Occurrence of tetracyclines in feedingstuffs – results of a two-year study within the official control of feed

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Abstract

The paper describes a microbiological method for the detection of antibacterial substances in feedingstuffs. The method allowed detection of the main antibiotic groups, including tetracyclines. In 2013-2014, a total of 171 feed samples were analysed to determine antibacterial substances. Among the analysed samples 84 (49.1%) were suspected to contain tetracyclines. Out of the 84 feeds analysed using chromatography, 28 (33.3%) contained undeclared tetracyclines, which were identified at concentrations ranging from 0.32 mg kg\(^{-1}\) to 48.98 mg kg\(^{-1}\).

Keywords: antibacterial substances, tetracyclines, feed analysis, microbial screening method, LC-MS.

Introduction

Under modern intensive animal production systems, antimicrobials are frequently incorporated in feedingstuffs to treat diseases (therapeutic use) or prevent diseases (prophylactic use). Tetracycline antibiotics are widely used in food-producing animals for the treatment of a variety of bacterial infections. They are commonly used because of their broad spectrum of activity, cost effectiveness, and some favourable pharmacokinetic properties. This group of antibiotics exerts a bacteriostatic effect in susceptible bacterial cells by inhibiting protein biosynthesis after binding to the 30S ribosomal subparticle (3).

According to different sources, tetracyclines (TCs) are the most widely used therapeutic antibiotics in farm animals. In 10 European countries, TCs accounted for 48% of the sales of veterinary antibacterial agents, whereas sulphonamides and trimethoprim constituted 17% and \(\beta\)-lactams 16% (4). In the United Kingdom and the Netherlands, the amount of TCs used in farm animals is nearly equal to the amount of all other administered antibiotics (8). In Poland, survey data concerning surveillance of the use of veterinary antimicrobials show that in 2010-2013, three groups of antibiotics were predominant in veterinary practice: TCs, penicillins, and sulphonamides (9, 10).

Antibacterials, including TCs, were known as antibiotic growth promoters (AGPs) and their use became common agricultural practice in Europe during the late 1950s and 1960s. In order to protect human health the authorisation of several antibiotics as feed additives has been withdrawn in the European Union since 1970s (TCs, lincomycin, streptomycin), through 1997-1999 (avoparcin, spiramycin, tylosin), till 2006, when the Regulation (EC) 1831/2003 laid down provisions phasing out the authorisations of AGPs in the European Union as from January 1, 2006 (2). The authorisation of AGPs as feed additives has been withdrawn because their contribution to bacterial resistance has been significantly larger than that from the use of these antibiotics for veterinary therapy alone (5).

The use of antibacterials in feed at low dosages aids the development of resistant bacteria in farm animals and of resistant microbes entering the food consumed by humans. Animal feeding has become an important economic activity and has a clear impact on food safety. Feedingstuffs must comply with legal limits regarding contaminants and antimicrobial agents. The latter substances can enter the feed via different routes, for
instance when added for therapeutic purposes at authorised level (medicated feed), but also due to cross-contamination in the feed mills or as a result of possible illegal use (e.g. growth promoters) (6).

Therefore, control laboratories have to manage a considerable volume of feed samples and analyse a large number of analytes. Microbial inhibition methods are the preferred methods for large scale surveillance programmes on the occurrence of veterinary medicines in food and feed (7, 13). In the past, microbial methods were the first choice for systematic detection of antibiotic residues in food. They were adopted for feed and now they still constitute mainstream screening methods. They allow to determine antibiotics and to identify specific antibiotic groups. When the results of these analyses indicate the presence of antibiotics or other antibacterial substances, the material is then examined by the appropriate chemical methods as microbial ones cannot be used to identify individual antibiotics. The results must always be verified and confirmed by chemical methods.

Because the use of antibiotics in feeds for non-medicinal purposes has been prohibited in EU, monitoring for undeclared or illegal use of these substances is conducted within national programmes of feed control. This paper reports the investigation on the contamination of feedingstuffs with antibacterial agents with particular consideration of TCs.

Material and Methods

Sample collection. During 2013-2014, a total of 171 samples of feedingstuffs were analysed. This number comprised five samples of cattle feed, 42 samples of pig feed, 47 samples of poultry feed, 13 samples of premixes, nine samples of feed materials, and 55 samples of compound feeds manufactured after medicated feeds on the same production line. The samples were taken as part of the national feed monitoring programme and in feed mills within internal control frameworks.

Screening method. Microbiological 8-plate screening method for detection of antibacterial substances in feedingstuffs was used (14). The method comprised eight plates: Bacillus subtilis BGA spore suspension - a ready-to-use suspension (Merk), Bacillus cereus ATCC 11778, Kocuria rhizopha ATCC 9341, and Escherichia coli ATCC 11303 - self-prepared bacterial suspensions prepared as described in the laboratory instruction.

Culture media. Antibiotic Medium No. 1 (Oxoid) and Standard II Nutrient Agar (Merk) were used as culture media.

Assay plates. Eight plates were prepared (Table 1). The melted media were inoculated with appropriate test microorganisms at adequate concentrations. The plate 4 was supplemented with trimethoprim to increase its sensitivity for sulphonamides. To prepare the plates, a volume of 20 mL of the inoculated media was poured into 120 mm Petri dish and left to harden at room temperature on a horizontal surface. When the agar was solidified, two 11 mm wells were punched by cork borer in each plate.

Sample assay. Ten gram portion of the samples was weighed into 200 mL Erlenmeyer flask. The samples were extracted with 50 mL of methanol/phosphate buffer (pH 8.0) mixture (1:1, v/v). The extraction time was 30 min. After the extraction, the samples were centrifuged for 10 min at 3000 × g and the supernatants were dispensed into wells on agar surface on the plates. Test plates were incubated for 18-24 h at 30°C (B. subtilis and B. cereus) and 37°C (K. rhizophila and E. coli).

Interpretation of results. The results of microbial method were evaluated qualitatively. They were obtained by analysing the effect of diffusion on agar medium inoculated with sensitive and resistant bacterial strains. The presence of antibacterial substances was shown by the formation of growth inhibition zones around the punch hole after overnight incubation. The diameter of the zones was measured. All samples showing growth inhibition zones on test plates (designed for TCs) were suspected to contain TCs and directly analysed and confirmed by the chemical method.

Chemical verification and confirmation. The samples showing the inhibition zones on plate 3 were subjected to chemical analysis using a method based on liquid chromatography with mass spectrometry. The procedure was developed for the determination of oxytetracycline (OTC), tetracycline (TC), chlorotetracycline (CTC), and doxycycline (DC).

The feed samples were ground. Each sample was mixed with 25 mL of McIlvaine buffer-Na$_2$EDTA solution (pH 4.0) in a 50 mL polypropylene centrifuge tube. The mixture was extracted for 45 min on a horizontal shaker and centrifuged for 20 min at 4000 × g.
Table 1. Composition of 8-plate screening test used in the study

<table>
<thead>
<tr>
<th>Plate</th>
<th>Test organism</th>
<th>Agar medium</th>
<th>pH</th>
<th>Incubation temperature (°C)</th>
<th>TMP µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>B. subtilis</em> strain BGA</td>
<td>Antibiotic Medium No. 1</td>
<td>6.0</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>K. rhizophila</em> ATCC 9341</td>
<td>Antibiotic Medium No. 1</td>
<td>6.0</td>
<td>37</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td><em>B. cereus</em> ATCC 11778</td>
<td>Antibiotic Medium No. 1</td>
<td>6.0</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td><em>B. subtilis</em> strain BGA</td>
<td>Antibiotic Medium No. 1</td>
<td>7.2</td>
<td>30</td>
<td>0.5</td>
</tr>
<tr>
<td>5</td>
<td><em>B. subtilis</em> strain BGA</td>
<td>Antibiotic Medium No. 1</td>
<td>8.0</td>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td><em>K. rhizophila</em> ATCC 9341</td>
<td>Antibiotic Medium No. 1</td>
<td>8.0</td>
<td>37</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td><em>E. coli</em> ATCC 11303</td>
<td>Standard II Nutrient Agar</td>
<td>6.0</td>
<td>37</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td><em>E. coli</em> ATCC 11303</td>
<td>Standard II Nutrient Agar</td>
<td>8.0</td>
<td>37</td>
<td>-</td>
</tr>
</tbody>
</table>

Fig. 1. The presence of growth inhibition zones on *B. cereus* ATCC 11778 plates

Then 5 mL of supernatant was loaded into the Strata-X-CW SPE cartridges (60 mg, 3 mL) and the drugs were eluted with 3 mL of methanol with 2% formic acid. The eluate was evaporated to dryness under nitrogen stream and the residue was reconstructed in 1 mL of 0.1% formic acid.

LC-MS analysis was performed on HP 1200 Series Agilent Technologies (Santa Clara, USA) liquid chromatography with a single mass spectrometer (Agilent Technologies, Santa Clara, USA). The HPLC system was coupled to a single MS equipped with an ESI interface operating in positive ion mode using a capillary voltage of 2000 V. The other optimum values of the ESI-MS in positive parameters were drying gas temperature 350°C, drying gas flow 13 L/min, nebulising gas pressure 40 psi.

LC-MS separation of TCs was achieved with the Kinetex C18 column (100 × 4.6 mm, 5 µm), (Phenomenex, USA). Eluent A was 0.1% formic acid in acetonitrile and eluent B was ultrapure water containing 0.1% formic acid (v/v). The flow rate of the mobile phase was 0.5 mL/min, and the column thermostat was set at 20°C. The injection volume was 15 µL and all compounds were eluted within 19 min.

Validation. The method validation was performed according to the recommendations of the European Commission Decision 2002/657/EC. Parameters such as linearity, specificity, precision (repeatability and within-laboratory reproducibility), accuracy, and detection (LOD) and quantification (LOQ) limits were established. Additionally, during the validation process, the decision limit (CCα) and detection capability (CCβ) were evaluated. The presented method was validated for four TCs: OTC, TC, CTC, and DC at three concentration levels: 300, 1000, and 5000 µg kg⁻¹. Linearity was tested by preparing matrix calibration curve in the range of 300 – 5000 µg kg⁻¹. To determine the specificity of this method, blank feed samples were analysed. The LOD and LOQ were calculated on the basis of signal to noise and were S/N = 3 for LOD and S/N=10 for LOQ. The LOD and LOQ values for four TCs in feeds ranged from 19.06 to 70.16 µg kg⁻¹ and 27.65 to 94.88 µg kg⁻¹ respectively. Repeatability and within-laboratory reproducibility, as well as accuracy calculated as recovery, were determined by analysing blank feed samples spiked with OTC, TC, CTC, and DC standard solutions at concentrations corresponding to 300, 1000, and 5000 µg kg⁻¹. The experiments were carried out on three consecutive days. The repeatability was determined by fortifying six blank samples, which were analysed on the same day with the same instrument and by the same operator, and the coefficient of variations (CV) was calculated. The within-laboratory reproducibility was determined by fortifying two other sets of blank samples at the same concentration levels of the analysed compounds as for
the repeatability and analysed by two different operators. The validation results were repeatable and reproducible with the repeatability CV and within-laboratory reproducibility CV lower than 12% for four TCs at all fortification levels. The mean TCs recovery for spiked samples was within the range of 79.77% and 98.81%. The CCα and CCβ were determined using the matrix calibration curve procedure. The CCα and CCβ values ranged from 134.22 µg kg⁻¹ to 143.22 µg kg⁻¹ and from 324.92 µg kg⁻¹ to 346.70 µg kg⁻¹ respectively.

**Results**

Among 171 feedingstuff samples analysed for antibacterial substances, 84 (49.1%) samples were suspected to contain TCs. The presence of TCs was confirmed in 28 (33.3%) samples. Overall, the percentage of positive samples for TCs among all the analysed samples was 16.4%. The results are presented in Table 2.

Doxycycline was the predominant TC identified, followed by chlortetracycline and oxytetracycline. The samples were identified at concentrations ranging from 0.32 mg kg⁻¹ to 48.98 mg kg⁻¹. Twelve of the samples contained TCs at the concentrations below 1.0 mg kg⁻¹.

Representative chromatograms of the sample spiked with the mixture of four TCs and the positive sample with doxycycline at a concentration of 4100 µg kg⁻¹ are shown in Figs 2 and 3 respectively.

**Table 2. The number of samples contaminated with TCs**

<table>
<thead>
<tr>
<th>Type of feedingstuff</th>
<th>Number of analysed samples</th>
<th>Number of samples suspected of TCs</th>
<th>Number and percentage of samples confirmed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle feed</td>
<td>5</td>
<td>1</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Pig feed</td>
<td>42</td>
<td>27</td>
<td>17 (63)</td>
</tr>
<tr>
<td>Poultry feed</td>
<td>47</td>
<td>39</td>
<td>5 (12.8)</td>
</tr>
<tr>
<td>Concentrates/Premixes</td>
<td>13</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Feed materials</td>
<td>9</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Compound feed (manufactured after medicated feed)</td>
<td>55</td>
<td>10</td>
<td>5 (50)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>171</strong></td>
<td><strong>84 (49.1%)</strong></td>
<td><strong>28 (33.3%)</strong></td>
</tr>
</tbody>
</table>

**Fig. 2.** LC-MS chromatogram of feed sample spiked with OTC, TC, CTC, and DC at a concentration of 300 µg kg⁻¹

**Fig. 3.** LC-MS chromatogram of feed sample, which revealed the presence of doxycycline at a concentration of 4100 µg kg⁻¹
Discussion

The effective and efficient control of the possible illegal use of antibacterial substances requires the availability of multi-screening and confirmatory methods. The plate microbial assays and liquid chromatography coupled to MS are essential techniques in food analysing laboratories and have been widely reported in the literature. However, there are fewer studies dealing with the analysis of antimicrobial substances in feeds. Multi-class methods for the analysis of antimicrobial agents in feed are still rare, because of the analytical challenges. For the control strategy, screening by microbiological inhibition is proposed, followed by additional confirmation by LC-MS (5). The primary aim of a screening assay is to sift out large numbers of samples. Microbial methods are relatively inexpensive, easy to perform, robust, do not require expensive equipment, and can be efficiently adopted by laboratory staff. These are the methods of choice for screening purposes as they allow qualitative detection of antibiotics in a sample and identification of antibiotic groups. This facilitates subsequent confirmation of specific antibiotics with chemical methods, because any positive or suspicious results should be verified and confirmed by chemical methods. Another important advantage, compared to the LC-MS system, is that microbiological tests can detect any antibiotic or metabolite with antibacterial activity, whereas LC-MS system is commonly applied to compounds previously selected as targets, so any other antibiotic would pass undetected (1). However, the lack of specificity is the most important drawback of the microbiological tests.

In the presented method we used a combination of eight plates which allowed to discriminate between different groups of antimicrobial compounds. Bacillus cereus ATCC 11778 was chosen as the sensitive strain for TCs group. Plate 3 was designed for the detection of TCs owing to its low pH and a sensitive bacterial strain. Originally the TCs family should be the only one which is detected on this plate. In the presence of TCs, this plate shows the largest inhibition zone around the well. However, using the described method it is possible to gain some information on the presence of other antibacterial substances. Our results showed quite a large number of samples which were suspected to contain TCs. A large number of poultry feed samples potentially containing TCs resulted, among others, from the presence of coccidiostats in poultry feed to which B. cereus ATCC 11778 is also sensitive, generating a large number of false-positive results. Another explanation is that poultry feeds are more often produced industrially than pig feeds, and consequently the latter feeds have a much less complicated composition. The proper analysis of feed is difficult, because of its complicated composition. Animal feed is a very complex matrix. There are differences not only in the composition between the species but also in the raw materials used for the production of different batches of feeds. Hence, each feed sample has almost its own characteristics, which makes the development of an effective diagnostic method even more challenging. There is a possibility to obtain false positive results due to the presence of different antibacterial substances included in feed: coccidiostats, organic acids, acidity regulators, preservatives, or any other non-specific antibacterial substances (inhibitors) which cause matrix interference. Lack of confirmed samples among concentrates/premixes shows that high concentrations of vitamins, minerals, amino acids, enzymes, or organic acids can impact the occurrence of false-positive results.

The presented chemical procedure was designed to obtain qualitative and quantitative verification and confirmation of the presence of the antibiotics belonging to TCs group. The need for confirmation of the results obtained by microbiological method, as well as for quantification purposes, led to the development of the method based on chromatography coupled to MS.

The presence of undeclared antibiotics in feed can occur as a result of the possible illegal use (AGPs) or as a result of cross-contamination of non-medicated feed either at the level of production in the feed mill or administration in farms, especially in the context of the use of medicated feed. The way of production of medicated feed poses a serious cross-contamination problem in the case of medicated feeds and unmedicated feeds processed on the same production line (15). Contamination of compound feeds with antibiotics depends on a number of factors including human error, production practices, and handling procedures in the feed mill. Contamination of non-medicated feed can also occur during transport to farms and, eventually, at the farm during storage, manipulation, and mixing operations (12, 16). However, there are few publications about contamination of feedingstuffs with antibiotics. A study conducted in Northern Ireland showed that a proportion of feeds was contaminated with antimicrobial drugs (11). Overall, contaminating antimicrobials were detected in 24.8% of the samples tested. Chlorotetracycline (15.2%), sulphonamides, penicillin, and ionophores were the most frequently identified contaminating antimicrobials. In a study carried out in Spain, non-medicated feed for breeding and growing pigs, produced after tiamulin-medicated feed, contained the antibiotic levels higher than 2.5 mg kg\(^{-1}\) (LOQ) but lower than 5 mg kg\(^{-1}\) (LOQ) as a result of cross-contamination (15). Another study concerning determination of antibacterials (belonging to sulphonamides and \(\beta\)-lactams) in feed revealed that among 21 samples of bovine feed, 13 samples contained at least one of the target compounds at a quantifiable level, five samples contained one or
more antibacterials at the levels below the corresponding LOD or LOQ, and three samples were free from antibacterials (7).

Our study showed that out of the 171 samples tested contaminating antimicrobials (TCs) were confirmed in 28 (16.4%) samples, and reveals that a proportion of feed was contaminated with antimicrobial substances. Some samples contained TCs at the concentrations below 1.0 mg kg\(^{-1}\). The explanation for the relatively low concentrations of antibiotics found in the samples would be cross-contamination, which could occur either during manufacturing of medicated feed, or during transport to farms, or even at the farm itself (storage, manipulation and mixing operations). Higher contamination could occur due to non-medicinal use of TCs (prophylaxis, growth promotion).

This paper has highlighted that contamination of feedingstuffs with antibiotics occurs. One must remember that contaminated feed may significantly contribute to the selection of antimicrobial resistant bacteria in food-producing animals, and may cause the occurrence of potentially harmful residues in meat and other products originating from food-producing animals fed the contaminated feed. For this reason further investigations are required and should be continued in the future.

**Conflict of Interests Statement:** The authors declare that there is no conflict of interests regarding the publication of this article.

**References**


