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# The determination of minimum inhibitory concentrations of selected antimicrobials for porcine *Haemophilus parasuis* isolates from the Czech Republic

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**Abstract**

*Haemophilus parasuis* isolates obtained from pigs in the Czech Republic were tested for their susceptibility to amoxicillin, penicillin, ceftiofur, enrofloxacin, tetracycline, and tulathromycin by determination of minimum inhibitory concentrations using the broth microdilution method. The *H. parasuis* isolates were mostly susceptible to majority of tested antimicrobials (amoxicillin 90%, penicillin 73.3%, enrofloxacin 83.3%, and tulathromycin 83.3%). All isolates were susceptible to ceftiofur. On the other hand, no isolate was susceptible to tetracycline, 30% of tested isolates were intermediately susceptible, and 70% were resistant. These findings indicate that tested antimicrobials with the exception of tetracycline should be the preferred option used for the treatment of infection caused by *H. parasuis* but due to the potential transmission of resistance from animals to humans, the use of ceftiofur is considered as a last resort option in antimicrobial treatment of animals.

*Bacterial infection, susceptibility, resistance, pigs*

*Haemophilus parasuis* may be the cause of great economic loss for breeders due to the cost of antibiotic therapy and piglet death in the acute forms of the disease (Oliveira et al. 2001). In conventional herds, *H. parasuis* is predominantly involved in the respiratory syndrome but can also cause acute septicaemia or Glässer's disease, systemic illness characterized by fibrinous polyserositis, polyarthritis, and meningitis (Amano et al. 1994). Infections caused by *H. parasuis* can be enzootic and may be acute or chronic, depending on the immunological situation of the breeding herd. If *H. parasuis* entered into farms with immunologically naive animals, it can cause serious acute illness and the main affected age categories are early weaned piglets about the age of 4–6 weeks (Nicolet 1992). A high antigenic heterogeneity exists among *H. parasuis* strains. According to the currently worldwide accepted classification, 15 serovars of *H. parasuis* (1–15) have been defined. However, it is necessary to say that a large number of non-typeable *H. parasuis* isolates also exist (Kielstein and Rapp-Gabrielson 1992).

Antibiotic treatment is one of the commonly used measures for the control of *H. parasuis* infections. However, the use of antimicrobial agents may lead to both selection and increase of resistance (Schwarz et al. 2001). Correct use of antimicrobial agents for treatment of bacterial infections requires the knowledge of the susceptibility of the infecting strain to antimicrobial agents to enhance efficacy and to prevent the emergence of resistance among other organisms, which may be causing respiratory diseases in pigs (De la Fuente et al. 2007). Moreover, continuous monitoring of the prevalence of drug resistance of bacterial pathogens to antimicrobials provides essential data on the state and development of the susceptibility in pathogens causing bacterial infections. These data are necessary for the prediction of an antimicrobial's efficacy in the initial treatment and to determine the position of individual substances as 'drugs of choice' or as alternative drugs in the therapy of infections. Susceptibility tests are therefore the basis of the formulation of objective antimicrobial treatment and prevention strategies (Nedbalcova et al. 2013).

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The objective of this study was to determine the minimum inhibitory concentrations (MICs) for selected antimicrobials (amoxicillin, penicillin, ceftiofur, enrofloxacin, tetracycline, and tulathromycin) in the Czech field strains of *Haemophilus parasuis* according to the latest guidelines of the Clinical and Laboratory Standards Institute (CLSI 2013a; CLSI 2013b).

## Materials and Methods

### Isolates

Thirty isolates of *H. parasuis* (1–30) were obtained from diseased pigs from pig herds in the Czech Republic from 2014 to 2015. *Haemophilus parasuis* isolates were isolated on Columbia blood agar (OXOID, England) plates with 5% sheep blood with a nurse strain of *Staphylococcus aureus* and then on chocolate blood agar (OXOID, England) plates with 5% sheep blood for β-nikotinamid adenin dinucleotide growth dependence of *H. parasuis* for 24–48 h at 37 °C, were confirmed by polymerase chain reaction test (Oliveira et al. 2001), and serotyped by modified co-agglutination test originally described for *Actinobacillus pleuropneumoniae* (Mittal et al. 1983). All isolates were stored at –80 °C in vials containing 0.25 ml 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). All isolates originated from animals without previous antimicrobial therapy during the last 3 weeks and only individual isolates from individual herds were included in this study.

### MIC determination

The MICs of selected antimicrobials for the isolates of *H. parasuis* were determined by the standardized dilution micro-method for *Actinobacillus pleuropneumoniae* and *Histophilus somni* (CLSI 2013a; CLSI 2013b).

### Preparation of microdilution trays

According to these documents, special microdilution trays were prepared. These trays included dilutions of tested antimicrobial agents (mg/l) in Veterinary Fastidious Medium (VFM) prepared according to CLSI (2013a). The tested antimicrobials and their concentrations are shown in Table 1.

Table 1. Microplate tray with concentrations of tested antimicrobials (mg/l).

| PNC | AMX | EFT | TUL | TET | ENR | PNC | AMX | EFT | TUL | TET | ENR |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 8 | 4 | 16 | 64 | 64 | 4 | 8 | 4 | 16 | 64 | 64 | PC |
| 4 | 2 | 8 | 32 | 32 | 2 | 4 | 2 | 8 | 32 | 32 | 2 |
| 2 | 1 | 4 | 16 | 16 | 1 | 2 | 1 | 4 | 16 | 16 | 1 |
| 1 | 0.5 | 2 | 8 | 8 | 0.5 | 1 | 0.5 | 2 | 8 | 8 | 0.5 |
| 0.5 | 0.25 | 1 | 4 | 4 | 0.25 | 0.5 | 0.25 | 1 | 4 | 4 | 0.25 |
| 0.25 | 0.125 | 0.5 | 2 | 2 | 0.125 | 0.25 | 0.125 | 0.5 | 2 | 2 | 0.125 |
| 0.125 | 0.06 | 0.25 | 1 | 1 | 0.06 | 0.125 | 0.06 | 0.25 | 1 | 1 | 0.06 |
| 0.06 | 0.03 | 0.125 | 0.5 | 0.5 | 0.03 | 0.06 | 0.03 | 0.125 | 0.5 | 0.5 | 0.03 |

PNC - penicillin; AMX - amoxicillin; EFT - ceftiofur; TUL - tulathromycine; TET - tetracycline; ENR - enrofloxacin; PC - positive growth control.

### Inoculum preparation, inoculation and incubation of trays

The culture of *H. parasuis* grown on blood agar was re-suspended in 5 ml CAMHB (Becton, Dickinson and Company, USA) and the density of the suspension was adjusted to a turbidity equivalent to that of a 0.5 McFarland standard and 5 µl of inoculum with the density of 0.5 McFarland standard were transferred into all of the wells of the micro-titre plates containing 100 ml of medium. The trays were incubated at 35°C in $CO_2$ for 24 h.

### Interpretation of results

The MICs values were read at the lowest concentration of an antimicrobial agent that inhibited the visible bacterial growth in the wells. The break-points of tested antimicrobials for *H. parasuis* are derived from break-points for *A. pleuropneumoniae* and *Pasteurella multocida* according to CLSI (2013b) and they are shown in Table 2.

### Quality control

Quality control of the results was performed with reference strains of *Actinobacillus pleuropneumoniae* (ATCC 27090). The acceptable quality control ranges for tested antimicrobials are in CLSI document Vet 01-S2 (2013b).

Table 2. The break-points of tested antimicrobials for *Haemophilus parasuis*.

| | S mg/l | I mg/l | R mg/l |
|---|---|---|---|
| Penicillin | ≤ 0.25 | 0.5 | ≥ 1 |
| Amoxicillin | ≤ 0.5 | 1 | ≥ 2 |
| Ceftiofur | ≤ 2 | 4 | ≥ 8 |
| Tulathromycin | ≤ 16 | 32 | ≥ 64 |
| Tetracycline | ≤ 0.5 | 1 | ≥ 2 |
| Enrofloxacin | ≤ 0.25 | 0.5 | ≥ 1 |

S – susceptible; I – intermediately susceptible; R – resistant

## Results

Distribution of MICs for penicillin, amoxicillin, ceftiofur, tulathromycin, tetracycline, and enrofloxacin in *H. parasuis* isolates, calculation of the $MIC_{50}$ and $MIC_{90}$ values and counts and percentages of susceptible, intermediately susceptible and resistant *H. parasuis* isolates to tested antimicrobials are in Table 3. As detected by the CLSI (2013a; 2013b)

Table 3. Interpretation of results

| | Concentration of antimicrobials | Isolates N (%) | C N (%) | I N (%) | R N (%) | $MIC_{50}$ (mg/l) | $MIC_{90}$ (mg/l) |
|---|---|---|---|---|---|---|---|
| Penicilin | ≤ 0.06 | 6 (20) | 22 (73.3) | 2 (6.7) | 6 (20) | 0.25 | 4 |
| | 0.125 | 3 (10) | | | | | |
| | 0.25 | 13 (43.3) | | | | | |
| | 0.5 | 2 (6.7) | | | | | |
| | 2 | 2 (6.7) | | | | | |
| | 4 | 1 (3.3) | | | | | |
| | 8 | 3 (10) | | | | | |
| Amoxicilin | ≤ 0.03 | 10 (33.3) | 27 (90) | 0 | 3 (10) | 0.06 | 0.25 |
| | 0.06 | 12 (40) | | | | | |
| | 0.125 | 2 (6.7) | | | | | |
| | 0.25 | 3 (10) | | | | | |
| | 4 | 3 (10) | | | | | |
| Ceftiofur | ≤ 0.125 | 16 (53.3) | 30 (100) | 0 | 0 | ≤ 0.125 | 0.5 |
| | 0.25 | 5 (16.7) | | | | | |
| | 0.5 | 6 (20) | | | | | |
| | 1 | 3 (10) | | | | | |
| Tetracycline | 1 | 9 (30) | 0 | 9 (30) | 21 (70) | 2 | 64 |
| | 2 | 15 (50) | | | | | |
| | 4 | 1 (3.3) | | | | | |
| | 32 | 1 (3.3) | | | | | |
| | 64 | 4 (13.3) | | | | | |
| Enrofloxacin | ≤ 0.03 | 17 (56.7) | 25 (83.3) | 0 | 5 (16.7) | ≤ 0.03 | 1 |
| | 0.06 | 2 (6.7) | | | | | |
| | 0.125 | 4 (13.3) | | | | | |
| | 0.25 | 2 (6.7) | | | | | |
| | 1 | 3 (10) | | | | | |
| | 2 | 2 (6.7) | | | | | |
| Tulathromycin | ≤ 0.5 | 7 (23.3) | 25 (83.3) | 1 (3.3) | 4 (13.3) | 2 | 64 |
| | 1 | 7 (23.3) | | | | | |
| | 2 | 4 (13.3) | | | | | |
| | 4 | 3 (10) | | | | | |
| | 8 | 2 (6.7) | | | | | |
| | 16 | 2 (6.7) | | | | | |
| | 32 | 1 (3.3) | | | | | |
| | 64 | 4 (13.3) | | | | | |

C – susceptibility; I – intermediate susceptibility; R – resistance; MIC – minimum inhibitory concentration
$MIC_{50}$ and $MIC_{90}$ presented the lowest concentration of antimicrobial substances in mg/l that inhibited the growth of 50% and 90% of isolates were determined by cumulative conversion (Schwarz 2010).

methods for MIC determination the majority of the tested isolates were susceptible to the tested antimicrobial substances at their $MIC_{50}$ values and to amoxicillin and ceftiofur also at their $MIC_{90}$ value, both below the break-point of resistance. The exception was tetracycline where the majority of isolates were resistant (70%), and the remaining 30% were only intermediately susceptible to tetracycline.

A higher level of resistance was detected to penicillin (20%) in comparison with amoxicillin (10%) and ceftiofur (0%); resistance to enrofloxacin was 16.7% and to tulathromycin 13.3%. The results of serotyping and MIC determination of 30 tested isolates against individual antimicrobials are summarized in Table 4. Nine isolates belonged to serotype 5, three isolates were determined as serotype 13, and three isolates were serotype 14. Two isolates were serotype 1 and other two isolates were serotype 12 and 4. A single isolate was identified as serotype 2 and another as serotype 15. Seven remaining isolates were serologically non-typable. The profiles of resistance of tested isolates are shown in Table 5.

Table 4. Minimum inhibitory concentration values (mg/l) of tested antimicrobials for *Haemophilus parasuis* isolates.

| Strain | Serotype | PNC | AMX | EFT | TUL | TET | ENR |
|---|---|---|---|---|---|---|---|
| 1 | 15 | ≤ 0.06 | ≤ 0.03 | ≤ 0.125 | 1 | 2 | ≤ 0.03 |
| 2 | NT | 0.25 | ≤ 0.03 | ≤ 0.125 | ≤ 0.5 | 2 | ≤ 0.03 |
| 3 | 13 | 0.25 | 0.06 | 1 | ≤ 0.5 | 2 | ≤ 0.03 |
| 4 | 2 | ≤ 0.06 | ≤ 0.03 | ≤ 0.125 | 4 | 2 | 1 |
| 5 | 1 | 0.25 | 0.06 | ≤ 0.125 | 1 | 1 | ≤ 0.03 |
| 6 | 5 | 0.25 | 0.06 | ≤ 0.125 | 1 | 1 | ≤ 0.03 |
| 7 | 1 | ≤ 0.06 | ≤ 0.03 | 0.25 | 2 | 2 | ≤ 0.03 |
| 8 | 4 | 0.25 | ≤ 0.03 | 1 | 16 | 2 | ≤ 0.03 |
| 9 | NT | 0.25 | 0.06 | ≤ 0.125 | 8 | 2 | ≤ 0.03 |
| 10 | NT | 2 | 0.25 | ≤ 0.125 | 64 | 32 | 2 |
| 11 | 13 | 0.5 | 0.125 | ≤ 0.125 | 1 | 2 | ≤ 0.03 |
| 12 | 4 | 0.125 | 0.06 | 1 | ≤ 0.5 | 2 | 0.25 |
| 13 | NT | 2 | 0.25 | 0.25 | 64 | 64 | 1 |
| 14 | NT | 8 | 4 | 0.5 | 16 | 64 | 2 |
| 15 | 14 | 0.25 | 0.06 | ≤ 0.125 | ≤ 0.5 | 1 | ≤ 0.03 |
| 16 | 5 | ≤ 0.06 | 0.06 | 0.25 | ≤ 0.5 | 1 | ≤ 0.03 |
| 17 | 14 | 0.25 | 0.06 | ≤ 0.125 | 2 | 2 | 0.125 |
| 18 | 12 | 0.25 | 0.06 | 0.25 | 64 | 2 | 0.25 |
| 19 | 5 | 0.25 | 0.06 | ≤ 0.125 | ≤ 0.5 | 2 | 0.06 |
| 20 | 12 | 0.25 | 0.06 | 0.5 | ≤ 0.5 | 1 | 0.125 |
| 21 | 5 | 0.25 | ≤ 0.03 | ≤ 0.125 | 1 | 1 | ≤ 0.03 |
| 22 | NT | 4 | 0.25 | ≤ 0.125 | 8 | 64 | 1 |
| 23 | 5 | 8 | 4 | 0.5 | 32 | 64 | 0.125 |
| 24 | 13 | 8 | 4 | ≤ 0.125 | 4 | 2 | ≤ 0.03 |
| 25 | 5 | 0.125 | ≤ 0.03 | 0.5 | 2 | 4 | ≤ 0.03 |
| 26 | 5 | ≤ 0.06 | ≤ 0.03 | 0.5 | 4 | 1 | ≤ 0.03 |
| 27 | 5 | 0.125 | ≤ 0.03 | 0.5 | 1 | 1 | ≤ 0.03 |
| 28 | 5 | ≤ 0.06 | ≤ 0.03 | 0.25 | 1 | 2 | 0.06 |
| 29 | 14 | 0.5 | 0.125 | ≤ 0.125 | 2 | 1 | ≤ 0.03 |
| 30 | NT | 0.25 | 0.06 | ≤ 0.125 | 64 | 2 | 0.125 |

PNC – penicillin; AMX – amoxicillin; EFT – ceftiofur; TUL – tulathromycine; TET – tetracycline; ENR – enrofloxacin; PC – positive growth control; NT – non-typable

We found 3 (10%) isolates resistant to three antimicrobials and isolates (10%) resistant to four antimicrobials.

Table 5. The profiles of resistance of isolates *Haemophilus parasuis* (n = 30).

| Frequency of resistance by active substance | Phenotypic profile of resistance | Number of isolates (%) | Number of isolates (%) cumulatively within groups |
|---|---|---|---|
| 0* | | 9 (30.0) | 9 (30.0) |
| 1 | TET | 12 (40.0) | 12 (40.0) |
| 2 | TET, ENR | 1 (3.3) | |
| 2 | TET, TUL | 2 (6.7) | 3 (10.0) |
| 3 | TET, PNC, AMX | 2 (6.7) | |
| 3 | TET, PNC, ENR | 1 (3.3) | 3 (10.0) |
| 4 | TET, PNC, TUL, ENR | 2 (6.7) | |
| 4 | TET, PNC, AMX, ENR | 1 (3.3) | 3 (10.0) |

TET – tetracycline; ENR – enrofloxacin; TUL – tulathromycin; PNC – penicillin; AMX – amoxicillin
*Isolates were susceptible to amoxicillin, penicillin, ceftiofur, enfofloxacin, tulathromycin, and intermediately susceptible to tetracycline.

## Discussion

A total of 15 serovars have been recognised in *H. parasuis* till now and a large number of non-typable isolates have been described. Besides serovar 5, which is considered as the most prevalent and virulent one, serovars 1, 2, 4, 8, 10, and 12–14 may lead to the death of infected animals and are considered virulent. Serovars 3, 6, 7, 9 and 11 are considered as avirulent (Kielstein and Rapp-Gabrielson 1992). The Czech isolates belong to serotypes 1, 2, 4, 5, 12, 13, 14, and 15, with the most frequent serovars isolated among them belonging to the virulent group of 5, 13, and 14 which are considered as highly virulent. Seven isolates (23%) were non-typable, which is similar to Germany (26%), but higher compared to Denmark and China (15% and 12% of isolates, respectively) (Angen et al. 2004; Kielstein and Rapp-Gabrielson 1992; Cai et al. 2005).

Antimicrobial treatment remains the most important tool to control *H. parasuis* infection under real field conditions due to the lack of commercially available, effective and broadly protective vaccines as the inconsistency of their effect is due to serovar diversity and a high number of non-typable isolates (Oliveira and Pijoan 2004).

Antibiotic-wise, tetracycline was an exception, as all of the tested isolates were either resistant or showed an intermediate sensitivity pattern (70% and 30%, respectively). High level of resistance to tetracycline is in agreement with findings in previous studies and our previously published results (Wissing et al. 2001; Pejsak et al. 2005; Dayao et al. 2014). In contrast, low level of resistance to tetracycline has been reported from China (3.6%) but a different breakpoint ≤ 8 μg/ml was used in the study (Zhou et al. 2010).

Surprisingly, we have found 5 (16.7%) isolates resistant to enrofloxacin with a bimodal distribution of MICs. This is the first evidence of enrofloxacin resistance reported in field isolates of *H. parasuis* in the Czech Republic. Fluoroquinolones are registered and used in the Czech Republic for the treatment of respiratory infections caused by *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae* as well as *H. parasuis*. Quinolone resistance typically develops in a stepwise manner and cross-resistance between different members of the group is common (Chen et al. 2011).

Similarly, strains with reduced susceptibility to fluoroquinolones have been described in Denmark (Aarestrup et al. 2004), and a high level of resistance was recently reported for enrofloxacin in China (70.9%) (Zhou et al. 2010). Fluoroquinolone resistance could be associated with virulence factors, thus highly virulent strains are more strongly exposed to antibiotics in their evolutionary history, and proper selection of strains during MIC monitoring of such serotypes is very important (Zhang et al. 2013).

According to the current interpretative criteria for respiratory pathogens (CLSI 2013a; CLSI 2013b) the authors have found four (13.3%) resistant isolates to tulathromycin. This finding is in accordance with our previous reported results from the Czech Republic (Nedbalcova et al. 2013). Taking into consideration the pharmacokinetic data characteristic for tulathromycin – the low level plasma concentration (0.62 μg tulathromycin/ml), the rapid concentration of tulathromycin into the lungs and the septicaemic character/nature of the pathogen, tulathromycin may be less effective for treatment and control of *H. parasuis* infection (Benchaoui et al. 2004; Evans 2005; Ritzmann and Heinritzi 2005). MIC data demonstrating a sensitivity and resistance pattern for *H. parasuis* and tulathromycin suggest that an epidemiological cut-off value occurs at 4.0 μg/ml (Godino et al. 2005). Based on this proposed criteria, 12 (40%) isolates from our study would belong to a resistance pattern. Tulathromycin resistance has recently been identified in *H. parasuis* isolates from Australia (Dayao et al. 2014). An experimental challenge study demonstrated a failure in protection and development of polyserositis with a high isolation rate of *H. parasuis* with the metaphylactic use of tulathromycin (Palzer at al. 2015).

A low degree of resistance was recorded to amoxicillin (3 strains/10%); all the rest of the tested isolates belong to a fully sensitive pattern, in contrast with another β-lactam antibiotic - penicillin, which has a comparatively higher degree of resistant isolates (20%). The level of resistance to aminopenicillins remains relatively stable and low in the Czech Republic in comparison with data from Spain and the United Kingdom where a higher level of resistance was found to aminopenicillins (66.7% and 21.1%, respectively) (de la Fuente et al. 2007). Aminopenicillins are also frequently used in other countries with reported high resistance to tetracycline in clinical strains *H. parasuis* (San Millan et al. 2007). Aminopenicillins remain one of the most important antimicrobials for treatment and control of *H. parasuis* infections in the Czech Republic's field conditions. All the tested strains were sensitive to ceftiofur.

In conclusion, this study showed that the Czech isolates of *H. parasuis* are generally susceptible to amoxicillin and ceftiofur and these antimicrobial agents should be the preferred option used for the treatment of infection caused by *H. parasuis*. Recently, the impact of third generation cephalosporins, such as ceftiofur, in veterinary medicine has been discussed and has led to the emergence and dissemination of resistant organisms producing extended-spectrum beta-lactamases (ESBLs). Also, potential transmission from animals to humans mainly via the food chain has been widely postulated (Hammerum et al. 2014), so the use of ceftiofur should be considered as a last resort option.

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