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Inhibition tests for detection and presumptive identification of tetracyclines, beta-lactam antibiotics and quinolones in poultry meat

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A combination of three plates, seeded with strains of Micrococcus luteus, Bacillus cereus *or* Escherichia coli, *can be used for detection of residues of beta-lactam antibiotics, tetracyclines and fluoroquinolones. The sensitivity of each plate is optimal for only one of these groups, resulting in detection limits (LOD) lower than the corresponding maximum residue limits (MRL) and in distinct inhibition patterns typical for each antibiotic family. Beta-lactam antibiotics such as penicillin G, ampicillin and amoxicillin give only inhibition zones on the plate with* M. luteus. *Tetracyclines are detected up to the MRL level with* B. cereus, *and fluoroquinolones with* E. coli. *The LODs of the antibiotics tested were as follows: penicillin G (PENG) 0.9 ng, ampicillin (AMPI) 0.6 ng and amoxicillin (AMOX) 1.0 ng on the plate with* M. luteus; *tetracycline (TET) 4 ng, oxytetracycline (OXY) 3 ng, doxycycline (DOX) 0.6 ng, and chlortetracycline (CHL) 0.3 ng on the plate with* B. cereus; *enrofloxacin (ENRX) 1.5 ng, ciprofloxacin (CIPX) 0.5 ng and flumequine (FLUM) 1.5 ng on the plate with* E. coli. *The combination of plates enables the laboratory to select appropriate chromatographic techniques for identification and quantification of the residues. On the other hand, the three groups can also be detected on one plate seeded with* Bacillus subtilis, *although the limits of detection are higher: PENG 0.4 ng, AMPI and AMOX 3 ng; TET 5 ng, OXY 8 ng, DOX 1 ng, CHL 0.5 ng, ENRX 4 ng, CIPX 10 ng and FLUM 4 ng. The test was applied to 228 broiler fillets and to 27 turkey thighs, originating from different poultry slaughterhouses. Nineteen broiler fillets contained inhibiting substances. The positive results of the inhibition tests were confirmed with a chromatographic technique. Doxycycline residues were found in 16 samples and amoxicillin in two.*

Keywords: antibitiotic residue, poultry, inhibition test

Introduction

Maximum residue limits (MRL) have been established for all antibiotics allowed for use in food-producing animals (Anadón and Martínez-Larrañaga 1999). Violation of the MRL regulation can only be proven with fully validated chromatographic methods such as high-performance liquid chromatography (HPLC) (Moats 1997), or by hyphenated techniques such as gas chromatography–mass spectrometry (GC–MS) (Pfennig *et al.* 1998) and liquid chromatography–mass spectrometry (LC–MS) (De Wasch *et al.* 1998). These methods require highly sophisticated equipment. Examination of routine samples for the presence of residues of all possible antibiotics with chromatographic methods is too expensive in practice: each antibiotic family requires a different extraction and detection procedure, and many chromatographic methods are not even able to detect all members of the same family up to the MRL level. Therefore, samples should first be screened with easy, quick and inexpensive methods, in order to select samples that contain or may contain violative levels of antibiotic residues from the great majority of samples that do not contain such residues.

Microbiological methods, used for detection of antibiotics in animal tissues, rely on their ability to inhibit

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growth of sensitive bacteria (Johnston *et al.* 1981). Simple inhibition tests, using only one test bacterium and one medium, have a serious disadvantage: there is no indication which group or groups of antibiotics is or are involved. After a sample has been found positive, group-specific tests can be carried out before chromatographic identification is performed (Nouws *et al.* 1999), but this procedure requires a large amount of work, money and time.

The European four-plate test (FPT) (Heitzman 1994) was originally intended to detect five different groups of antibiotics: beta-lactam antibiotics, tetracyclines, sulphonamides, aminoglycosides and macrolides. In practice, the method does not detect sulphonamides, and is not reliable for detection of aminoglycosides or macrolides in meat (Okerman *et al.* 1998a, Korsrud *et al.* 1998). Moreover, the test does not indicate which one of the remaining groups is involved, because *Bacillus subtilis* is sensitive to tetracyclines, beta-lactam antibiotics and fluoroquinolones, which may all be found in poultry. This corresponds with earlier observations that all confirmed positives in 4795 samples from different animal species inhibited the plate seeded with *B. subtilis* at pH 6 (Okerman *et al.* 1998b).

Beta-lactam antibiotics, tetracyclines and quinolones may all be used as antibacterials in broilers, administered via the drinking water or incorporated in the feed. Active substances belonging to these families may occur as residues in poultry meat. In the present study, we compared a combination of three inhibition tests, based on three different test bacteria, with an inhibition test based on one plate seeded with *B. subtilis* as the test organism. With the combination of three plates, the LODs of the most important beta-lactam antibiotics, quinolones and tetracyclines are lower than the corresponding MRL levels, and the inhibition pattern allows differentiation between those groups. Both screening methods were evaluated with field samples obtained from different poultry slaughterhouses.

Materials and methods

Media used for maintenance of Escherichia coli *and* Micrococcus luteus *strains and for preparation of inocula*

Tryptone soya agar (TSA) (Oxoid CM131, Basingstoke, England) and tryptone soya broth (TSB) (Oxoid CM129), were prepared and autoclaved as indicated by the manufacturers.

Mist desiccans (MD) was used as a medium intended for conservation of bacterial strains at – 20 °C and was prepared as follows: 6 g of dextrose was added to 20 ml Brain Heart Infusion Broth (Difco 0037-17-08; Detroit, USA), prepared according to the manufacturer's instructions but not autoclaved. This solution was sterilized by filtration and added to 60 ml inactivated horse serum. The medium was kept frozen for a maximum of 12 months before use.

Bacterial suspensions

B. subtilis (BGA) spore suspension (Merck, Darmstadt, Germany, No. 10649): this is a ready-to-use spore suspension.

M. luteus bacterial suspension: the ATCC9341 strain was prepared as described in the *Manual of Reference Materials and Methods to Detect Veterinary Drug Residues* (Heitzman 1994), but MD was used instead of culture broth to maintain the stock inoculum. A few colonies were suspended in 0.5 ml MD in sterile Eppendorf tubes; these tubes were kept frozen until needed.

E. coli suspension: a freeze-dried strain of *E. coli* Bayer 14 (kindly provided by Robrecht Froyman, Bayer, Leverkusen, Germany) was reconstituted and inoculated onto TSA in a Petri dish. The plate was incubated for 24 h at 37 °C and inspected for purity. Sterile Eppendorf tubes with 0.5 ml MD were inoculated with several colonies of the *E. coli* strain. This stock inoculum was kept at – 20 °C for a maximum of 2 years. When needed, the stock inoculum was thawed and inoculated onto a TSA plate. After overnight incubation at 37 °C the plate was inspected for purity. Ten ml of TSB were inoculated with several colonies obtained on the TSA plate and incubated overnight. The TSB culture, which contained at least 5×10^8 colony forming units (cfu) of the *E. coli* strain per ml, was diluted 1 to 10 in sterile TSB before adding to the final medium.

Bacillus cereus spore suspension: the ready-to-use spore suspension (Bacto cereus spore suspension, Difco) was used according to the manufacturer's instructions.

Media for residue testing

Four batches of test agar pH 6 (Merck; dehydrated medium 10663) were prepared and autoclaved. After cooling to 45–50 °C, one of the above mentioned bacterial or spore suspensions were added, each to one of the batches:

- to the general medium, included for comparative purposes: 0.1 ml of *B. subtilis* spore suspension in 100 ml of medium;
- to the medium intended for detection of beta-lactam antibiotics: *M. luteus* ATCC9341. The final bacterial density in the medium should be between 4 and 6×10^4 cfu;
- to the medium intended for detection of tetracyclines: 0.1 ml of *B. cereus* spore suspension to 100 ml of medium;
- to the medium intended for detection of quinolones: 0.05 ml of the diluted *E. coli* Bayer 14 suspension to 100 ml of medium.

Petri dishes of 90 mm diameter were filled with 5 ml of the prepared and seeded media, as prescribed for the four-plate test (Heitzman 1994). The plates were cooled to 4 °C immediately thereafter, and kept at that temperature until use (maximum 4 days). As the amount of medium is very small, the plates should be filled carefully in order to obtain a thin, even layer of agar.

Determination of LODs

LODs of 14 different antibiotics, belonging to three different antibiotic families, were determined: the beta-lactam antibiotics penicillin G (PENG), ampicillin (AMPI), amoxicillin (AMOX); the tetracyclines tetracycline (TET), oxytetracycline (OXY), chlortetracycline (CLT) and doxycycline (DOX); the fluoroquinolones flumequine (FLUM), enrofloxacin (ENRX), and ciprofloxacin (CIPX). All standards were purchased from Sigma, with the exception of the quinolones ENRX and CIPX, which were obtained from Bayer.

Stock solutions of 1 mg/ml were prepared of each standard: PENG was dissolved in distilled water; AMOX and AMPI in a 0.1 M phosphate buffer, pH 8; tetracyclines in methanol; quinolones in 0.1 N NaOH. The LODs were first estimated roughly by previous testing of ten-fold dilutions of the antibiotics, and thereafter determined by testing two-fold dilutions. Ten µl of the two-fold dilutions in distilled water were placed upon four paper disks, diameter 6-mm, which were laid on the agar layers at a distance of approximately 1 cm from the edges of the plates. The concentration range was chosen around the expected LOD, in order to obtain zones ranging from less than 12 mm up to 25 mm. These concentrations were different for each antibiotic and each plate. Very high LODs were not determined exactly, because such high residue concentrations are unlikely to occur in field samples. The plates were all incubated overnight at 30 °C, except those seeded with *M. luteus*, which were incubated for 20–24 h at 37 °C. The diameters of the zones were measured. A regression line of the zone diameters vs log(ng/disk) enabled to calculate the LOD, this is the concentration producing zones with a diameter of 12 mm.

Testing of routine samples

Two hundred and twenty-eight fillets from broiler chickens and 27 turkey thighs were collected in different slaughterhouses in Belgium and the Netherlands. They were frozen immediately after arrival and kept at – 20 °C in the laboratory until analysis. Meat was sampled while still frozen. Cylinders of frozen meat, sampled as described earlier (Okerman *et al.* 1998b), were cut into eight disks, each *ca* 2 mm thick and weighing approximately 0.1 g. Two disks of a sample were placed on opposite ends of each of the four plates, at a distance of approximately 1 cm from the edges. All plates were subjected to a quality control: paper disks were placed in the middle and 10 µl of PENG (2 IU/ml) was placed upon plates seeded with *B. subtilis* and with *M. luteus*; OXY (2 µg/ml) or FLUM (2 µg/ml) were placed respectively on plates seeded with *B. cereus* and with *E. coli.* The plates inoculated with *B. cereus*, *B. subtilis* and *E. coli* were incubated overnight at 30 °C, and the plates seeded with *M. luteus* were incubated at 37 °C for 24 h. Then they were inspected for inhibition zones around the control disks and around the samples. When both meat disks on a plate showed complete inhibition zones of 2 mm or more in width, the result was recorded as positive.

Samples giving positive results on the plate inoculated with *M. luteus* were retested in the presence of

penicillinase. Penicillinase is an enzyme that breaks down the penicillins PENG, AMPI and AMOX into microbiologically inactive products, by cleaving the beta-lactam ring (Moats *et al*. 1998). Four meat disks of each positive sample were prepared as described and brought upon inoculated plates. Paper disks impregnated with 10 µl penicillinase solution (Bacto penase, Difco 0345-63-8) were laid at a distance of approximately 2 mm from two sample pieces. The plates were examined after 24 h of incubation at 37 °C for the presence of inhibition zones around the meat pieces and for the absence of inhibition around the disk impregnated with penicillinase solution.

Chromatographic identification and quantification of residues in inhibition-positive samples

Confirmation and quantification of presumptively identified tetracyclines was performed using a validated HPLC method with fluorescence detection. The method has been described in detail in a previous report (Croubels *et al*. 1997). All but one sample with zones larger than 2 mm on the plate seeded with *B. cereus* were tested with this technique. One sample was not analysed because the amount of tissue left was too small. In brief, this procedure involved placing 3 g of minced tissue in a centrifuge tube. The samples were spiked at this point with the internal standard, demethylchlortetracycline. The contents were homogenized with 20 ml of a 0.1 M sodium succinate buffer (pH 4.0) and shaken for 10 min on a horizontal shaker. Next, 20 ml of methanol were added, followed by ultrasonication for 5 min and centrifugation for 10 min at 2600 rpm. The clean-up of the filtered supernatant was done with the metal-chelate affinity chromatography (MCAC) technique. Further concentration of the MCAC eluate was performed on an Empore® extraction membrane with reversed-phase and cation-exchange properties. After application of the pH-adjusted MCAC eluate, the membrane was washed with 1 ml of 0.1 M HCl and DOX was eluted with 4 × 250 µl of HPLC-grade methanol containing concentrated (25%) ammonia (97:3, v/v). The extract was evaporated to dryness under nitrogen (40 °C). The dried sample was reconstituted with 250 µl of 0.01 M oxalic acid in HPLC-grade water, vortexed and ultrasonicated. A 20 µl aliquot was injected into the HPLC system. A reversed-phase polymeric column (type PLRP-S, 100 Å, 8 µm, 250 × 4.6 mm i.d.) was used in combination with a PLRP-S guard cartridge of 5 × 3.0 mm. The mobile phase contained 0.01 M oxalic acid in water (A), acetonitrile (B) and methanol (C). A gradient solvent programme with the following conditions was run: 0 to 5 min (A–B–C, 80:15:5); 5 to 20 min (A–B–C, 40:20:40); 20 to 25 min (A–B–C, 80:15:5) and at 35 min the next injection was done. These conditions made it possible to separate DOX and 4-EDOX. The flow rate was set at 1 ml/min. The fluorescence was induced by complexation of DOX with the zirconium cation (5% (m/v) zirconyl chloride octahydrate in HPLC-grade water), which was added post-column to the HPLC eluate. This fluorescence detection made it possible to detect residues below the MRL of 100 µg/kg in muscle. The limit of quantification (LOQ) of the proposed method is below one-half of the MRL, since the LOQ is 10.0 µg/kg for DOX in muscle tissue. This LOQ is defined as the lowest concentration for which the method is validated with an accuracy and precision that fall within the ranges recommended by the EU (Heitzman 1994). The LOD is defined as three times the peak-to-peak noise determined at the retention time of DOX.

Two samples, one which inhibited *M. luteus* and one which inhibited *B. subtilis*, were selected for chromatographic detection of AMOX and AMPI. The method will be published in detail soon and is described briefly hereafter, (De Baere *et al*., manuscript in preparation). Cephadroxyl was added as an internal standard to a concentration of 250 ng/g. The tissue was extracted twice with phosphate buffer (pH 4.5), followed each time by a 10 min centrifugation step. After deproteinization with trichloroacetic acid, the supernatant was further purified using a C18 solid-phase extraction column. The sample was eluted with acetonitrile and evaporated until dryness. The dry extract was redissolved in 200 µl of water and a 50 µl aliquot was injected onto the LC–MS. The chromatographic separation was performed using a Waters Alliance 2690 Separations Module and a reversed-phase Nucleosil column (Chrompack). The mobile phase consisted of 20 mm pentafluoropropionic acid in water and acetonitrile (60/40, v/v) and was delivered to the HPLC column at a flow rate of 200 µl/min. An isocratic elution was performed. Detection was performed using a Quattro Ultima mass spectrometer (Micromass, Manchester, UK), equipped with an electrospray ionization source, which was operated in the positive ion MS/MS mode. The multiple reaction monitoring (MRM) transitions used were $m/z = 366.1 > m/z = 207.4$ for amoxicillin and $m/z = 350.30 > m/z = 05.9$ for ampicillin. The

LOQ values were 25 ng/g and the LOD values 5 ng/g for both compounds.

Results

In table 1, the LODs of 10 antibiotics on four different plates are compared. The method is designed to analyse intact pieces of muscle tissue and should detect an amount of residue equal to the MRL in 100 mg of sample. In table 1, LODs are not expresssed as concentrations but as absolute quantities, and, because the matrix may influence detection, these should be lower than the corresponding MRLs. MRLs are usually given in µg/kg (Anonymous 2000), but in order to compare the LODs with the MRL, table 1 contains MRL values in ng/100 mg. All tested antibiotics can be found on the plate seeded with *B. subtilis*, but the LODs are not optimal. The three other plates, seeded with respectively *M. luteus*, *E. coli* and *B. cereus*, are each optimally sensitive for one group and much less sensitive for the other antibiotic families. The different antibiotic families give a distinct inhibition pattern on the three plates. The beta-lactam antibiotics PENG, AMPI and AMOX are easily detected with *M. luteus*, and not on both other plates. The tetracycline family is the only one which is detected far below the MRL on the plate seeded with *B. cereus*. Low concentrations of fluoroquinolones can be found on the plate seeded with *E. coli*, less easily on the plate seeded with *B. cereus*, and not on the plate seeded with *M. luteus*.

The results of the inhibition tests of 228 broiler breast fillets and 27 turkey thighs are given in table 2 and table 3. In table 2, the number of samples with the different inhibition is given, while the width of the zones and the results of the chromatographic identifications of the individual positive samples are given in table 3.

None of the turkey samples reacted positive, but 19 out of the 228 broilers contained inhibiting substances. Eighteen were positive with the discriminating combination, while only 12 gave an inhibition zone on the *B. subtilis* plate. Seventeen samples inhibited *B. cereus*, and DOX was detected in the 16 samples that were investigated with HPLC with fluorescence detection. One sample with small zones was not analysed with HPLC, because the amount of tissue left was insufficient. The concentration was higher than the MRL of 100 ng/g in four samples, and below the limit of quantification (LOQ) of 10 ng/g in two samples. The width of the inhibition zones of the samples with DOX concentrations exceeding the MRL was larger than 4.5 mm on the plates seeded with *B. cereus* as well as with *B. subtilis* and this observation confirms the high correlation between doxycycline residue concentration and inhibition zones found earlier (De Wasch *et al.* 1998). In

Table 1. Limits of detection (LOD) of penicillins, tetracyclines and quinolones on pH 6 media seeded with different test bacteria.

			Concentrations (ng/disk) producing zones with a diameter of 12 mm on plates seeded with			
Group	Compound	MRL in ng/100 mg[a]	*B. subtilis* (BGA)	*M. luteus* ATCC9341	*E. coli* Bayer 14	*B. cereus*
Penicillins	Penicillin G	5	0.4	0.9	> 80	> 80
	Ampicillin	5	3	0.6	> 80	> 80
	Amoxicillin	5	3	1.0	> 80	> 80
Tetracyclines	Tetracycline	10	5	40	20	4
	Oxytetracycline	10	8	30	10	3
	Doxycycline	10	1	20	20	0.6
	Chlortetracycline	10	0.5	10	7	0.3
Quinolones	Enrofloxacin	10[b]	4	> 80	1.5	40
	Ciprofloxacin	10[b]	10	> 80	0.5	30
	Flumequine	40	4	> 400	1.5	40

[a] Approximately 100 mg of intact tissue is tested.
[b] Sum of enrofloxacin and ciprofloxacin (enrofloxacin is metabolized *in vivo* into ciprofloxacin).

Table 2. Screening field samples for antibiotic residues: results of the B. subtilis *general test compared with a combination of three plates used for detection and presumptive identification of antibiotic families.*

Possible inhibition patterns		Presumptive identification	Number of samples	
B. subtilis	Combination of three plates		Broiler fillets	Turkeys
Positive[a]	Negative	?	1	0
	Positive on plate with *B. cereus*	Tetracyclines	11	0
	Positive on plate with *M. luteus*	Beta-lactam	0	0
	Positive on plate with *E. coli*	Fluoroquinolones	0	0
Negative	Positive on plate with *B. cereus*	Tetracyclines	6	0
	Positive on plate with *M. luteus*	Beta-lactam	1	0
	Positive on plate with *E. coli*	Fluoroquinolones	0	0
Total number of positives/number investigated			19/228	0/27

[a] Positive: all samples giving zones with a width of 2 mm or more around both meat disks.

general, the zones were slightly smaller with *B. subtilis* than with *B. cereus*, and five samples with low levels of DOX residues were not found with *B. subtilis*.

Low levels of AMOX residues were detected in the two other samples. One sample inhibited *M. luteus* and one sample inhibited *B. subtilis*, with large zones. The inhibition zone around the first sample was affected by penase; zones with *B. subtilis* were not retested with penase.

None of the investigated samples inhibited *E. coli* Bayer 14.

Discussion

Considering the LODs of antibiotics belonging to different groups, it appears that the combination of

Table 3. Individual results of positive broiler breast fillets: inhibition zones on four different plates and quantitative results of confirmation techniques.

Width of inhibition zones (mm) on plates seeded with				
B. subtilis	*B. cereus*	*M. luteus*	*E. coli*	HPLC (tetracyclines) or LC-MS/MS (beta-lactam) confirmation
3.6/2.5	3.6/3.9	< 2/< 2	< 2/< 2	Doxycycline 22 ng/g
4.1/4.8	5.7/4.9	< 2/< 2	< 2/< 2	Doxycycline 58 ng/g4
0/4.2	6.2/5.6	< 2/< 2	< 2/< 2	Doxycycline 63 ng/g
7.7/7.7	8.1/8.5	< 2/< 2	< 2/< 2	Doxycycline 472 ng/g
2.7/2.8	3.9/3.7	< 2/< 2	< 2/< 2	Doxycycline 30 ng/g
< 2/< 2	< 2/< 2	10.0/10.3	< 2/< 2	Amoxicillin (< LOQ[a])
4.4/3.0	4.4/4.0	< 2/< 2	< 2/< 2	Doxycycline 64 ng/g
< 2/< 2	2.9/2.1	< 2/< 2	< 2/< 2	Doxycycline 69 ng/g
4.8/4.9	5.5/5.6	< 2/< 2	< 2/< 2	Doxycycline 107 ng/g
5.5/4.8	6.2/4.7	< 2/< 2	< 2/< 2	Doxycycline 114 ng/g
< 2/< 2	2.6/2.7	< 2/< 2	< 2/< 2	Doxycycline 32 ng/g
5.4/6.2	5.6/6.1	< 2/< 2	< 2/< 2	Doxycycline 116 ng/g
2.9/3.8	3.6/4.0	< 2/< 2	< 2/< 2	Doxycycline 67 ng/g
< 2/< 2	2.1/3.6	< 2/< 2	< 2/< 2	Doxycycline (< LOQ)
< 2/< 2	3.8/3.4	< 2/< 2	< 2/< 2	Doxycycline (< LOQ)
4.1/3.7	5.1/6.2	< 2/< 2	< 2/< 2	Doxycycline 65 ng/g
< 2/< 2	3.1/3.3	< 2/< 2	< 2/< 2	Not analysed
< 2/< 2	2.9/3.0	< 2/< 2	< 2/< 2	Doxycycline 18 ng/g
11.4/5.7	< 2/< 2	< 2/< 2	< 2/< 2	Amoxicillin (< LOQ)

[a] LOQ = limit of quantification; LOQ of doxycycline = 10 ng/g; LOQ of amoxicillin = 25 ng/g.

three plates seeded with different bacterial strains, i.e. *M. luteus* ATCC 9341, *E. coli* Bayer 14, and *B. cereus* has two important advantages when compared with one plate seeded with *B. subtilis*. First, the LODs of most antibiotics are lower with the combination. Second, the sensitivity of each plate of the system is optimal for only one of the antibiotic families tested. A technical note by Valset and Yndestad (1989) prescribes an identical combination of test bacteria, *B. cereus*, *M. luteus* and *E. coli* ATCC11303, for detection of antibiotics and chemotherapeutics in fish. The different patterns of the 10 products tested can be deduced from their LODs given in table 1. The penicillins are only detected with *M. luteus*. The plate seeded with *E. coli* detects the quinolones, and the tetracyclines are the only group detected effectively with *B. cereus*. The discriminating power of the combination was confirmed with naturally-contaminated samples. Even the field sample with the highest concentration of DOX, 472 ng/g, did not inhibit *M. luteus* nor *E. coli*, although the LODs of DOX on the other two plates are sufficiently low to detect high levels of these drugs,

The presence of PENG, AMPI and AMOX can be confirmed microbiologically with the use of penicillinase. The commercial penase solution can be incorporated into the medium but this requires the preparation of a new batch of medium (Ferrini *et al.* 1997). Therefore we retested the positive samples without and with paper disks impregnated with penicillinase, deposited at a distance of 2 mm from the samples. A penase test was recorded as positive when the inhibition zone was interrupted around the penase disk. Penase breaks down penicillin as well as ampicillin and amoxicillin. Cephalosporins are much less sensitive to this enzyme (Moats *et al.* 1998), but cephalosporins are not used in industrial broilers because these products are expensive and not available for oral therapy of poultry. Only one sample inhibited *M. luteus*, with a positive penase effect. AMOX was identified in the sample, but this antibiotic was also found in another sample which inhibited *B. subtilis* and not *M. luteus*. Possible explanations for this discrepancy are: inactivation of the compound during incubation at 30 or 37 °C, uneven distribution of AMOX in the samples, differences in diffusion into the medium, or an effect of the matrix. Beta-lactam residues can be detected with *B. subtilis* as well as with *M. luteus*, but the inconsistent results indicate that false negatives might occur with both plates. On the other hand, the concentration of AMOX in both samples was below the MRL.

No inhibiting substances were detected in the relatively small number of turkeys tested. A previous survey on a larger number of turkeys revealed that inhibiting residues were commonly found in Britain and rarely in west and central Europe (Okerman *et al.* 1998b). On the other hand, the percentage of samples from broilers that contained antibiotic residues was high: DOX residues were found in approximately 7.5% of samples and beta-lactam antibiotics in less than 1%. The majority of poultry carcasses with inhibiting substances contained DOX residue levels below the MRL. Such meat is not considered unfit for consumption, but our results are an indication that DOX is not always administered to broilers according to 'good veterinary practices'. These positive results should not be considered as false positives. The high number of samples found with DOX residues below the MRL is due to the higher sensitivity of *B. cereus* and *B. subtilis* to this product, as compared with other tetracyclines such as OXY and TET. It is impossible to develop screening tests which detect all samples with residue levels above the MRL, and none with levels beneath the MRL. This is especially the case with multi-residue tests, because differences in LOD between substances are common. The sensitivity of a screening test has been defined as the probability of finding positive a sample fortified with a specified concentration of the analyte (most often the MRL). High sensitivities are only obtained when LODs are below the MRL.

No fluoroquinolones were found in this selection of routine samples. Fluoroquinolones, such as ENRX, are indicated for several diseases in poultry, but the drug is rather expensive and probably not often used in broilers at the end of the fattening period. Earlier experiments have shown that high levels of enrofloxacin and its active metabolite ciprofloxacin can be detected with *B. subtilis* (Okerman *et al.* 1998b). Also we have found FLUM residues in other samples from poultry with the plate seeded with *E. coli* (unpublished data). All observations indicate that the presence of quinolone residues is not as common as DOX residues in Belgian and Dutch broilers.

The discriminative method is intended for detection of residues of beta-lactam antibiotics, quinolones and tetracyclines. Three other groups, which may be administered to broilers and turkeys, are not found: aminoglycosides, sulphonamides and macrolides. Aminoglycosides, such as streptomycin and neomycin, are not absorbed from the gut and thus unlikely to be present as residues in the muscles of poultry.

Routine screening tests used in Europe and in the US do not detect sulphonamides and macrolides up to the MRL level in intact muscle tissue (Korsrud *et al.* 1998). Recently, an inhibition test with low LODs for sulphonamides, beta-lactam antibiotics and macrolides has been commercialized for use with meat samples. The test is carried out with fluid from meat, in tubes containing a medium seeded with *Bacillus stearothermophilus* and an indicator for growth. Its performance with incurred or field samples has not yet been determined.

The combination of plates described herein should be compared with other similar methods. Several plates and test bacteria, or combinations of plates, which can be used for detection of antibiotic residues in meat, have been described previously (Ellerbroek 1991, Ellerbroek *et al.* 1997, Ferrini *et al.* 1997), but the performance of such methods with samples obtained from slaughterhouses or retail outlets has seldom been evaluated. Most methods claim to detect a large range of substances and a critical evaluation requires that the presence of these drugs is confirmed by another, unrelated method, such as EIA or chromatography. Johnston *et al.* (1981) developed an inhibition test with *B. subtilis* and tested 351 muscle samples from different animal species, including poultry, with this assay. They found 46 positive results. The inhibiting substances were not identified. Myllyniemi *et al.* (1999) used a combination of 18 plates in order to identify inhibiting substances found in muscle or kidney tissue with *B. subtilis*. Nine different test bacteria, four media, and three additives were needed to prepare all 18 plates, making this combination unsuited as a first step in a screening procedure, but, in fact, the method was developed as a microbiological identification procedure, and performed well for that purpose. Their collection of inhibitor-positive samples originated from large farm animals, and only PENG, OXY, ENRX + CIPX, AMPI and streptomycin were identified in the kidney samples. The influence of the pH of the medium on detection of antimicrobials was less clear when examining muscle tissue than with standard solutions, which resulted in less pronounced inhibition patterns. The pH of intact muscle is usually lower than 6, and a high medium pH will not be maintained around the samples during incubation in the thin agar layer. Our much less complex combination of three plates uses only one medium, with a low pH, and the distinct inhibition patterns are only dependent on the test strains. Moreover, all substances that were identified with the 18-plate method can also be detected with our three plates, with the exception of streptomycin, but this product does not occur in muscle tissue from poultry.

We concluded that the combination of test bacteria can be used as a routine screening method for the presence of tetracycline, beta-lactam and quinolone residues in poultry meat. However, when the cost of using three plates is considered to be too high, one plate seeded with *B. subtilis* is a good alternative; positive samples can then be tested with the combination of plates for a presumptive identification of the three groups mentioned above.

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