoint for susceptible *Brachyspira* strains is MIC ≤ 1 μg/ml, for resistance > 4 μg/ml. According to this interpretation 89.9% of *B. hyodysenteriae* strains in the present study were susceptible to valnemulin, one (5.2%) strain was intermediately susceptible and one (5.2%) was resistant. The valnemulin-resistant strain was also resistant to tiamulin. Similarly, Dünser and Schweighart (1998) found that 93 of *Brachyspira* strains (91.2%) in Austria were sensitive (inhibited by 1 μg/ml of valnemulin) and 9 strains (8.8%) were resistant (inhibited by 5 μg/ml of valnemulin).

Isolation and identification of *B. hyodysenteriae* is essential to confirm the diagnosis of swine dysentery. The present results show that the culture technique and selective culture medium used in this study for the detection of *B. hyodysenteriae* were successful. Typing of isolated strains by PCR is a rapid method for identification of spirochaetes strains. Although the strains tested for antimicrobial sensitivity in this paper represent a material collected only from the central part of Bohemia, the results can serve as a guide to the susceptibility of *B. hyodysenteriae* isolates to 3 commonly used antibiotics for treatment and prevention of swine dysentery also in other regions of the Czech Republic and in other countries.

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