Antimicrobial susceptibility of *Brachyspira hyodysenteriae* in Switzerland

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Abstract

*Brachyspira (B.) hyodysenteriae* is the causative agent of swine dysentery (SD), a severe mucohaemorrhagic diarrheal disease in pigs worldwide. So far, the antimicrobial susceptibility patterns of *B. hyodysenteriae* in Switzerland have not been investigated. Therefore, a panel of 30 porcine *B. hyodysenteriae* isolates were tested against 6 antimicrobial agents by using the VetMIC Brachy panel, a broth microdilution test. Tiamulin and valnemulin showed high antimicrobial activity inhibiting all isolates at low concentrations. The susceptibility testing of doxycycline revealed values from $\leq 0.25 \mu g/ml$ (47%) to 2 µg/ml (10%). The MIC values of lincomycin ranged between $\leq 0.5 \mu g/ml$ (30%) and 32 µg/ml (43%). For tylosin, 57% of the isolates could not be inhibited at the highest concentration of $\geq 128 \mu g/ml$. The MIC values for tylvalosin were between $\leq 0.25 \mu g/ml$ (10%) and 8 µg/ml (20%). These findings reveal Switzerland’s favourable situation compared to other European countries. Above all, tiamulin and valnemulin are still effective antimicrobial agents and can be further used for the treatment of SD.

Keywords: *Brachyspira hyodysenteriae*, antimicrobial susceptibility testing, resistance, swine dysentery, Switzerland

Antimikrobielle Empfindlichkeit von *Brachyspira hyodysenteriae* in der Schweiz

*Brachyspira (B.) hyodysenteriae* ist der Erreger der Schweinedysenterie (SD), einer schweren mucohaemorrhagischen Durchfallerkrankung bei Schweinen weltweit. Bisher ist das Empfindlichkeitsmuster von *B. hyodysenteriae* gegenüber Antibiotika in der Schweiz nicht untersucht worden. Daher wurde eine Auswahl von 30 porzinen *B. hyodysenteriae* Isolaten auf ihre Empfindlichkeit gegenüber 6 antimikrobiellen Wirkstoffen mittels VetMIC Brachy panel, einem Mikrodilutionstest, getestet. Tiamulin und Valnemulin zeigten eine starke antimikrobielle Aktivität und hemmten alle Isolate bei niedrigen Konzentrationen. Die Empfindlichkeitsprüfung von Doxycyclin ergab Werte von $\leq 0.25 \mu g/ml$ (47%) bis 2 µg/ml (10%). Die MHK-Werte von Lincomycin reichten von $\leq 0.5 \mu g/ml$ (30%) bis 32 µg/ml (43%). Für Tylosin konnten 57% der Isolate nicht bei der höchsten Konzentration von $\geq 128 \mu g/ml$ gehemmt werden. Die MHK-Werte für Tylvalosin lagen zwischen $\leq 0.25 \mu g/ml$ (10%) und 8 µg/ml (20%). Diese Befunde zeigen die günstige Situation der Schweiz verglichen mit anderen europäischen Ländern. Besonders Tiamulin und Valnemulin sind noch wirksame antimikrobielle Substanzen, die weiterhin für die Behandlung von SD eingesetzt werden können.

Schlüsselwörter: *Brachyspira hyodysenteriae*, Empfindlichkeitsprüfung, Resistenz, Schweinedysenterie, Schweiz
Introduction

Brachyspira (B.) hyodysenteriae is an anaerobic intestinal spirochaete causing swine dysentery (SD), a disease associated with mucohaemorrhagic diarrhea in pigs (Taylor and Alexander, 1971; Glock and Harris, 1972; Harris et al., 1972b). It is one of the most important diseases in pig production worldwide. In Switzerland, SD was etiologically confirmed for the first time in 2008 (Speiser, 2008). Since then, the pathogen has been spreading throughout the country (Prohaska et al., 2012). Currently, B. hyodysenteriae is detected in nearly 2% of all pig herds served by the Swiss Pig Health Service (Figi et al., 2014). Up to now, antimicrobial therapy remains the only treatment available. The selection of antimicrobial agents admitted for the treatment of SD in Switzerland is restricted to 3 antimicrobial agents (tiamulin, valnemulin and lincomycin). Since culturing of B. hyodysenteriae is difficult and time-consuming, antimicrobial susceptibility testing is not performed routinely in diagnostics. Therapy of clinical SD is mostly started without a final diagnosis and information about the susceptibility of the isolate. An increase in resistance is observed all over Europe (Rohde et al., 2004; Hidalgo et al., 2009; Pringle et al., 2012). Until now, no data of antimicrobial susceptibility of B. hyodysenteriae exist for Switzerland. The testing in this study was performed with a broth microdilution test developed for Brachyspira spp. This is the first investigation of the antimicrobial susceptibility of Swiss B. hyodysenteriae isolates.

Material and Methods

Isolation and differentiation

A panel of 30 porcine B. hyodysenteriae isolates from 8 different Swiss cantons collected from 30 different farms between 2009 and 2015 were tested (Fig. 1). The isolation and differentiation of the strains is described elsewhere (Prohaska et al., 2014). In short, faecal swabs were streaked on a selective agar (Dünser et al., 1997) and incubated anaerobically (TRILAB, bioMérieux, Switzerland) for 2 to 7 days at 42°C. Samples were investigated using native microscopy. If positive, they were subcultured on Columbia blood agar (Oxoid, Switzerland) and analysed by a duplex PCR targeting the nox gene for B. hyodysenteriae and the 16S rRNA gene for B. pilosicoli (La et al., 2003). B. hyodysenteriae isolates were stored at −80°C in brain heart infusion with glycerine and fetal calf serum. The frozen isolates were restreaked on Columbia blood agar, controlled for contamination by native microscopy, and all isolates were additionally analysed by a multiplex real-time PCR targeting the 23S rRNA gene to avoid culture of different Brachyspira spp.

Susceptibility testing

Antimicrobial susceptibility testing was performed using the VetMIC Brachy panel (Statens veterinärmedicinska anstalt, Sweden), a broth microdilution test including the pleuromutilins tiamulin and valnemulin, the tetracycline doxycycline, the macrolide tylosin and its derivate tyvalosin (avilnosin) as well as lincomycin, a lincosamide. The VetMIC Brachy panel provides a 48-well plate coated with the 6 different antimicrobials in arithmetically decreasing concentrations. The inoculation of the VetMIC Brachy panel was done with modifications to the manufacturer’s instructions and is described as follows: The pure isolates were incubated anaerobically at 42°C for 3 days. Cultures were suspended in brain heart infusion with 10% fetal calf serum (BHI + FCS) and diluted up to an OD600 of 0.6 to 0.8 E (BioPhotometer, eppendorf, Switzerland). The suspension was diluted 1:1000 in BHI + FCS, corresponding to 10⁵ bacterial cells per ml. The VetMIC Brachy plates were inoculated with 500 µl of the diluted bacterial suspension per well. Additionally, a control plate without antimicrobials and a negative control with BHI + FCS only was included. The plates were incubated in an anaerobic jar (AnaeroGen 2.5 l, Thermo Scientific, Switzerland) shaking at 37°C for 4 days. All cultures were controlled twice for contamination by native microscopy and by streaking on a Columbia blood agar plate (aerobic and anaerobic at 37°C) before and after the incubation of the VetMIC Brachy panel. For control purposes, B. hyodysenteriae ATCC strain 27164 was tested in parallel at every approach. The evaluation was done by controlling the wells for growth with a mirror. A well was considered positive for growth if a macroscopically visible turbidity could be detected. The minimal inhibitory concentration (MIC) was the lowest concentration at which no visible growth in a well could be detected.

Figure 1: B. hyodysenteriae isolates investigated in this study are sampled from the red-coloured cantons of Switzerland.
Results

The antimicrobial susceptibility testing of the control strain B. hyodysenteriae ATCC 27164 revealed a good reproducibility of the assay. The distribution of the MICs for all isolates and agents as well as the concentrations used for the different antimicrobial agents is shown in Figure 2. The pleuromutilins, tiamulin and valnemulin, were able to inhibit growth at very low concentrations. For tiamulin, all isolates could be inhibited at a concentration of 0.125 µg/ml and 70% already at a concentration of ≤0.063 µg/ml. Therefore, the MIC inhibiting 90% of the isolates (MIC90) was 0.125 µg/ml. Valnemulin suppressed growth at concentrations of ≤0.031 µg/ml with the exception of 2 isolates which had higher MICs of 0.063 µg/ml and 0.125 µg/ml, respectively. For both agents, a clear unimodal distribution of the isolates can be observed (Fig. 2A/B). In the case of doxycycline, 10% of the isolates showed growth up to concentrations of 2 µg/ml. Nevertheless, the MIC inhibiting 50% of the isolates (MIC50) was 0.25 µg/ml and MIC90 was 1 µg/ml. Nearly half of the isolates (47%) were inhibited at a low concentration of ≤0.125 µg/ml.

A bimodal distribution is seen in Figure 2C, parting the isolates into two populations with the major part being inhibited at lower concentrations. A wider range of MICs can be observed for the lincosamides and macrolides. For lincomycin, isolates grew up to a concentration of 32 µg/ml (43% of the tested strains). One third of the isolates was already inhibited at a concentration of ≤0.5 µg/ml, but MIC50 was 16 µg/ml and MIC90 was 32 µg/ml. Isolates can be separated into 2 populations (Fig. 2D). For tylosin, nearly 60% of the isolates were able to grow at the highest concentration tested (128 µg/ml, Fig. 2E). Thus, tylosin had the highest MIC90 (>128 µg/ml) and the widest distribution of MICs ranging from ≤2 µg/ml to >128 µg/ml compared to the other antimicrobial agents tested. Isolates with increased MICs for tylosin also showed high MICs for lincomycin. For tylvalosin, a tylosin derivate, the MIC values ranged from ≤0.25 µg/ml to 8 µg/ml. Since 37% of the isolates had a MIC of 4 µg/ml, MIC50 could be set at this concentration and MIC90 was 8 µg/ml. No growth was detected at the 2 highest concentrations (16 µg/ml and 32 µg/ml) tested (Fig. 2F). Both macrolides showed a bimodal distribution of isolates (Fig. 2E/F).

Discussion

This is the first study on the antimicrobial susceptibility of Swiss B. hyodysenteriae isolates. Antimicrobial therapy remains the only treatment option for SD. Facing progressive resistance in B. hyodysenteriae isolates all over Europe (Rohde et al., 2004; Sperling et al., 2011; Hidalgo et al., 2011) and due to the restricted selection of therapeutics admitted for the treatment of SD, an evaluation of the situation in Switzerland is more than necessary. The antimicrobial agents currently licensed for treatment of SD in Switzerland are the pleuromutilins tiamulin and valnemulin and the lincosamide lincomycin. Hence, all these antimicrobial agents were included in the study. The remaining antimicrobial agents additionally tested in our study are the tetracycline doxycycline and the macrolides tylosin and its derivate tylvalosin. At the moment, there is no product containing tylvalosin available in Switzerland. Products containing doxycycline or tylosin have a Swiss license for the treatment in swine. For the therapy of SD the use of these products is not allowed except for cases where it has to be assumed that authorised products are ineffective, for example because of resistance. Therefore, knowledge about antimicrobial susceptibility patterns of the pathogen is required.

A major restriction of antimicrobial susceptibility testing of B. hyodysenteriae is the difficulty of interpreting the results due to a lack of standardised established breakpoints. An overview of the classification of the isolates according to the established breakpoints is given in Table 1. For tiamulin, an initially proposed clinical breakpoint is >4 µg/ml (Ronne and Szancer, 1990). However, the susceptible wild-type population had MIC values below 0.5 µg/ml (Karlsson et al., 2003). Thus, a revision of the tiamulin breakpoint to <0.5 µg/ml and <1 µg/ml, respectively, is postulated (Karlsson et al., 2003; Lobová et al., 2004; Burch, 2005; Vyt and Hom...
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Table 1: Distribution and classification of the isolates according to clinical breakpoints. The range of the measured concentrations is coloured light grey.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>MIC in μg/ml (number of isolates)</th>
<th>Interpretation (isolates in %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>s0.031 0.063 0.125 0.25 0.5 1 2 4 8 16 32 64 128</td>
<td>Rønne and Szancer, 1990 Burch, 2005</td>
</tr>
<tr>
<td>tiamulin</td>
<td>21 9 0 0 0 0 0 0 0</td>
<td>0 100 0 0 100 0</td>
</tr>
<tr>
<td>valnemulin</td>
<td>28 1 1 0 0 0 0</td>
<td>/ / / / 100 0</td>
</tr>
<tr>
<td>doxycycline</td>
<td>14 7 2 4 3 0 0 0</td>
<td>/ / / / 100 0</td>
</tr>
<tr>
<td>lincomycin</td>
<td>9 0 1 1 0 6 13 0</td>
<td>0 37 63 0 100 0</td>
</tr>
<tr>
<td>tylosin</td>
<td>3 5 1 1 1 0 0 0 0</td>
<td>10 0 17 73 33 67</td>
</tr>
<tr>
<td>tylosin</td>
<td>3 5 2 3 11 6 0 0</td>
<td>/ / / / 100 0</td>
</tr>
</tbody>
</table>

Irrespective of the breakpoint used, all Swiss isolates tested in this study were susceptible to tiamulin. For valnemulin, a breakpoint of >0.125 μg/ml is the only one available (Burch, 2005), and all isolates investigated in this study were fully susceptible to valnemulin. Less is known about the resistance mechanism of pleuromutilins, but it develops slowly and stepwise (Karlsson et al., 2001; Lobová et al., 2004) indicating the involvement of several genes. Alterations of the ribosomal binding sites induced by mutations of the L3 and 23S rRNA genes are connected to reduced susceptibility to tiamulin and valnemulin (Pringle et al., 2004; Hidalgo et al., 2011; Hillen et al., 2014). For doxycycline, an investigation of German and Swedish isolates parted population into a more susceptible group with MICs from 0.125 μg/ml to 0.25 μg/ml and a group showing decreased susceptibility with MICs between 1 μg/ml to 4 μg/ml. A bimodal distribution of isolates is an indication for an acquired resistance mechanism and genetic investigation of the less susceptible strains revealed a mutation in the 16S rRNA gene (Pringle et al., 2007). Due to the lack of clinical breakpoints for doxycycline, Zmudzki et al. (2012) used the MIC of ≥1 μg/ml as a cut-off value. The tested Swiss isolates showed the same bimodal distribution and applying the cut-off value, the major group of nearly 80% of the isolates can be considered as susceptible to doxycycline and about 20% of the isolates showed decreased susceptibility. The results for the lincosamides and macrolides revealed a broader range of concentrations but also a bimodal distribution of the isolates. According to the breakpoints of Rønne and Szancer (1990), 37% of the isolates were in the susceptible range (≤4 μg/ml) for lincomycin, and the remaining strains can be classified as intermediate (>4 μg/ml to ≤16 μg/ml). None of the isolates was found to be resistant. However, therapy outcome by using lincomycin is not always in accordance with the in-vitro susceptibility testing in B. hyodysenteriae, since treatment was efficient for strains classified as resistant (Smith, 1990; Vyt and Hommez, 2006). Therefore, Burch (2005) suggests a clinical breakpoint of >50 μg/ml. In this case, all isolates can be classified as susceptible to lincomycin. For tylosin, nearly two thirds of the isolates are resistant to tylosin (Rønne and Szancer, 1990; Burch, 2005). According to Rønne and Szancer (1990), 17% of the isolates are categorised as intermediate. Three isolates had a MIC of ≤2 μg/ml and cannot be classified. Applying the breakpoint proposed by Burch (2005), the remaining third of the isolates is susceptible to tylosin. For tylvalosin, the tylosin derivative, there is only one breakpoint of >16 μg/ml available (Burch, 2005), and all tested isolates of the study were susceptible. The mechanism of macrolide and lincosamide resistance is well known. In general, both antimicrobial classes have the same binding site at the 50S subunit of the ribosome and cross-resistance is quite common (Vester and Douthwaite, 2001). For B. hyodysenteriae, a point mutation in the 23S rRNA gene is responsible for decreased susceptibility (Karlsson et al., 1999). Additionally, resistance development has been facilitated by the former use of tylosin as a growth promoter and the ongoing preventive administration for respiratory diseases in countries with less rigidly regulated antimicrobial application. Thus, high MIC values for lincomycin and tylosin were already observed at the beginning of antimicrobial susceptibility testing of B. hyodysenteriae (Kitai et al., 1979; Messier et al., 1990; Rønne and Szancer, 1990) and is still common all over the world (Karlsson et al., 1999; Clothier et al., 2011; Zmudzki et al., 2012). Referring to the situation in Switzerland, the high rate of resistance against tylosin is not surprising. Cross-resistance between tylosin and lincomycin was observed as well, since isolates with high MICs to tylosin showed also increased MICs to lincomycin. In addition to the present reduced susceptibility, further development of resistance can be anticipated. In Poland, a rapid increase of lincomycin-resistant isolates was observed within 10 years (Binek, 1994). Trends towards lower susceptibility are also seen in the pleuromutilins (Lobová et al., 2004; Rohde et al., 2004; Hidalgo et al., 2011). In Hungary, tiamulin-resistant B. hyodysenteriae isolates occurred in the early 1990s after an observation period in which no resistant isolate was found (Molnár, 1996). Up to now, tiamulin resistance is present in many countries worldwide (Novotna and Skardova, 2002; Rohde et al., 2004; Clothier et al., 2011).
2011) with rates of up to 17.6% of the isolates in Spain (Hidalgo et al., 2009). Moreover, multiresistant isolates occur in Spain and the Czech Republic (Sperling et al., 2011; Hidalgo et al., 2011). The confrontation with progressive resistance leads to another problem. Culture of the slow-growing *B. hyodysenteriae* is fastidious and takes a long time. Therapy of SD with clinical symptoms must be started before the agent is diagnosed and results of antimicrobial susceptibility testing are present. Consequently, an optimised and accelerated diagnosis of SD as well as a genetic screening method for resistance is highly required and needs further research. At the moment, the situation in Switzerland is still favourable. Nevertheless, to maintain the status quo, a responsible use of antimicrobial agents and a continuous monitoring are essential.

### Sensibilità antimicrobienne de *Brachyspira hyodysenteriae* en Suisse

*Brachyspira* (*B.*) *hyodysenteriae* est l’agent de la dysenterie porcine, une affection diarrhéique muco-hémorragique grave des porcs connue dans le monde entier. Jusqu’à ce jour, la sensibilité aux antibiotiques *B. hyodysenteriae* n’avait pas été étudiée en Suisse. C’est pour cela qu’on a examiné, au moyen du test de micro dilution VetMIC Brachy panel, un choix de 30 isolats porcins de *B. hyodysenteriae* quant à leur sensibilité face à 6 substances antimicrobiennes. La Tiamuline et la Valnémuline ont montré une activité antimicrobienne élevée, bloquant tous les isolats à de faibles concentrations. Les tests de sensibilité vis-à-vis de la Doxycilline ont donné des valeurs comprises entre ≤0.25 µg/ml (47%) et 2 µg/ml (10%). Les valeurs de CMI de la Lincomycine variaient entre ≤0.5 µg/ml (30%) et 32 µg/ml (43%). Avec la Tylosine, 57% des isolats n’ont pas pu être bloqués avec la concentration la plus élevée de ≥128 µg/ml. Les valeurs de CMI pour la Tylovlosine se situaient entre ≤0.25 µg/ml (10%) et 8 µg/ml (20%). Ces résultats montrent que la situation suisse est favorable en regard d’autres pays européens. La Tiamuline et la Valnémuline en particulier restent des substances antimicrobiennes efficaces qui peuvent continuer à être utilisées pour lutter contre la dysenterie porcine.

### Sensibilità antimicrobica della *Brachyspira hyodysenteriae* in Svizzera

La *Brachyspira* (*B.*) *hyodysenteriae* è l’agente eziologico della dissenteria dei suini (SD), una grave malattia diarrhoea muco-emorragica nei suini in tutto il mondo. Finita, il modello di sensibilità di *B. hyodysenteriae* verso gli antibiotici non è ancora stato analizzato in Svizzera. Pertanto, una selezione di 30 suini con isolati di *B. hyodysenteriae* sono stati testati per la loro sensibilità a 6 principi attivi antimicrobici dal pannello VetMIC Brachy, un test di microdiluizione. Tiamulina e valnemulina erano caratterizzate da una forte attività antimicrobica e hanno inibito tutti gli isolati a basse concentrazioni. Il test di sensibilità della doxiciclina ha dato valori da ≤0.25 g/ml (47%) a 2 ug/ml (10%). I valori di MIC della lincomicina si situavano da ≤0.5 g/ml (30%) a 32 ug/ml (43%). Per la tilosina non si è potuto inibire il 57% degli isolati alla più alta concentrazione di ≥128 ug/ml. I valori di MIC della tivalosina si situavano da ≤0.25 g/ml (10%) a 8 ug/ml (20%). Questi risultati dimostrano la situazione favorevole della Svizzera rispetto ad altri Paesi europei. Particolarmente la tiamulina e la valnemulina sono ancora sostanze antimicrobiche efficaci che possono continuare ad essere utilizzate per il trattamento della SD.

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