



## Article

# Susceptibility of *Actinobacillus pleuropneumoniae*, *Pasteurella multocida* and *Streptococcus suis* Isolated from Pigs in Hungary between 2018 and 2021

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**Abstract:** Porcine respiratory disease complex (PRDC) has been a major animal health, welfare, and economic problem in Hungary; therefore, great emphasis should be put on both the prevention and control of this complex disease. As antibacterial agents are effective tools for control, antibiotic susceptibility testing is indispensable for the proper implementation of antibacterial therapy and to prevent the spread of resistance. The best method for this is to determine the minimum inhibitory concentration (MIC) by the broth microdilution method. In our study, we measured the MIC values of 164 *Actinobacillus pleuropneumoniae*, 65 *Pasteurella multocida*, and 118 *Streptococcus suis* isolates isolated from clinical cases against the following antibacterial agents: amoxicillin, ceftiofur, cefquinome, oxytetracycline, doxycycline, tylosin, tilmicosin, tylvalosin, tulathromycin, lincomycin, tiamulin, florfenicol, colistin, enrofloxacin, and sulfamethoxazole-trimethoprim. Outstanding efficacy against *A. pleuropneumoniae* isolates was observed with ceftiofur (100%) and tulathromycin (100%), while high levels of resistance were observed against cefquinome (92.7%) and sulfamethoxazole-trimethoprim (90.8%). Ceftiofur (98.4%), enrofloxacin (100%), florfenicol (100%), and tulathromycin (100%) were found to be highly effective against *P. multocida* isolates, while 100% resistance was detected against the sulfamethoxazole-trimethoprim combination. For the *S. suis* isolates, only ceftiofur (100%) was not found to be resistant, while the highest rate of resistance was observed against the sulfamethoxazole-trimethoprim combination (94.3%). An increasing number of studies report multi-resistant strains of all three pathogens, making their monitoring a high priority for animal and public health.

**Keywords:** *Actinobacillus pleuropneumoniae*; *Pasteurella multocida*; *Streptococcus suis*; MIC; antibacterial agents; swine



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## 1. Introduction

Porcine Respiratory Disease Complex (PRDC) is most commonly found in weaning and weaning-to-finishing pigs and is a complex disease, i.e., in most cases caused by multiple pathogens or facultative pathogens. The primary pathogenic agents of PRDC are *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, and *Bordetella bronchiseptica*, as well as viruses causing various respiratory pathologies [1,2]. *Pasteurella multocida*, *Streptococcus suis*, *Glässerella parasuis*, and *Salmonella Choleraesuis* are usually considered

secondary pathogens [3]. These primary and secondary pathogens together form PRDC, whose severity gradually increases until it is out of control [4–6].

The continuous monitoring of antibacterial agents is a very important task, as it is not only of animal health, animal welfare, and economic importance but also of paramount public health importance. The European Committee on Antimicrobial Susceptibility Testing [7] plays an important role in this, not only by testing pathogens coming from public health but also by testing pathogens of animal origin. This includes the antibiotic susceptibility of bacteria involved in the development of PRDC. There are further proposals for the establishment of an EU-wide antimicrobial resistance (AMR) monitoring system [8,9].

Determining the minimum inhibitory concentration (MIC) of PRDC-causing bacteria not only allows us to measure the prevalence of different resistant strains but also provides a quantitative result that helps determine the exact antibacterial therapy. The MIC value can also be used to perform pharmacokinetic/pharmacodynamic analysis, which is important in determining the dose of antibacterial agents. The dose helps select the appropriate route of drug administration and the frequency of antibacterial agent use, i.e., the time interval between two administrations [10,11]. There have been fewer studies on the antibiotic susceptibility of PRDC pathogens in the last five years than in the early 2010s, which further strengthens the need for this [12].

In the present study, we investigated the antibiotic susceptibility of three bacterial species most frequently responsible for respiratory tract infections in pigs in Hungary. *Actinobacillus pleuropneumoniae*, *Pasteurella multocida*, and *Streptococcus suis* strains were evaluated for their susceptibility to amoxicillin, ceftiofur, cefquinome, oxytetracycline, doxycycline, tylosin, tilmicosin, tylvalosin, tulathromycin, lincomycin, tiamulin, florfenicol, colistin, enrofloxacin, and sulfamethoxazole-trimethoprim.

Amoxicillin, ceftiofur, and cefquinome belong to the beta-lactam group of antibiotics that are used extensively in pig medicine. According to the EMA classification, amoxicillin is one of the antimicrobials that should be the first choice in veterinary medicine, thus reducing the use of more important public health agents such as ceftiofur and cefquinome [13–17].

Oxytetracycline, doxycycline, and sulfamethoxazole-trimethoprim, like amoxicillin, should be prioritised in animal health. Unfortunately, antimicrobial resistance to these agents is the highest. For this reason, it is of paramount importance to assess the antibiotic susceptibility of pathogenic bacteria prior to the use of these products [14,18–21].

Macrolides (tylosin, tilmicosin, tylvalosin, and tulathromycin), lincomycin, tiamulin, and florfenicol are some of the most important agents in the treatment of respiratory tract infections in pigs, so the assessment of resistance to them is very important in pig medicine. They are also of public health importance, as cross-resistance between macrolides and oxazolidinones (linezolid), which are important for public health, can occur [14,18,22–31].

Colistin is a less important antibacterial agent in respiratory diseases of pigs, but its public health importance is paramount, so it is important to confirm or exclude the presence of resistance to it in all Gram-negative pathogens [14,32–34].

The susceptibility of bacteria to enrofloxacin is important at both the public health and pig health levels, as it is still commonly used in most countries, despite its public health importance being as high as that of colistin [14,35–40].

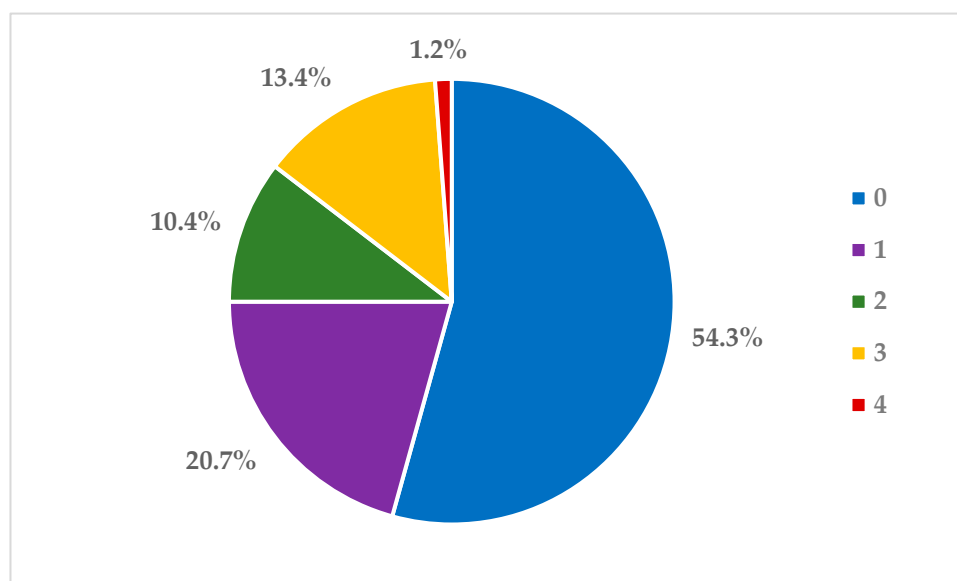
## 2. Results

### 2.1. Susceptibility of *Actinobacillus pleuropneumoniae* Isolates

The susceptibility of 164 *A. pleuropneumoniae* clinical isolates collected by us was tested against 15 antibacterial agents. The clinical breakpoint and epidemiological cut-off value for MIC set by the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) are available for the following antibacterial agents: amoxicillin, ceftiofur, cefquinome, oxytetracycline, tilmicosin, tulathromycin, tiamulin, florfenicol, enrofloxacin, and sulfamethoxazole-trimethoprim [7,41]. Thus, sensitivity was only determined for these agents. High susceptibility to ceftiofur

(100%), tilmicosin (95.7%), tulathromycin (100%), and florfenicol (98.8%) was observed for the tested isolates [41], with low MIC<sub>90</sub> values (0.125 µg/mL, 8 µg/mL, 16 µg/mL, and 4 µg/mL, respectively). In contrast, a lower percentage of the tested isolates were sensitive to amoxicillin (64.0%), oxytetracycline (44.4%), tiamulin (70.5%), and enrofloxacin (47.5%) [41]. For amoxicillin, oxytetracycline, and tiamulin, MIC<sub>90</sub> values of 64 µg/mL were determined in all cases, while for enrofloxacin, the MIC<sub>90</sub> value was 4 µg/mL. Only 7.3% and 9.8% of the isolates were found to be sensitive to cefquinome and sulfamethoxazole-trimethoprim, respectively, taking into account the epidemiological cut-off values set by EUCAST [7]. These agents were associated with MIC<sub>90</sub> values of 0.25 µg/mL and 64 µg/mL. For tylvalosin, the MIC values were 64 µg/mL for both MIC<sub>50</sub> and MIC<sub>90</sub>. For tylosin and lincomycin, similar MIC values were observed (MIC<sub>50</sub> 32 µg/mL and MIC<sub>90</sub> 64 µg/mL). For the other drugs, MIC values were the following: for doxycycline, MIC<sub>50</sub> was 1 µg/mL, MIC<sub>90</sub> was 16 µg/mL, and for colistin, MIC<sub>50</sub> was 0.015 µg/mL, and MIC<sub>90</sub> was 1 µg/mL. The susceptibility of *A. pleuropneumoniae* strains to the tested drugs is summarised in Tables 1 and 2.

Comparing the susceptibility of the tested isolates with the clinical breakpoints defined by CLSI, 89 isolates (54.3%) did not show resistance to any antimicrobial agent. A total of 34 isolates (20.7%) showed resistance to only one antibiotic, while 17 isolates (10.4%) showed resistance to two antibiotics. A total of twenty-four isolates proved to be multidrug resistant (MDR), of which twenty-two isolates (13.4%) showed resistance to three antibiotics and two isolates (1.2%) showed resistance to four antibiotics (Figure 1).



**Figure 1.** Percentage distribution of isolates according to the number of antimicrobials to which they show resistance.

**Table 1.** Distribution of MIC values for the clinical isolates of *Actinobacillus pleuropneumoniae* tested and the MIC<sub>50</sub> and MIC<sub>90</sub> values. The top row shows the number of pieces, and the bottom row shows the percentage distribution per substance. The red line indicates concentrations that are already resistant to the antibacterial agent, based on CLSI (\*) and EUCAST (\*\*) recommendations. +MIC values are relative to the trimethoprim component in the 20:1 sulfamethoxazole/trimethoprim combination.

| Antibacterial Agents               | Isolates Tested (pcs) | Breakpoints (µg/mL) | Distribution of Bacterial Strains (pcs and %) by Dilution Series (µg/mL) |            |            |            |            |            |            |            |            |            |            |            |            |            | MIC <sub>50</sub> (µg/mL) | MIC <sub>90</sub> (µg/mL) |
|------------------------------------|-----------------------|---------------------|--|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|---------------------------|---------------------------|
|                                    |                       |                     | 128  | 64         | 32         | 16         | 8          | 4          | 2          | 1          | 0.5        | 0.25       | 0.125      | 0.06       | 0.03       | 0.015      |                           |                           |
| Amoxicillin                        | 164                   | ≥2 *                |  | 36<br>22.0 | 18<br>11.0 | 1<br>0.6   | 3<br>1.8   | 1<br>0.6   |            | 8<br>4.9   | 9<br>5.5   | 48<br>29.3 | 13<br>7.9  | 13<br>7.9  | 14<br>8.5  |            | 0.25                      | 64                        |
| Ceftiofur                          | 144                   | ≥8 *                |  |            |            |            |            | 6<br>4.1   | 2<br>1.4   | 2<br>1.4   |            | 3<br>2.1   | 14<br>9.7  | 24<br>16.7 | 32<br>22.2 | 61<br>42.4 | 0.03                      | 0.125                     |
| Cefquinome                         | 41                    | ≥0.03 **            |  |            |            |            |            |            |            | 2<br>4.9   | 2<br>4.9   | 3<br>7.3   | 6<br>14.6  | 15<br>36.6 | 10<br>24.4 | 3<br>7.3   | 0.06                      | 0.25                      |
| Oxytetracycline                    | 45                    | ≥2 *                | 4<br>8.9   | 18<br>40.0 | 2<br>4.4   |            |            |            | 1<br>2.2   |            |            | 2<br>4.4   | 10<br>22.2 | 1<br>15.6  | 7          |            | 32                        | 64                        |
| Doxycycline                        | 109                   | -                   |  | 1<br>0.9   | 8<br>7.3   | 23<br>21.1 | 3<br>2.8   | 2<br>1.8   | 8<br>7.3   | 30<br>27.5 | 17<br>15.6 | 8<br>7.3   | 3<br>2.8   | 6<br>5.5   |            |            | 1                         | 16                        |
| Tylosin                            | 78                    | -                   | 6<br>7.7   | 17<br>21.8 | 36<br>46.2 | 4<br>5.1   | 8<br>10.3  |            | 1<br>1.3   | 1<br>1.3   |            |            | 2<br>2.6   | 3<br>3.8   |            |            | 32                        | 64                        |
| Tilmicosin                         | 138                   | ≥32 *               | 2<br>1.4   | 3<br>2.2   | 1<br>0.7   | 3<br>2.2   | 43<br>31.2 | 20<br>14.5 | 19<br>13.8 | 29<br>21.0 | 2<br>1.4   | 2<br>1.4   | 2<br>1.4   | 12<br>8.7  |            |            | 4                         | 8                         |
| Tylvalosin                         | 58                    | -                   | 1<br>1.8   | 37<br>63.8 | 7<br>12.1  |            | 1<br>1.7   | 1<br>1.7   |            |            |            |            | 2<br>3.4   | 4<br>6.9   |            |            | 64                        | 64                        |
| Tulathromycin                      | 139                   | ≥128 *              |  | 1<br>0.7   | 1<br>0.7   | 23<br>16.5 | 18<br>12.9 | 8<br>5.8   | 4<br>2.9   | 8<br>5.8   | 23<br>16.5 | 27<br>19.4 | 2<br>1.4   | 24<br>17.3 |            |            | 0.5                       | 16                        |
| Lincomycin                         | 70                    | -                   | 5<br>7.1   | 27<br>38.6 | 13<br>18.6 | 15<br>21.4 | 4<br>5.7   |            |            |            |            |            |            | 6<br>8.6   |            |            | 32                        | 64                        |
| Tiamulin                           | 112                   | ≥32 *               | 9<br>8.0   | 6<br>5.4   | 18<br>16.1 | 23<br>20.5 | 14<br>12.5 | 14<br>12.5 | 10<br>8.9  | 9<br>8.0   | 1<br>0.9   |            | 2<br>1.8   | 6<br>5.4   |            |            | 8                         | 64                        |
| Florfenicol                        | 164                   | ≥8 *                |  |            | 1<br>0.6   |            | 1<br>0.6   | 19<br>11.6 | 21<br>12.8 | 54<br>32.9 | 52<br>31.7 | 1<br>0.6   | 3<br>1.8   | 6<br>3.7   |            |            | 1                         | 4                         |
| Colistin                           | 88                    | -                   |  |            | 3<br>3.4   |            |            |            |            | 8<br>9.1   |            |            | 3<br>3.4   |            | 23<br>26.1 | 51<br>58.0 | 0.015                     | 1                         |
| Enrofloxacin                       | 141                   | ≥1 *                | 3<br>2.1   | 1<br>0.7   |            | 2<br>1.4   | 7<br>5.0   | 11<br>7.8  | 25<br>17.7 | 25<br>17.7 | 2<br>1.4   | 2<br>1.4   | 12<br>8.5  | 2<br>1.4   | 9<br>6.4   | 40<br>28.4 | 1                         | 4                         |
| Sulfamethoxazole/<br>Trimethoprim+ | 61                    | ≥0.125 **           | 5<br>8.2   | 3<br>4.9   |            | 2<br>3.3   | 7<br>11.5  | 12<br>19.7 | 13<br>21.3 | 5<br>8.2   | 3<br>4.9   | 1<br>1.6   | 4<br>6.6   |            | 6<br>9.8   |            | 2                         | 64                        |

**Table 2.** Percentage of susceptibility of clinical isolates of *Actinobacillus pleuropneumoniae* to the antibacterial agents tested, based on CLSI (\*) and EUCAST (\*\*) recommendations. +MIC values are relative to the trimethoprim component in the 20:1 sulfamethoxazole/trimethoprim combination.

| Antimicrobial Agents           | Strains Tested (pcs) | Breakpoints (µg/mL) | Sensitive (%) | Resistant (%) |
|--------------------------------|----------------------|---------------------|---------------|---------------|
| Amoxicillin                    | 164                  | ≥2 *                | 64.0          | 36.0          |
| Ceftiofur                      | 144                  | ≥8 *                | 100.0         | 0.0           |
| Cefquinome                     | 41                   | ≥0.03 **            | 7.3           | 92.7          |
| Oxytetracycline                | 45                   | ≥2 *                | 44.4          | 55.6          |
| Tilmicosin                     | 138                  | ≥32 *               | 95.7          | 4.3           |
| Tulathromycin                  | 139                  | ≥128 *              | 100.0         | 0.0           |
| Tiamulin                       | 112                  | ≥32 *               | 70.5          | 29.5          |
| Florfenicol                    | 164                  | ≥8 *                | 98.8          | 1.2           |
| Enrofloxacin                   | 141                  | ≥1 *                | 47.5          | 52.5          |
| Sulfamethoxazole/Trimethoprim+ | 61                   | ≥0.125 **           | 9.8           | 90.8          |

In total, 13 AMR profiles were identified among *A. pleuropneumoniae* isolates. Of these, five profiles were identified where the isolates showed resistance to three or more antibiotics (MDR profiles). A total of 24 (14.6%) isolates belonged to these MDR profiles. Half of these isolates were in one profile (profile 1). Of the other half of the isolates, eight isolates were in profile two, two isolates were in profile three, and one isolate was in both profiles four and five (Table 3).

**Table 3.** MDR profiles detected in *A. pleuropneumoniae* isolates.

| Profile Number | Number of Isolates | MDR Profile *     |
|----------------|--------------------|-------------------|
| 1              | 12                 | AM, TIA, ENR      |
| 2              | 8                  | AM, OTC, TIA      |
| 3              | 2                  | AM, OTC, TIA, FLO |
| 4              | 1                  | AM, OTC, TILM     |
| 5              | 1                  | OTC, TILM, TIA    |

\* AM = amoxicillin, TIA = tiamulin, ENR = enrofloxacin, OTC = oxytetracycline, FLO = florfenicol, and TILM = tilmicosin.

## 2.2. Susceptibility of *Pasteurella multocida* Isolates

The 65 *P. multocida* clinical isolates were tested for 12 antibacterial agents. Clinical breakpoints or epidemiological cut-off values defined by CLSI or EUCAST are available for most of the agents we tested [7,41]. Exceptions are tylosin and lincomycin, for which identical MIC<sub>50</sub> (16 µg/mL) and MIC<sub>90</sub> (32 µg/mL) values were determined, and colistin, for which lower MIC values were found compared to the two antibacterial agents (MIC<sub>50</sub> 0.03 µg/mL and MIC<sub>90</sub> 0.06 µg/mL). Compared to the CLSI clinical breakpoints and the EUCAST epidemiological cut-off values, doxycycline was found to be the least effective, with only 69.2% of *P. multocida* isolates being susceptible and a MIC<sub>90</sub> of 2 µg/mL. For ceftiofur, tilmicosin, and tiamulin, >90% of the isolates were sensitive (MIC<sub>90</sub> 0.125 µg/mL, 8 µg/mL, and 16 µg/mL), and all isolates were sensitive for amoxicillin, tulathromycin, florfenicol, and enrofloxacin (MIC<sub>90</sub> 1 µg/mL, 0.5 µg/mL, 1 µg/mL, and 0.06 µg/mL). The susceptibility of *P. multocida* isolates to the tested agents is summarised in Tables 4 and 5.

**Table 4.** Distribution of MIC values for the *Pasteurella multocida* clinical isolates tested and the MIC<sub>50</sub> and MIC<sub>90</sub> values. The top row shows the number of pieces, and the bottom row shows the percentage distribution per substance. The red line indicates concentrations that are already resistant to the antibacterial agent, based on CLSI (\*) and EUCAST (\*\*) recommendations. +MIC values are relative to the trimethoprim component in the 20:1 sulfamethoxazole/trimethoprim combination.

| Antibacterial Agents               | Isolates Tested (pcs) | Breakpoints (µg/mL) | Distribution of Bacterial Strains (pcs and %) by Dilution Series (µg/mL) |     |      |      |      |      |      |      |      |      |      |       |      |      | MIC <sub>50</sub> (µg/mL) | MIC <sub>90</sub> (µg/mL) |       |   |
|------------------------------------|-----------------------|---------------------|--|-----|------|------|------|------|------|------|------|------|------|-------|------|------|---------------------------|---------------------------|-------|---|
|                                    |                       |                     | 256  | 128 | 64   | 32   | 16   | 8    | 4    | 2    | 1    | 0.5  | 0.25 | 0.125 | 0.06 | 0.03 |                           |                           | 0.015 |   |
| Amoxicillin                        | 65                    | ≥2 *                |  |     |      |      |      |      |      | 13   | 15   | 21   | 4    | 10    | 2    |      | 0.25                      | 1                         |       |   |
|                                    |                       |                     |  |     |      |      |      |      |      |      | 20.0 | 23.1 | 32.3 | 6.2   | 15.4 |      |                           |                           | 3.1   |   |
| Ceftiofur                          | 63                    | ≥8 *                |  |     |      |      | 1    |      |      | 2    | 1    | 2    | 4    | 7     | 14   | 32   | 0.015                     | 0.125                     |       |   |
|                                    |                       |                     |  |     |      | 1.6  |      |      | 3.2  | 1.6  | 3.2  | 6.3  | 11.1 | 22.2  | 50.8 |      |                           |                           |       |   |
| Doxycycline                        | 65                    | ≥1 **               |  |     |      | 2    |      | 1    | 3    | 5    | 9    | 19   | 7    | 6     | 13   |      | 0.5                       | 2                         |       |   |
|                                    |                       |                     |  |     |      | 3.1  |      | 1.5  | 4.6  | 7.7  | 13.8 | 29.2 | 10.8 | 9.2   | 20.0 |      |                           |                           |       |   |
| Tylosin                            | 29                    | -                   |  |     | 2    | 11   | 13   | 3    |      |      |      |      |      |       |      |      |                           | 16                        | 32    |   |
|                                    |                       |                     |  |     | 6.9  | 37.9 | 44.8 | 10.3 |      |      |      |      |      |       |      |      |                           |                           |       |   |
| Tilmicosin                         | 42                    | ≥32 *               |  |     |      | 2    | 1    | 2    | 2    | 11   | 8    | 12   | 4    |       |      |      |                           |                           | 1     | 8 |
|                                    |                       |                     |  |     |      | 4.8  | 2.4  | 4.8  | 4.8  | 26.2 | 19.0 | 28.6 | 9.5  |       |      |      |                           |                           |       |   |
| Tulathromycin                      | 59                    | ≥64 *               |  |     |      |      | 1    |      | 1    |      | 1    | 5    | 9    | 17    | 23   | 2    | 0.125                     | 0.5                       |       |   |
|                                    |                       |                     |  |     |      |      | 1.7  |      | 1.7  |      | 1.7  | 8.5  | 15.3 | 28.8  | 39.0 | 3.4  |                           |                           |       |   |
| Lincomycin                         | 19                    | -                   |  |     | 2    | 6    | 6    | 1    |      | 2    | 2    |      |      |       |      |      |                           | 16                        | 32    |   |
|                                    |                       |                     |  |     | 10.5 | 31.6 | 31.6 | 5.3  |      | 10.5 | 10.5 |      |      |       |      |      |                           |                           |       |   |
| Tiamulin                           | 44                    | ≥64 **              |  |     | 1    | 2    | 13   | 22   | 5    | 1    |      |      |      |       |      |      | 8                         | 16                        |       |   |
|                                    |                       |                     |  |     | 2.3  | 4.5  | 29.5 | 50.0 | 11.4 | 2.3  |      |      |      |       |      |      |                           |                           |       |   |
| Florfenicol                        | 65                    | ≥8 *                |  |     |      |      |      |      | 2    | 2    | 11   | 30   | 16   | 1     | 3    |      | 0.5                       | 1                         |       |   |
|                                    |                       |                     |  |     |      |      |      | 3.1  | 3.1  | 16.9 | 46.2 | 24.6 | 1.5  | 4.6   |      |      |                           |                           |       |   |
| Colistin                           | 25                    | -                   |  |     |      |      |      |      |      |      | 1    |      |      |       | 8    | 10   | 6                         | 0.03                      | 0.06  |   |
|                                    |                       |                     |  |     |      |      |      |      | 4    |      |      |      |      | 32    | 40   | 24   |                           |                           |       |   |
| Enrofloxacin                       | 41                    | ≥1 *                |  |     |      |      |      |      |      |      |      |      |      |       | 9    | 12   | 20                        | 0.03                      | 0.06  |   |
|                                    |                       |                     |  |     |      |      |      |      |      |      |      |      |      | 22.0  | 29.3 | 48.8 |                           |                           |       |   |
| Sulfamethoxazole/<br>Trimethoprim+ | 54                    | ≥0.125 **           | 3  | 2   |      | 9    | 5    | 4    | 10   | 19   | 2    |      |      |       |      |      |                           | 4                         | 32    |   |
|                                    |                       |                     | 5.6  | 3.7 |      | 16.7 | 9.3  | 7.4  | 18.5 | 35.2 | 3.7  |      |      |       |      |      |                           |                           |       |   |

**Table 5.** Percentage of susceptibility of clinical isolates of *Pasteurella multocida* to the antibacterial agents tested based on CLSI (\*) and EUCAST (\*\*) recommendations. +MIC values are relative to the trimethoprim component in the 20:1 sulfamethoxazole/trimethoprim combination.

| Antimicrobial Agents           | Strains Tested (pcs) | Breakpoints (µg/mL) | Sensitive (%) | Resistant (%) |
|--------------------------------|----------------------|---------------------|---------------|---------------|
| Amoxicillin                    | 65                   | ≥2 *                | 100.0         | 0.0           |
| Ceftiofur                      | 63                   | ≥8 *                | 98.4          | 1.6           |
| Doxycycline                    | 65                   | ≥1 **               | 69.2          | 30.8          |
| Tilmicosin                     | 42                   | ≥32 *               | 95.2          | 4.8           |
| Tulathromycin                  | 59                   | ≥64 *               | 100.0         | 0.0           |
| Tiamulin                       | 44                   | ≥64 **              | 97.7          | 2.3           |
| Florfenicol                    | 65                   | ≥8 *                | 100.0         | 0.0           |
| Enrofloxacin                   | 41                   | ≥1 *                | 100.0         | 0.0           |
| Sulfamethoxazole/Trimethoprim+ | 54                   | ≥0.125 **           | 0.0           | 100.0         |

### 2.3. Susceptibility of *Streptococcus suis* Isolates

The susceptibility of 118 clinical isolates of *S. suis* to 11 antibacterial agents was determined. One hundred percent of the isolates were sensitive to ceftiofur, with a MIC<sub>90</sub> of 1 µg/mL. For florfenicol, 75.4% of isolates (MIC<sub>90</sub> 8 µg/mL) and for enrofloxacin, 82.9% of isolates (MIC<sub>90</sub> 8 µg/mL) were sensitive. The *S. suis* isolates tested were highly resistant to the combination sulfamethoxazole-trimethoprim, with only 5.7% being sensitive and a MIC<sub>90</sub> of 256 µg/mL. The MIC values of the other tested substances were variable. Amoxicillin had the lowest MIC value (MIC<sub>50</sub> 0.06 µg/mL and MIC<sub>90</sub> 4 µg/mL). Doxycycline had a MIC<sub>50</sub> of 8 µg/mL and a MIC<sub>90</sub> of 32 µg/mL. The MIC<sub>50</sub> values for tylosin, tilmicosin, tulathromycin, and lincomycin differed (32 µg/mL, 8 µg/mL, 2 µg/mL, and 32 µg/mL, respectively), but their MIC<sub>90</sub> values were the same (128 µg/mL). The sensitivity of the isolates to the tested drugs is summarised in Tables 6 and 7.

**Table 6.** Distribution of MIC values for the *Streptococcus suis* clinical isolates tested and the MIC<sub>50</sub> and MIC<sub>90</sub> values. The top row shows the number of pieces, and the bottom row shows the percentage distribution per substance. The red line indicates concentrations that are already resistant to the antibacterial agent, based on CLSI (\*) and EUCAST (\*\*) recommendations. +MIC values are relative to the trimethoprim component in the 20:1 sulfamethoxazole/trimethoprim combination.

| Antibacterial Agents                | Isolates Tested (pcs) | Breakpoints (µg/mL) | Distribution of Bacterial Strains (pcs and %) by Dilution Series (µg/mL) |      |      |      |      |      |      |      |      |      |      |       |      |      |       | MIC <sub>50</sub> (µg/mL) | MIC <sub>90</sub> (µg/mL) |
|-------------------------------------|-----------------------|---------------------|--|------|------|------|------|------|------|------|------|------|------|-------|------|------|-------|---------------------------|---------------------------|
|                                     |                       |                     | 256  | 128  | 64   | 32   | 16   | 8    | 4    | 2    | 1    | 0.5  | 0.25 | 0.125 | 0.06 | 0.03 | 0.015 |                           |                           |
| Amoxicillin                         | 116                   | -                   |  |      | 4    | 2    | 4    | 1    | 2    |      | 4    | 3    | 4    | 12    | 37   | 43   |       | 0.06                      | 4                         |
|                                     |                       |                     |  |      | 3.4  | 1.7  | 3.4  | 0.9  | 1.7  |      | 3.4  | 2.6  | 3.4  | 10.3  | 31.9 | 37.1 |       |                           |                           |
| Ceftiofur                           | 117                   | ≥8 *                |  |      |      |      |      |      | 2    | 3    | 8    | 4    | 10   | 3     | 13   | 17   | 57    | 0.03                      | 1                         |
|                                     |                       |                     |  |      |      |      |      |      | 1.7  | 2.6  | 6.8  | 3.4  | 8.5  | 2.6   | 11.1 | 14.5 | 48.7  |                           |                           |
| Doxycycline                         | 100                   | -                   |  |      | 3    | 24   | 18   | 6    | 8    | 7    | 2    | 5    | 4    | 8     | 15   |      |       | 8                         | 32                        |
|                                     |                       |                     |  |      | 3    | 24   | 18   | 6    | 8    | 7    | 2    | 5    | 4    | 8     | 15   |      |       |                           |                           |
| Tylosin                             | 41                    | -                   |  | 15.0 |      | 12.0 |      | 2.0  |      |      | 3.0  | 2.0  | 1.0  |       | 6.0  |      |       | 32                        | 128                       |
|                                     |                       |                     |  | 36.6 |      | 29.3 |      | 4.9  |      |      | 7.3  | 4.9  | 2.4  |       | 14.6 |      |       |                           |                           |
| Tilmicosin                          | 86                    | -                   |  | 21   | 2    | 11   | 7    | 3    | 1    | 4    | 16   | 3    | 7    | 4     | 7    |      |       | 8                         | 128                       |
|                                     |                       |                     |  | 24.4 | 2.3  | 12.8 | 8.1  | 3.5  | 1.2  | 4.7  | 18.6 | 3.5  | 8.1  | 4.7   | 8.1  |      |       |                           |                           |
| Tylvalosin                          | 89                    | -                   |  | 17   | 1    |      | 14   | 6    | 4    | 7    | 3    | 5    | 8    | 4     | 16   | 2    | 2     | 2                         | 128                       |
|                                     |                       |                     |  | 19.1 | 1.1  |      | 15.7 | 6.7  | 4.5  | 7.9  | 3.4  | 5.6  | 9.0  | 4.5   | 18.0 | 2.2  | 2.2   |                           |                           |
| Lincomycin                          | 40                    | -                   |  | 17   | 3    | 4    | 2    |      |      |      |      | 2    | 4    |       | 8    |      |       | 32                        | 128                       |
|                                     |                       |                     |  | 42.5 | 7.5  | 10.0 | 5.0  |      |      |      |      | 5.0  | 10.0 |       | 20.0 |      |       |                           |                           |
| Tiamulin                            | 33                    | -                   |  | 8    | 7    | 1    | 1    | 6    | 1    |      | 1    | 4    | 1    | 1     | 2    |      |       | 16                        | 128                       |
|                                     |                       |                     |  | 24.2 | 21.2 | 3.0  | 3.0  | 18.2 | 3.0  |      | 3.0  | 12.1 | 3.0  | 3.0   | 6.1  |      |       |                           |                           |
| Florfenicol                         | 118                   | ≥8 *                |  |      | 8    |      | 4    | 17   | 24   | 38   | 9    | 5    | 3    | 5     |      | 5    |       | 2                         | 8                         |
|                                     |                       |                     |  |      | 6.8  |      | 3.4  | 14.4 | 20.3 | 32.2 | 7.6  | 4.2  | 2.5  | 4.2   |      | 4.2  |       |                           |                           |
| Enrofloxacin                        | 70                    | ≥2 *                |  |      |      |      | 4    | 5    | 1    | 2    | 12   | 12   | 7    | 11    | 6    | 4    | 6     | 0.5                       | 8                         |
|                                     |                       |                     |  |      |      |      | 5.7  | 7.1  | 1.4  | 2.9  | 17.1 | 17.1 | 10.0 | 15.7  | 8.6  | 5.7  | 8.6   |                           |                           |
| Sulfamethoxazole/<br>Trimethoprim + | 105                   | ≥0.25 **            | 15   | 17   | 2    | 9    | 10   | 13   | 18   | 3    | 2    | 9    | 1    | 6     |      |      |       | 16                        | 256                       |
|                                     |                       |                     | 14.3   | 16.2 | 1.9  | 8.6  | 9.5  | 12.4 | 17.1 | 2.9  | 1.9  | 8.6  | 1.0  | 5.7   |      |      |       |                           |                           |



**Table 7.** Percentage of susceptibility of clinical isolates of *Streptococcus suis* to the antibacterial agents tested, based on CLSI (\*) and EUCAST (\*\*) recommendations. +MIC values are relative to the trimethoprim component in the 20:1 sulfamethoxazole/trimethoprim combination.

| Antimicrobial Agents           | Strains Tested (pcs) | Breakpoints (µg/mL) | Sensitive (%) | Resistant (%) |
|--------------------------------|----------------------|---------------------|---------------|---------------|
| Ceftiofur                      | 117                  | ≥8 *                | 100.0         | 0.0           |
| Florfenicol                    | 118                  | ≥8 *                | 75.4          | 24.6          |
| Enrofloxacin                   | 105                  | ≥2 *                | 82.9          | 17.1          |
| Sulfamethoxazole/Trimethoprim+ | 116                  | ≥0.25 **            | 5.7           | 94.3          |

### 3. Discussion

The continuous monitoring of antibacterial agents is an extremely important task for veterinarians, as it has not only a veterinary and economic purpose but is also beneficial for public health. Continuous monitoring of the susceptibility to antibacterial products used in food-producing farm animals helps us optimise therapeutic management and inhibit the spread of antimicrobial resistance. In order to reduce resistant strains of bacteria, Hungary has already tightened the conditions of antibiotic use [42], based on the European Union (EU) Directive 2001/82/EC [43], and from 8 January 2022, the stricter use of antibiotics has been regulated at EU level [44]. By monitoring the resistance profile of bacterial strains on livestock farms, we can successfully determine the antibacterial agents of choice at the herd level, further reducing the chance of selection of resistant strains.

In order to set clinical breakpoints at the national level to assist veterinarians working in livestock farms, it would be necessary to carry out harmonised susceptibility testing using the same methodology to determine the susceptibility of bacteria in different farms. The development of such a test method and the creation of a central database would be a valuable addition to national veterinary practice and, if harmonised at an international level, would open up new ways of combating resistant strains of bacteria. To implement the previous proposal, our research group applied for and won a grant to establish a National Laboratory for Animal Infectious Diseases, Antimicrobial Resistance, Veterinary Public Health, and Food Chain Safety, which will allow this work to be carried out.

#### 3.1. Susceptibility of *Actinobacillus pleuropneumoniae* Isolates

The isolates of *A. pleuropneumoniae* we tested were 100% sensitive to ceftiofur and tulathromycin, which is in agreement with the previous literature [6,8,45–48]. However, 92.7% of the isolates we tested were resistant to cefquinome. Cefquinome is a frequently used antibacterial agent in the pig farming industry in Hungary. Unfortunately, the use of cefquinome may increase the chance of the selection of bacteria resistant to beta-lactam antibiotics [14,16,17]. Based on our results, it would be advisable to greatly reduce its use in animal health. This would presumably have positive results, both in animal health and in public health. To florfenicol, 98.8% of isolates were sensitive, and to tilmicosin, 95.7% of isolates were sensitive; the results are in agreement with the data previously published by others [6,8,45–49]. However, it was observed that while in previous studies more than 95% of the isolates were sensitive to tiamulin [6,8,46,48], only 70.5% of the isolates tested in our study were sensitive to this antibiotic. To amoxicillin, 64.0% of the isolates were sensitive, which is moderate compared to that observed by Vilaró et al. (72.2%) [48]. There is a divergent picture in the literature on the sensitivity of *A. pleuropneumoniae* isolates to tetracyclines. While in a Danish study by Holmer et al., 92.4% of isolates were sensitive to the antibacterial agent [8], only 76% of isolates were sensitive in a Czech Republic study by Kucerova et al. [46], and 70% of isolates were sensitive in a European monitoring programme by El Garch et al. [6]. In the Spanish survey by Gutiérrez-Martín et al., only 26.2% of the strains were sensitive to tetracyclines [49]. In the North American survey by Portis et al., less than 7.4% of the isolates were sensitive to tetracyclines [45], while in the North American survey by Sweeney et al., all strains were resistant [47]. A total of 44.4% of our isolates were sensitive, which is in line with what has been described

previously in Central and Eastern Europe. For enrofloxacin, in most cases, more than 95% of strains showed sensitivity [6,45,47], with only Vilaró et al. describing a lower sensitivity (72.2%) [48]. In comparison, 47.5% of the isolates we collected were sensitive, which is strikingly low compared to previous results. The case is even more severe for the sulfamethoxazole-trimethoprim combination, where 90.8% of the isolates were resistant, while the same value in the literature is below 12% [6,48].

For doxycycline, our MIC values (MIC<sub>50</sub> 1 µg/mL and MIC<sub>90</sub> 16 µg/mL) were higher than previously reported by Vilaró et al. (MIC<sub>50</sub> 0.5 µg/mL and MIC<sub>90</sub> 4 µg/mL) [48] and Yuan et al. (MIC<sub>90</sub> 8 µg/mL) [50]. The same can be said for lincomycin, where the MIC<sub>50</sub> and MIC<sub>90</sub> of the isolates we collected were 32 µg/mL and 64 µg/mL, while values of 16 µg/mL and 32 µg/mL were reported in the literature for the same values [6,51]. For tylosin, the MIC<sub>90</sub> of our isolates was the same as that reported in the El Garch et al. study (64 µg/mL) [6]. For colistin, MIC values were lower (MIC<sub>50</sub> 0.015 µg/mL and MIC<sub>90</sub> 1 µg/mL) than those reported in the Swiss study by Matter et al. (MIC<sub>50</sub> 1 µg/mL and MIC<sub>90</sub> 2 µg/mL) [52].

Based on these data, the most effective antibacterial agents for *A. pleuropneumoniae* infection will be ceftiofur and tulathromycin. Florfenicol and tilmicosin also have outstanding efficacy against the bacteria. Nationally, enrofloxacin and tiamulin have been shown to be less effective, so these antibiotics should only be used after sensitivity testing. The moderate susceptibility of amoxicillin and oxytetracycline is in line with the literature, while cefquinome and sulfamethoxazole-trimethoprim have been found to have high resistance in the national context and should be used only in exceptional cases after sensitivity testing.

### 3.2. Susceptibility of *Pasteurella multocida* Isolates

The *P. multocida* isolates we tested were all sensitive to amoxicillin, enrofloxacin, florfenicol, and tulathromycin. Similar results can be found in the literature for all four antibacterial agents [6,47,48,53], but less pronounced susceptibilities have been reported for enrofloxacin and florfenicol (93.1% [54] and 81.5% [53]). Our result for ceftiofur, where 98.4% of isolates were sensitive, is similar to that described by Oh et al. [53], but most of the literature data suggest that 100% of the isolates tested are usually sensitive to ceftiofur [6,45,47,48,54]. To tilmicosin, 95.2% of the isolates were sensitive, which is in agreement with the results reported in the literature [6,45,47,48,53], while to tiamulin, 97.7% of the isolates were sensitive, which is higher than that reported by Vilaró et al. (60.8%) [48]. For amoxicillin (100.0% of isolates) and for doxycycline (69.2% of isolates), we found a higher sensitivity than Vilaró et al. (96.2% and 51.5%, respectively) [48]. For sulfamethoxazole-trimethoprim, El Garch et al. [6] and Oh et al. [53] reported a sensitivity above 90% for the tested isolates, whereas Vilaró et al. [48] reported a sensitivity of only 74.7%. In contrast, the isolates collected by us showed 100% resistance to this substance.

For tylosin, our MIC<sub>90</sub> value was 32 µg/mL, which is in agreement with that reported by Oh et al. [53] and lower than that reported by El Garch et al. [6] (64 µg/mL). In relation to lincomycin, the MIC<sub>90</sub> value for the isolates collected by us was 32 µg/mL, which is the same as described by El Garch et al. [6], but higher than that published by Cuevas et al. (16 µg/mL) [54].

These suggest that in the case of *P. multocida* infection, all isolates will show sensitivity to ceftiofur and tulathromycin, similar to that of *A. pleuropneumoniae* infection, as well as to amoxicillin, enrofloxacin, and florfenicol. Approximate sensitivities were also observed for tilmicosin and tiamulin, which are higher for the latter agent than previously reported in the literature. Doxycycline was only effective in half of the isolates, so its empirical use should be avoided, while in the national context, the combination sulfamethoxazole-trimethoprim showed complete resistance to the isolates we tested, so its use should only be considered after a sensitivity study and only if the results are favourable.

### 3.3. Susceptibility of *Streptococcus suis* Isolates

In our study, 100% of *S. suis* isolates were susceptible to ceftiofur, which is in agreement with the data published in the literature [6,8,45,47,55–57], but it is worth noting that in most cases, researchers have already reported a few percent resistance, compared to *A. pleuropneumoniae* and *P. multocida* isolates, where 100% of isolates were sensitive in most published results. In the case of enrofloxacin and florfenicol, only 82.9% and 75.4% of our isolates were sensitive, respectively, which is lower than that reported in the literature. A sensitivity of over 90% of isolates for florfenicol and over 95% for enrofloxacin has been previously described by researchers [6,8,45,47,55–57]. For sulfamethoxazole-trimethoprim, in most cases more than 90% of the isolates were sensitive [8,55,57], but in the study by Hernandez et al., only 79% of the isolates were sensitive [56]. In contrast, 94.3% of the isolates we studied were resistant to potentiated sulfonamide.

In the case of amoxicillin, the MIC<sub>90</sub> of the isolates we tested was 4 µg/mL, whereas previously in the literature a value of 0.06 µg/mL or less was described [6,56]. Doxycycline also had a higher MIC<sub>90</sub> (32 µg/mL) than previously described (16 µg/mL) [56]. The MIC<sub>90</sub> value obtained for tylosin, tilmicosin, tulathromycin, and lincomycin was consistently 128 µg/mL, which is in agreement with results published by others [6,8,45,47,55–57], but the MIC<sub>90</sub> value obtained for tiamulin (128 µg/mL) was higher than previously described (16 µg/mL and 32 µg/mL) [6,56].

In conclusion, ceftiofur will be the best choice against *S. suis* isolates in the national context, while enrofloxacin and florfenicol, which have shown excellent efficacy in the literature, show more moderate efficacy among the national isolates. In the case of sulfamethoxazole-trimethoprim, outstanding resistance among national isolates was observed, and this antibacterial agent can only be used after sensitivity testing.

## 4. Materials and Methods

### 4.1. Laboratory Participants and Isolate Characterisation

From clinical cases collected in Hungary between 2018 and 2021, isolates of *A. pleuropneumoniae*, *P. multocida*, and *S. suis* were obtained from the Department of Epidemiology and Microbiology, University of Veterinary Medicine Budapest, and SCG Diagnostics Ltd. (East Hartford, CT, USA).

The isolates were collected and processed continuously throughout the study. Identification to species level was carried out based on their culture, morphological characteristics, and biochemical characteristics using commercially available kits (such as API Microbial Identification Kits, bioMérieux, Durham, NC, USA). Each isolate was stored in a mixture of 800 µL of tryptone soy broth (PharmaBio® Tryptone Soy Broth, Biolab Zrt., Budapest, Hungary) and 200 µL of sterile glycerol (Glycerol 87% P.A., Lach-Ner, Ltd., Neratovice, Czech Republic) at −80 °C until antimicrobial susceptibility testing was performed.

### 4.2. Determination of Minimal Inhibitory Concentration Values

The in vitro susceptibility tests were performed at the Department of Pharmacology and Toxicology, University of Veterinary Medicine Budapest. All MICs were performed in tryptone soy broth (PharmaBio® Tryptone Soy Broth, Biolab Zrt., Budapest, Hungary), and for *A. pleuropneumoniae* strain I, NAD (β NAD, Biolab Zrt., Budapest, Hungary) supplementation was used, following the Clinical and Laboratory Standards Institute (CLSI) specifications, so that our results are reproducible and internationally comparable with those of other laboratories. For each isolate, the final concentration of bacteria in the broth dilution was  $5 \times 10^5$  colony-forming units per mL (CFU/mL). Soup dilutions were performed on 96-well microplates (96-well BRANDplates—F—pureGrade S, VWR International, Radnor, PA, USA). The 96-well microplate was used to prepare a two-based dilution series of the following antibacterial agents: amoxicillin, ceftiofur, cefquinome, oxytetracycline, doxycycline, tylosin, tilmicosin, tylvalosin, tulathromycin, lincomycin, tiamulin, florfenicol, colistin, enrofloxacin, and sulfamethoxazole-trimethoprim. The concentration

range of antibacterial agents was selected based on quality control principles and clinical limits [7,41].

#### 4.3. Number of Isolates Tested per Antibiotic

In this study, we tested the susceptibility of 164 clinical isolates of *A. pleuropneumoniae*, 65 clinical isolates of *P. multocida*, and 118 clinical isolates of *S. suis*, for a total of 15 substances. The number of isolates tested per agent is summarised in Table 8.

**Table 8.** Number of *A. pleuropneumoniae*, *P. multocida*, and *S. suis* isolates tested, by substance and in total.

| Antibacterial Agent                   | <i>A. pleuropneumoniae</i> (pcs) | <i>P. multocida</i> (pcs) | <i>S. suis</i> (pcs) |
|---------------------------------------|----------------------------------|---------------------------|----------------------|
| Amoxicillin                           | 164                              | 65                        | 116                  |
| Ceftiofur                             | 144                              | 63                        | 117                  |
| Cefquinome                            | 41                               | -                         | -                    |
| Oxytetracycline                       | 45                               | -                         | -                    |
| Doxycycline                           | 109                              | 65                        | 100                  |
| Tylosin                               | 78                               | 29                        | 41                   |
| Tilmicosin                            | 138                              | 42                        | 86                   |
| Tylvalosin                            | 58                               | -                         | -                    |
| Tulathromycin                         | 139                              | 59                        | 89                   |
| Lincomycin                            | 70                               | 19                        | 40                   |
| Tiamulin                              | 112                              | 44                        | 33                   |
| Florfenicol                           | 164                              | 65                        | 118                  |
| Colistin                              | 88                               | 25                        | -                    |
| Enrofloxacin                          | 141                              | 41                        | 70                   |
| Sulfamethoxazole: Trimethoprim (20:1) | 61                               | 54                        | 105                  |
| Total (pcs)                           | 164                              | 65                        | 118                  |

#### 4.4. Data Analysis

MIC<sub>50</sub> and MIC<sub>90</sub> values were determined for all clinical isolates. Where possible, these values were compared with the clinical breakpoints and epidemiological cut-off values set by CLSI and EUCAST. The percentage of the number of susceptible and resistant strains expressed as a percentage was determined (Tables 1 and 3–7).

Multidrug-resistant bacteria were defined as having acquired non-susceptibility to at least one agent in three or more antimicrobial categories [58]. For the *A. pleuropneumoniae* isolates, AMR profiles of the MDR bacteria were performed against those agents for which CLSI clinical breakpoints were available [41] (Table 2, Figure 1). For *P. multocida* and *S. suis*, CLSI breakpoints were available for only a few antimicrobial agents, and outstanding efficacy was observed against the isolates for these agents, so AMR profiling was not feasible.

## 5. Conclusions

To summarise the results of our study, ceftiofur will be the most effective antibacterial agent, showing maximum efficacy in most cases against *A. pleuropneumoniae*, *P. multocida*, and *S. suis* isolates. Florfenicol, tulathromycin, and tilmicosin can be highly effective against both *A. pleuropneumoniae* and *P. multocida* infections. For *P. multocida* isolates, amoxicillin, enrofloxacin, and tiamulin have also shown high efficacy, whereas for *A. pleuropneumoniae* isolates, these agents show more moderate efficacy. Tetracyclines showed only moderate sensitivity against both *A. pleuropneumoniae* and *P. multocida* isolates. Enrofloxacin and florfenicol also showed only moderate sensitivity against *S. suis* isolates. The national isolates showed outstanding resistance to the combination sulfamethoxazole-trimethoprim for all three bacteria. However, the high level of resistance to cefquinome in *A. pleuropneumoniae* strains, which is of major public health importance, should be highlighted.

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