

## Comparison of various assays used for detection of beta-lactam antibiotics in poultry meat

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### Abstract

In this study, microbiological tests for the detection of beta-lactam antibiotics in meat and meat products were evaluated. The traditional FPT (four plate test, containing *Bacillus subtilis* and *Kocuria rhizophila*), BsDA (*Bacillus stearothermophilus* disc assay) and a newly developed microbiological test, Premi<sup>®</sup>Test (containing *Bacillus stearothermophilus*) were included in the study. The limit of detection (LOD) of the Premi<sup>®</sup>Test was compared with the LOD of the traditional methods. The detection limits of the tests were determined by using beta-lactam antibiotic standards dissolved in meat juice, as well as meat tissue obtained from laying hens after experimental administration of amoxicillin. Positive samples, based on inhibition of growth of the organism in the test, were confirmed by high performance liquid chromatography (HPLC). Growth inhibition in the traditional tests is visible as a clear zone on the plate, whereas for Premi<sup>®</sup>Test, this is based on the absence of a colour change of the test. The LODs of antibiotics tested were as follows: Penicillin G (PENG) 5 µg kg<sup>-1</sup>, amoxicillin (AMOX) 10 µg kg<sup>-1</sup>, ampicillin (AMP) 25 µg kg<sup>-1</sup>, oxacillin (OXA) 30 µg kg<sup>-1</sup>, and cloxacillin (CLOX) 30 µg kg<sup>-1</sup> on the plate with *Bacillus stearothermophilus*. Beta-lactam antibiotics can be detected also on one plate seeded with *Kocuria rhizophila*, although the LODs are higher: PENG 10 µg kg<sup>-1</sup>, AMOX 25 µg kg<sup>-1</sup>, AMP 30 µg kg<sup>-1</sup>, OXA 50 µg kg<sup>-1</sup>, and CLOX 50 µg kg<sup>-1</sup>. Premi<sup>®</sup>Test was performed according to the Standard Operating Procedure intended for detection of beta-lactam antibiotics in poultry tissues with following LODs: PENG 4 µg kg<sup>-1</sup>, AMOX 5 µg kg<sup>-1</sup>, AMP 5 µg kg<sup>-1</sup>, OXA 40 µg kg<sup>-1</sup>, CLOX 50 µg kg<sup>-1</sup>. All tests are able to detect beta-lactam antibiotics such as penicillin G, ampicillin, amoxicillin, oxacillin and cloxacillin below the maximum residue level (MRL). However, the detection limits of the Premi<sup>®</sup>Test for PENG, AMOX and AMP were below the limits of BsDA and the plate containing *Kocuria rhizophila*.

**Keywords:** BsDA, Premi<sup>®</sup>Test, beta-lactam antibiotics

### Introduction

The administration of antimicrobial drugs to food producing animals may lead to the presence of residues in edible products. Growing concern among consumers and public health authorities regarding the presence of antibiotic residues in food products demands a better control in the use of antimicrobials in animal production. As long as antibiotics are used on a large scale in the intensive poultry farming, we have to be aware of the risk of their occurrence in the food chain. Several concerns regarding the use of antibiotics are the development of resistance due to the transfer of antibiotic resistance genes to human pathogens, the effect on the composition of the human intestine microflora, potential allergic reactions in sensitized individuals, direct toxicity and technological problems of fermented meat products (Stark 2000).

Beta-lactam antibiotics are one of the most widely used antibiotics for human and animal therapy. Drug residues may be present in slaughter animals and this situation leads to more strict regulations for the occurrence of antibiotic residues in animal food products (Directive EC 96/23 1996). Therefore, maximum residue limits are set by the European Council (EEC Directive 2377/90 and amendments) for residues of veterinary drugs in foodstuff from animal origin. There is a consumer's expectation that the food products do not contain concentrations of antibiotics over the set limits.

Antibiotic residues in animal tissues are traditionally determined by microbiological tests. Microbiological inhibition tests are considered as multi-residue screening tests for antibiotics in milk, meat and other animal tissues. Tests like BsDA and FPT have been used to analyse meat samples, which are laid directly on the agar medium, which is

inoculated with an antibiotic sensitive bacterial strain.

Methods based on inhibition are useful for the detection of an antibiotic or a group of antibiotics. For these tests, the micro-organism *Bacillus spp.* is often used. However, inhibition tests may be of high sensitivity but they lack specificity. They cannot distinguish among different forms of beta-lactam antibiotics, therefore every inhibition test can only be used as a tool for screening. Some of the microbiological methods may be very time-consuming and cumbersome.

The FPT is described in the Manual of Reference Materials and Methods to Detect Veterinary Drug Residues (Heitzman 1994). It is intended to detect residues of beta-lactams and other groups of antibiotics in muscle tissue of slaughtered animals. The FPT is qualitative and recommended for screening only. The FPT is based on the inhibition of growth of the micro-organism, which is included in the test. This inhibition becomes visible as a clear zone around the sample; in the case antibiotic substances are present (at or above the LOD of the plate). The size of this zone is dependent on the antibiotic concentration: concentrations above the LOD will lead to larger zones.

An essential tool in assuring the safety of food products is the availability of a simple and reliable screening system for the detection of antibiotics. The procedures for conducting the screening tests need to be reliable, simple and inexpensive, and require very simple equipment. They have been used as methods of first choice when a qualitative decision is needed, in order to determine if a sample is contaminated with an antibiotic or not. Recently, a new broad spectrum screening test for the detection of antimicrobial residues in meat, Premi<sup>®</sup>Test, has been developed by DSM and is used for the detection of antibiotics in meat (Reybroeck 2000a). The Premi<sup>®</sup>Test is based on the inhibition of growth of *Bacillus stearothermophilus*, a thermophilic bacterium sensitive to many antibiotics (Stead et al. 2004).

The purpose of a microbiological inhibition test, like the plate tests and Premi<sup>®</sup>Test is to screen for samples which may contain amounts of antibiotic residues above certain limits. In routine

residue testing, the great majority of samples are evaluated with screening tests. Nevertheless, confirmation and quantification should be performed by using a more sensitive and sophisticated immunochemical and/or chromatographic method, like a validated HPLC method. These methods require sophisticated equipment and examination of routine samples for the presence of residues of all possible antibiotics, as well as different extraction and detection procedures. A chromatographic method should meet reasonable standards of precision, accuracy, specificity, and above all, it must be practical in use. Beta-lactams are separated in forms as derivatives with pre-column derivatization. Beta-lactams are able to form mercury mercaptide derivatives and specifically aminopenicillins are determined as fluorescent derivatives, which can be detected by UV detection at 325 nm. They are analysed by reversed phase liquid chromatography with fluorescence detection (Ang et al. 1996; Luo et al. 1997). Methods must be able to determine residues at the concerned or tolerance level as well as at one half and twice the tolerance level.

In our experiment, the detection limit of the Premi<sup>®</sup>Test was established for five beta-lactams at different concentrations. The results were compared with the standard microbiological FPT and BsDA methods. All samples found to be positive were confirmed by validated HPLC technique.

## Materials and methods

### Chemicals and reagents

Beta-lactams, including penicillin, ampicillin, amoxicillin, oxacillin, and cloxacillin were purchased from Sigma (St Louis, MO, USA). Separate stock solutions of penicillin, ampicillin, amoxicillin, oxacillin, and cloxacillin were prepared at a concentration of 1 mg per ml, by dissolving 25 mg of the antibiotic into 25 ml of deionized water. Working standard solutions (WSS) and meat juice samples for the antibiotics to be tested are prepared as described in Table I. Meat juice samples were obtained from antibiotic free poultry meat, using a garlic press for the extraction of the samples. This meat juice was used as a zero control. WSS were

Table I. Concentration ranges of tested antibiotics in meat juice samples.

Antibiotic	Concentration ranges ( $\mu\text{g kg}^{-1}$ )
Penicillin G	1; 2; 3; 4; 5; 10; 15; 25; 30; 40; 50; 100; 150; 200; 250; 500
Ampicillin	1; 2; 3; 4; 5; 10; 15; 25; 30; 40; 50; 100; 150; 200; 250; 500
Amoxicillin	1; 2; 3; 4; 5; 10; 15; 25; 30; 40; 50; 100; 150; 200; 250; 500
Oxacillin	10; 20; 30; 40; 50; 100; 150; 200; 250; 300; 400; 500; 1000
Cloxacillin	10; 20; 30; 40; 50; 100; 150; 200; 250; 300; 400; 500; 1000

stored in a cool place (2–4°C), protected from light for a maximum of five days.

### Methods

The FPT (four plate test) is described in the Manual of Reference Materials and Methods to Detect Veterinary Drug Residues (Heitzman 1994). It is intended to detect residues of beta-lactams and other groups of antibiotics in muscle tissues of slaughtered animals. The FPT consists of plates with *Bacillus subtilis* (BGA) spore suspension (Merck, Germany) and *Kocuria rhizophila* bacterial suspension (Merck, Germany). Media used for residue testing: Test agar pH 6.0 (Merck, dehydrated medium 10 663), test agar pH 7.2 with addition of trimetoprim (Merck, dehydrated medium 15 787), and test agar pH 8.0 (Merck, dehydrated medium 10 664). Media were prepared according to the manufacturer's instructions. After cooling down the agar to 45–55°C, cell and spore suspensions are added to the appropriate media. Sterile Petri dishes (diameter 90 mm) are filled with 8 ml of the inoculated media and stored at 2–5°C for a maximum of five days.

*Bacillus stearothermophilus* var. *calidolactis* disc assay (BsDA) consists of a plate utilizing a spore suspension of *Bacillus stearothermophilus* var. *calidolactis* C 953 (Merck, Germany). The spores are inoculated into indicator agar (Oxoid CM 261, England). Sterile Petri dishes (diameter 90 mm) are filled with 8 ml of the inoculated media and stored at 2–5°C for a maximum of five days.

Paper discs soaked with spiked meat juice samples have been tested with the FPT and BsDA. The paper discs (Whatman 1, diameter 12 mm) were soaked with 100 µl of sample and placed on the surface of the agar, at a minimum distance of 10 mm from the edge of the plate. Each antibiotic concentration was tested in fourfold and discs were placed two by two at opposite sites on the plate. The different plates used in the FPT, were incubated as follows: *B. subtilis* at 30 ± 1°C for 18–24 hours, *K. rhizophila* at 37 ± 1°C for 18–24 hours and *B. stearothermophilus* at 64 ± 1°C for 3 hours. After incubation, plates were read visually by measuring the clear zones around the sample discs with a ruler. Inhibition zones are indicated in mm. Sample discs containing zones of 2 mm and up are interpreted as positive.

Every series of the prepared plates for determination of antibiotics has to be tested. Testing is performed by applying a reference concentration of antibiotic standards spiked in meat juice on the plate used for determining its presence. After incubation, the diameter of the growth inhibition zone of the test culture has been measured. Since minimal difference in the diameter of the inhibition

zone of the test culture occurred (denser or thinner growth of the test culture), a so-called correction factor has been determined for every series of plates to their use.

Premi®Test (DSM, the Netherlands) was developed as a new method for antibiotic residue detection. Premi®Test is an ampoule, containing an agar medium, imbedded spores of *Bacillus stearothermophilus* var. *calidolactis* and a colour indicator. Premi®Test combines the principle of an agar diffusion test with colour change of the indicator. In case of an active metabolism of the included micro-organism, the test will change colour from purple to yellow. In case growth of the micro-organism is inhibited (due to presence of an antibiotic at or above the LOD) the test will remain purple. A total of 100 µl of the meat juice was placed onto the agar in the ampoule and incubated for 20 minutes at room temperature for pre diffusion. The meat juice remained onto agar and the ampoules were then incubated for 3 hours at 64 ± 1°C. The colour of all ampoules is read at the moment the negative control changed colour from purple to yellow. Inconclusive samples are repeated and confirmed by HPLC. In order to determine the LOD of the Premi®Test, each concentration was tested in fourfold.

Confirmation and quantification of amoxicillin is performed by using a validated HPLC method with fluorescence detection. This HPLC method was developed for the determination of aminopenicillins in muscle tissues of chicken (Ang et al. 1996; Luo et al. 1997). In this procedure, a sample extraction is included. Muscle tissues were blended with a food processor and 2 g of aliquot sample was homogenized with 5 ml of 0.01 M phosphate buffer (pH 6.5). Proteins were precipitated with the addition of 1 ml trichloroacetic acid (30%) followed by centrifugation. After filtration, 1 ml of the supernatant was derivatized with 50 µl of formaldehyde for 45 minutes at 100°C in a water bath. The supernatant was extracted twice with diethyl ether and extracts were evaporated until dryness. The dried sample was reconstituted with in 1 ml of mobile phase and a 20 µl aliquot was injected into HPLC system. The amoxicillin derivate was then analysed by reversed phase HPLC with fluorescence detection at an excitation wavelength of 346 nm and an emission wavelength of 422 nm. The mobile phase contained phosphate buffer and acetonitrile (75:25, v/v) and the flow rate was set at 1 ml min<sup>-1</sup>. The limit of detection and limit of quantification for amoxicillin in the tissues were 5 µg kg<sup>-1</sup> and 20 µg kg<sup>-1</sup>, respectively. This limit of quantification (LOQ) is defined as the lowest concentration for which the method is validated, while LOQ is below half MRL (50 µg kg<sup>-1</sup>) in muscle tissue.

### Experimental administration of Amoxicillin to laying hens

On the basis of approval of official authorities 30 laying hens (ISA brown) with an average weight of 2.3 kg were used in our experiment. Amuril plv.sol. (10 g of amoxicillinum trihydrate in 100 g of powder) was purchased from Lek (Slovenia) and administered in a dosage 0.5 g per laying hen and day with probe per orally 4 times in 24 hour intervals. Zero control hens were slaughtered prior to starting administration. Experimental hens were slaughtered daily (four hens daily) and continued until the time when all samples were tested negative.

Samples of muscle tissues (breast and thigh muscle), organs (kidney, heart, liver), and meat juice (derived from breast muscle) were tested. Breast muscle juice samples were extracted by using a garlic press. The meat juice from antibiotic free poultry was used as a zero control. Four pieces of meat and organ samples (size: 7 × 7 × 7 mm) were directly laid onto the surface of the agar. Four paper discs (Whatman 1, diameter 12 mm) were soaked with the sample (100 µl) to be tested and laid onto the surface of the agar. For both series, agar inoculated with *B. stearothermophilus* was used. Samples were placed at a minimum distance of 10 mm from the edge of the plate. All samples were also analysed with the Premi®Test. Results are confirmed with the previously mentioned HPLC method.

Data are analysed by Win Episcopo 2.0 using test agreement and kappa value for the comparison of various assay systems. Data were also analysed by GraphPad Prism 3.0 using *t*-test for the comparison the means of sample replications (*p* value).

### Results and discussion

The meat juice samples with the addition of standard solutions of the beta-lactam antibiotics were analysed by using of BsDA, FPT, and Premi®Test. The results, in which different concentration of penicillin

G, ampicillin, amoxicillin, oxacillin, and cloxacillin were added to poultry meat juice, are summarised in Table II.

When considering the FPT, it was concluded that the plates seeded with *Bacillus subtilis*, intended for determination of beta-lactam antibiotics are not sensitive enough to detect the selected beta-lactams. The spiking method presented by Okerman et al. (1998a), demonstrated that the FPT is not suited for detection of many antibiotics in muscle tissues. Originally, the FPT was developed to identify five different groups of antibiotics: beta-lactam antibiotics, tetracyclines, sulphonamides, aminoglycosides and macrolides. In the practice, the method does not detect sulphonamides, and is not reliable for detection of aminoglycosides or macrolides in meat (Okerman et al. 2001).

Considering the LODs of antibiotics belonging to the beta-lactams family, it appears that the method is not suitable for screening all beta-lactams. All tested beta-lactams could be detected with the plate containing *B. subtilis* (pH 6.0), but the LODs are not optimal, although the LOD of penicillin G on the plate seeded with *Bacillus subtilis* (pH 6.0) is sufficiently low to detect the presence of this antibiotic at the level of MRL. The plates containing *K. rhizophila* and *B. stearothermophilus* are both sensitive for beta-lactams. The plates inoculated with *K. rhizophila* were able to detect the presence of the tested antibiotics at the following levels: penicillin G (PENG) 10 µg kg<sup>-1</sup>, amoxicillin (AMOX) 25 µg kg<sup>-1</sup>, ampicillin (AMP) 30 µg kg<sup>-1</sup>, cloxacillin (CLOX) 50 µg kg<sup>-1</sup>, oxacillin (OXA) 50 µg kg<sup>-1</sup>.

The LOD of the plates inoculated with *B. stearothermophilus* (BsDA) were as follows: PENG 5 µg kg<sup>-1</sup>, AMP 10 µg kg<sup>-1</sup>, AMOX 25 µg kg<sup>-1</sup>. Higher LODs were obtained with CLOX and OXA estimated 30 µg kg<sup>-1</sup> for both antibiotics, respectively.

The Premi®Test could detect the presence of penicillin G at lower levels as the *K. rhizophila* plate and BsDA (LOD 4 µg kg<sup>-1</sup>) and similar results were

Table II. Limit of detection of beta-lactam antibiotics using fortified poultry meat juice tested with various assays.

Compound	Limit of detection for various assays (µg kg <sup>-1</sup> )						MRL (µg kg <sup>-1</sup> )
	BsDA	<i>Bacillus subtilis</i>			<i>Kocuria rhizophila</i>	Premi® Test	
		6.0	7.2	8.0			
Penicillin G	5	50	200	100	10	4	50
Amoxicillin	10	150	500	200	25	5	50
Ampicillin	25	150	500	200	30	5	50
Oxacillin	30	NS	NS	NS	50	40	300
Cloxacillin	30	NS	NS	NS	50	50	300

NS – no sensitivity for tested concentrations.

recorded also for ampicillin and amoxicillin ( $5 \mu\text{g kg}^{-1}$ ) and for cloxacillin and oxacillin (40 and  $50 \mu\text{g kg}^{-1}$  respectively).

Both methods, detected the presence of beta-lactam residues in poultry meat juice below the permitted maximum residue limits established by EEC Directive 2377/90 ( $50 \mu\text{g kg}^{-1}$ ). However, it must be noted that the tests are "too sensitive" for these antibiotics, i.e., a LOD much below the respective MRL, which is in some countries a drawback, not a benefit.

*Bacillus stearothermophilus* disc assay (BsDA) is a standard test recommended by official authorities for antibiotic residues detection in food and the food-stuffs of animal origin. As an alternative to BsDA, Premi<sup>®</sup>Test was prepared. This easy-to-use test should guarantee that producers could prevent the risk of penalties due to positive meat samples. The principle of the test is similar to Delvo<sup>®</sup>Test SP which is routinely used for antibiotic residue detection in milk in the milk industry. Premi<sup>®</sup>Test should be applied in the same way in meat processing facilities. Reybroeck (2000a) tested concentrations of residues of different substances in different naturally contaminated meat samples. Each meat sample was originated from a different chicken and Premi<sup>®</sup>Test fulfilled the conditions of European legislation. The Premi<sup>®</sup>Test was used by Reybroeck (2000b) also for spiked meat juice samples and the LODs of the Premi<sup>®</sup>Test for ampicillin ( $5 \mu\text{g kg}^{-1}$ ), amoxicillin ( $5 \mu\text{g kg}^{-1}$ ), oxacillin ( $25 \mu\text{g kg}^{-1}$ ), and cloxacillin ( $40 \mu\text{g kg}^{-1}$ ) were comparable with our results ( $5, 5, 40, 50 \mu\text{g kg}^{-1}$ , respectively). An improvement of the screening for antimicrobial drug residues was performed and minimum detection levels became  $2.5 \mu\text{g kg}^{-1}$  for penicillin G, and  $5 \mu\text{g kg}^{-1}$  for ampicillin and amoxicillin.

Proper use of Premi<sup>®</sup>Test will contribute to less positive animals, safer products and better consumer protection. This will assure the quality of animal food products in this respect, which is beneficial for the consumers and the producers.

In our animal study the presence of amoxicillin residues after experimental administration in laying hens was evaluated. All results are summarized in Table III. Microbial inhibition tests were intended for screening of antibiotic residues in poultry muscle tissues and organs. The tests are originally designed for the analysis of intact pieces of muscle tissues.

Amoxicillin residues were detected in meat juice derived from the breast muscles, breast and thigh muscles, kidney, heart, and liver using BsDA. The inhibition zones of meat juice samples and muscles are compared, after they were placed directly onto the agar medium and incubated. It is shown that meat juice produced significantly ( $p < 0.005$ ) larger zones than muscle samples. Antibiotics do not diffuse completely into the medium, when they are present in naturally contaminated samples. In meat juice samples, lower levels of amoxicillin could be detected, in comparison with incurred tissue. This can be due to a different diffusion of this antibiotic into the agar medium. Nevertheless, inhibition zones become larger in thin agar layers, with every matrix (Okerman et al. 1998b).

Premi<sup>®</sup>Test was used for residue detection in meat juice from breast muscles. Amoxicillin that could be detected with the BsDA, could also be detected with Premi<sup>®</sup>Test. The last positive results of BsDA were recorded 24 hours after the finishing of administration, last positives with Premi<sup>®</sup>Test were found after 48 hours. This means, in comparison with BsDA, Premi<sup>®</sup>Test is able to detect amoxicillin residues in poultry muscles for a 24-hour longer period after treatment ( $\kappa < 0.6$ ).

All positive results were confirmed by using the liquid chromatography method, and we found a fair correlation among selected tests. HPLC analysis of breast muscle samples demonstrates that the highest value of ampicillin ( $92 \mu\text{g kg}^{-1}$ ) was on the last day of drug administration. Consequently, concentrations constantly decreased and the last positive sample was recorded 48 hours after finishing of administration ( $21 \mu\text{g kg}^{-1}$ ).

Table III. Comparison of mean values of BsDA and Premi<sup>®</sup>Test confirmed with HPLC after experimental administration of Amuril plv. sol. in laying hens.

Time (hours)	BsDA (mm)					Premi <sup>®</sup> Test	HPLC ( $\mu\text{g kg}^{-1}$ )
	Breast**	Thigh	Kidney	Heart	Liver		
0*	0/0	0	0	0	0	–	0
24*	3.3/6.7	1.7	1.7	0	2.3	+	26
48*	4.7/7.7	1.7	1.3	1.3	2.0	+	44
72*	1.8/4.7	1.7	1.7	0.7	2.0	+	92
96	2.3/4.0	1.7	0.3	0	1.7	+	29
120	0.7/1.3	0.3	0.3	0	1.0	+	21
144	0.3/0.7	0.3	0.7	0.3	1.3	–	<5
168	0/0	0	0	0	0	–	0

\*Amuril plv. sol. administration; \*\*Zones of inhibition from breast muscles/breast muscles juice.

The results of BsDA were not in accordance with the results of the Premi<sup>®</sup>Test ( $\kappa < 0.6$ ). Data obtained with the Premi<sup>®</sup>Test did not confirm the results of the other microbiological tests, but were in accordance with the results of HPLC ( $\kappa = 0.6$ ). Results of BsDA, Premi<sup>®</sup>Test and HPLC on the third day after the finishing of drug administration were negative. BsDA analysis of heart and kidney showed that all zones of inhibition were below 2 mm and must be considered as negative. The liver only produced positive results at the time of Amuril plv. sol. administration. On the basis of these results, the methods did not exceed withdrawal period recommended by producer (2 days).

We concluded that the test organisms *Kocuria rhizophila*, *Bacillus stearothermophilus* and Premi<sup>®</sup>Test can be used as routine screening methods for the presence of beta-lactam residues in poultry meat. The purpose of the tests is to detect the presence of positive samples, which should be confirmed by more sophisticated chromatographic methods. Screening tests should be simple, cheap, and practical in routine residue testing. However, when the cost of plates is considering being too high, using Premi<sup>®</sup>Test is a good alternative. The great advantage of the Premi<sup>®</sup>Test is the short time of the analysis (<4 hours) and besides, it is able to detect some antibiotics below MRL, which is desirable for some labs. As a screening test, Premi<sup>®</sup>Test offers a first tool to detect the presence of relevant residues of beta-lactam antibiotics. Positive samples can then be confirmed for a presumptive identification of beta-lactam residues by HPLC. Premi<sup>®</sup>Test relieves the chemical analytical resources of food inspection laboratories and may contribute to more attention paid for residues of veterinary drugs in animal production.

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