Quantitative determination of florfenicol in pig manure by LC-MS/MS to assess the exposure of the gut microbiota to florfenicol

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Introduction
The current posology of veterinary antimicrobial drugs is mainly established solely on clinical efficacy, without taking resistance selection of pathogenic or commensal bacteria into account. Moreover, dosage regimens of antimicrobials in animal husbandry commonly show considerable variability, even between manufacturers. In order to assess the effect of different dosage regimens (administration route and dosage) on resistance selection in the intestinal commensal microbiota, data on the exposure are mandatory. Florfenicol is a broad-spectrum antimicrobial, increasingly used in veterinary medicine. When administered in pigs, florfenicol has a complete oral and intramuscular bioavailability and it is mainly renally excreted, even though a significant part (up to 24%) is excreted via the faeces. This is interesting with regards to exposure of the gut microbiota to the antimicrobial active molecule both after oral and intramuscular administration.

Hence, the objective of this study was to develop and validate a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to quantify florfenicol in intestinal content of pigs.

Materials & Methods
Sample pre-treatment
A rapid and effective sample pre-treatment (figure 1) was developed based on liquid-liquid extraction (LLE). One gram of faeces was homogenized and diluted tenfold in PBS. A 1.0 g subsample of this dilution was spiked with 25.0 µL internal standard solution (10.00 µg/ml florfenicol-d3). For optimal extraction, 20.0 µL of 1M NaOH was also added (pH 10). After vortex mixing and equilibrating, 7.5 mL of EtOAc was added, followed by roller mixing and centrifugation. A clear supernatant was subjected to a nitrogen stream until dryness. The extract was reconstituted in a vial and an aliquot of 10.0 µL was injected onto the LC-MS/MS instrument.

Instrumentation and analysis
Liquid chromatography was performed using a Hypersil Gold™ column (Reversed Phase, 50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column (10 mm x 2.1mm i.d., dp: 5µm). Mobile phases for chromatographic separation consisted of 0.1% acetic acid in H2O (A) and ACN (B) following a gradient elution. Flow rate was set at 300 µL/min. This LC system was connected to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, with electrospray ionization in negative mode (Figure 2). Acquisition was performed in the selected reaction monitoring mode. For florfenicol and its internal standard, following transitions were followed (*quantification ion): m/z 356.0 > 185.00/336.00*, florfenicol-d3: m/z 359.00 > 188.00, 339.00*.

Table 1. Validation results for within- and between-run accuracy and precision.

<table>
<thead>
<tr>
<th>Theoretical concentration (µg/g)</th>
<th>Mean concentration ± SD (µg/g)</th>
<th>Precision (RSD %)</th>
<th>Accuracy (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00 10-5</td>
<td>1.03 ± 0.03 ± 1.23 × 10^-5</td>
<td>10.9</td>
<td>3.0</td>
</tr>
<tr>
<td>1.00 10-4</td>
<td>1.10 ± 0.04 ± 4.88× 10^-5</td>
<td>4.4</td>
<td>9.9</td>
</tr>
<tr>
<td>1.00 10-3</td>
<td>0.94 ± 0.05 ± 1.01 × 10^-4</td>
<td>5.4</td>
<td>5.8</td>
</tr>
<tr>
<td>1.00 10-2</td>
<td>0.99 ± 0.06 ± 1.01 × 10^-3</td>
<td>10.1</td>
<td>1.3</td>
</tr>
<tr>
<td>1.00 10-1</td>
<td>0.90 ± 0.08 ± 1.01 × 10^-2</td>
<td>6.5</td>
<td>9.9</td>
</tr>
</tbody>
</table>

*Within-run accuracy and precision (n=6)

Results & Conclusion
All validation procedures were carried out compliant with European and international guidelines1-2. All crucial validation parameters were evaluated, namely linearity, accuracy, precision, limit of quantification (LOQ), limit of detection (LOD), extraction recovery (Re) and signal suppression and enhancement (SSE). A matrix-matched approach was used for calibration of the method in manure. The calibration curve for florfenicol (1/x^2 weighing) was linear over the working concentration range (1.00-10^-2 - 1.00 µg/g). Linearity was evaluated based on correlation coefficient (r) and goodness-of-fit (g) as seen in figure 3. Next the LOQ level was determined at 1.00-10^-3 µg/g and the LOD at 2.9-10^-3 µg/g. Accuracy and precision were determined at different concentration levels (each n=6); namely the LOQ-level, an intermediate level and a high level; an overview is given in table 1.

In conclusion, a fast, accurate and precise method was developed for the quantitative determination of florfenicol in pig manure. This method will be further used to assess intestinal florfenicol concentrations in duodenum, jejunum, ileum, cecum, colon and rectum, after antimicrobial therapy in pigs. Determining the concentrations of the drug in intestinal samples is necessary with regards to assessing the exposure of the gut microbiota to florfenicol and evaluating the magnitude of possible antimicrobial resistance selection.