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1 **Antimicrobial susceptibility of *Actinobacillus pleuropneumoniae* isolates from clinical**
2 **outbreaks of porcine respiratory diseases**

3

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26 ABSTRACT

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28 Limited data regarding the susceptibility of *Actinobacillus pleuropneumoniae* to
29 antimicrobials has been published during recent years. Accordingly, the aim of the present
30 study was to investigate the distribution of MICs for the isolates of *A. pleuropneumoniae*
31 from diseased pigs in the Czech Republic between 2007 and 2009.

32

33 A total of 242 isolates were tested for susceptibility to 16 antimicrobial agents by a broth
34 microdilution method. A low degree of resistance was observed for florfenicol (0.8 %),
35 amoxicillin and clavulanic acid (0.8 %), tilmicosin (1.2 %), tiamulin (1.7 %) and ampicillin
36 (3.3 %), whereas resistance to tetracycline was detected more frequently, 23.9 % of
37 isolates. Interestingly, resistance to florfenicol has not yet been reported in any study
38 investigating antimicrobial resistance of *A. pleuropneumoniae*. By PCR the presence of the
39 *floR* gene was confirmed in all florfenicol resistant isolates.

40

41 Keywords: Antimicrobials; Minimal inhibition concentration; Pig; Pleuropneumonia

42

43 INTRODUCTION

44

45 *Actinobacillus pleuropneumoniae* is one of the main etiologic agents of contagious
46 bacterial diseases in swine. It causes pleuropneumonia, a severe contagious pulmonary
47 disease of pigs resulting in high morbidity and mortality worldwide (Sebunya et al., 1983).

48

49 Variations in antimicrobial use for the control of *A. pleuropneumoniae* infections from one
50 country to another and variations in methodology can contribute to evident differences in
51 antimicrobial susceptibility of *A. pleuropneumoniae*.

52

53 In accordance with the recommendation of Schwarz et al. (2010) on the requirement of
54 application of the same methodology and interpretive criteria (which would allow for re-
55 evaluation of the original data if the interpretive criteria change over time), only a limited
56 number of recent studies were conducted with the use of microdilution method (Gutiérrez-
57 Martín et al., 2006; Matter et al., 2007; Godinho, 2008).

58

59 Due to the fact that there is a lack of recent results obtained by *A. pleuropneumoniae*
60 testing for antimicrobial susceptibility which could be compared with results from
61 subsequent years, the purpose of the current study was to assess the distribution of the
62 minimum inhibition concentration (MIC) of 16 antimicrobial agents for *A.*
63 *pleuropneumoniae* isolates, using the broth microdilution method.

64

65 This paper presents the results obtained by evaluation of the distribution of the MICs,
66 clinical resistance rates, and phenotypic drug resistance profiles for 242 isolates of *A.*
67 *pleuropneumoniae* from diseased pigs in the Czech Republic between 2007 and 2009.
68 Furthermore, presence of the *floR* gene was confirmed in florfenicol resistant isolates.

69

70 MATERIALS AND METHODS

71

72 Sampling

73

74 All *A. pleuropneumoniae* isolates used in this study were derived from the lungs of
75 growing pigs (body weight from 18 to 50 kg) that died of acute respiratory diseases. The
76 isolates were obtained from 127 herds across the Czech Republic in 2007-2009.

77 Not more than one isolate of *A. pleuropneumoniae* from the same farm per six-month
78 period was included in the study. Isolates from animals that had been treated with
79 antimicrobials during the two weeks prior to sampling were not included in this study.

80

81 Bacterial isolates

82

83 Two hundred and forty-two isolates were isolated on Columbia blood agar plates, using a
84 strip of *Staphylococcus aureus* culture to display the positive CAMP reaction. After an
85 additional passage through Columbia chocolate agar, cultures were identified and
86 serotyped using serological and molecular techniques as described previously (Mittal et al.,
87 1983; Gram et al., 2000). All isolates were stored at -80°C in vials containing 0.25 ml of
88 Foetal Bovine Serum Gold (PAA Laboratories GmbH, Austria) and 0.25 ml of Cation
89 Adjusted Mueller Hinton Broth II (CAMHB) (Becton, Dickinson and Company, USA).

90

91 Antimicrobial susceptibility testing

92

93 All *A. pleuropneumoniae* isolates were investigated for their *in vitro* susceptibility by the
94 microdilution broth method using veterinary fastidious medium (VFM) according to the
95 CLSI standard M31-A3 (CLSI, 2008). MIC-determinations were performed using a
96 commercially prepared microtitre plates (Trek Diagnostic Systems, East England and
97 Trios, Czech Republic). The antimicrobial agents tested and their dilution ranges are
98 shown in Table 1. In categorizing the MIC results, CLSI breakpoints of resistance for

99 swine respiratory disease pathogens were generally used (CLSI, 2008). The interpretive
100 criteria for *A. pleuropneumoniae* taken from a proposal of clinical breakpoints for
101 amoxicillin (Schwarz et al., 2008) were used for ampicillin and amoxicillin/clavulanic acid
102 and breakpoints of *A. pleuropneumoniae* for tulathromycin (Godinho, 2008) were
103 accepted. The breakpoints used are indicated in Table 1. *A. pleuropneumoniae* ATCC
104 27090 was used as reference strain for quality-control (QC) testing each batch of the plates
105 and lot of VFM. QC testing was also performed simultaneously in each series of
106 investigated isolates. The expected MIC values (CLSI, 2008) for the control strain were
107 established: tetracycline ≤ 0.5 mg/L; gentamicin 8-16 mg/L;
108 trimethoprim/sulfamethoxazole $\leq 0.5/9.5$ mg/L; ceftiofur ≤ 0.5 mg/L; cefquinome ≤ 0.25
109 mg/L; tilmicosin 4-8 mg/L; tiamulin 8-16 mg/L; tulathromycin 16-32 mg/L; florfenicol
110 0.25-0.5 mg/L.

111

112 Polymerase chain reaction (PCR)

113

114 The primers for the detection of the *floR* gene were 5' GCG ATA TTC ATT ACT TTG
115 GC 3' and 5' TAG GAT GAA GGT GAG GAA TG 3' (Faldynova et al., 2003). To obtain
116 a template DNA, a loop of bacterial culture was re-suspended in 50 μ l of water and boiled
117 for 20 min. The suspension was spun for 1 min and 2 μ l of the supernatant was used for the
118 reaction. The PCR was carried out in 20 μ l volumes using 10 pmol of each primer and
119 PCR Master Mix (Qiagen, Germany). PCR cycling consisted of 35 cycles of 40 s at 95 °C,
120 45 s at 55 °C and 1 min at 72 °C. The amplification products were separated by
121 electrophoresis in 1.5% agarose gel, stained with ethidium bromide and visualized under
122 UV light.

123

124 Sequencing of the *floR* gene

125

126 The PCR product obtained by amplification with primers covering the whole sequence of
127 the *floR* gene (5' ACC ACC ACA CGC CCC GCG TG 3' and 5' GAC GAC TGG CGA
128 CTT CTC GG 3') was cloned into pCR2.1-TOPO (Invitrogen, USA) according to the
129 manufacturer's instructions. The insert was then sequenced using the BigDye Terminator
130 v1.1 Sequencing Standard Kit (Applied Biosystems, USA) in ABI 310 Genetic Analyser
131 (Applied Biosystems, USA) with M13 forward and reverse primers. The sequences
132 obtained were compared at the GenBank web site using BLAST.

133

134 RESULTS

135

136 Results of susceptibility testing of 242 *A. pleuropneumoniae* isolates to 16 antimicrobial
137 agents between 2007 and 2009 presented as the MICs distribution data, MIC₅₀, MIC₉₀
138 values and percentage of resistance are shown in Table 1.

139

140 Based on the established MIC breakpoints, the 242 *A. pleuropneumoniae* isolates showed
141 low levels of antimicrobial drug resistance, except for tetracycline for which, on the
142 contrary, was frequent, i.e. in 58 (23.9 %) isolates. Resistance to florfenicol and tiamulin
143 was detected in two (0.8 %) and four (1.7 %) isolates, respectively. Regarding β -lactams,
144 low-levels of ampicillin and amoxicillin/clavulanic acid resistance were observed, in eight
145 (3.3 %) and two (0.8 %) isolates, respectively. Resistance to ceftiofur was not detected in
146 any isolate of *A. pleuropneumoniae*. With regard to macrolide resistance, a low-level
147 resistance to tilmicosin was exhibited by three (1.2 %) isolates, whereas resistance to
148 tulathromycin was not observed.

149

150 The two *A. pleuropneumoniae* isolates exhibiting resistance (MIC 8 mg/L) to florfenicol,
151 were analysed for the presence of the *floR* gene. The presence of the *floR* gene was
152 confirmed in both isolates. In one of them, the DNA sequence was further confirmed by
153 DNA sequencing and obtained sequence was 99 % identical to *Escherichia coli*
154 florfenicol-resistance *flo* gene (accession number AF252855).

155

156 Due to the fact that resistance breakpoints for swine bacterial respiratory pathogens are not
157 available for all antimicrobial agents tested, phenotypes of antimicrobial resistance profiles
158 for the isolates were constructed, taking into account ampicillin, amoxicillin and clavulanic
159 acid, ceftiofur, tulathromycin, tilmicosin, florfenicol, tetracycline and tiamulin (Table 2).
160 One hundred and seventy-three isolates (71.5 %) were fully susceptible to the selected
161 antimicrobial agents. The occurrence of resistance to at least one antimicrobial agent was
162 observed in 69 isolates (28.5 %), which were grouped into eight resistance profiles. The
163 most frequently occurring profile of resistance to one antimicrobial agent was found in 63
164 isolates (26.0 %) being followed - at a long interval - by 6 isolates (2.5 %) resistant to two
165 or three antimicrobial agents from two antimicrobial classes. Multidrug resistance to three
166 or more antimicrobial classes was not found among the isolates in the present study.

167

168 Regarding serovar distribution of the isolates, the most prevalent were serovar 9 (93, 38.6
169 %) and serovar 2 (88, 36.4 %), followed by serovar 11 (23, 9.5 %) and a group of serovars
170 9 and 11 (26, 10.7 %), which could not be differentiated by either serological or molecular
171 techniques used. Serovars 3, 7, 8 and 12 were only represented by a small number of
172 isolates, in proportions equal or lower than 1.7 %. No correlation was detected between the
173 distributions of the resistances to antimicrobials and the serovars of *A. pleuropneumoniae*.

174

175 DISCUSSION

176

177 This study reports on the antimicrobial susceptibility to 16 antimicrobial agents in *A.*
178 *pleuropneumoniae* isolates collected from diseased pigs in Czech Republic between 2007
179 and 2009.

180 Based on the established MIC breakpoints, the 242 *A. pleuropneumoniae* isolates showed a
181 low level of resistance to antimicrobial agents, except for tetracycline. Following the
182 recommendation for the use of the same methodology and interpretive criteria because it
183 would allow for re-analysis of the original data if interpretive criteria changed (Schwarz et
184 al., 2010), three recent studies of susceptibility of *A. pleuropneumoniae* to antimicrobial
185 agents (Gutiérrez-Martin et al., 2006; Matter et al., 2007; Godinho, 2008) were suitable for
186 comparison with our data.

187

188 In the first study (Gutiérrez-Martin et al., 2006), *in vitro* susceptibility of 11 antimicrobial
189 agents was determined for 229 *A. pleuropneumoniae* isolates collected in Spain between
190 1997-2004. Using equivalent breakpoints of resistance to amoxicillin (Schwarz et al.,
191 2008) and tetracycline for swine respiratory disease pathogens (CLSI, 2008) the
192 percentages of resistant Spanish isolates to tetracycline (85.6 %) and amoxicillin (27.9 %)
193 were much higher than those of tetracycline and ampicillin found in the present study.

194

195 The second study (Matter et al., 2007) described *in vitro* susceptibility to 20 antimicrobial
196 agents in 83 *A. pleuropneumoniae* isolates collected in Switzerland between 2002 and
197 2004. Using equivalent currently accepted breakpoints, our study showed a close similarity
198 in the percentage of isolates resistant to ampicillin and tilmicosin. In contrast, the

199 percentage of isolates resistant to amoxicillin/clavulanic acid (100 %) and tiamulin (10.8
200 %) was higher and the percentage of isolates resistant to tetracycline (8.4 %) was lower in
201 the Swiss study compared to the values obtained in the present study.

202

203 In the third study (Godinho, 2008) *in vitro* susceptibility to tulathromycin was detected in
204 bacterial isolates obtained from field outbreaks of bovine and porcine respiratory disease in
205 a number of European countries between 2004 and 2006. Their MIC₉₀ and MIC₅₀ values
206 for tulathromycin against 53 *A. pleuropneumoniae* isolates were in accordance with those
207 of the present study.

208

209 Whilst resistance to florfenicol was observed in two (1.2 %) isolates, no resistance of *A.*
210 *pleuropneumoniae* to florfenicol has been reported for isolates tested so far by other
211 authors (Priebe and Schwarz, 2003; Gutierrez et al., 2006; Matter et al., 2007). A number
212 of genes, referred to in the published literature as *pp-flo*, *omlA*-like, *floSt*, *flo*, or *floR*,
213 mediate combined resistance of different bacterial species to chloramphenicol and
214 florfenicol. Despite their varying designations, these genes are closely related and show
215 96-100 % identity in their nucleotide sequences (Schwarz et al., 2004). The similar results
216 were obtained in this study. The presence of *floR* was confirmed in florfenicol resistant
217 isolates and 99 % identity of *floR* to *Escherichia coli* florfenicol-resistance *flo* gene was
218 detected.

219

220 In conclusion, based on the MIC breakpoints, we have detected a new phenotype of
221 resistance to florfenicol and a low degree of resistance to other antimicrobials, with the
222 exception of tetracycline, among *A. pleuropneumoniae* isolates from the Czech Republic.
223 The *floR* gene was identified in florfenicol resistant isolates.

224

225 CONFLICT OF INTEREST

226

227 None of the authors have any relationship with any commercial entity that has interest in
228 the subject matter of this manuscript.

229

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231

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235

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Table 1

MICs for 16 antimicrobial agents of the *Actinobacillus pleuropneumoniae* (n = 242) isolates identified in this study

Antimicrobial agent	Number of isolates with MIC of (mg/L)											MIC ₅₀	MIC ₉₀	Percentage of resistance	
	0.12	0.25	0.5	1	2	4	8	16	32	64	128				256
Ampicillin			229	5	2	0	1	1	1	0	3		≤0.5	≤0.5	3.3
Amoxicillin/clavulanic acid ^a			238	2	2	0	0	0	0	0			≤0.5	≤0.5	0.8
Ceftiofur			241	0	0	1	0	0	0	0			≤0.5	≤0.5	0.0
Cefquinome		238	3	1	0	0	0	0	0				≤0.25	≤0.25	-
Tulathromycin			0	2	19	51	145	23	2	0			8	16	0.0
Tilmicosin				3	11	107	115	3	1	1	0	1	4	8	1.2
Chloramphenicol		197	23	6	3	4	4	3	2				≤0.25	0.5	-
Florfenicol		88	137	3	8	4	2	0	0				0.5	0.5	0.8
Oxolinic acid			190	14	4	5	9	7	7	3	3		≤0.5	8	-
Flumequine			211	4	4	13	4	0	2	4			≤0.5	2	-
Enrofloxacin	223	11	6	2	0	0	0	0					≤0.12	≤0.12	-
Tetracycline			153	31	10	11	6	8	13	6	4		≤0.5	16	24.0
Tiamulin			3	3	13	63	126	30	3	1			8	16	1.7
Gentamicin			17	34	94	79	13	1	0	1	3		2	4	-
Streptomycin				6	16	90	102	15	2	1	5	5	8	16	-
Trimethoprim/sulfamethoxazole ^b		215	6	9	6	3	1	0	2				≤0.25	0.5	-

^a Amoxicillin and clavulanic acid in the ratio 2:1; test ranges are expressed as the amoxicillin concentration. ^b Trimethoprim and sulfamethoxazole in the ratio 1:19; test ranges are expressed as the trimethoprim concentration. The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, breakpoints of resistance used are indicated with vertical black lines. Cross: No breakpoint for *A. pleuropneumoniae* is available.

Table 2

Resistance phenotypes of 242 *Actinobacillus pleuropneumoniae* isolates

Resistance phenotype	Number of antimicrobial agents	Number of isolates
No antimicrobial resistance	0	173
TET	1	53
AMP	1	3
TIA	1	3
TIL	1	2
FFN	1	2
AMP + TET	2	3
TIL + TIA	2	1
AMP + AMC + TET	3	2
Total		242

Abbreviations: AMC, amoxicillin and clavulanic acid; AMP, ampicillin; FFN, florfenicol; TET, tetracycline; TIA, tiamulin; TIL, tilmicosin.