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

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ABSTRACT

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

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

Keywords: Antimicrobials; Minimal inhibition concentration; Pig; Pleuropneumonia

INTRODUCTION

*Actinobacillus pleuropneumoniae* is one of the main etiologic agents of contagious bacterial diseases in swine. It causes pleuropneumonia, a severe contagious pulmonary disease of pigs resulting in high morbidity and mortality worldwide (Sebunya et al., 1983).
Variations in antimicrobial use for the control of A. pleuropneumoniae infections from one country to another and variations in methodology can contribute to evident differences in antimicrobial susceptibility of A. pleuropneumoniae.

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

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

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

MATERIALS AND METHODS

Sampling
All *A. pleuropneumoniae* isolates used in this study were derived from the lungs of growing pigs (body weight from 18 to 50 kg) that died of acute respiratory diseases. The isolates were obtained from 127 herds across the Czech Republic in 2007-2009. Not more than one isolate of *A. pleuropneumoniae* from the same farm per six-month period was included in the study. Isolates from animals that had been treated with antimicrobials during the two weeks prior to sampling were not included in this study.

Bacterial isolates

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

Antimicrobial susceptibility testing

All *A. pleuropneumoniae* isolates were investigated for their *in vitro* susceptibility by the microdilution broth method using veterinary fastidious medium (VFM) according to the CLSI standard M31-A3 (CLSI, 2008). MIC-determinations were performed using a commercially prepared microtitre plates (Trek Diagnostic Systems, East England and Trios, Czech Republic). The antimicrobial agents tested and their dilution ranges are shown in Table 1. In categorizing the MIC results, CLSI breakpoints of resistance for
Swine respiratory disease pathogens were generally used (CLSI, 2008). The interpretive criteria for *A. pleuropneumoniae* taken from a proposal of clinical breakpoints for amoxicillin (Schwarz et al., 2008) were used for ampicillin and amoxicillin/clavulanic acid and breakpoints of *A. pleuropneumoniae* for tulathromycin (Godinho, 2008) were accepted. The breakpoints used are indicated in Table 1. *A. pleuropneumoniae* ATCC 27090 was used as reference strain for quality-control (QC) testing each batch of the plates and lot of VFM. QC testing was also performed simultaneously in each series of investigated isolates. The expected MIC values (CLSI, 2008) for the control strain were established: tetracycline $\leq 0.5$ mg/L; gentamicin 8-16 mg/L; trimethoprim/sulfamethoxazole $\leq 0.5/9.5$ mg/L; ceftiofur $\leq 0.5$ mg/L; cefquinome $\leq 0.25$ mg/L; tilimicosin 4-8 mg/L; tiamulin 8-16 mg/L; tulathromycin 16-32 mg/L; florfenicol 0.25-0.5 mg/L.

Polymerase chain reaction (PCR)

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

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

RESULTS

Results of susceptibility testing of 242 *A. pleuropneumoniae* isolates to 16 antimicrobial agents between 2007 and 2009 presented as the MICs distribution data, MIC$_{50}$, MIC$_{90}$ values and percentage of resistance are shown in Table 1.

Based on the established MIC breakpoints, the 242 *A. pleuropneumoniae* isolates showed low levels of antimicrobial drug resistance, except for tetracycline for which, on the contrary, was frequent, i.e. in 58 (23.9 %) isolates. Resistance to florfenicol and tiamulin was detected in two (0.8 %) and four (1.7 %) isolates, respectively. Regarding β-lactams, low-levels of ampicillin and amoxicillin/clavulanic acid resistance were observed, in eight (3.3 %) and two (0.8 %) isolates, respectively. Resistance to ceftiofur was not detected in any isolate of *A. pleuropneumoniae*. With regard to macrolide resistance, a low-level resistance to tilmicosin was exhibited by three (1.2 %) isolates, whereas resistance to tulathromycin was not observed.
The two *A. pleuropneumoniae* isolates exhibiting resistance (MIC 8 mg/L) to florfenicol, were analysed for the presence of the *floR* gene. The presence of the *floR* gene was confirmed in both isolates. In one of them, the DNA sequence was further confirmed by DNA sequencing and obtained sequence was 99 % identical to *Escherichia coli* florfenicol-resistance *flo* gene (accession number AF252855).

Due to the fact that resistance breakpoints for swine bacterial respiratory pathogens are not available for all antimicrobial agents tested, phenotypes of antimicrobial resistance profiles for the isolates were constructed, taking into account ampicillin, amoxicillin and clavulanic acid, ceftiofur, tulathromycin, tilmicosin, florfenicol, tetracycline and tiamulin (Table 2).

One hundred and seventy-three isolates (71.5 %) were fully susceptible to the selected antimicrobial agents. The occurrence of resistance to at least one antimicrobial agent was observed in 69 isolates (28.5 %), which were grouped into eight resistance profiles. The most frequently occurring profile of resistance to one antimicrobial agent was found in 63 isolates (26.0 %) being followed - at a long interval - by 6 isolates (2.5 %) resistant to two or three antimicrobial agents from two antimicrobial classes. Multidrug resistance to three or more antimicrobial classes was not found among the isolates in the present study.

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

This study reports on the antimicrobial susceptibility to 16 antimicrobial agents in A. pleuropneumoniae isolates collected from diseased pigs in Czech Republic between 2007 and 2009. Based on the established MIC breakpoints, the 242 A. pleuropneumoniae isolates showed a low level of resistance to antimicrobial agents, except for tetracycline. Following the recommendation for the use of the same methodology and interpretive criteria because it would allow for re-analysis of the original data if interpretive criteria changed (Schwarz et al., 2010), three recent studies of susceptibility of A. pleuropneumoniae to antimicrobial agents (Gutiérrez-Martin et al., 2006; Matter et al., 2007; Godinho, 2008) were suitable for comparison with our data.

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

The second study (Matter et al., 2007) described in vitro susceptibility to 20 antimicrobial agents in 83 A. pleuropneumoniae isolates collected in Switzerland between 2002 and 2004. Using equivalent currently accepted breakpoints, our study showed a close similarity in the percentage of isolates resistant to ampicillin and tilmicosin. In contrast, the
percentage of isolates resistant to amoxicillin/clavulanic acid (100 %) and tiamulin (10.8 %) was higher and the percentage of isolates resistant to tetracycline (8.4 %) was lower in the Swiss study compared to the values obtained in the present study.

In the third study (Godinho, 2008) in vitro susceptibility to tulathromycin was detected in bacterial isolates obtained from field outbreaks of bovine and porcine respiratory disease in a number of European countries between 2004 and 2006. Their MIC$_{90}$ and MIC$_{50}$ values for tulathromycin against 53 A. pleuropneumoniae isolates were in accordance with those of the present study.

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

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

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

ACKNOWLEDGEMENTS

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Table 1

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

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Number of isolates with MIC of (mg/L)</th>
<th>MIC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>MIC&lt;sub&gt;90&lt;/sub&gt;</th>
<th>Percentage of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.12  0.25  0.5  1  2  4  8  16  32  64  128  256</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>229 5 2 0 1 1 1 0 3</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>3.3</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid&lt;sup&gt;a&lt;/sup&gt;</td>
<td>238 2 2 0 0 0 0 0</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Ceftiofur</td>
<td>241 0 0 1 0 0 0 0</td>
<td>≤0.5</td>
<td>≤0.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Cefquinome</td>
<td>238 3 1 0 0 0 0</td>
<td>≤0.25</td>
<td>≤0.25</td>
<td>-</td>
</tr>
<tr>
<td>Tilmicosin</td>
<td>197 23 6 3 4 4 3 2</td>
<td>≤0.25</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>197 23 6 3 4 4 3 2</td>
<td>≤0.25</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>Florfenicol</td>
<td>88 137 3 8 4 2 0 0</td>
<td>0.5</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>Oxolinic acid</td>
<td>190 14 4 5 9 7 7 3 3</td>
<td>≤0.5</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>Flumequine</td>
<td>211 14 4 13 4 0 2 4</td>
<td>≤0.5</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>223 11 6 2 0 0 0</td>
<td>≤0.12</td>
<td>≤0.12</td>
<td>-</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>153 31 10 11 6 8 13 6 4</td>
<td>≤0.5</td>
<td>16</td>
<td>24.0</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>3 3 13 63 126 30 3 1</td>
<td>8</td>
<td>16</td>
<td>1.7</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>17 34 94 79 13 1 0 1 3</td>
<td>2</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>5 16 90 102 15 2 1 5 5</td>
<td>8</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>Trimethoprim/sulfamethoxazole&lt;sup&gt;b&lt;/sup&gt;</td>
<td>215 6 9 6 3 1 0 2</td>
<td>≤0.25</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Amoxicillin and clavulanic acid in the ratio 2:1; test ranges are expressed as the amoxicillin concentration. <sup>b</sup> 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

<table>
<thead>
<tr>
<th>Resistance phenotype</th>
<th>Number of antimicrobial agents</th>
<th>Number of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antimicrobial resistance</td>
<td>0</td>
<td>173</td>
</tr>
<tr>
<td>TET</td>
<td>1</td>
<td>53</td>
</tr>
<tr>
<td>AMP</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>TIA</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>TIL</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>FFN</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>AMP + TET</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>TIL + TIA</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>AMP + AMC + TET</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>242</td>
</tr>
</tbody>
</table>

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