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The analysis of animal faeces as a tool to monitor antibiotic usage

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ABSTRACT

The analysis of antibiotics in animal faeces is important to obtain more insight in the possible formation of bacterial resistance in the animals' gut, to learn about the dissemination of antibiotics to the environment, to monitor trends in antibiotic usage and to detect the illegal and off-label use of antibiotics. To facilitate these studies a comprehensive method for the analysis of trace levels of 44 antibiotic compounds including tetracyclines, quinolones, macrolides and sulfonamides in animal faeces by liquid chromatography in combination with tandem mass spectrometric (LC–MS/MS) detection is reported. The method is fully validated according to European regulation and showed satisfactory quantitative performance according to the stringent criteria adopted, with the exception of some of the macrolide compounds, which can be analysed with somewhat high measurement uncertainty. A large survey was carried out monitoring swine and cattle faeces and the outcomes were striking. In 55% of the swines, originating from 80% of the swine farms and in 75% of the calves, originating from 95% of the cattle farms, antibiotics were detected. Oxytetracycline, doxycycline and sulfadiazine were the most detected antibiotics, followed by tetracycline, flumequine, lincomycin and tylosin. Over 34% of the faeces samples contained two or more different antibiotics with a maximum of eight. Possible explanations for these findings are given and the effects are discussed.

1. Introduction

Antibiotics are medical agents that either kill bacteria or inhibits their growth. Antibiotics can be produced by microorganisms but also be synthetically produced and can be used for both human and veterinary purposes. Antibiotics are excessively used in both human and animal health and as a negative side-effect, bacteria become resistant. Nowadays, bacterial resistance is a major threat for human healthcare: in the USA, 23,000 people per year are dying due to bacterial infections which cannot be cured by antibiotic treatment [1]. Therefore, policies on the use of antibiotics have become more stringent within most countries aiming to decrease bacterial resistance and its dissemination. The focus is mainly on the decrease of antibiotic usage in human health as well as in veterinary practices, because resistant bacteria are easily transferred from animal to human [2]. In The Netherlands, a specific measure was the implementation of mandatory registration of antibiotics delivered to and used by farmers to prevent off-label usage.

After administration of antibiotics, 30–90% of the dose given is excreted in non-metabolized form or as active metabolites [3–5] and as a result high levels of antibiotics and active metabolites are expected to be present in faeces [6]. As a matter of fact, the presence of veterinary antibiotics in liquid waste from swines was previously confirmed [7]. Because slurry is used to optimise the growth and harvest in agriculture, antibiotics are unwillingly disseminated throughout the environment and are found in surface water, soil and crops [8–15] which is expected to contribute to adverse ecotoxicological effects [4,5,16,17] and the emergence of microbial resistance [18–23].

Analysing antibiotics in animal faeces is expected to be effective in several ways. First to obtain more insight in the possible formation of bacterial resistance in the animals' gut, leading to valuable information on the relation between residues and resistance. Second to learn about the dissemination of antibiotics to the environment and possible ecotoxicological effects. Third to monitor trends in antibiotic usage at the farms using non-invasive sampling and last to enforce policies on the use of antibiotics and thus to prevent the illegal and off-label use of antibiotics. We conclude that the analysis of antibiotics in faeces could be very informative and therefore a multi-method to detect a wide range of veterinary antibiotics in faeces is needed.

Multiple analytical methods for the analysis of antibiotics are available focussing on regulatory limits established for food products. Only a few multi-analyte methods on the analysis of antibiotics in faeces are published [12,24–31]. Most of these

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studies solely focussed on samples taken in Asia and they cover only a minor part of the relevant veterinary antibiotics, up to 17 in total. No fully validated multi-methods applying liquid chromatography (LC) coupled to tandem-mass spectrometry (MS/MS) for the analysis of a wide range of antibiotics in animal faeces is available and no data is available on the antibiotic content of faeces of swines and calves in Dutch animal breeding.

We present a multi-method for the analysis of veterinary antibiotics in faeces aiming for the detection, quantification and confirmation of 4 tetracyclines, 18 sulfonamides, 14 macrolides and 10 quinolones using LC–MS/MS. The method was fully validated for swine faeces according to Commission Decision 2002/657/EC [32]. Using this method 340 different swine faeces samples and 340 calf faeces samples obtained from 20 swine respectively 20 cattle farms were monitored for the presence of antibiotics. Results are presented here.

2. Material and methods

2.1. Reagents and equipment

HPLC grade methanol (MeOH) and acetonitrile (ACN) was obtained from Biosolve (Valkenswaard, The Netherlands). Milli-Q water was prepared using a Milli-Q system at a resistivity of at least 18.2 MΩ cm^{-1} (Millipore, Billerica, MA, USA). Formic acid, ammonium formate, citric acid monohydrate, disodiumhydrogenphosphate (Na_2HPO_4) dihydrate and disodium ethylenediaminetetraacetate (Na_2EDTA) were obtained from VWR International (Darmstadt, Germany) and lead acetate from Sigma Aldrich (St. Louis, MO, USA).

The reference standard of oxytetracycline hydrate, tetracycline HCl, chlortetracycline HCl, doxycycline hyclate, ciprrofloxacin, danofloxacin, enrofloxacin, flumequine, marbofloxacin, nalidixinic acid, norfloxacin, oxolinic acid, sarafloxacin HCl trihydrate, dapsone, sulfacetamide, sulfachloorpyridazine, sulfadiazine, sulfadimidin, sulfadimethoxin, sulfadoxin, sulfamerazine, sulfamethoxazole, sulfamethizole, sulfamethoxypyridazine, sulfamonomethoxin, sulfaphenazole, sulfapyridin, sulfaquinoxalin Na, sulfathiazole, sulfisoxazole, erythromycin, gamithromycin, josamycin, lincomycin HCl, natamycin, pirlimycin HCl, spiramycin, tildipirosin, tulathromycin, tylosin tartrate, valnemulin, amoxicillin, ampicillin, penicillin G Na, penicillin V K, cloxacillin Na hydrate, dicloxacillin Na H_2O, nafcillin Na hydrate, oxacillin Na hydrate were obtained from Sigma-Aldrich, gamithromycin from Hovione (Loures, Portugal), tylvalosin tartrate from ECO Animal Health (London, United Kingdom), tilmicosin and tiamulin fumarate from LGC standards (Teddington, Middlesex United Kingdom) and tildipirosin from MSD Animal Health (Boxmeer, The Netherlands).

Ciprofloxacin d_8 HCL hydrate, difloxacin d_3 HCl hydrate, enrofloxacin d_5 HCl, nalidixic acid d_5, norfloxacin d_5, oxolinic acid d_5, sarafloxacin d_8 HCl, lincomycin d_3, spiramycin d_3, sulfachloropyridazine $^{13}C_6$, sulfadimidine $^{13}C_6$, sulfadimethoxine d_6, sulfadoxine d_3, sulfamerazine $^{13}C_6$, sulfamethoypyridazine d_3, sulfapyridine $^{13}C_6$, sulfaquinoxaline $^{13}C_6$, sulfathiazole $^{13}C_6$, sulfisoxazole $^{13}C_6$, penicillin G d_7 and penicillin V d_5 were obtained from Witega (Beril, Germany), danofloxacin d_3 mesylate, flumequin $^{13}C_3$, gamithromycin d_4, sulfadiazine d_4, sulfamethoxazole d_4, dapson d_8, from Toronto Research Chemicals (Toronto, ON, Canada), erythromycin ^{13}C d_3 from Cachesyn (Mississauga, ON, Canada), tetracycline d_6 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and demeclocycline and lomefloxacin from Sigma Aldrich.

Stock solutions of the tetracyclines, sulfonamides, penicillins were prepared in MeOH, of the quinolones in ammoniated MeOH and of the macrolides in ACN at 1000 mg L^{-1}. Mix solutions of the individual antibiotic groups were prepared at 10 mg L^{-1} in MeOH and a spiking solution was prepared containing 0.25 μg L^{-1} of the individual sulfonamides 1.0 μg L^{-1} of the individual quinolones, macrolides and sulfonamides, and 2.5 μg L^{-1} of the individual penicillins in MeOH. A mixture of the internal standards was prepared at 5 μg L^{-1} in MeOH.

Citric acid solution (0.1 M) was prepared by dissolving 21 g citric acid monohydrate in 1 L of water. Phosphate solution (0.2 M) was prepared by Na_2HPO_4 in 1 L of water. EDTA solution (0.2 M) was prepared by dissolving 74.4 g Na_2EDTA in 1 L of water. McIlvain–EDTA buffer (0.1 M, pH 4.0) was prepared by mixing 500 ml citric acid solution with 280 ml phosphate solution. 74.4 g Na_2EDTA was added. Stir using a magnetic bar for 30 min. Check the pH and if needed set to pH 4.0 by adding either citric acid solution or phosphate solution. Add water to a total volume is 2 L. Lead acetate solution was prepared by dissolving 100 g lead acetate in 500 mL of water. Ammonium formate solution (1 M) was prepared by dissolving 6.3 g ammonium formate in 100 mL water. Mobile phase A was prepared by diluting 2 ml ammonium formate solution, 160 μL formic acid to 1 L with water. Mobile phase B was prepared by diluting 2 ml ammonium formate solution, 160 μL formic acid to 1 L with MeOH.

2.2. Sample preparation

The faeces samples were homogenised by stirring with a disposable wooden rod, 2.0 g was weighted into a 50 mL poly propylene (PP) centrifuge tube and 80 μL internal standard solution was added. 4 mL of McIlvain–EDTA buffer and 1 mL ACN were added to the sample. The sample was vigorously shaken by hand and placed in a rotary tumbler for 15 min. After shaking 2 mL of lead acetate solution was added and again the sample was vigorously shaken by hand. After centrifugation (3500 g, 10 min) the extract was transferred into a clean test tube and diluted by adding 13 ml 0.2 M EDTA solution. A Phenomenex (Torrance, CA, USA) Strata-X RP 200 mg/6 mL reversed phase solid phase extraction (SPE) cartridge was conditioned with 5 mL MeOH and 5 mL water. The complete extract was applied onto the SPE cartridge which was subsequently washed with 5 mL of water and dried by applying vacuum for 5 min. The antibiotics were eluted from the cartridge using 5 mL MeOH followed by evaporation of the solvent (40 °C, N_2). The residue was redissolved in 200 μL MeOH and subsequently 300 μL water was added before transferring the final extract into an LC–MS/MS sample vial.

2.3. LC–MS/MS

The LC system consisted of a Waters (Milford, MA, USA) model Acquity with a Phenomenex Kinetex C_{18} analytical column of 2.1 × 100 mm^2, 1.7 μm, placed in a column oven at 40 °C. The gradient was: 0–0.5 min, 1% mobile phase B, 0.5–5.0 min, linear increase to 100% B with a final hold of 1.0 min and an equillibration time of 3.5 min, operating at a flow rate of 0.3 mL min^{-1}. The injection volume was 10 μl. Detection was carried out by LC–MS/MS using an AB Sciex (Ramingham, MA, USA) Q-Trap 6500 mass spectrometer in the positive electrospray ionisation (ESI) mode. The operating parameters were: capillary voltage, 2.0 kV; cone voltage, 25 V; source offset, 20 V; source temperature, 150 °C; desolvation temperature, 550 °C; cone gas flow, 150 L h^{-1}; and desolvation gas, 600 L h^{-1}. The antibiotics were fragmented using collision induced dissociation (N_2) and the scheduled Selected Reaction Monitoring (SRM) transitions (20 s window) are presented in Table 1. Data was processed using Multiquant software V2.1.1 (AB Sciex).