Detection of residues of tetracycline antibiotics in pork and chicken meat: correlation between results of screening and confirmatory tests†

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Residues of the tetracycline group of antibiotics were quantified in pork and chicken muscle tissue that had previously been screened with a microbiological inhibition test and an immunological method. Pieces of frozen pork and chicken meat were screened on a pH 6 culture medium seeded with *Bacillus subtilis*. An aqueous extract of the inhibitor-positive samples was then screened with a group-specific commercial ELISA kit, able to detect levels of oxytetracycline, chlortetracycline, tetracycline and doxycycline corresponding with the European MRL or lower. The cut-off value of the ELISA was set at a B/B_0 value of 75%. Finally, confirmation and quantification were performed using a validated HPLC method with fluorescence detection. The fluorescence was induced by complexation of the tetracyclines with the zirconium cation which is added post-column to the HPLC eluate. This fluorescence makes it possible to quantitate residues below one-half of the MRL. To gain additional qualitative information some samples were also analysed with LC-MS-MS.

ELISA analysis demonstrated the presence of residues of tetracyclines in 12 out of 19 inhibitor-positive pork samples and in 19 out of 21 inhibitor-positive chicken samples. Doxycycline was detected with HPLC in 10 of these 12 pork samples and in 18 out of 19 chicken samples. The two other ELISA positive pork samples contained oxytetracycline, while no tetracyclines were found in one ELISA positive chicken meat sample. The correlation between the ELISA B/B_0 values and the actual levels determined with the HPLC method was poor, whereas a better correlation was observed between the inhibition zones and the doxycycline levels. Our results indicate that an inhibition test with a medium at pH 6 and *B. subtilis* as test organism is well suited to screen pork and chicken muscle tissue for residues of tetracycline antibiotics. Since many positive samples contained doxycycline levels below the MRL, a confirmatory method is necessary to quantify the residues.

1. Introduction

Although introduced in veterinary medicine more than 40 years ago, tetracyclines are probably still the most frequently used antibiotics in animal husbandry.[1,2] Tetracyclines have a broad spectrum activity against G^+ and G^- bacteria. In 1968, their use as growth promoters was discussed, because scientists were alarmed by the high resistance status in gram-negatives.[3] This led to the ban of different antibiotics, including the tetracycline family, which were used as therapeutics in animal and human medicine, as food additives in Europe. In the US tetracyclines are still accepted as growth promoters.[4,5]

As a consequence, residues of the commonly used tetracyclines, oxytetracycline, tetracycline, chlortetracycline and doxycycline are often found in meat. Okerman *et al.*[6] reported that 1.2% of chicken meat samples and 2.7% of pork meat samples, purchased from retail outlets, inhibited media seeded with *B. subtilis*. The majority of these samples contained residues belonging to the tetracycline family. The EU legislation[7] on veterinary drug residues has laid down an MRL for tetracyclines in pigs and poultry, including its 4-epimer, at 100 $\mu g\ kg^{-1}$ in muscle.

Screening tests are often used to detect residues of legally and illegally used chemicals in farm animals. Microbiological inhibition tests are less specific than ELISA tests: ELISA tests detect one substance or a group of related chemicals, while theoretically all antibiotics and all naturally occurring contaminants which possess antibacterial activity can cause growth inhibition of susceptible bacteria.

Ideal antibiotic detection systems are often proposed as a microbiological screening, followed by an ELISA test which detects one family of antibiotics.[8] The final confirmation and quantification step consists of a suitable chemical technique, such as HPLC with fluorescence, UV or MS detection or GC-MS. In the experiments described in the present paper, such a strategy was followed to detect tetracycline residues in pork and chicken muscle tissue. The aim of this study was to evaluate the necessity of using a double screening, a microbiological inhibition test combined with ELISA, and to correlate the results with the quantitative confirmation of the HPLC-fluorescence detection.

2. Materials and Methods

Samples

During 1998, 1768 samples of chicken breast meat and 523 samples of pork muscle were examined for inhibitory substances. Most samples were frozen when they arrived in the

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laboratory; if not, they were cooled immediately to −20 °C. They were investigated for inhibitory substances within 2 weeks after arrival. Only a minority of the inhibitor-positive samples, and no inhibitor-negative samples, with the exception of the negative controls, were further investigated with ELISA and HPLC fluorescence or MS-MS detection.

Detection of inhibitory substances

Inhibitory substances were detected on one of the plates, which are used for the so-called Four Plate Test (FPT) described in the *Manual of Reference Materials and Methods To Detect Veterinary Drug Residues*.[9] In previous experiments, we had found that tetracyclines were best detected on the medium at pH 6 and seeded with *B. subtilis*.[6]

Test agar pH 6 (Merck, Darmstadt, Germany; dehydrated medium 10 663) was prepared and autoclaved. After cooling to 45 °C, 100 ml of a ready-to-use spore suspension (Merck; catalogue No. 10649) was added to 100 ml of medium. Sterile petri dishes (diameter, 90 mm) were filled with 5 ml inoculated medium, cooled immediately, and kept at 2–4 °C for a maximum of 3 d. When unknown samples were analysed, all plates were subjected to a quality control. Paper disks (6 mm diameter, filters Durieux, Paris, France; catalogue No. 268) were placed in the center of the petri dish; 0.0002 units of penicillin were deposited upon these disks. Detection limits of tetracyclines were as follows: oxytetracycline, 8 ng; tetracycline, 5 ng; chlortetracycline, 0.5 ng; doxycycline, 1 ng.

Meat was sampled while still frozen. A cylinder of frozen meat was removed with a cork borer of 8 mm diameter, and cut into 2 disks, each 2 mm thick. Two disks of a meat sample were placed on opposite ends of an inoculated plate. Three meat samples were analysed on each plate, for a total of 6 disks per plate. The plates were incubated overnight at 30 °C, and then inspected for inhibition zones around the meat disks. When both meat disks showed inhibition rings of 2 mm or more in width, the result was recorded as positive. The area of the inhibition zone was calculated as πr^2. A positive result corresponds with an area of 113 mm^2. The mean area of both inhibition zones was calculated.

Detection of tetracyclines with ELISA

Twenty one chicken samples and 19 pork samples showing inhibition zones wider than 2 mm were examined further for tetracyclines with a commercially available ELISA kit (Foodscan TC Microwell Test Kit®, Idetek, Sunnyvale, CA). Extracts were prepared in phosphate buffer and tested according to manufacturer's instructions. The buffer and a series of 3 oxytetracycline standards, diluted in buffer, were tested with each group of FPT-positive samples, together with at least 2 blank meat samples found negative in the FPT, and 1 sample spiked with 100 μg kg^{-1} doxycycline. Results were read with a microtiter plate reader (Titertek Multiscan MCC/340, Flow Laboratories, Lugano, Switzerland). The B_0 value was the mean optical density of at least 2 duplicate analyses of extraction buffer, run together with the sample extracts. All samples with B/B_0 values lower than 75% were recorded as positive; values between 75 and 80% were recorded as suspect. At a B/B_0 value of 75%, the kit is 95% specific and detects > 99.98% of samples spiked with 100 μg kg^{-1} oxytetracycline, but only 80% of samples spiked with 100 μg kg^{-1} doxycycline (results of own validation procedure, 20 assays at 20 different occasions).

Detection of tetracyclines with HPLC

All chemicals used were of analytical grade from Merck (Darmstadt, Germany). Tetracycline standards were obtained from Sigma (St. Louis, MO, USA). Stock solutions (1 mg ml^{-1}) were prepared in MeOH and stored at 220 °C. Working standard solutions were prepared in MeOH.

The extraction and clean-up procedure, based on metal–chelate affinity chromatography technique (MCAC), and additional concentration step prior to the detection was described by Croubels *et al*.[10]

The internal standard was demeclocycline spiked at a concentration of 100 μg kg^{-1}. Since the MRL of tetracyclines in meat is 100 μg kg^{-1}, each batch of samples was accompanied with a spike at the MRL level and a blank. The calibration line was based on fortified blank samples at five concentrations: 10, 50, 100, 150, 200 μg kg^{-1}.

For the fluorescence detection a RP polymeric column type PLRP-S (100 Å, 8 μm, 250 × 4.6 mm id) was used in combination with a PLRP guard cartridge of 5 × 3.0 mm (Polymer Laboratories, Church Stretton, UK). The mobile phase contained 0.01 M oxalic acid in water (A) and acetonitrile (B). A gradient solvent program was run [85(A) : 15(B), v/v to 60(A) : 40(B), v/v in 16 min]. The flow rate was set at 1 ml min^{-1}. The fluorescence was induced by complexation of the tetracyclines with the zirconium cation [5% (m/v) zirconyl chloride octahydrate in HPLC grade water] which is added post-column to the HPLC eluate.

For MS-MS detection a different mobile phase was used. To prevent clogging of the heated capillary the use of involatile buffers was avoided. The analytes were eluted using a Symmetry C18 column (5 μm, 150 × 2.1 mm, Waters, Milford, USA). The mobile phase consisted of a mixture of MeOH and 0.069% TFA (A) (30 : 70, v/v) pumped at a rate of 0.3 ml min^{-1}. A linear gradient was run (100% A to 100% MeOH in 10 min). Over the following five minutes 100% MeOH was pumped through to force the late eluting compounds to come off.

The pump and autosampler used for both detection methods were from the same supplier [a P4000 quaternary pump and an AS3000 autosampler of TSP (CA, USA)]. The MS-detector was an LCQ ion trap from Finnigan MAT (CA, USA) working with an electrospray interface in positive ion mode MS-MS full scan.

3. Results

Out of a total of 1768 chicken samples, 103 were found to contain a substance inhibiting *B. subtilis* at pH 6; similarly, 38 out of 523 pork meat samples were positive with the same test. Nineteen pork samples and 21 chicken samples were analysed further with the ELISA test: B/B_0 values lower than 75% were found in 19 of the 21 chicken samples and in 12 of the 19 pork samples. A B/B_0 value of 80% was found in two other chicken samples. The mean area of both inhibition zones and the B/B_0 values of ELISA positive and ELISA suspected chicken and pork samples are mentioned in Tables 1 and 2.

Oxytetracycline was found after HPLC analysis in 2 of the 12 ELISA positive pork samples and in none of the 21 chicken samples (Tables 1 and 2). One sample contained levels higher than 100 μg kg^{-1}, which is the maximum residue limit for tetracyclines in muscle tissue. Doxycycline was found in 18 out of 19 inhibitor-positive, ELISA positive chicken samples. No tetracyclines were found in the remaining 3 samples, one of which was ELISA positive. The levels of doxycycline ranged from 63 μg kg^{-1} to 1033 μg kg^{-1} in a pork sample and from 10 μg kg^{-1} to 1680 μg kg^{-1} in a chicken sample (Tables 1 and 2). Levels higher than 100 μg kg^{-1} were found in 7 pork samples and in 6 chicken samples. In all samples with doxycycline or

oxytetracycline levels above the MRL, both inhibition areas were wider than 3 mm (area of 154 mm²). In all but one of the samples with doxycycline levels of 100 μg kg^{-1} or more, the width of the area was more than 5 mm corresponding to an area of 254 mm².

The 21 positive chicken samples were analysed with LC-MS-MS. Three samples were negative and the remaining 18 were positive for doxycycline. The negative samples were also negative with HPLC fluorescence detection.

The correlation between the mean area of the inhibition zones and the doxycycline levels found in 28 samples which contained doxycycline was 0.82; the correlation between the ELISA results and the doxycycline levels in the same samples was 0.73. The correlation between the doxycycline levels and the mean inhibition area was higher in the 18 chicken samples than in the 10 pork meat samples (Table 3).

Table 1 Comparison between the mean area of growth inhibition (mm²), B/B_0 value (%) of an ELISA test and concentration of doxycycline (μg kg^{-1}), found in chicken meat samples

Sample	Mean area of inhibition zone/mm²	B/B_0 value of ELISA (%)	Concentration of doxycycline/μg kg^{-1}	LC-MS-MS confirmation
1	155	72	< LOD	< LOD
2	119	80	< LOD	< LOD
3	121	80	< LOD	< LOD
4	214	72	10	Doxycycline
5	132	71	32	Doxycycline
6	129	68	41	Doxycycline
7	141	61	46	Doxycycline
8	240	73	50	Doxycycline
9	204	68	51	Doxycycline
10	224	66	52	Doxycycline
11	228	68	56	Doxycycline
12	210	73	62	Doxycycline
13	170	68	64	Doxycycline
14	275	64	65	Doxycycline
15	321	64	91	Doxycycline
16	186	60	114	Doxycycline
17	331	65	163	Doxycycline
18	315	62	231	Doxycycline
19	314	64	302	Doxycycline
20	412	74	347	Doxycycline
21	819	47	1680	Doxycycline

Table 2 Comparison between the mean area of growth inhibition (mm²), B/B_0 value (%) of an ELISA test and concentration of doxycycline (μg kg^{-1}), found in pork meat samples

Sample	Mean area of inhibition zones/mm²	B/B_0 value of ELISA (%)	Concentration of doxycycline/μg kg^{-1}	Concentration of oxytetracycline μg kg^{-1}
1	143	55	< LOD	52
2	269	35	< LOD	253
3	230	72	63	< LOD
4	214	67	63	< LOD
5	263	65	73	< LOD
6	334	67	102	< LOD
7	434	62	105	< LOD
8	423	68	105	< LOD
9	427	69	110	< LOD
10	449	67	130	< LOD
11	693	52	529	< LOD
12	586	58	1033	< LOD
13	113	11	Not analysed	Not analysed
14	127	98	Not analysed	Not analysed
15	143	92	Not analysed	Not analysed
16	161	80	Not analysed	Not analysed
17	178	97	Not analysed	not analysed
18	189	81	Not analysed	Not analysed
19	449	88	Not analysed	Not analysed

4. Discussion

An inhibition test using only 1 plate with a medium at pH 6, seeded with *B. subtilis*, is a cheap screening method, especially when a large number of samples is analysed. On the other hand, ELISA tests are more specific and provide faster results but the ready-to-use kits cost more and the reagents are not stable for a long time.

Screening tests can be validated by comparing test results of blank and spiked samples. In the case of an ELISA, the number of expected false-negatives and false-positives can be predicted using a statistical evaluation of B/B_0 values of the negative and positive samples.[11] The ELISA test for tetracyclines was validated according to this principle. Such an approach is however impossible for inhibition tests.

Reliabilities of both the ELISA and the inhibition test can be estimated by evaluating the correlation between their respective quantitative results and the actual amount of analyte found with HPLC. The highest correlation found was 0.94 and was between the inhibition area produced by the lean chicken breast tissue and the doxycycline level. This correlation was lower with pork muscle, probably because it contained more fat which could have influenced the diffusion of the antibiotic into the inoculated medium.

Low detection limits can be obtained with microbiological inhibition tests, when antibiotic standards are applied in an aqueous solution. It is not sure if low concentrations present in muscle tissue will be detected as easily, this depends on the diffusion of the compound into the medium, and on the effects of the matrix. Recently, we have shown that the matrix does not influence the detection of tetracyclines substantially, contrary to other antibiotics.[11] The high correlation that we found between the inhibition zones of lean chicken meat and the actual content of doxycycline suggests that most, if not all, of the antibiotic is released from the tissue.

It is not probable that the inhibition test missed any samples with doxycycline levels above the MRL, because all but one of such samples had large inhibition zones (rings wider than 5 mm or mean area larger than 254 mm²). The number of samples with oxytetracycline (detection limit of 8 ng) was too low to draw a similar conclusion; but one of the two samples detected with the inhibition test contained 52 μg kg^{-1}, while the MRL is 100 μg kg^{-1}. The detection limit of chlortetracycline and tetracycline, which were not found in our series of samples, are respectively 0.5 ng and 5 ng, corresponding with 5 ng and 50 μg kg^{-1} tissue.

The correlation between the B/B_0 values of doxycycline containing samples and the doxycycline concentration was 0.73, which is a similar value as for the pork tissue and the doxycycline concentration. According to the manufacturer's instructions, a B/B_0 value of 50% is obtained with 20 μg kg^{-1} oxytetracycline, 34 μg kg^{-1} tetracycline, 117 μg kg^{-1} chlortetracycline and 161 μg kg^{-1} doxycyline. Quantitative interpretation of ELISA results are thus impossible when the nature of the tetracycline residue is not known, which is always the case in residue screening.

The purpose of the ELISA test was to obtain information on the nature of the substance that caused inhibition. The ELISA

Table 3 Correlation between the results of two screening tests and the level of doxycycline found in chicken and pork muscle tissue

	Chicken	Pork	Both species
Number of samples	18	10	28
Correlation between area of inhibition and concentration of doxycycline	0.94	0.72	0.81
Correlation between B/B_0 value and concentration of doxycycline	0.74	0.72	0.73

results were positive in 31 out of 40 samples that inhibited *B. subtilis* at pH 6. Residues of the tetracycline family were found in 30 of these samples. As the majority of samples were found positive for tetracyclines, it turned out that the second screening had been an unnecessary cost in our series of samples. In earlier experiments, muscle tissue was screened with the FPT as described by Heitzman; tetracyclines were also the most frequent cause of inhibition of *B. subtilis*.

To confirm the results obtained with the ELISA test and to supply quantitative data in relation to the established MRL level, HPLC analysis with fluorescence detection was performed on the suspect and positive samples. The detection limits of the method were estimated at 0.42 μg kg^{-1} of oxytetracycline, 0.49 μg kg^{-1} of tetracycline, 0.66 μg kg^{-1} of chlortetracycline and 1.38 μg kg^{-1} of doxycycline in pork muscle, using signal to noise ratios of 4 : 1. The same values are also applicable to chicken muscle. A chromatogram of a positive and negative sample is given. (Fig. 1, 2).

The LC-MS-MS results confirmed the results obtained with HPLC fluorescence detection. A chromatogram and spectrum of a positive sample is given (Fig. 3). Since the purpose of this analysis was only to gain additional qualitative information, no quantitative parameters were calculated.

Pork and chicken tissue can be screened for the presence of tetracycline antibiotics with an inhibition test, using a solid medium at pH 6 and *B. subtilis* as a test organism. We have found that a second screening with an ELISA test for tetracyclines seems to be not necessary as only a minority of the samples we analysed did not contain tetracyclines. Confirmation with HPLC or a similar technique is always necessary to quantify the residue in order to determine the concentration in relation to the MRL-level.

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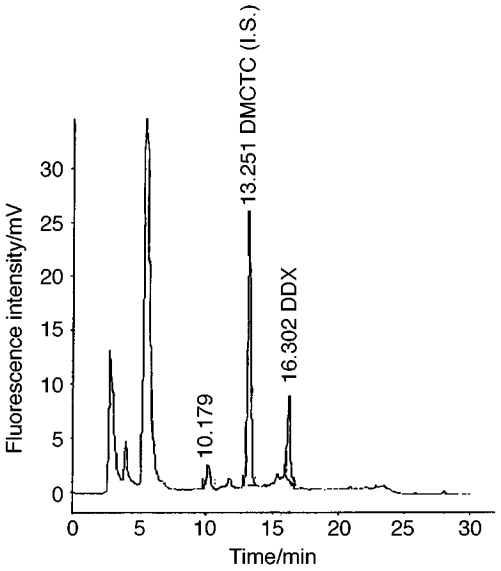

Fig. 1 LC-fluorescence chromatogram of a doxycycline positive sample (t_R internal standard : 13.2 min, t_R doxycycline = 16.3 min)

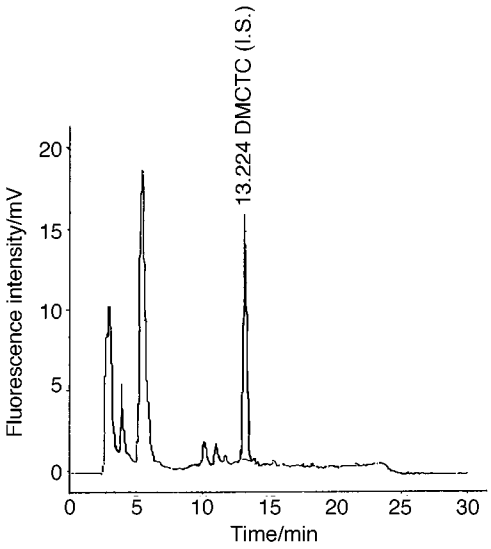

Fig. 2 LC-fluorescence chromatogram of a negative sample (t_R internal standard = 13.2 min)

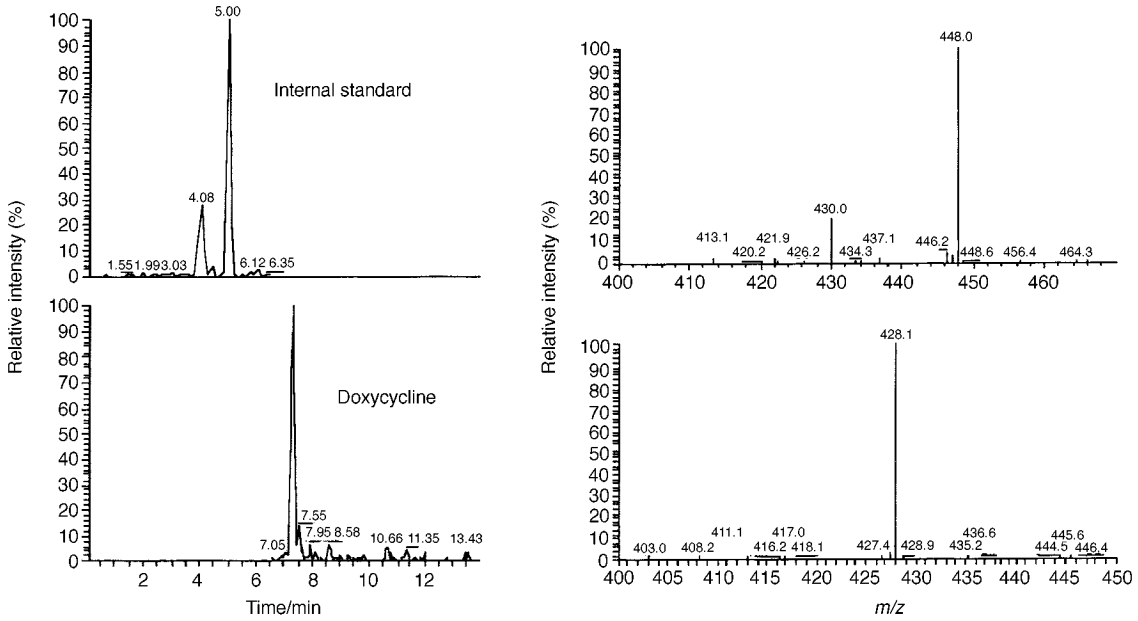

Fig. 3 LC-MS-MS chromatogram of a doxycycline positive sample (upper mass trace with spectrum: demeclocycline, lowest mass trace with spectrum: doxycycline)

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