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Märit Pringle, Claes Fellström, Karl-Erik Johansson. Decreased Susceptibility to Doxycycline Associated with a 16S rRNA Gene Mutation in. *Veterinary Microbiology*, 2007, 123 (1-3), pp.245. 10.1016/j.vetmic.2007.02.019 . hal-00532214

**HAL Id: hal-00532214**

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Submitted on 4 Nov 2010

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## Accepted Manuscript

Title: Decreased Susceptibility to Doxycycline Associated with a 16S rRNA Gene Mutation in *Brachyspira hyodysenteriae*

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PII: S0378-1135(07)00105-8  
DOI: doi:10.1016/j.vetmic.2007.02.019  
Reference: VETMIC 3603

To appear in: *VETMIC*

Received date: 15-1-2007  
Revised date: 19-2-2007  
Accepted date: 21-2-2007

Please cite this article as: Pringle, M., Fellström, C., Johansson, K.-E., 2007. Decreased Susceptibility to Doxycycline Associated with a 16S rRNA Gene Mutation in *Brachyspira hyodysenteriae*, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2007.02.019

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Revised version

Short communication

Decreased Susceptibility to Doxycycline Associated with a  
16S rRNA Gene Mutation in *Brachyspira hyodysenteriae*

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## 1 Abstract

2 The objective of this study was to assess whether nucleotide substitutions in the 16S rDNA  
3 sequence of selected *Brachyspira hyodysenteriae* isolates could explain differences in  
4 doxycycline minimal inhibitory concentrations (MICs). The main part of the 16S rRNA gene was  
5 sequenced and compared for 19 isolates with different doxycycline MICs. A mutation in the 16S  
6 rRNA gene at the position corresponding to 1058 in *Escherichia coli* has been shown to cause  
7 tetracycline resistance in other bacteria. In the *B. hyodysenteriae* sequences a G1058C mutation  
8 was found for all isolates with increased doxycycline MICs whereas all susceptible isolates had  
9 the wild type sequence.

10  
11 *Keywords:* Antibiotic resistance, *Brachyspira hyodysenteriae*, Tetracycline, 16S rRNA, Swine  
12 dysentery

## 15 1. Introduction

16  
17 The anaerobic spirochete *Brachyspira hyodysenteriae* is the causative agent of swine dysentery  
18 (Taylor and Alexander, 1971), a major disease of pigs world-wide. Doxycycline susceptibility  
19 tests of *B. hyodysenteriae* isolates reveal two groups with different minimal inhibitory  
20 concentrations (MICs), one highly susceptible (MIC 0.125-0.25 µg/ml) and one with decreased  
21 susceptibility (MIC 1-4µg/ml) (Karlsson et al., 2004). Tetracyclines bind to the 30S ribosomal  
22 subunit and thereby inhibit the elongation step during the protein synthesis. Recently, when  
23 complexes of tetracycline and the 30S subunit from *Thermus thermophilus* were analysed by X-  
24 ray crystallography, multiple binding sites were identified (Brodersen et al., 2000; Pioletti et al.,

25 2001). At the binding site with the highest degree of tetracycline occupancy, designated Tet-1,  
26 the attachment of aa-tRNA to the A-site is blocked (Pioletti et al., 2001). A G→C mutation in the  
27 16S rRNA gene corresponding to the position 1058 in *Escherichia coli* has been shown to cause  
28 tetracycline resistance in cutaneous propionibacteria (Ross et al., 1998). This nucleotide position  
29 is close to Tet-1, but does not affect the binding site directly. It has been suggested that this  
30 mutation could hinder the base pairing of G1058 with U1199, thus altering conformation and  
31 impede binding of tetracycline (Pioletti et al., 2001). An eightfold increase in MIC of tetracycline  
32 has been shown for an *E. coli* G1058C mutant constructed by site-directed mutagenesis and the  
33 binding of the drug to the ribosome was decreased to less than half compared to the wild type  
34 (Nonaka et al., 2005). Besides altering or protecting the ribosomal target structures other  
35 mechanisms conferring tetracycline resistance include efflux pumps and enzymatic inactivation  
36 of the drug.

37  
38 No genetic background for decreased susceptibility to tetracyclines has previously been described  
39 for *Brachyspira* spp. To assess whether any nucleotide changes could explain the doxycycline  
40 MIC differences, 16S rDNA sequences were analysed for *B. hyodysenteriae* isolates with  
41 different doxycycline MICs.

42

43

## 44 **2. Materials and methods**

45

46 The main part of the 16S rDNA sequence (1434 positions) was determined for 19 *B.*  
47 *hyodysenteriae* isolates. Two reference strains, twelve Swedish field isolates and five German  
48 field isolates were used. The field isolates with low doxycycline MICs were from clinical

49 samples taken at different sampling occasions from herds throughout Sweden. Isolates with  
50 increased doxycycline MICs are uncommon in Sweden and therefore German field isolates were  
51 also included. The DNA sequencing and the antimicrobial susceptibility tests were performed as  
52 described previously (Johansson et al., 2004; Karlsson et al., 2003).

53

54

### 55 **3. Results and discussion**

56

57 The doxycycline MICs and the sequence adjacent to position 1058 (*E. coli* numbering) for the 19  
58 isolates of *B. hyodysenteriae* are presented in Table 1. All isolates with elevated doxycycline  
59 MICs had a G1058C mutation whereas all susceptible isolates had the wild type sequence. An  
60 alignment of the 19 sequences showed a total of 10 other nucleotide differences, not within the  
61 described tetracycline binding sites. However, three German isolates (T23, A 5677/96 and A  
62 4974/96) with decreased doxycycline susceptibility also had a C1200A mutation, next to U1199,  
63 the pairing nucleotide to G1058. Despite the closeness to position G1058 there was no difference  
64 between the doxycycline MICs for these isolates compared to the other G1058C mutated isolates.  
65 No difference in vigour when grown on agar plates was observed between the wild type and  
66 mutated isolates. The sequences were deposited in GenBank (accession nos. see Table 1).

67

68 In *Helicobacter pylori* and laboratory derived mutants of *E. coli*, mutations in position 965-967  
69 cause tetracycline resistance (Dailidienė et al., 2002; Gerrits et al., 2002; Nonaka et al., 2005;  
70 Trieber and Taylor, 2002). No nucleotide differences were found at, or nearby, these positions in  
71 the *Brachyspira* sequences.

72

73 There is only one copy each of the ribosomal RNA genes in *B. hyodysenteriae* (Zuerner and  
74 Stanton, 1994), and single step mutations in 16S rRNA consequently dominate. To our  
75 knowledge, the only bacterial genus in which the mutation in position 1058 has been described  
76 before is *Propionibacterium* (Ross et al., 1998). Other common features for propionibacteria and  
77 *Brachyspira* spp. are the low number of ribosomal RNA (*rrn*) operons and their mechanism for  
78 resistance to macrolides and lincosamides. In both genera, this resistance is caused by mutations  
79 in 23S rDNA (Karlsson et al., 1999; Ross et al., 1997). Conversely bacteria with a high copy  
80 number of *rrn* operons are more likely to have transferable genes causing resistance to antibiotics  
81 that bind to the ribosome. The relationship between the number of *rrn* operon and macrolide  
82 resistance mechanisms has been reviewed by Vester and Douthwaite (Vester and Douthwaite,  
83 2001). Thus the mutation described herein could well be found in other bacteria with a low  
84 number of *rrn* operons, such as other spirochetes or *Mycoplasma* spp.

85  
86 In conclusion a G1058C mutation in the 16S rRNA gene is associated with low level resistance in  
87 *B. hyodysenteriae* and spread of this resistance determinant will depend on the mutation rate and  
88 the ability of mutant clones to multiply (vertical transmission) with no risk for co-transfer of, and  
89 selection for, other resistance mechanisms.

## 91 **Acknowledgements**

92  
93 We thank Judith Rohde for supplying field isolates from Germany and Marianne Persson for  
94 technical assistance.

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Table 1

Antimicrobial susceptibility to doxycycline and a 30 nucleotides fragment from 16S rRNA gene sequences for 19 strains/isolates of *Brachyspira hyodysenteriae*. The nucleotide position 1058 is shaded

Strain/isolate	Doxycycline MIC ( $\mu\text{g/ml}$ )	16S rDNA sequence	Acc no.
B78 <sup>1</sup> (ATCC 27164 <sup>1</sup> )	0.25	ATAGGTGCTG CATGGCTGTC GTCAGCTCGT	U14930
174/92 <sup>a</sup>	0.25	ATAGGTGCTG CATGGCTGTC GTCAGCTCGT	U14931
1357/02 <sup>a</sup>	0.125	ATAGGTGCTG CATGGCTGTC GTCAGCTCGT	DQ922553
2899/02 <sup>a</sup>	0.25	ATAGGTGCTG CATGGCTGTC GTCAGCTCGT	DQ922554
3105/02 <sup>a</sup>	0.25	ATAGGTGCTG CATGGCTGTC GTCAGCTCGT	DQ922555
3155/02 <sup>a</sup>	0.25	ATAGGTGCTG CATGGCTGTC GTCAGCTCGT	DQ922556
3542/02 <sup>a</sup>	0.25	ATAGGTGCTG CATGGCTGTC GTCAGCTCGT	DQ922557
3724/02 <sup>a</sup>	0.25	ATAGGTGCTG CATGGCTGTC GTCAGCTCGT	DQ922558
3729/02 <sup>a</sup>	0.125	ATAGGTGCTG CATGGCTGTC GTCAGCTCGT	DQ922559
B 204 (ATCC 31212)	2	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	U14932
2835/02 <sup>a</sup>	2	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	DQ922560
2869/02 <sup>a</sup>	2	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	DQ922561
3563/02 <sup>a</sup>	1	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	DQ922562
3678/02 <sup>a</sup>	2	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	DQ922563
84193-2x/99 (T4) <sup>b</sup>	2	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	DQ922564
91507-8x/01 (T21) <sup>b</sup>	2	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	DQ922565
91507-13x/01 (T23) <sup>b</sup>	4	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	DQ922566
A 5677/96 <sup>b</sup>	1	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	DQ974209
A 4974/96 <sup>b</sup>	1	ATAGGTGCTG CATGCCTGTC GTCAGCTCGT	AF172986

<sup>a</sup> Swedish field isolates.

<sup>b</sup> German field isolates.