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Evaluation and establishing the performance of different screening tests for tetracycline residues in animal tissues

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Four methods intended for screening muscle tissue for residues belonging to the tetracycline group were compared using artificially contaminated as well as incurred samples. Two agar diffusion methods were studied: one with Bacillus subtilis *as a test strain, the second with* Bacillus cereus. *Two variants of each method were compared: thin plates for analysis of intact or minced meat, and thick plates for analysis of meat fluid. The thin plate variants could not be evaluated with artificially contaminated samples because it was impossible to prepare homogeneously spiked, undiluted meat. The thick plates were suited for doxycycline and chlortetracycline, but they did not detect oxytetracycline or tetracycline in spiked meat fluid. The results of these tests done on incurred meat were very good for doxycycline and satisfying or just failing for oxytetracycline, while the best detection capability was obtained when intact frozen meat was examined on thin plates seeded with* B. cereus. *Two commercially available screening tests were also evaluated. The Premi® test, an inhibitor test with* Bacillus stearothermophilus *as a test strain and an indicator for growth, was not suited for detection of tetracyclines up to the maximum residue limit. Tetrasensor®, a receptor test specific for tetracyclines, proved a quick and simple test able to detect meat samples artificially contaminated with tetracycline, oxytetracycline, doxycycline or chlortetracycline, as well as meat incurred with oxytetracycline or doxycycline.*

Keywords: tetracycline residues, animal products, screening tests, performance evaluation

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Introduction

Tetracyclines are probably the most widely used therapeutic antibiotics in food animals because of their broad-spectrum activity and cost effectiveness. In a review on contaminants occurring in animal feed and causing residues in feedstuffs, McEvoy (2002) states that tetracyclines account for more than 50% of all in-feed antibiotics sold for use in food animals in the UK. In 1990, the amount of tetracyclines used for farm animals in the Netherlands nearly equalled that of all other antibiotics (Van den Bogaard *et al.* 1994). In 1991, about 12.2% of marketed pigs in Ontario, Canada, had been exposed to tetracyclines in their food, and 20% had been injected with tetracyclines during the fattening period (Dunlop *et al.* 1998). In Belgium and the Netherlands, doxycycline residues have frequently been found in broiler chickens, oxytetracycline in veal calves, and oxytetracycline and doxycycline in pork meat (De Wasch *et al.* 1998, Okerman *et al.* 2001). Injection sites of adult cattle often contain oxytetracycline (N. Van Hoof, personal communication, 2003). A Japanese survey revealed residues of tetracyclines in the kidneys of nearly one-third of the animals that did not pass the inspection (Oka *et al.* 2001). The number of antibiotics used in fish farming is restricted, but oxytetracycline is also approved for oral use in these species.

Efficient control of residues requires good screening tests, which must be cheap, easy to perform, allow simultaneous analysis of large numbers of samples and give rapid results. According to European legislation, a screening method is used to detect the presence of a substance or class of substances at the level of interest (Anon 2002). In the case of permitted substances, the maximum residue limits (MRL) can be considered as the levels that should be detected. Screening methods have the capability for a high sample throughput and are used to sift large numbers of samples for suspect or potential non-compliant results. They are specifically designed to avoid false compliant results (Anon 2002). Thus, the number of

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so-called false-negative results of a screening test should be as low as possible, while a few false-positive results can be accepted as long as all positive results of the screening test are confirmed with a chromatographic method.

Traditionally, antibiotic residues are detected with inhibition tests, mostly agar diffusion tests, such as the Four Plate Test (FPT), but also fast inhibition tests using an indicator for growth. The latter are most frequently used for milk. Specified products or groups of related antibiotics can be detected with immunological tests such as enzyme-linked immunosorbent assays (ELISA), solid-phase fluorescence immunoassays (SPFIA) or with receptor tests. A combination of four SPFIA tests, for example, enables the detection in one run of: (1) the group of the tetracyclines, (2) ceftiofur and cefquinome, (3) penicillin, ampicillin and amoxicillin, and (4) cephapirin, in milk or in kidney tissue (Okerman *et al*. 2003).

The probability that the tested sample is truly non-compliant, although a compliant (or negative) measurement has been obtained, is called the ß error. The detection capability (CCß) means the smallest content of the substance that can be detected, identified and/or quantified in a sample with an error probability of ß. European legislation requires that the CCß of screening tests is equal to or below the MRL, while the ß deviation is below 5%. Thus, a screening method will be approved for official surveys when none of 20 samples with a residue concentration equal to or lower than the MRL give a compliant, or negative, result.

This principle was followed and the authors tried to validate four different methods for detection of oxytetracycline, doxycyline, tetracycline and chlortetracycline in meat: two plates filled with pH 6 medium seeded, respectively, with *B. subtilis* (part of the FPT) (Heitzman 1994) and *B. cereus*; the Premi® test, a commercially available inhibition test intended as a general test for antibiotic residues in meat; and Tetrasensor®, a receptor test intended specifically for tetracyclines. This test uses TetR as a receptor, a cytoplasmatic protein of *Escherichia coli* that plays a role in bacterial tetracycline resistance based on an efflux pump mechanism. Therefore, samples spiked were analysed at the MRL level of $100\,\mu g\,kg^{-1}$ (or $ng\,g^{-1}$). The agar diffusion tests intended for analysis of intact meat could not be evaluated with spiked meat samples because it is impossible to prepare intact or minced meat with an even distribution of the analyte. Therefore, two variants of these methods were evaluated with spiked meat fluid, prepared as for the Premi® test, and the liquid was added into wells cut in the 2-cm thick layer of medium. The thicker layer was necessary in order to analyse 100 µl of fluid, corresponding to 100 µg of the intact tissue that is investigated with the thin plates.

Finally, seven samples of chicken meat incurred with doxycycline and 12 samples of veal meat incurred with oxytetracycline were investigated with all the tests described above.

Materials and methods

Preparation of spiked samples

The inhibition tests were validated with spiked tissue fluid. Large amounts of antibiotic-free tissue fluid were obtained by freezing and thawing chicken breast muscle obtained from five different animals. The meat was inhibitor-negative and had previously been tested with a combination of four inhibition tests: the two agar diffusion tests described above, the Premi® test, and an agar diffusion test with *E. coli* Bayer 14 as a test strain (Okerman *et al*. 2001). To 1 ml fluid, 100 ng of the respective tetracyclines were added, corresponding with the MRL of $100\,ng\,g^{-1}$ meat. The chemical standards tetracycline, oxytetracycline dihydrate, chlortetracycline hydrochloride and doxycycline hydrochloride were obtained from Sigma (ref. T-3258, O-5750, C-4881, D-9891) (St Louis, MO, USA). Stock solutions of $1\,mg\,ml^{-1}$ were prepared in methanol, kept at $-20^{\circ}C$ for a maximum of 6 months, and diluted in distilled water 1/200 (W/V) to a concentration of $5\,\mu g\,ml^{-1}$.

For the Tetrasensor® test, muscle tissue from different animal species (chicken, pork, beef, veal and shrimp meat) was ground with a kitchen blender and 5 g were weighed in the stomacher bags provided with the kit. One of the respective standards was added to a concentration of $100\,ng\,g^{-1}$ and the samples were diluted with 3 ml buffer (provided with the kit) g^{-1} tissue.

All spiked samples were further processed in the same way as the naturally contaminated samples.

Origin and pretreatment of incurred samples

The seven chicken fillets were from broilers treated *per os* with doxycycline in the weeks before slaughter and had been found positive with the inhibition test with *B. subtilis*. The veal calves had received two intramuscular injections with oxytetracycline at a $20\,mg\,kg^{-1}$ dose, and were slaughtered at 4 ($n=4$), 14 ($n=4$) and 21 ($n=4$) days after the end of the treatment. All the samples had been ground with a Moulinette mixer for the extraction and purification of the chromatographic method (see below) and had been kept at $-20°C$. Meat fluid was obtained from the naturally contaminated samples as follows: a 2-cm-long cylinder of frozen meat was obtained using the same cork borer as for the intact meat, and centrifuged for 5 min at 20 000 *g* in 2-ml Eppendorf tubes.

Test procedures

The four methods that will be compared were all intended for screening. A summary of their characteristics is given in table 1.

The agar diffusion tests for the examination of intact meat have already been described (Heitzman 1994, Okerman *et al.* 2001). In short, 90-mm plates were filled with 5 ml test medium, pH 6, seeded with *B. subtilis* or *B. cereus*. The sensitivities for the four tetracyclines had been tested with 6-mm disks impregnated with 10 µl of a series of twofold dilutions of the analytes in distilled water, and the concentrations producing zones of 12 mm diameter were calculated. Pieces of approximately 100 mg intact frozen meat or frozen ground meat obtained with an 8 mm cork borer were laid 1 cm from the edges of the plates. The limits of detection (LODs) of oxytetracycline, tetracycline, chlortetracycline and doxycycline were respectively 8, 5, 0.5 and 1 ng on the *B. subtilis* plate, and 3, 4, 0.3 and 0.6 ng on the *B. cereus* plate (Okerman *et al.* 2001). The plates were incubated overnight at 30°C and then controlled for zones of inhibition around the samples. The zones were measured from the edge of the meat to the first visible colonies and noted. Zones $\geqslant 2$ mm were considered positive.

Plates filled with 14 ml of the same seeded media (variants of the above described methods) were used for investigation of meat fluid. In each plate, four holes of 1 cm diameter were cut into the agar layer and the agar disks were removed. The holes were then filled with 100 µl tissue fluid obtained from untreated animals from the incurred samples or from the samples spiked with one of the four tetracyclines. Twenty or more assays were done with the spiked fluid on the plates with 2-mm thick medium. The plates were incubated and read as described above; the zones were measured from the edges of the holes to the first visible colonies. Zones $\geqslant 1$ mm were considered positive.

The Premi® test, commercialized by DSM food specialities (Delft, The Netherlands), uses *B. stearothermophilus* as a test strain and contains an indicator for growth that turns from deep blue to yellow. The tests were done with 100 µl tissue fluid obtained as described above. For each tetracycline, three spiked samples were analysed with the Premi® test. After pre-incubation for 20 min at room temperature, the remaining fluid was washed away and the tubes were closed with tape and incubated at $64\pm1°C$. Two negative control samples (tissue fluid from chicken fillet obtained from animals not treated with antibiotics) were analysed together with each series. Usually after 150–165 min, the negative control tubes start changing colour from blue to yellow. The colour of each test tube was noted at that time and again 15 and 30 min later. Tubes still blue after 30 min were reported as positive (bacterial growth inhibited). All others were considered as negative.

Tetrasensor® is a competitive receptor test commercialized by Unisensor (Liege, Belgium) intended for quick detection of tetracyclines. Frozen tissue was cut finely and diluted in buffer 1/3 (w/v) as prescribed by the manufacturer. After homogenizing for 2 min with a stomacher, 2 ml fluid were centrifuged at 20 000 g for 1 min and the supernatant was used for the test. A total of 200 µl was transferred into the vial containing the receptor and the contents were mixed gently until the dried pellet was dissolved completely. The strip was then dipped into the vial and the whole was incubated for 10 min at room temperature. Meanwhile, the fluid mounts the strip and passes the two green lines on it, turning their colour into red. The first line binds the remaining free receptor, the second the excess receptor. The result is read by comparing the colour intensities of the first and the second line. The interpretation can be done by visual inspection or with the aid of a Unisensor camera and Unisensor software, which calculates the ratio of the colour intensity of the test line/colour intensity of

Table 1. Characteristics of the screening tests used.

Method	Variant	Antibiotics detected in muscle tissue	Equipment*	Cost of the reagents	Sample pretreatment	Incubation or reaction time	Throughput of samples
Agar diffusion, *B. subtilis*	thin plate for intact meat	tetracyclines, quinolones, beta-lactam antibiotics, high concentrations of other groups	A	very low	none	overnight	very high
Agar diffusion, *B. subtilis*	thick plate for meat fluid		A	very low	minimal	overnight	high
Agar diffusion, *B. cereus*	thin plate for intact meat	tetracyclines, high concentrations of other groups	A	very low	none	overnight	very high
Agar diffusion, *B. cereus*	thick plate for meat fluid		A	very low	minimal	overnight	high
Premi test		all groups, quinolones only at high concentrations	A or B	fair	minimal	3–4 h	high
Tetrasensor		tetracyclines	centrifuge, stomacher plus B	highest	few	10 min	high

*A, normal equipment of bacteriological laboratories: incubator, centrifuge, autoclave, laminar air flow, etc.; B, specific equipment provided by the manufacturers of the commercial test systems.

the control line. Ratios < 1.00 are reported as positive, the others as negative.

Quantification of tetracyclines in the incurred samples

The confirmatory method consisted of a multiresidue LC-ESI-MS/MS method (liquid chromatography-electrospray ionization tandem mass spectrometry) capable of quantifying all four tetracyclines as well as their 4-epimers (Cherlet *et al*. 2003). LODs ranged from 0.5 to 4.5 ng g^{-1} and the LOQs were at half the MRLs.

Results

The results of the analysis of spiked tissue fluid are shown in table 2. The agar diffusion tests with thick plates could not detect the four tetracyclines at the required levels: doxycycline and chlortetracycline were detected on both plates, but tetracycline and oxytetracycline were not found on the plate seeded with *B. subtilis*, and not always on the plate seeded with *B. cereus*. None of the four tetracyclines was detected at MRL levels with the Premi® test.

Tetrasensor® reported all the spiked samples as positives. The ratios of the colour intensities of the test lines over the colour intensities of the control lines of all the spiked samples were below 1.00, while the ratios of the blanks were above 2.73. The largest range was observed with the shrimps spiked with oxytetracycline. The ratios of meat samples spiked with doxycycline and chlortetracycline were very low (table 3).

All but one of the chicken fillets incurred with doxycycline were compliant with the MRL legislation, as the concentrations were only in one case above the MRL, but nearly all samples reacted positively with the agar diffusion tests (table 4). The results also showed that the inhibition zones were larger when examining intact meat compared with ground meat and meat fluid. The Premi® test could not detect doxycycline levels around the MRL. Tetrasensor reported all the samples as positive, even the sample with 14 ng g^{-1}.

Table 2. Results of three inhibition tests with 100 µl tissue fluid from chicken muscle spiked with 100 ng ml^{-1} oxytetracycline, doxycycline, chlortetracycline or tetracycline.

		14-ml plates seeded with *B. subtilis*		14-ml plates seeded with *B. cereus*	
	Premi test®, number of positive samples	Range of inhibition zones (mm)	Number of positive results (zones > 1 mm)	Range of inhibition zones (mm)	Number of positive results (zones > 1 mm)
Blanks	0/3	0–0	0/5	0–0	0/5
Tetracycline	0/3	0–< 1	0/20	0–5.1	13/32
Oxytetracycline	0/3	0–< 1	0/20	0–2.3	22/32
Doxycycline	0/3	1.0–3.0	20/20	4.9–7.8	20/20
Chlortetracycline	0/3	3.6–4.8	20/20	8.6–10.0	20/20

Table 3. Results of the Tetrasensor® test on muscle tissue spiked with 100 ng g^{-1} oxytetracycline, doxycycline, chlortetracycline or tetracycline.

	Number of muscle samples and animal species	Range of Tetrasensor® ratio	Number of positive samples
Blanks	4 poultry, 4 pork, 4 veal, 4 shrimps, 4 beef	2.73–14.76	0/20
Tetracycline	10 beef	0.17–0.53	10/10
Oxytetracycline	10 shrimp	0.18–0.95	10/10
	10 veal	0.21–0.60	10/10
Doxycycline	10 poultry	0.01–0.13	10/10
Chlortetracycline	10 pork	0.00–0.12	10/10

Table 4. Analysis of chicken breast muscle incurred with doxycycline: results of seven screening tests.

Doxycycline concentration ($ng\,g^{-1}$)	Diameters of inhibition zones (mm)					Premi® test [1]	Tetrasensor® ratio and interpretation [2]
	Intact muscle	Minced muscle		Fluid from meat			
	B. subtilis, thin plate	*B. subtilis*, thin plate	*B. cereus*, thin plate	*B. subtilis*, thick plate	*B. cereus*, thick plate		
108.8	7.3/7.9	6.2/6.3	5.6/5.8	2.4/2.6	5.3/5.7	N	0.01 (P)
95.0	7.9/7.4	6.4/6.3	5.9/5.5	2.4/2.3	5.5/5.2	N	0.08 (P)
84.7	6.1/6.2	4.5/4.1	5.3/5.0	1.6/1.4	4.3/4.6	N	0.07 (P)
82.7	6.3/5.8	4.7/4.0	5.5/4.1	1.3/1.2	3.8/4.0	N	0.05 (P)
76.6	6.2/5.6	3.5/3.5	4.7/4.3	1.1/1.3	3.2/3.6	N	0.05 (P)
47 [3]	5.5/5.3	3.4/4.2	3.9/4.1	1.1/1.0	3.1/3.0	N	0.05 (P)
14	4.8/5.6	3.0/2.5	3.9/3.3	0/0	3.4/3.2	N	0.43 (P)

[1] N, bacterial growth not inhibited; I, bacterial growth inhibited.
[2] P, positive result.
[3] Values $< 50\,ng\,g^{-1}$ are below the LOQ of the LC-MS/MS method and estimated concentrations.

Table 5. Analysis of minced veal muscle incurred with oxytetracycline: results of six screening tests.

Concentration ($ng\,g^{-1}$)		Diameters of inhibition zones (mm)					
		Minced muscle		Fluid from meat			
Mother molecule	4-Epi metabolite	*B. subtilis*, thin plate	*B. cereus*, thin plate	*B. subtilis*, thick plate	*B. cereus*, thick plate	Premi® test [1]	Tetrasensor® ratio and interpretation [2]
673.4	88.7	7.3/7.2	9.6/8.7	6.8/6.6	8.7/8.7	I	0.00 (P)
627.1	96.6	7.1/7.2	9.0/8.8	6.9/7.0	9.4/8.7	I	0.05 (P)
561.5	83.7	5.6/5.7	8.4/8.1	5.3/4.9	7.4/7.5	I	0.01 (P)
427.5	63	6.2/5.9	8.8/8.5	5.9/6.0	7.8/8.2	I	0.00 (P)
192.8	23.8	3.6/2.9	5.7/6.0	1.0/1.0	4.5/5.0	N	0.01 (P)
165	20.6	3.0/3.0	5.6/5.7	0/0	4.3/4.3	N	0.11 (P)
140.1	24	2.4/2.5	5.4/5.0	0/0	4.4/4.3	N	0.00 (P)
52.2	8.4	0/0	2.3/2.4	0/0	0/0	N	0.40 (P)
37 [3]	6.8	0/0	0/0	0/0	0/0	N	0.82 (P)
26	6.2	0/0	0/0	0/0	0/0	I	2.14
23	4.8	0/0	0/0	0/0	0/0	I	2.10
16	8.8	0/0	0/0	0/0	0/0	I	3.18

[1] N, bacterial growth not inhibited; I, bacterial growth inhibited.
[2] P, positive result.
[3] Values $< 50\,ng\,g^{-1}$ are below the LOQ of the LC-MS/MS method and estimated concentrations.

The range of the oxytetracycline concentrations in the incurred veal samples was large enough to permit good evaluation of all the methods for oxytetracycline (table 5). Concentrations below $50\,ng\,g^{-1}$ were not detected with agar diffusion tests. Only the thin plate inoculated with *B. cereus* could detect one sample with a residue concentration lower than the MRL. All samples with concentrations $> 100\,ng\,g^{-1}$ were detected with the thin *B. subtilis* plate, and with the thick plates inoculated with *B. cereus*. Two samples with concentrations between 100 and $200\,ng\,g^{-1}$ were not detected when meat fluid was analysed on the plate inoculated with *B. subtilis*. The Premi® test gave inconsistent results with samples incurred with oxytetracycline in repeated tests. The four samples with the highest levels and the three samples with the lowest levels of oxytetracycline inhibited growth of *B. stearothermophilus*. On the other hand, the five samples with intermediate oxytetracycline levels were negative. Finally, the receptor test Tetrasensor® could detect all veal samples with oxytetracycline concentrations equal to or above $44\,ng\,g^{-1}$.

A summary of all the tests done on the different spiked and incurred samples and of the results of this tests is given in table 6.

Table 6. Suitability of the methods tested for detection of four tetracyclines in spiked or incurred muscle tissue.

Method	Sampling	Samples spiked at 100 ng g^{-1}				Incurred samples (range of concentrations)	
		Oxytetracycline	Doxycycline	Chlortetracycline	Tetracycline	Oxytetracycline	Doxycycline
Agar diffusion test							
B. subtilis, thin	intact meat disk						OK
B. subtilis, thin	minced meat disk					OK	OK
B. cereus, thin	minced meat disk					OK	OK
B. subtilis, thick	meat fluid	N	OK	OK	N	N	OK
B. cereus, thick	meat fluid	N	OK	OK	N	OK	OK
Fast inhibition test							
Premi test	meat fluid	N	N	N	N	N	N
Receptor test							
Tetrasensor	extract 1/3 (w/v)	OK	OK	OK	OK	OK	OK

OK, all samples with residue concentrations at or above the MRL detected; N, not all samples with residue concentrations at or above the MRL detected.

Discussion

According to EC 2002/657/EC (Anon 2002), reports of residue tests should not mention positive and negative results, but instead the terms 'non-compliant' and 'compliant' should be used. A screening test result can be either compliant or suspect. However, the result can only be considered as compliant when the detection capability of the screening test is below the MRL for a given analyte. The actual multiresidue tests relying on inhibiting characteristics of antibiotics do not detect all antibiotics at MRL levels, and as long as the test is not validated for a given antibiotic or group of antibiotics, it is not known if the result is compliant or not. For example, a negative Premi® test result does not permit one to decide that the sample is compliant for tetracyclines, and a negative FPT result does not mean that the sample is compliant for sulphonamides (Korsrud *et al.* 1998), although both tests are intended as general screening tests for antibiotics. Indeed, they do not detect samples contaminated with the respective analytes at MRL levels. Therefore, as the terms 'suspect', 'compliant' and 'not compliant' are to be considered as juridical rather than scientific, the present authors preferred to use the terms 'positive' and 'negative'.

Validation of residue screening tests is most often done using samples with the analyte or analytes added at the required concentration, because it is impossible to produce incurred samples from different animal species with a specified concentration of residue. Nevertheless, this poses a problem when intact meat has to be analysed, as is prescribed for the FPT (Heitzman 1994). To avoid the difficulty of producing spiked undiluted samples, the inhibition zones obtained with meat fluid spiked with different tetracyclines were measured. For practical reasons, it was supposed that the drug concentration in the fluid was approximately equal to the drug concentration in the whole tissue, and 100 ng of the respective antibiotic standards were added to 1 ml fluid. The amount of test material is more standardized compared with the meat disks tested in the FPT, and this seemed another advantage. Meat disks can be analysed on thin plates, less than 0.4 mm thick, while a depth of 2 mm is needed in order to add 100 µl fluid in wells with 1 cm diameter. However, the analysis of the incurred samples demonstrated that the detection capability was not enhanced compared with analysis of intact meat. Moreover, not all tetracyclines were detected at MRL levels in spiked fluid: the method was satisfactory for doxycycline and chlortetracycline but not for the other two. The detection capabilities of the thin plates are probably more favourable because the analytes diffuse only in one direction, and larger zones are obtained in this way. The thickness of the agar plates is an important parameter influencing the quality and performance of such tests.

The detection capability of the plates seeded with *B. cereus* for tetracyclines was better than that of the plates seeded with *B. subtilis*. It must be remembered that *B. subtilis* media detect other antibiotic families as well as tetracyclines, while plates seeded

with *B. cereus* are more specific for tetracyclines (Okerman *et al.* 2001). This selectivity for specified groups is only observed with the usually low residue concentrations; very high concentrations that can be found in injection lesions will also produce growth inhibition zones on the more selective plates (unpublished data). In practice, most of the muscle samples that inhibit *B. subtilis* contain tetracyclines (Okerman *et al.* 1998b). This is probably due to the frequent use of this group compared with others and to the high tissue affinity and slow elimination of some tetracyclines, especially doxycycline in poultry. The *B. subtilis* plate also detects fluoroquinolones, but only a minority of the positive poultry samples contains fluoroquinolone residues (unpublished data).

The incurred meat samples had been minced before they were subjected to the series of screening tests, and only the chicken samples with doxycycline had been analysed previously as intact meat on plates seeded with *B. subtilis* (table 4). The zones obtained with coarsely minced meat in this series were smaller than the zones obtained with intact meat on the same plates. Minced meat has a higher water-binding capacity than intact meat, and this may be an explanation of this finding.

Considering all results of agar diffusion tests of the incurred samples, it can be concluded that: (1) intact meat produces larger zones than minced meat; (2) *B. cereus*-seeded plates detect lower levels than *B. subtilis* plates; and (3) analysing undiluted meat fluid instead of whole tissue is not an advantage, as the detection capability is not enhanced and the sample preparation takes more time (table 6).

A practical conclusion can be made when evaluating the results of the agar diffusion tests done on the incurred samples. The LODs of doxycycline are much lower than the MRL, but the LODs of oxytetracycline are borderline. Thus, most samples inhibiting *B. subtilis* or *B. cereus* that are contaminated with doxycycline will contain levels below or even far below the MRL (table 4). This can be frustrating, because only a minority of the meat that fails the initial screening will eventually be condemned. On the other hand, it cannot be recommended to change the cut-off value of the zones, for example to 4 or 5 mm, because in that case the tests will fail to detect all samples with oxytetracycline levels equal to the MRL. The same remark holds for Tetrasensor®. In practice, all positive results of screening tests should be confirmed by using a quantitative chromatographic method (Anon 2002).

The manufacturers of the Premi® test claim that the LODs of three tetracyclines, oxytetracycline, chlortetracycline and tetracycline, are at MRL levels of $100\,ng\,g^{-1}$. In the authors' hands, the Premi® test failed to detect these levels in spiked meat fluid (table 2). Moreover, inconsistent results were obtained with the veal samples incurred with oxytetracycline. The variation in time needed for a colour change of the indicator probably depends on other characteristics of the meat, such as its fat and water content, and may be large enough to produce false-positive as well as false-negative results. Five of the incurred chicken samples contained doxycycline levels around the MRL, and none was detected with the Premi® test, not even a sample with $108.8\,ng\,g^{-1}$. Thus, the results with the spiked and incurred samples indicate that the Premi® test is not suited for detection of tetracyclines. The main advantage of the Premi® test is its good detection capability of sulfonamides and some macrolides compared with the FPT, which fails for these groups (Okerman *et al.* 1998a). The Premi® test should certainly not be considered as a miraculous tool that detects all antibiotics in all kinds of meat.

Tetrasensor®, the receptor test based on the TetR protein receptor, which is specific for tetracyclines, detected all samples spiked with tetracycline, oxytetracycline, doxycycline and chlortetracycline. The principle of Tetrasensor® is new, as receptor tests have not been used up to now for tetracyclines. The Charm II system, which is partly based on bacterial receptors for detection of different groups of antibiotics, includes a radioimmunoassay for tetracyclines (Korsrud *et al.* 1994, Operator's Manual of Charm II Test 1994). Tetrasensor® was the only screening test that detected all samples spiked or incurred with one of the tetracyclines (table 6) at levels equal to or above the MRL, and thus can be considered as fully validated for the whole group.

Another simple way to screen for tetracyclines was described by Kühne *et al.* (2001). All tetracyclines accumulate in bone tissue resulting in a yellow fluorescence of the long bones when examined under long wave ultraviolet light (365 nm). The technique has been recommended for large slaughter animals but can also be applied to the long bone of the pelvic limb, the tibiotarsus, or to the wing bones (radius, ulna and humerus) of broilers treated with doxycycline (unpublished data). Two practical objections can be made: only samples containing bones can be inspected for this characteristic and it is very difficult

to prove that all non-compliant samples will be detected.

Conclusions

The choice of the most suitable screening method for the whole group of tetracyclines depends on the practical situation in the laboratory. When large numbers of samples have to be analysed and immediate results are not needed, classical agar diffusion tests with thin plates and performed as prescribed for the FPT still seem the most economical choice. However, the plate seeded with *B. subtilis* might fail to detect all non-compliant samples contaminated with oxytetracycline or with tetracycline (table 6). The detection capability of plates seeded with *B. cereus* is more appropriate and most probably also performing adequately for oxytetracycline, as was shown with incurred samples. The receptor test Tetrasensor® is recommended for official surveys and also in cases when immediate results are needed. Unlike the inhibition tests, the use of this receptor test does not require a well-equipped laboratory and is more suited for the meat industry. The Premi® test failed to detect tetracyclines at MRL levels in muscle tissue, and is not recommended for that purpose.

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