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Sequence types and pleuromutilin susceptibility of *Brachyspira hyodysenteriae* isolates from Italian pigs with swine dysentery: 2003-2012

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Highlights

- Isolates of *Brachyspira hyodysenteriae* recovered from 2003 to 2012 in Italy were analysed.
- Susceptibility to pleuromutilins (tiamulin and valnemulin) was significantly associated with genetic group.
- Susceptibility to pleuromutilins (tiamulin and valnemulin) was significantly associated with year of isolation.
- Isolates in clonal clusters 2 and 7 were more than five times more likely to be sensitive than those in the other clonal clusters.

Abstract

Swine dysentery is a mucohaemorrhagic colitis of pigs caused by infection with *Brachyspira hyodysenteriae*. The disease can be controlled by treatment with antimicrobial agents, with the pleuromutilins tiamulin and valnemulin being widely used. In recent years, the occurrence of *B. hyodysenteriae* with reduced susceptibility to these drugs has been increasing. The aim of this study was to determine temporal changes in genetic groups and pleuromutilin susceptibility amongst *B. hyodysenteriae* isolates from Italy. Multilocus sequence typing (MLST) was performed on 108 isolates recovered from 87 farms in different regions of Italy from 2003 to 2012, and their minimum inhibitory concentrations (MICs) for tiamulin and valnemulin were determined. Logistic regression was performed to assess associations between susceptibility to the two antimicrobial agents and genetic group, year and region of isolation. The isolates were allocated to 23 sequence types (STs), with five clonal clusters (Ccs) and seven singletons. More than 50% of isolates were resistant to both pleuromutilins (MIC > 2.0 µg/mL for tiamulin and > 1.0 µg/mL for valnemulin). All 10 isolates in ST 83 were resistant; these were first isolated in 2011 and came from

nine farms, suggesting recent widespread dissemination of a resistant strain. Significant associations were found between the proportion of pleuromutilin susceptible isolates and the genetic group and year of isolation. Although resistant isolates were found in all Ccs, isolates in Ccs 2 and 7 were over five times more likely to be susceptible than those in the other Ccs. A significant trend in reduction of susceptibility over time also was observed.

Keywords: Swine dysentery; *Brachyspira hyodysenteriae*; Multilocus sequence typing; Pleuromutilin susceptibility

Introduction

Swine dysentery (SD) is a severe mucohaemorrhagic colitis affecting pigs primarily during the grower-finisher period (Hampson, 2012). The most common aetiological agent is the anaerobic intestinal spirochaete *Brachyspira hyodysenteriae*, although '*Brachyspira hampsonii*' occasionally may be involved (Rubin et al., 2013). Infection spreads amongst pigs in the same or different herds by direct or indirect contact with infected faecal material.

Control and treatment of SD are based mainly on the use of antimicrobial agents, particularly the pleuromutilins tiamulin and valnemulin (van Duijkeren et al., 2014). Reduced susceptibility of *B. hyodysenteriae* strains to these antibiotics has been reported (Karlsson et al., 2003, 2004; Lobova et al., 2004; Rohde et al., 2004; Hidalgo et al., 2011; Sperling et al., 2011). As a consequence, temporal monitoring of antimicrobial susceptibility in clinical isolates of *B. hyodysenteriae* is important (Hidalgo et al., 2009). Data from susceptibility testing can be correlated with

information about the origin and genetic identity of individual strains, providing a better understanding of factors associated with the evolution of resistance.

Numerous methods have been developed for molecular typing of *B. hyodysenteriae* strains, of which multilocus sequence typing (MLST) is especially useful (Råsbäck et al., 2007; La et al., 2009; Osorio et al., 2012). Data from *B. hyodysenteriae* isolates from throughout the world have been deposited at the PubMLST site[1]. The aim of this study was to determine the genetic diversity of *B. hyodysenteriae* isolates from Italy and to investigate relationships with year of origin, local place of origin and susceptibility to pleuromutilins.

Materials and methods

Brachyspira hyodysenteriae isolates

B. hyodysenteriae isolates (n = 108) were obtained from the collection of the Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna (IZSLER) in northern Italy. The isolates originated from diagnostic submissions received in 2003-2012 from pigs with SD on 87 farms in eight regions of Italy (see Appendix: Supplementary Table 1); 2-8 isolates were obtained in the same or different years from 13 farms.

Pleuromutilin susceptibility testing

The susceptibility of 103 *B. hyodysenteriae* isolates to tiamulin and valnemulin was determined by using the micro-broth dilution test performed with VetMIC Brachy version 2 (SVA National Veterinary Institute). Minimal inhibitory

[1] See: http://pubmlst.org (accessed 22 October 2014).

concentrations (MIC) were established for each isolate and interpreted according to Pringle et al. (2012). Isolates were classified as being resistant (MIC > 2 µg/mL), intermediate (MIC > 0.25 µg/mL) or susceptible (MIC ≤ 0.25 µg/mL) to tiamulin, or resistant (MIC > 1 µg/mL), intermediate (MIC > 0.125 µg/mL) or susceptible (MIC ≤ 0.125 µg/mL) to valnemulin.

Multilocus sequence typing

Isolates were incubated at 37 °C for 5 days in trypticase soy broth (Oxoid) with 10% fetal bovine serum in an anaerobic atmosphere. One millilitre of each culture (~10^8 cells/mL) of *B. hyodysenteriae* was centrifuged at 5000 *g* for 5 min. Cells were suspended in sterile distilled water and chromosomal DNA was extracted according to the Gram-negative bacterial protocol of the DNeasy Blood and Tissue kit (Qiagen).

MLST was performed with minor modifications from La et al. (2009). Seven MLST loci were used, consisting of the genes encoding alcohol dehydrogenase (*adh*), alkaline phosphatase (*alp*), esterase (*est*), glutamate dehydrogenase (*gdh*), glucose kinase (*glpK*), phosphoglucomutase (*pgm*) and acetyl-CoA acetyltransferase (*thi*). PCRs were performed in 30 µL reaction mixtures using the GoTaq Hot Start Colorless Master Mix (Promega). Each PCR reaction set included DNA from *B. hyodysenteriae* field strain Izler MO 371/2011 as a positive control and double distilled water as a negative control. The PCR conditions were 95 °C for 2 min, followed by 33 cycles at 95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, then 5 min at 72 °C before cooling at 4 °C. The PCR products were purified with the Ampure XP PCR purification kit (AgenCourt). Purified PCR products were

sequenced using the GenomeLab DTCS Quick Start Kit (Beckman Coulter). Oligonucleotide primers were those described by Råsbäck et al. (2007), except for *pgm*, for which a sequence reaction with a new primer PGM-F685 (5'-TATACTCCTATTCATGGTTCCG-3') was used in addition to PGM-F172.

Sequencing was performed with a CEQ 8000 sequencer (Beckman Coulter) and the results were analysed and assembled using the 'Sequencing' and 'Investigator' packages of CEQ 8000 version 8.0 software. For each locus the sequences were aligned with the original *B. hyodysenteriae* strain WA1 sequence (GenBank EF488202) using the CLUSTAL W application of BioEdit version 7.0.8.0 (Hall, 1999). The aligned loci sequences were trimmed as reported by La et al. (2009) and used for MLST analysis.

Statistical analysis

The aligned sequences for each of the seven MLST loci were analysed using BioEdit version 7.0.8.0 (Hall, 1999) to identify identical sequences. Each unique sequence obtained for each locus was checked in the *B. hyodysenteriae* MLST Database[2] to obtain the correspondent allele number. The allelic profile for each isolate was determined and consisted of a line listing the allele number for each locus in turn. Isolates were assigned a sequence type (ST) according to their allelic profiles. Isolates were considered to be genetically identical and belonging to the same ST if their sequences were identical at all seven loci. Clonal clusters sharing six or more loci were also identified using the BURST algorithm in START2 (Jolley et al., 2001).

[2] See: http://pubmlst.org (accessed 22 October 2014).

An MLST dendrogram was constructed from the data matrix of allelic mismatches using the unweighted-pair group method with allelic arithmetic means (UPGMA) method with 1000 bootstrap replicates. A minimum spanning tree was generated using the Bionumerics Software version 7.1 (Applied Maths) and versions were colour-coded according to pleuromutilin susceptibility and year of isolation to assist with visualisation of the results.

Isolates were divided in two groups for statistical analysis of MIC data for both antibiotics: (1) fully susceptible (MIC ≤ 0.25 µg/mL for tiamulin and ≤ 0.125 µg/mL for valnemulin); and (2) isolates with decreased susceptibility (MIC > 0.25 µg/mL for tiamulin and > 0.125 µg/mL for valnemulin). Logistic regression was performed to assess associations between pleuromutilin susceptibility and the independent variables geographic origin, year of isolation and genetic group (Cc).

The probability that strains belonging to a Cc were fully susceptible was expressed as an odds ratio with a 95% confidence interval (95% CI). A trend in reduction of susceptibility to both antibiotics over the period 2003 to 2012 was tested with Pearson's correlation and an extension of the Wilcoxon rank-sum test for the trend. All statistical analyses were performed using Intercooled Stata 7.0 software (Stata) and the significance level was set at $P < 0.05$.

Results

MLST analysis

Allelic frequencies over the seven loci ranged from four (*adh*, *est*) to 10 (*glpK*), with a mean of 6.71. A total of 23 profiles (STs) was obtained (see Appendix:

Supplementary Table 1). Twenty-one STs (STs 74-87, 97-103) containing 104 isolates (96.3%) were newly described at the time they were deposited in PubMLST. ST 8 and ST 52 had been reported previously and contained isolates from other European countries (La et al., 2009; Osorio et al., 2012). ST 77 contained the greatest number of isolates (*n* = 25), followed by ST 76 (*n* = 12), STs 75 and 78 (*n* = 11 each) and STs 79 and 83 (*n* = 10 each). The other STs contained 1-5 isolates. The five most prominent STs (STs 75-79), each containing > 10 isolates, originated from 55 farms in five regions where more than 80% of Italian pig production is undertaken.

An UPGMA dendrogram showing the relative relationship amongst the isolates is presented as Fig. 1. The isolates were broadly distributed across the tree; this distribution was reflected in the minimum spanning tree, which identified the five Ccs and was colour-coded to show susceptibility to tiamulin (Fig. 2a) and valnemulin (Fig. 2b), along with temporal changes (Fig. 3).

Of the five Ccs, the largest was Cc 4 and this included 42 isolates from five STs that were only identified in Italy. Cc 3 and Cc 7 also contained STs described in other European countries (Fig. 2). Isolates from 2003-2006 were present amongst all five Ccs, while isolates belonging to STs 83 and 99-103 were only isolated in 2011-2012 (Fig. 3).

In 10/13 farms where multiple *B. hyodysenteriae* isolates were available, all isolates belonged to the same ST, although in four cases they varied in their pleuromutilin susceptibility (farms 3, 15, 26 and 34; see Appendix: Supplementary Table 1). In the other three farms (farms 8, 20 and 77) more than one ST was

identified; on farm 8, these isolates also varied in susceptibility. Isolates from Farms 8 and 20 had different STs, but belonged to the same Ccs.

Pleuromutilin susceptibility

Susceptibility results obtained for 103/108 isolates are recorded in Supplementary Table 1 (see Appendix). No data was available for five isolates due to overgrowth with contaminating microorganisms. Among these 103 isolates, 55 (53.4%) were resistant, 21 (20.4%) were intermediate and 27 (26.2%) were susceptible to tiamulin, while 59 (57.3%) were resistant, 20 (19.4%) were intermediate and 24 (23.3%) were susceptible to valnemulin. The susceptibility classifications were identical for the two antimicrobial agents for 86/103 (83.5%) isolates, but the patterns varied for 17/103 (16.5%) isolates (STs 52, 74-79, 84, 100, 102 and 103; Fig. 2).

No significant association was found between the two susceptibility groups (isolates fully susceptible to both antibiotics or isolates with reduced susceptibility; MIC $\geq$ 0.25 µg/mL for tiamulin and 0.125 µg/mL for valnemulin) or by geographical distribution of the isolates. Genetic group was significantly associated with the proportion of fully susceptible isolates ($P < 0.05$) for both antibiotics. In particular, isolates belonging to Ccs 7 and 2 had an odds of being fully susceptible to tiamulin and valnemulin that was greater than five times (odds ratio 5.5; 95% CI 1.9-16.0; $P < 0.01$) and six times (odds ratio 6.3; 95% CI: 2.1-18.9; $P < 0.01$) higher than isolates from the other Ccs. Year of isolation was significantly associated with the proportion of isolates that were fully susceptible to tiamulin ($P < 0.01$) and a significant trend was observed in reduction of susceptibility from 2003 to 2012 (67% and 33% of fully

susceptible isolates, respectively; Pearson's correlation r = -0.30; extension of the Wilcoxon rank-sum test for the trend, $P < 0.01$). This association was not significant for valnemulin ($P = 0.08$).

Discussion

The aim of this study was to gain insight into the genetic background of Italian *B. hyodysenteriae* isolates from different regions and to examine temporal changes in pleuromutilin susceptibility patterns. Using previously published criteria, more than half of *B. hyodysenteriae* isolates were resistant to tiamulin and/or valnemulin in vitro, with others showing reduced susceptibility and only about a quarter being fully susceptible.

The two pleuromutilins target the domain V of the 23S rRNA gene and/or the ribosomal protein L3 gene; reduced susceptibility in *B. hyodysenteriae* involves point mutations in these regions (Pringle et al., 2004; Hidalgo et al., 2011). Our results suggest that there are likely to be differences in binding sites for the two pleuromutilins that result in different susceptibilities.

Resistance to the pleuromutilins develops in a step-wise manner, suggesting that multiple mutations are needed to achieve high level resistance (Karlsson et al., 2001). Low level resistance (or decreased susceptibility, recorded here as 'intermediate') is likely to develop into higher MICs and full resistance. Consistent with this, a temporal decrease in susceptibility between 2003 and 2012 was observed in this study.

Although resistance in *B. hyodysenteriae* has been recorded previously in Italy (Bonilauri et al., 2004; Merialdi et al., 2006) and other European countries (Karlsson et al., 2003, 2004; Lobova et al., 2004; Rohde et al., 2004; Vyt and Hommez, 2006), this study confirms that increasing resistance to pleuromutilins is an ongoing problem in Italy.

The 108 isolates had 23 different allelic profiles (STs), of which 21 were newly described and two (ST 52 and ST 8) had been detected previously in other European countries. Isolates in ST 52 have been detected in Germany, Belgium and Spain, while those in ST 8 have been recorded in the UK (La et al., 2009), with this being the predominant ST in Spain (Osorio et al., 2012). Although not isolated in Italy, ST 9 contains isolates from Sweden and belongs to the same Cc that included two Italian STs (74 and 99).

Together, these observations support the likelihood that trans-national spread of resistant *B. hyodysenteriae* isolates has accompanied regular trade of pigs within Europe. In addition, local selection and spread of clonal groups in Italy is likely, as shown by the existence of Ccs (2, 4 and 17) that only included Italian strains. A better understanding of these features will emerge as additional isolates from widespread locations are analysed and added to the PubMLST database.

On 10/13 farms where more than one *B. hyodysenteriae* isolate from the same or different years was examined, they belonged to the same ST. In the case of the isolates from different years, this suggests persistence of a single strain on these farms; on four farms (farms 8, 15, 26, and 34) isolates developed reduced

susceptibility with time. This is likely to have occurred under the selection pressure of pleuromutilin use, although specific records about such usage were not available due to the retrospective nature of this study.

Different STs occurred on another three farms; on two farms (farms 8 and 20), these belonged to the same Ccs, whilst on farm 77 the isolates were more genetically distinct. These findings could involve transmission of new strains to the farms, although it might also be explained by the emergence of variants of the original strain (Atyeo et al., 1999; La et al., 2009). This seems most likely to have occurred on farm 8, where the two isolates differed by only one nucleotide substitution. This is the first description of ST 83, which was not part of a Cc. The 10 isolates in this ST were recovered from nine Italian farms in 2011 and 2012; all were resistant to both pleuromutilins. These data suggest recent widespread dissemination of this resistant strain in Italy.

There was a significant association between susceptibility to pleuromutilins and the allelic profiles of the isolates in Ccs 2 and 7. These findings imply that if a strain acquires a mutation in a conserved housekeeping gene and a trait of antibiotic resistance, the subsequent antibiotic selection pressure could be sufficient to promote expansion of such clones on infected farms and eventual dissemination.

Conclusions

MLST analysis showed the existence of several clonal groups and STs of *B. hyodysenteriae* in Italy that have not previously been described, as well as evidence that some strains have been spread amongst European countries. More than half of the

Italian isolates of *B. hyodysenteriae* were resistant to pleuromutilins and there was a significant trend for this to have increased in the last 10 years. Such increases in MICs for tiamulin and valnemulin against porcine *B. hyodysenteriae* isolates in Italy and other European countries are of concern, since there are only a limited number of other antimicrobial agents still available and effective for the treatment of SD. There is a need to include *B. hyodysenteriae* in national antimicrobial resistance monitoring programmes, linking changes in susceptibility to MLST data to trace the national and international spread of resistant clones.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi: ...

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Figure legends

Fig. 1. UPGMA dendrogram depicting genetic relationships amongst the 23 Italian *Brachyspira hyodysenteriae* STs identified in the study (underlined) and 110 STs from PubMLST. The tree was constructed from combined individual distance matrices of sequences from seven MLST loci (*adh, alp, est, gdh, glpK, pgm, thi*). The length of the scale bar represents 1 nucleotide substitution in 100 base pairs of the sequenced gene fragment. The five clonal clusters of Italian STs sharing six or more common *loci* are indicated as Cc

Fig. 2. Minimum spanning tree analysis showing (a) tiamulin and (b) valnemulin susceptibility of 103 isolates of *Brachyspira hyodysenteriae* represented by 23 sequence types (ST).

Fig. 3. Minimum spanning tree analysis comparing the year of isolation of 108 Italian isolates with the 23 sequence types (ST) they represent. Year of isolation has been grouped into four-year intervals. Each node of the MST indicates a different ST (labelled), its size reflects the number of isolates and the colour represents the period in which the strain was isolated. Each node indicates a different ST (labelled), its size indicates the number of isolates in the ST and the colour represents the susceptibility of the isolate to tiamulin. The width of the branches indicates the allelic difference between two STs; heavy lines link single locus variants (SLVs), thin lines link double locus variants (DLVs) and dotted lines link STs differing by more than two loci. The five clonal clusters of STs sharing six or more common loci are indicated by shading

441 in grey. Isolates from other countries that belong in the same ST or Cc are marked:

442 DE, Germany; BE, Belgium; SP, Spain; SE, Sweden; IT, Italy.

Appendix: Supplementary Table 1

Details of 108 *Brachyspira hyodysenteriae* isolates from Italy included in the study.

Isolate	Farm of origin	Region	Year of isolation	Clonal cluster	Sequence type	Tiamulin MIC (µg/mL) [a]	Valnemulin MIC (µg/mL) [b]
295-2012_77	1	Lombardy	2012	4	77	>8.0 (R)	>4.0 (R)
297-2012_77	1	Emilia-Romagna	2012	4	77	>8.0 (R)	>4.0 (R)
232-2009	2	Lombardy	2009	2	75	ND	ND
136-2005_S	3	Lombardy	2005	4	77	<0.063 (S)	0.031 (S)
136-2005_I	3	Lombardy	2005	4	77	1.0 (I)	2.0 (R)
148-2006	4	Lombardy	2006	2	82	<0.063 (S)	<0.063 (S)
203-2005	5	Lombardy	2005	7	74	<0.063 (S)	<0.063 (S)
243-2011	6	Lombardy	2011	NA	83	>8.0 (R)	2.0 (R)
247-2011	7	Piedmont	2011	3	79	>8.0 (R)	>4.0 (R)
248-2011	8	Piedmont	2011	3	79	>8.0 (R)	>4.0 (R)
310-2010	8	Piedmont	2010	3	79	0.5 (I)	0.5 (I)
318-2010	8	Piedmont	2010	3	80	0.5 (I)	0.5 (I)
96i-2008	9	Lombardy	2008	4	77	<0.063 (S)	<0.031 (S)
110i-2008	10	Lombardy	2008	4	77	0.5 (I)	0.5 (I)
128-2008	11	Lombardy	2008	2	75	0.5 (I)	0.5 (I)
137-2005	12	Lombardy	2005	2	81	ND	ND
240-2009	13	Lombardy	2009	17	85	0.25 (S)	0.063 (S)
245-2011	14	Lombardy	2011	4	77	>8.0 (R)	>4.0 (R)
271-2009	15	Lombardy	2009	4	77	0.25 (S)	0.5 (I)
338-2010	15	Lombardy	2010	4	77	>8.0 (R)	>4.0 (R)
246-2011	16	Emilia-Romagna	2011	2	75	>8.0 (R)	>4.0 (R)
155-2006	17	Lombardy	2006	NA	84	0.25 (S)	0.25 (I)
160-2006	18	Lombardy	2006	17	78	>8.0 (R)	>4.0 (R)
108i-2008	19	Lombardy	2008	2	75	0.25 (S)	<0.031 (S)
153-2006	20	Lombardy	2006	4	76	8.0 (R)	>4.0 (R)
150-2006	20	Lombardy	2006	4	86	8.0 (R)	>4.0 (R)
156-2008	21	Lombardy	2008	4	77	>8.0 (R)	>4.0 (R)
167-2003	22	Lombardy	2003	17	78	<0.063 (S)	<0.031 (S)
170-2003	23	Lombardy	2003	2	75	8.0 (R)	>4.0 (R)
190-2009	24	Lombardy	2009	7	74	0.5 (I)	0.5 (I)
154-2006	25	Emilia-Romagna	2006	17	78	>8.0 (R)	>4.0 (R)
254-2011	26	Emilia-Romagna	2011	4	77	>8.0 (R)	>4.0 (R)
257-2011	26	Emilia-Romagna	2011	4	77	>8.0 (R)	>4.0 (R)
259-2011	26	Emilia-Romagna	2011	4	77	8.0 (R)	>4.0 (R)
156m-2006	26	Emilia-Romagna	2006	4	77	0.25 (S)	0.125 (S)
286-2011	26	Emilia-Romagna	2011	4	77	0.5 (I)	0.5 (I)
7-2012	26	Emilia-Romagna	2012	4	77	>8.0 (R)	>4.0 (R)
MO520-2012	26	Emilia-Romagna	2012	4	77	>8.0 (R)	>4.0 (R)
MO521-2012	26	Emilia-Romagna	2012	4	77	<0.063 (S)	<0.031 (S)
302-2010	27	Lombardy	2012	4	77	>8.0 (R)	>4.0 (R)
30i-2005	28	Umbria	2005	3	52	0.25 (S)	<0.031 (S)
293-2009	29	Umbria	2009	3	79	>8.0 (R)	>4.0 (R)
157-2006	30	Basilicata	2006	NA	87	0.25 (S)	0.063 (S)
143-2006	31	Emilia-Romagna	2006	2	75	0.25 (S)	0.5 (I)
146-2006	32	Emilia-Romagna	2006	7	74	0.125 (S)	0.125 (S)
151-2006	33	Emilia-Romagna	2006	4	76	>8.0 (R)	>4.0 (R)
152-2006	33	Emilia-Romagna	2006	4	76	>8.0 (R)	>4.0 (R)
158-2006	34	Emilia-Romagna	2006	3	79	0.5 (I)	0.25 (I)
192-2007	34	Emilia-Romagna	2007	3	79	2.0 (I)	4.0 (R)
169-2003	35	Emilia-Romagna	2003	2	75	ND	ND
172-2003	36	Emilia-Romagna	2003	2	75	0.25 (S)	0.063 (S)
244-2011	37	Emilia-Romagna	2011	4	77	>8.0 (R)	>4.0 (R)
NC1 -2011	38	Emilia-Romagna	2011	4	77	>8.0 (R)	>4.0 (R)
145-2006	39	Toscana	2006	3	79	ND	ND
135-2005	40	Veneto	2005	17	78	ND	ND
159-2006	41	Lombardy	2006	4	76	>8.0 (R)	>4.0 (R)
161-2006	42	Emilia-Romagna	2006	17	78	2.0 (I)	>4.0 (R)
166-2006	43	Emilia-Romagna	2006	4	76	>8.0 (R)	>4.0 (R)
123-2005	44	Piedmont	2005	2	97	0.5 (I)	0.5 (I)
125-2005	45	Lombardy	2005	4	76	0.25 (S)	0.5 (I)
126-2005	46	Lombardy	2005	4	76	0.25 (S)	0.5 (I)
134-2005	47	Emilia-Romagna	2005	2	75	0.125 (S)	<0.031 (S)
138-2006	48	Emilia-Romagna	2006	4	77	>8.0 (R)	>4.0 (R)
141-2006	49	Veneto	2006	4	77	0.25 (S)	0.125 (S)
142-2006	50	Lombardy	2006	7	74	0.25 (S)	0.25 (I)
191-2007	51	Lombardy	2007	4	77	>8.0 (R)	4.0 (R)
196-2007	52	Lombardy	2007	4	76	1.0 (I)	2.0 (R)
198-2007	53	Emilia-Romagna	2007	3	79	>8.0 (R)	>4.0 (R)
201-2007	54	Emilia-Romagna	2007	4	76	1.0 (I)	1.0 (I)
203-2007	55	Lombardy	2007	4	98	>8.0 (R)	>4.0 (R)

212-2008	56	Toscana	2008	3	79	2.0 (I)	4.0 (R)
227-2009	57	Emilia-Romagna	2009	3	52	4.0 (R)	2.0 (I)
228-2009	58	Emilia-Romagna	2009	NA	8	<0.063 (S)	<0.031 (S)
250-2011	59	Lombardy	2011	17	78	>8.0 (R)	>4.0 (R)
252-2011	60	Emilia-Romagna	2011	17	78	>8.0 (R)	>4.0 (R)
262-2011	61	Emilia-Romagna	2011	17	78	8.0 (R)	2.0 (R)
263-2011	62	Emilia-Romagna	2011	4	76	>8.0 (R)	>4.0 (R)
264-2011	63	Emilia-Romagna	2011	17	78	>8.0 (R)	2.0 (R)
266-2011	64	Marche	2011	NA	83	>8.0 (R)	>4.0 (R)
268-2011	65	Campania	2011	NA	8	>8.0 (R)	>4.0 (R)
270-2011	66	Lombardy	2011	NA	83	>8.0 (R)	2.0 (R)
271-2011	67	Emilia-Romagna	2011	7	99	<0.063 (S)	<0.031 (S)
272-2011	68	Lombardy	2011	NA	83	>8.0 (R)	>4.0 (R)
273-2011	69	Lombardy	2011	7	74	>8.0 (R)	>4.0 (R)
274-2011	70	Veneto	2011	3	79	>8.0 (R)	>4.0 (R)
275-2011	71	Emilia-Romagna	2011	NA	100	1.0 (I)	2.0 (R)
277-2011	72	Emilia-Romagna	2011	NA	101	<0.063 (S)	<0.031 (S)
278-2011	72	Emilia-Romagna	2011	NA	101	0.5 (I)	0.5 (I)
279-2011	73	Lombardy	2011	17	78	0.5 (I)	0.25 (I)
280-2011	74	Lombardy	2011	4	76	>8.0 (R)	>4.0 (R)
281-2011	75	Emilia-Romagna	2011	17	78	4.0 (R)	1.0 (I)
284-2011	76	Lombardy	2011	2	102	0.50 (I)	0.125 (S)
282-2011	77	Lombardy	2011	17	85	>8.0 (R)	>4.0 (R)
285-2011	77	Lombardy	2011	NA	83	>8.0 (R)	4.0 (R)
290-2011	78	Lombardy	2011	NA	83	>8.0 (R)	>4.0 (R)
291-2012	78	Lombardy	2012	NA	83	>8.0 (R)	>4.0 (R)
292-2012	79	Emilia-Romagna	2012	NA	83	>8.0 (R)	>4.0 (R)
293-2012	80	Lombardy	2012	NA	83	>8.0 (R)	>4.0 (R)
295-2012_103	81	Lombardy	2012	4	103	>8.0 (R)	>4.0 (R)
296-2012	82	Lombardy	2012	4	77	>8.0 (R)	>4.0 (R)
297-2012_76	83	Emilia-Romagna	2012	4	76	>8.0 (R)	>4.0 (R)
337-2012	84	Lombardy	2012	2	75	0.125 (S)	<0.031 (S)
339-2012	84	Lombardy	2012	2	75	0.125 (S)	<0.031 (S)
348-2012	85	Lombardy	2012	4	103	0.125 (S)	<0.031 (S)
350-2012	85	Lombardy	2012	4	103	2.0 (I)	0.063 (S)
351-2012	85	Lombardy	2012	4	103	2.0 (I)	<0.031 (S)
100-2012	86	Emilia-Romagna	2012	NA	83	>8.0 (R)	>4.0 (R)
283-2011	87	Lombardy	2011	NA	84	0.5 (I)	0.25 (I)

NA, not appropriate (not part of a clonal cluster); ND, not determined; MIC, minimum inhibitory concentration.

[a] Tiamulin susceptibility: R, resistant (MIC > 2 µg/mL); I, intermediate (MIC > 0.25µg/mL); S, susceptible (MIC ≤ 0.25).

[b] Valnemulin susceptibility: R, resistant (MIC > 1 µg/mL); I, intermediate (MIC > 0.125µg/mL); S, susceptible (MIC ≤ 0.125).