A review of the current state of antimicrobial susceptibility test methods for Brachyspira

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A review of the current state of antimicrobial susceptibility test methods for *Brachyspira*

D.G.R. S. Kulathunga¹, J.E. Rubin¹*

¹Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Canada
D.G.R.S. Kulathunga - rsd351@mail.usask.ca
J.E. Rubin - joe.rubin@usask.ca

*Corresponding Author
52 Campus Drive
Department of Veterinary Microbiology
Phone: (306) 966-7246
Fax: (306) 966-7244

Email: joe.rubin@usask.ca
Abstract

The re-emergence of swine dysentery (*Brachyspira*-associated muco-haemorrhagic colitis) since the late 2000's has illuminated diagnostic challenges associated with this genus. The methods used to detect, identify and characterize *Brachyspira* from clinical samples have not been standardized and laboratories frequently rely heavily on in-house techniques. Particularly concerning is the lack of standardized methods for determining and interpreting the antimicrobial susceptibility of *Brachyspira* spp. The integration of laboratory data into a treatment plan is a critical component of prudent antimicrobial usage, the lack of standardized methods is therefore an important limitation to the evidence based use of antimicrobials. This review will focus on describing the methodological limitations and inconsistencies between current susceptibility testing schemes employed for *Brachyspira*, provide an overview of what we do know about the susceptibility of these organisms and suggest future directions to improve and standardize diagnostic strategies.

Keywords: *Brachyspira*, susceptibility test, method standardization, veterinary diagnostics, swine dysentery
1.0 Introduction

*Brachyspira* are a genus of Gram-negative, oxygen-tolerant anaerobic spirochetes which colonize the intestinal tracts of wild and domestic animals and humans (Hampson and Ahmed 2009). Seven species with standing in nomenclature are currently recognized within the genus *Brachyspira*, including the causative agents of swine dysentery and porcine, avian and human intestinal spirochetosis. Swine dysentery, characterized by profuse muco-hemorrhagic colitis, was first described in 1921 in the United States, although the causative agent (*B. hyodysenteriae*) was not isolated until the early 1970s (Harris et al. 1972; Whiting et al. 1921). Since the late 2000s, several additional distinct taxa ('Brachyspira hampsonii' and 'Brachyspira suanatina') have been found to causally associated with this syndrome (Burrough 2017). Swine dysentery, which primarily affects grower-finisher pigs, is the most economically damaging disease associated with *Brachyspira* attributable to both mortality and production limiting sub-optimal feed conversion (Alvarez-Ordonez et al. 2013). *Brachyspira pilosicoli* has a broad host spectrum including pigs, other domestic animals, wildlife and humans (Trivett-Moore et al. 1998). In pigs, intestinal spirochetosis caused by *Brachyspira pilosicoli* typically affects pigs within 7 to 14 days post-weaning (Trott et al. 1996). Porcine intestinal spirochetosis is characterized by diarrhea often described as having a "wet cement" consistency (Hampson and Duhamel 2006). In chickens, *Brachyspira intermedia* and *pilosicoli* are associated with diarrhea, decreased production and fecal staining of eggs (Mappley et al. 2014). Finally, human intestinal spirochetosis caused by *B. pilosicoli* or *B. aalborgi*, is an infrequent cause of diarrhea most commonly affecting children and immunocompromised individuals (Helbling et al. 2012; Tateishi et al. 2015).
Since the late 2000s the re-emergence of *Brachyspira* associated disease in pigs in North America and the emergence of 'Brachyspira hampsonii' in Europe have been described (Mahu et al. 2014; Martinez-Lobo et al. 2013; Rubin et al. 2013a). This re-emergence has included the identification of novel taxa causing a dysentery like disease ('Brachyspira hampsonii' and 'Brachyspira suanatina') which have been effectively but not validly described (Chander et al. 2012; Mirajkar et al. 2016b; Mushtaq et al. 2015; Perez et al. 2016). The specific etiological diagnosis of *Brachyspira* associated disease is challenging and relies heavily on PCR and culture based assays; it was therefore not surprising that the emergence of 'B. hampsonii' has been associated with substantial diagnostic challenges. The economic and animal welfare implications of *Brachyspira* associated disease have led to renewed interest in improving the diagnostic methods used for *Brachyspira*, including antimicrobial susceptibility test methods to facilitate the evidence based use of antimicrobials. The purpose of this review is therefore to summarize the current state of knowledge regarding the susceptibility of *Brachyspira* to antimicrobials, the intrinsic methodological limitations associated with *Brachyspira* and to highlight future research avenues to improve the reproducibility and reliability of *Brachyspira* antimicrobial susceptibility testing.

### 2.0 General information about *Brachyspira*

#### 2.1 Taxonomy

The genus *Brachyspira* is within the order Brachyspirales, and was recognized as a distinct genus from *Treponema* in 1983 (Gupta et al. 2013; Hovind-Hougen et al. 1982; Oren and Garrity 2014). There are currently seven species with standing in the nomenclature: *Brachyspira hyodysenteriae, B. pilosicoli, B. murdochii, B. innocens, B. intermedia, B. aalborgi* and *B.
alvinipulli (Euzeby 1997; Parte 2017). *Brachyspira* require specialized 'non-standard' culture conditions, don't typically form colonies on agar, grow unpredictably in broth and are poorly characterized phenotypically compared to other organisms such as *E. coli*; accurate etiological identification is therefore challenging. In the 1970s pathogenic *Brachyspira* (then *Treponema hyodysenteriae*) were identified as strongly hemolytic on blood containing media while weakly hemolytic spirochetes were considered to be non-pathogens (Kinyon et al. 1977). The identification of multiple, phylogenetically distinct weakly hemolytic *Brachyspira* highlights the limitations of this approach. Molecular tools have proven to be extremely useful diagnostically, although care must be taken to interpret test results considering the limitations of the test such as primer sensitivity and specificity. Recently, MALDI-TOF has been explored for the identification of *Brachyspira* species and shows promise (Calderaro et al. 2013; Prohaska et al. 2014; Warneke et al. 2014). Identification by phylogenetic analysis, particularly of the NADH oxidase (*nox*) gene has proven useful and provides comparable, objective data which is portable between labs. Sequence analysis at present is considered the gold standard for species level identification (Perez et al. 2016; Rohde et al. 2014). DNA sequencing has revealed phylogenetic diversity within the genus *Brachyspira*, including the novel strain 'B. hampsonii', and a large number of other strains which have not yet been characterized but appear to be distinct from recognized species (Patterson et al. 2013; Rubin et al. 2013b).

2.2 Treatment

In pigs *Brachyspira* associated disease has been effectively treated with the pleuromutilins, macrolides/lincosamides and carbadox while metronidazole has been effectively used in people with intestinal spirochetosis (Hampson 2012; Helbling et al. 2012). However, the ability to use
some of these agents in food producing animals is restricted in many jurisdictions; metronidazole
is banned for use in food animals in North America and the European Union, and carbadox is
banned in Canada, the EU and the UK and recently had its approval rescinded in the United
States (FDA 2016). The pleuromutilins (tiamulin and valnemulin) and the
macrolide/lincosamides (tylosin, lincomycin and tylvalosin) are the mainstay of anti-Brachyspira
therapy in swine medicine, although a number of other antimicrobial including bacitracin,
 virginiamycin and gentamicin are labeled for the treatment or prevention of Brachyspira
associated diseases in pigs (Table 1) (Hampson 2012). The heavy reliance on mechanistically
similar drugs, to which resistance in other organisms has been shown to develop by a common
mechanism highlights the potential impact of resistance emergence and the clinical value of
laboratory test guided therapeutic selection. Although there is no consensus method for
determining the antimicrobial susceptibility of Brachyspira, trends of decreasing susceptibility
among B. hyodysenteriae for the macrolides and pleuromutilins have been described in Europe
and the United States (Aarestrup et al. 2008; Lobova et al. 2004; Mirajkar et al. 2016a; Prasek et
al. 2014; Rugna et al. 2015).

3.0 Antimicrobial susceptibility testing
3.1 Importance of susceptibility testing to the prudent use of antimicrobials
3.1.1 Antimicrobial resistance
The term "antimicrobial resistance" is often used imprecisely. The categorization of an organism
as susceptible or resistant based on an in vitro test is designed to be clinically predictive, viewing
bacterial drug susceptibility from the patient's perspective. Susceptibility indicates a high
likelihood of clinical success, while resistance suggests a low likelihood of treatment success.
Clinical breakpoints are therefore necessarily predicated on assumptions including the species being treated, target pathogen, site of infection and treatment regimen (dose, dosing frequency and route of administration) (Dalhoff et al. 2009; Mouton et al. 2012).

Bacterial drug resistance can be intrinsic or acquired, and mechanistically falls into one of four broad categories: (i) enzymatic inactivation of the antibiotic (ii) decreased permeability of the cell wall (iii) active efflux of the antimicrobial from within the cell and (iv) alteration or absence of drug binding sites (Boerlin and White 2013). Intrinsic resistance is constitutive to the physiology of a particular organism, examples include penicillin resistance among Enterobacteriaceae and cephalosporin resistance in Enterococcus spp. Conversely, organisms with a susceptible wild-type can become resistant as a result of chromosomal mutation or the acquisition of resistance genes through transformation, transduction or conjugation (Boerlin and White 2013).

3.1.2 Importance of susceptibility testing for resistance surveillance

Beyond the clinical applicability of antimicrobial susceptibility test results, there is value in collecting this data for resistance surveillance. The purpose of resistance surveillance is to identify temporal changes in antimicrobial susceptibility and to detect the emergence of novel resistance phenotypes or genotypes. Globally, harmonization of test methodology, drug panels tested and interpretive criteria, is recognized as a critical step to ensure that data generated in different laboratories is comparable (OIE 2003). Substantial obstacles facing the detection of emerging resistance in Brachyspira therefore include the lack of standardized test methods and interpretive criteria, and consensus regarding which drugs should be included in test panels and
what concentrations should be tested. The description of the emergence of resistance to anti-
Brachyspira therapies has at best relied upon small scale, geographically limited, single
laboratory studies, and at worse the spurious comparison of data generated using different
methods.

Epidemiological cut-offs based on distributions of MICs of bacterial populations, may prove to
be useful for resistance surveillance. Epidemiological cut-offs, based on MIC distributions of
bacterial populations, are useful for detecting organisms with acquired resistance that
differentiates them from the wild type population but does not address questions of clinical
efficacy because they do not consider host factors (Dalhoff et al. 2009).

3.2 General information on susceptibility testing methods

Early in the antibiotic era, the development of antimicrobial susceptibility test methods grew
from the need to select the most appropriate therapy in the face of emerging resistance and the
availability of new, mechanistically distinct compounds. The requirement for high quality
information led to the standardization of test methods which improved comparability of results
between labs, and allowed the development of uniform interpretive criteria (when to classify an
isolate as susceptible or resistant) (Ericsson and Sherris 1971). Regularly updated internationally
recognized standardized methods are now published by the Clinical and Laboratory Standards
Institute (CLSI) in the United States, and the European Committee on Antimicrobial
Susceptibility Testing (EUCAST) in Europe. Because test results vary with divergences from
standard methodologies, test guidelines are necessarily prescriptive in terms of the density and
growth phase of the test culture, test media composition including pH and cation concentration,
incubation temperature, time and atmosphere and endpoint definition (CLSI 2012). The effects of test chemistry on MIC are well recognized but vary by drug and organism. For example test media with too low a pH may yield decreased penicillin MICs or increased quinolone MICs (CLSI 2017). Other test factors are intuitively more universal; for instance low MICs may be an artifact of too low a test inoculum, while high MICs result from testing too many organisms (CLSI 2017). Although test methods specific to a wide variety of anaerobic and fastidious organisms (ex. HACEK group organisms, Helicobacter, Listeria, Moraxella, Campylobacter, Actinobacillus pleuropneumoniae) are available, no standardized protocols (human or veterinary) addressing the unique requirements of Brachyspira have been published (CLSI 2015, 2016, 2017). The methods described by the CLSI and EUCAST all rely on phenotypic indicators of susceptibility, either growth or inhibition of growth in the presence of antimicrobial. Unambiguous and standardized endpoint definition is therefore essential (Jenkins and Schuetz 2012).

Molecular techniques (PCR) are increasingly used to identify the presence of resistance genes and therefore infer resistance. PCR has the advantage of detecting resistance without requiring classical phenotypic susceptibility test methods, a potential benefit when characterizing Brachyspira. However, the use of molecular methods requires knowledge of resistance genes or resistance conferring mutations. It must also be recognized that while these methods can identify resistance, they are insufficient to confirm susceptibility due to the potential presence of novel resistance genes or resistance conferring mutations not included in the assay.

4.0 Specific challenges to susceptibility testing of Brachyspira
The ability to assess the antimicrobial susceptibility of *Brachyspira* is hindered by two proximate causes: 1. the growth conditions required for *Brachyspira* are different than those standardized for susceptibility testing and 2. the inability to use typical susceptibility test endpoints. Although *Brachyspira* are reported to grow within the range of conditions prescribed by the CLSI for antimicrobial susceptibility testing, no single set of conditions have been standardized for testing these organisms. The unusual growth characteristics of *Brachyspira* sp. are at the root of many of these challenges. On solid media *Brachyspira* do not form distinct colonies, growth is typically inferred by the presence of hemolytic zones on blood containing agar (Figure 1). This property complicates/precludes the application of the concept of the colony forming unit, a foundational bacteriological principle which allows viable cells to be enumerated, and genetic clones to be isolated. The inability to differentiate whether apparently distinct hemolytic zones represent unique founding organisms or multiple individuals is a critical limitation. Pure cultures are a prerequisite for antimicrobial susceptibility testing, tests of mixed cultures yield cumulative results which may not reflect the susceptibility of either organism individually. Furthermore, this characteristic potentially precludes reliable test endpoint definition; whereas an antimicrobial inhibiting the formation of a colony is taken to indicate growth inhibition, failure to observe haemolysis in the presence of an antimicrobial may simply reflect inhibition of haemolysin production with or without affects on microbial growth. This is particularly germane for *Brachyspira* where protein synthesis inhibiting drugs are the primary agents of interest. Creative approaches to address endpoint validity including microscopic evaluation or sub-culture of media may prove useful in identifying viable organisms in media containing supra-MIC drug concentrations.
Broth culture of *Brachyspira* spp. also presents some unique challenges. Anecdotal observations suggest that broth cultures require a high starting inoculum and so failure to grow an organism in broth may simply reflect an insufficient initial inoculum density. The complicates two steps of the susceptibility testing process: 1. growing an initial broth culture used to inoculate test media, and 2. interpreting broth based susceptibility tests - did the culture not grow because it was inhibited by the drug, or because the culture was insufficiently dense?

5.0 Summary of susceptibility test methods which have been used for *Brachyspira*

The English language literature through 2017 was searched and studies describing antimicrobial susceptibility testing of *Brachyspira* were reviewed. Both agar and broth dilution techniques were used although there was substantial variability in the methods reported (Table 2). Incubation temperatures ranged from 37 – 42 °C with incubation times ranging from 2 to 5 days for agar dilution, and 37 – 38 °C and 3-5 days for broth dilution respectively. Where reported, highly variable inoculum densities were described: $1 \times 10^5$ - $5 \times 10^6$ CFU/ml for broth dilution and $10^4$ - $10^6$ CFU/spot for agar dilution tests. Finally, where reported atmospheres with varying composition were used. According to the manufacturers all anaerobic systems yield a final O$_2$ concentration of $\leq 1\%$, but yield highly variable CO$_2$ concentrations (7- >15%). A 2016 study has also described the use of doxycycline gradient strips although this method is not widely used (Mirajkar and Gebhart 2016). Further complicating the interpretation of susceptibility test results, is the lack of agreed upon interpretive criteria. With the lack of CLSI and EUCAST resistance breakpoints, investigators have relied upon a number of divergent sets of researcher proposed breakpoints (Burch 2005; Duhamel et al. 1998; Pringle et al. 2012; Ronne and Szancer 1990; SVARM 2014).
The introduction of the VetMIC *Brachyspira* broth micro-dilution system, first described in 2001, has led to substantial progress towards test standardization (Karlsson et al. 2004a; Karlsson et al. 2001). This product includes serial two-fold dilutions of dried antimicrobials in tissue culture trays. Testing is done by inoculating wells with 500µl of broth culture and incubating anaerobically at 37°C for 4 days with agitation (SVA 2011). Antimicrobial MICs as determined using this method have been shown to be reproducible, although interestingly typically yield a doubling dilution lower MIC than agar dilution tests (Rohde et al. 2004).

### 6.0 Challenges in comparing susceptibility data generated using non-standard tests

Because, there are no established standard test methods, interpretive criteria comparison of results generated by different labs must only be done with extreme caution. This inability to compare data was exemplified by a 2005 study where eight European laboratories participated in a ring test of *Brachyspira* diagnostics (Rasback et al. 2005). Each laboratory received samples of pig feces seeded with a previously identified *Brachyspira* isolate at specific concentrations and two pure cultures of known *Brachyspira* for susceptibility testing. Antibiotic susceptibility test methods differed by media composition and type, incubation temperature and time. Only a very limited description of the methods used by each laboratory for susceptibility testing and interpretation was presented. Not surprisingly results were highly variable; errors including failure to detect or identify isolates or discordant susceptibility test results occurred 32% of the time (Rasback et al. 2005). The varying concentration of antimicrobials tested made comparison of MICs between labs impossible even when the results were not inconsistent (ex. different labs reported lincomycin MIC of ≤4, ≤2 and ≤0.5 for the same isolate). The lack of validated
Brachyspira specific susceptibility test methods has resulted in the development of a variety of "in-house" techniques, consequently susceptibility data are not reliably comparable between labs. Further confounding susceptibility data portability is the lack of a consensus scheme for determining species level identification (Rasback et al. 2005). Researchers have used combinations of conventional methods (ex. culture and biochemical tests) and a variety of molecular methods (ex. nox and 16S-rDNA sequencing and nox-RFLP) which are recognized to have varying levels of sensitivity and specificity.

7.0 Objective measures of resistance

Genetic associations with decreased susceptibility to a number of drugs have been elucidated in several Brachyspira spp. (Table 3). Most published studies have focused on the association between single nucleotide polymorphisms (SNPs) in genes encoding ribosomal RNA and ribosomal proteins and increased MIC to drugs which act by inhibiting protein synthesis inhibitors including macrolides, lincosamides, pleuromutilins, doxycycline, chloramphenicol and florfenicol (Hidalgo et al. 2011; Hillen et al. 2014; Pringle et al. 2007; Pringle et al. 2004; Verlinden et al. 2011). Narrow spectrum β-lactamases including 13 enzymes related to OXA-63 have also been identified in B. pilosicoli (La et al. 2015; Meziane-Cherif et al. 2008; Mortimer-Jones et al. 2008). Differences in intrinsic resistance, even between closely related bacteria can inform treatment decisions based on accurate organism identification. For example, differences in the intrinsic resistance to vancomycin of Enterococcus gallinarum but not Enterococcus faecalis or faecium (CLSI 2017). Among Brachyspira species differences in susceptibility have also been observed, one study reported significant differences between species (Clothier et al.
2011). Although this study was small, it highlights the possibility of identifying taxa-specific differences in intrinsic resistance which may prove useful to clinicians for therapeutic selection.

8.0 Summary and future directions

The lack of standardized antimicrobial susceptibility test methods and interpretive criteria for *Brachyspira* is an important limitation to the evidence based use of antimicrobials for treating clinical disease. These diagnostic limitations have been made acutely problematic by the re-emergence of *Brachyspira* associated disease in North America, and the emergence of novel taxa in Europe. Furthermore, the increasing public scrutiny of antimicrobial usage in agriculture provides constant pressure to ensure that prescribing practices are evidence based and prudent. Unfortunately, for the reasons described here, we do not recommend that diagnostic laboratories routinely report categorical susceptibility test results for *Brachyspira* spp. Instead, we encourage our colleagues to continue their research describing temporal changes in MIC and archiving *Brachyspira* culture collections to facilitate the eventual development of agreed upon test methodology and interpretive criteria.

The lack of consensus on how to identify an isolate to the species level is the most immediate obstacle to improving laboratory diagnostics for *Brachyspira*. A common ontology is critical for researchers and diagnosticians to make simple comparisons of laboratory prevalence, describe the distribution of particular taxa or to compare the drug susceptibility of two organisms. The next most urgent need is an enriched understanding the genetic determinants of resistance. These genes or mutations have the potential to serve as an objective data point against which phenotypic susceptibility can be benchmarked, potentially serving as a pillar of quality control.
Finally, we call for additional collaboration in the global *Brachyspira* diagnostic/research communities. Collaboration and consensus building is going to be a critical step in identifying and recommending best practices for *Brachyspira*. This leadership was exemplified by Rasback et al., 2005 where the results of a multi-centre ring test were described; such investigations will be essential not only for ensuring method portability and objectivity, but also as a feedback mechanism to identify areas for improvement in individual laboratories.

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PONE-D-12-28322 [pii].


Figure 1.

Hemolytic zones associated with the growth of Brachyspira hyodysenteriae ATCC 27164 on CVS agar. Note the lack of colony formation on the surface of the media.
Table 1. Antimicrobials with label claims for the treatment/prevention/control of *Brachyspira* associated disease

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<th>Drug Name</th>
<th>United States</th>
<th>Dosage*</th>
<th>Canada</th>
<th>Dosage‡</th>
<th>European Union</th>
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<td>Tylosin</td>
<td>YES</td>
<td>Water - 250mg/gallon Feed - 40-100g/kg feed</td>
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<td>Feed - 44-110 mg/kg</td>
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<tr>
<td>Lincomycin</td>
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<td>Feed - 40-100g/kg</td>
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<td>Feed - 44-110 mg/kg</td>
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<td>NO</td>
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<td>Feed - 250g/ton</td>
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*administered dosage is per mass or volume of feed or water, ‡ administered dosage is per mass of pig receiving medication
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<tr>
<th>Method</th>
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<td>Various</td>
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<td>0.1 x 10^6 / spot</td>
<td>GENbox</td>
<td>TSA5SB</td>
<td>Karlsson et al. 2003</td>
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<tr>
<td></td>
<td>Japan</td>
<td>2004</td>
<td>BH</td>
<td>Unclear, 37-38°C</td>
<td>24 hours</td>
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<td>TSA5SB</td>
<td>Uezato et al. 2004</td>
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<td>Czech</td>
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<td>BH</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
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<td>WO5SB</td>
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<td>37°C</td>
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<td>Karlsson et al. 2004</td>
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<td>2011</td>
<td>Various</td>
<td>42°C</td>
<td>48-96 hours</td>
<td>1.6-4 x 10^5 / spot</td>
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<td>TSA5SB</td>
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<td>Various</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>TSA5SB0</td>
<td>Miraijkar et al. 2016a</td>
</tr>
<tr>
<td>Broth Dilution</td>
<td>Sweden</td>
<td>1999</td>
<td>BH</td>
<td>37°C</td>
<td>up to 96 hours</td>
<td>5 x 10^9 - 10^10 CFU/ml</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Karlsson et al. 1999</td>
</tr>
<tr>
<td></td>
<td>Sweden</td>
<td>§2001</td>
<td>BH</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Karlsson et al. 2001</td>
</tr>
<tr>
<td></td>
<td>Australia</td>
<td>2002</td>
<td>BH</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Karlsson et al. 2002</td>
</tr>
<tr>
<td></td>
<td>Sweden</td>
<td>§2003</td>
<td>Various</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Karlsson et al. 2003</td>
</tr>
<tr>
<td></td>
<td>Germany</td>
<td>§2004</td>
<td>BP</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Karlsson et al. 2004a</td>
</tr>
<tr>
<td></td>
<td>European</td>
<td>2005</td>
<td>BH</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Rohde et al. 2004</td>
</tr>
<tr>
<td></td>
<td>Spain</td>
<td>§2006</td>
<td>BP</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>BBD GasPak</td>
<td>BHIS10</td>
<td>Pringle et al. 2006</td>
</tr>
<tr>
<td></td>
<td>Spain</td>
<td>§2009</td>
<td>BH</td>
<td>38°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Hidalgo et al. 2009</td>
</tr>
<tr>
<td></td>
<td>Spain</td>
<td>§2011</td>
<td>BH</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Hidalgo et al. 2011</td>
</tr>
<tr>
<td></td>
<td>Sweden</td>
<td>§2012</td>
<td>BH, BP</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Pringle et al. 2012</td>
</tr>
<tr>
<td></td>
<td>Poland</td>
<td>§2012</td>
<td>BH</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Zmudzki et al. 2012</td>
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<tr>
<td></td>
<td>Germany</td>
<td>2014</td>
<td>BH</td>
<td>37°C</td>
<td>120 hours</td>
<td>10^6 CFU/ml</td>
<td>GENbox</td>
<td>BHIS20</td>
<td>Herbst et al. 2014</td>
</tr>
<tr>
<td></td>
<td>Italy</td>
<td>§2015</td>
<td>BH</td>
<td>Various</td>
<td>37°C</td>
<td>24 hours</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Rupa et al. 2015</td>
</tr>
<tr>
<td></td>
<td>USA</td>
<td>2016</td>
<td>Various</td>
<td>37°C</td>
<td>24 hours</td>
<td>0.1 x 10^6 / spot</td>
<td>No details reported</td>
<td>BHIS10</td>
<td>Miraijkar et al. 2016a</td>
</tr>
</tbody>
</table>

BH - B. hyodysenteriae; BP - B. pilosicoli

Media: Tryptic soy agar + 5% sheep blood: TSA 5 SB; Tryptic soy agar + 10% sheep blood: TSA 10 SB; Tryptic soy agar + 5% ox blood: TSA OX; Tryptic soy agar + 10% bovine blood: TSA10BB; Wilkins-Chargren + 5% sheep blood: WC 5SB; Mueller-Hinton agar + 5% sheep blood: MHA 5SB; Brain heart infusion + 10% fetal calf serum: BHIS 10; Brain heart infusion + 20% fetal calf serum: BHIS 20; Atmosphere: AnaeroGen Sachets Oxoid: AnaeroGen; Anaerobic gas pack GasPak BD: GasPak; anaerobic atmosphere generator biomerieux GENbox: GENbox; Anaerobic gas generator envelopes BBL gas pak: BBD GasPak

*GFU - authors report "growth forming units" as opposed to CFU*Studies which reported the use of the VetMIC Brachy panel
Table 3. Genetic associations with decreased antimicrobial susceptibility in *Brachyspira*

<table>
<thead>
<tr>
<th>Organisms Investigated</th>
<th>Phenotypic Resistance</th>
<th>Genetic Mechanism</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. hyodysenteriae</em></td>
<td>macrolides, lincosamides, streptogramins, tiamulin</td>
<td>Single nucleotide polymorphisms at positions 2032 and 2058 of the 23S ribosomal subunit</td>
<td>(Hidalgo et al. 2011; Karlsson et al. 1999; Karlsson et al. 2004b)</td>
</tr>
<tr>
<td><em>B. pilosicoli</em></td>
<td>tiamulin</td>
<td>Mutation in ribosomal protein L2, L3, L4, L22 and 23S rRNA in the peptidyl transferase region</td>
<td>(Hillen et al. 2014; Pringle et al. 2004)</td>
</tr>
<tr>
<td><em>B. hyodysenteriae</em></td>
<td>tiamulin</td>
<td>Single nucleotide polymorphism at position 1058 of the 16S ribosomal subunit</td>
<td>(Pringle et al. 2007; Verlinden et al. 2011)</td>
</tr>
<tr>
<td><em>B. intermedia</em></td>
<td>doxycycline</td>
<td>β-lactamases (OXA-63, OXA-136, OXA-137 and OXA-470 - OXA-479)</td>
<td>(La et al. 2015; Meziane-Cherif et al. 2008)</td>
</tr>
<tr>
<td><em>B. pilosicoli</em></td>
<td>penicillin, ampicillin and oxacillin</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Hemolytic zones associated with the growth of Brachyspira hyodysenteriae ATCC 27164 on CVS agar. Note the lack of colony formation on the surface of the media.

180x179mm (300 x 300 DPI)