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Collaborative study of a microbiological screening method (three-plates) for the banned antimicrobial growth promoters tylosin, virginiamycin, spiramycin, zinc bacitracin and avoparcin in animal feed

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Collaborative study of a microbiological screening method (three-plates) for the banned antimicrobial growth promoters tylosin, virginiamycin, spiramycin, zinc bacitracin and avoparcin in animal feed

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Additives/Contaminants:	Veterinary drug residues - antibiotics
Food Types:	Animal feedingstuffs

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Table I: Description of the solvent of the stock-solutions of the used antibiotics.

Antibiotic	Supplier	Solvent
Spiramycin	Merial	Methanol
Virginiamycin	Pfizer	Methanol
Tylosin	Sigma	Methanol + 0.01M phosphate buffer pH 8.0
Avoparcin	American Cyanamid Company	Acetone: 0.1M HCl (6:4)
Zinc Bacitracin	Sigma	0.1 M HCl:phosphate buffer pH 6.5: water (1:1:8)

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Table II: Bioassay conditions for the different antibiotics/bacteria using the extraction conditions as described in "Methods and materials"

Antibiotic	Test strain	Inoculum* (cfu/ml agar)	Agar	pH	Incubation temp (°C)
Avoparcin	<i>B. megatherium</i> ATCC 10778	10 ⁶	Plate Count agar	6	25
Zinc bacitracin	<i>M. luteus</i> ATCC 10240	10 ⁶	Antibiotic medium 1 + 1 µg ml ⁻¹ neomycin base	6.5	30
Spiramycin	<i>M. luteus</i> ATCC 9341	10 ⁶	Antibiotic medium 1 + 20 g l ⁻¹ K ₂ HPO ₄ + 7.5 µg l ⁻¹ tylosin	8	30
Tylosin					
Virginiamycin					

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* cfu = colony forming units

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Table III: Classification of the laboratories in the training period (T) and Collaborative study (C) in satisfactory performance

(+), intermediate performance (±) or unsatisfactory performance (-)

Labo- ratory	Tylosin/virginamycin/spiramycin			Avoparcin			Zinc bacitracin			Results excluded
	T	C	Specification	T	C	Specification	T	C	Specification	
1	±	±		±	±	C2: Incorrect inc. temperature	±	±		Avo (C2)
2	±	-	C: No results, growth problems	±	±	T&C: blanks positive	±	±	T&C: blanks positive	Tyl/Vir/Spir/Bac/Avo
3	±	±		±	±		±	±		-
4	±	±		±	±	T: blanks positive	±	±		-
5	±	±		±	±		±	±	T: blanks positive	-
6	±	±	T: Sample exchange	±	±		±	±		-
7	±	±		±	±		±	±	T&C: blanks positive	Bac
8	±	±		±	±		±	±	T&C: blanks positive	Bac
9	±	±		±	±		±	±		-
10	±	±		±	±		±	±		-
11	±	±		±	±		±	±		-
12	±	±	T&C: Sensitivity bio-assay	±	±	T: Sensitivity bio-assay	-	n.a.	T: Sensitivity bio-assay	Tyl/Vir/Spir/Bac
13	±	±		±	±		±	±		-
14	±	±	T&C: Sensitivity bio-assay	±	±		±	±		Tyl/Vir/Spir
15	±	±		±	±	T: Sensitivity bio-assay	±	±		-
16	±	-	T&C: No results, growth problems	±	±		-	n.a.	T: Sensitivity bio-assay	Tyl/Vir/Spir/Bac
17	±	±	T: No results, C: Sens. bio-assay	±	±	C1: Incorrect inc. temperature	±	±		Tyl/Vir/Spir/Avo (C1)
18	±	-	C: withdrawn	±	-	C: withdrawn	±	-	C: withdrawn	n.a.

C1 = regular avoparcin study, C2 = additional avoparcin study n.a. = not applicable

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Table IV: Final validation results of the materials spiked with tylosin, spiramycin and virginiamycin (results from 12 laboratories).

Level (mg.kg ⁻¹)	Total amount of samples	Tylosin Feed	positive results			Virginiamycin Feed	positive results			Spiramycin Feed	positive results			Blank Feed	positive results	
			n	%	mean%*		n	%	mean%*		n	%	mean%*		total	n
1	48	Calf	45	94	97	Poultry	48	100	100	Cattle	42	88	94	Calf	24	0
2	48	Pig	48	100	99	Pig	48	100	100	Poultry	48	100	100	Pig	24	0
5	48	Calf	48	100	100	Calf	48	100	100	Poultry	48	100	100	Cattle	24	2
	48	Cattle	48	100	100	Cattle	48	100	100	pig	48	100	100			

*mean% at each level

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Table V: Final validation results of the materials spiked with zinc bacitracin (n= 12 laboratories).

Level (mg kg ⁻¹)	Total amount of samples	Zinc bacitracin Feed	positive results			Blank Feed	Positive results	
			n	%	mean%*		Total amount samples	n
1	48	Poultry	15	31	23	Calf	23	6
	48	Cattle	7	15		Pig	22	
2	48	Calf	29	60	55	Poultry	24	2
	48	Pig	24	50		Cattle	24	2
3	48	Poultry	48	100	88			
	48	Pig	36	75				
5	48	Calf	47	98	96			
	48	Cattle	45	94				

*mean% at each level

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Table V: Final validation results of the materials spiked with avoparcin, (n = 15 laboratories). The feed samples marked with * are those from the additional avoparcin study

Level (mg kg ⁻¹)	Total amount of samples	Avoparcin Feed	positive results			Blank Feed	Positive results		
			n	%	mean%**		Total amount	samples	n
1	68	Calf	14	21	#	Pig*	45	2	
▲	60	Calf*	53	88	96	Poultry	30	-	
▲	60	Pig*	60	100		Cattle	30	-	
▲	59	Cattle	59	100		Calf*	45	2	
2	59	Poultry	59	100	100				
▲	60	Cattle	60	100					
3	59	Calf*	55	93	93				
5	60	Calf*	60	100	100				
▲	60	Poultry	60	100					
▲	60	Pig	60	100					
**mean% at each level		# data not used							

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Table VJJ: Accuracy (AC), Sensitivity (SE) and Specificity (SP) for tylosin, virginiamycin, spiramycin, avoparcin and zinc bacitracin at different concentrations.

Level (mg kg ⁻¹)	compound	AC	SE	SP
1	Tylosin	97.4	96.9	97.9
	Virginiamycine	98.9	100	97.9
	Spiramycine	95.8	93.7	97.9
	Zinc Bacitracin	55.5	22.9	89.2
	Avoparcin	96.7	96.0	97.4
2	Tylosin	98.4	98.9	97.9
	Virginiamycin	98.9	100	97.9
	Spiramycin	98.9	100	97.9
	Zinc Bacitracin	71.9	55	89.2
	Avoparcin	98.5	100	97.4
3	Zinc Bacitracin	87.5	87.5	89.2
	Avoparcin	96.2	93.2	97.4
5	Tylosin	98.9	100	97.9
	Virginiamycin	98.9	100	97.9
	Spiramycin	98.9	100	97.9
	Zinc Bacitracin	92.5	95.8	89.2
	Avoparcin	98.8	100	97.4

Accuracy AC = $\frac{PA+NA}{PA+ND+PD+NA}$

PA = positive agreements (correctly identified positives)

NA = negative agreements (correctly identified negatives)

ND = negative deviations (false negatives)

PD = positive deviations (false positives)

Sensitivity SE = $\frac{PA}{PA+ND}$

Specificity SP = $\frac{NA}{PD+NA}$

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Table VIII: Results of discrimination study

compound	Level (mg kg ⁻¹)	Total amount of samples	Classification results (amount of samples)				
			Tyl/virg/spi	All	Avo/bac	Blank	No result
Tylosin	2	24	24	-	-	-	-
	5	12	11	1	-	-	-
Virginiamycin	2	24	23	1	-	-	-
	5	12	11	1	-	-	-
Spiramycin	2	24	23	1	-	-	-
	5	12	12	-	-	-	-
Zinc Bacitracin	2	12	-	-	5	6	1
	3	12	-	-	7	4	1
	5	24	-	-	21	-	3
Avoparcin	2	24	-	-	22	-	2
	5	12	-	-	11	-	1
Blanks	-	48	-	-	6	37	5

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6 **Collaborative study of a microbiological screening method**
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8 **(three-plate) for the banned antimicrobial growth promoters**
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10 **tylosin, virginiamycin, spiramycin, zinc bacitracin and**
11 **avoparcin in animal feed**
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Abstract

A microbiological screening method (three-plate) for detection of the antimicrobial growth promoters tylosin, spiramycin, virginiamycin, zinc bacitracin and avoparcin in animal feed has been developed and validated successfully. A collaborative study involving 18 laboratories receiving 172 samples was carried out to verify the performance characteristics. The detection level for tylosin/virginiamycin/spiramycin, expressed in microbiological activity, was 1 mg kg⁻¹ (false positives 2%, false negatives 3, 0, and 6% respectively). Avoparcin could be detected at 1 mg/kg in feed in general (false positives 2%, false negatives 0%). However, in calf feed, the sensitivity was lower. The percentage of false negatives was found to be 12%, 7%, 0% at 1, 3 and 5 mg kg⁻¹ respectively (false positives 4%). The limit of detection for zinc bacitracin was 3 - 5 mg kg⁻¹ (false positives 5-10%, false negatives 77% at 1 mg kg⁻¹, 45% at 2 mg kg⁻¹, 12% at 3 mg kg⁻¹ and 4% at 5 mg kg⁻¹). The method allowed for distinction between the groups of antibiotics: avoparcin/zinc bacitracin vs. tylosin/virginiamycin/spiramycin. This definitely gives added value to the method in the framework of a follow-up of positive screening results by post-screening and confirmatory analysis.

Key words: Microbiological screening method, validation, animal feed, antibiotics, collaborative study, spiramycin, virginiamycin, tylosin, zinc bacitracin, avoparcin

Introduction

Antimicrobial growth promoters have been related to increased development of antibiotic resistance in bacterial strains. For instance use of avoparcin, which is structurally related to the human medicines vancomycin and teicoplanin, leads to selection and amplification of vancomycin-resistant pathogens (Acar et al, 2000). Because of possible transfer of resistance genes from animal enteric flora to human bacteria and pathogens and increased appearance of cross resistance, the medical effectiveness of antibiotics reserved for human treatment could be impaired. In accordance with consumers demand for more safe and healthy foods, the European Union has therefore withdrawn the authorisation of several antimicrobial growth promoters as feed additives. Avoparcin, zinc-bacitracin, spiramycin, tylosin and virginiamycin, were banned from use as animal feed additives in 1997 and 1998 (European Community, 1997, 1998).

In order to effectively and efficiently control the possible illegal use of these substances multi-screening and confirmation methods with sufficiently low detection limits are required. The available methods of analysis (adopted in the EC) at that time were based on microbiological inhibition tests (European Community, 1972, 1981, 1984a, 1984b). These specific methods mainly focussed on checking the content of one single compound at the registered additive level, and were therefore not suited as broad screening method for all substances (Higgins et al. 1999). Gafner described a multi-analyte method using TLC and bio-autography (Gafner 1999), however that method was not able to detect antibiotics at low levels, viz. five times lower than the contents formerly described in Council Directive 70/524/EEC (European Community, 1970).

Within the European project: Screening and Identification Methods for official control of

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4 Banned Antibiotics and Growth promoters in FEEDingstuffs (SIMBAG FEED) various
5 analytical methodologies were developed and validated, including methods for
6 screening and confirmatory purposes. The developed methods should preferably have
7 a multi-analyte approach and should meet the required specificity and sensitivity; the
8 target limit for the antimicrobial growth promoters was 1 mg kg⁻¹ (de Jong et al, 2006).
9 After development, the analytical methods were subjected to a single-laboratory
10 validation. Due to lack of appropriate validation guidelines for the feed area, the
11 validation of this method was carried out according to guidelines for determination of
12 residues in food matrices (European community 2002), afterwards the transferability of
13 the method to other laboratories was evaluated. Only methods successfully passing
14 both these tests, were allowed to enter the validation phase consisting of an
15 interlaboratory study. One of the major objectives of this collaborative study was to
16 verify the performance characteristics of the developed microbiological screening
17 method when used for identical samples by different laboratories.
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37 This paper describes the interlaboratory validation of a microbiological screening
38 method (van Egmond et al. 2005). The method is based on plate inhibition tests,
39 aiming at the detection of avoparcin, zinc bacitracin, tylosin, spiramycin and
40 virginiamycin at detection limit of 1 mg kg⁻¹, expressed in microbiological activity (van
41 Egmond 2005) The purpose of the method is to screen samples for the *presence* of
42 one or more of the target analytes in feed without giving a quantitative statement on
43 their concentration.
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Method and materials

Design of the study

The collaborative study was jointly conducted by RIKILT Institute for Food Safety in corporation with the European Commission's Joint Research Centre, Institute for Reference Materials and Measurements (JRC-IRMM) for overall coordination. JRC-IRMM also prepared the blank feed samples. The study was conducted with a step-by-step approach, where the laboratories were first involved in a training period consisting of specific training on the method through audiovisual material and known and unknown test samples. Laboratories showing good results were asked to participate in the final inter-comparison trail to validate the method.

The training period.

The training period was designed as a specific exercise for the candidate laboratories in order to make them (more) familiar with the method protocol. In addition, their performance and their rigorous application of the protocol were evaluated. First, an audiovisual support (CD-ROM) containing the method description was sent to the participants, along with a specific training reporting sheet, a feedback questionnaire and some test samples and standards.

The first training period contained known and unknown samples of all target analytes. A second training period proved to be necessary for avoparcin and zinc bacitracin to be able to have sufficient experienced laboratories for the actual validation phase of the collaborative study.

Collaborative study.

In total eighteen laboratories participated in the two training periods. Laboratories that successfully implemented the method were invited to join the collaborative study. All

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eighteen labs were selected for the analysis of tylosin/spiramycin/virginiamycin and avoparcin in the collaborative study, two labs were disqualified for the analysis of zinc bacitracin. The participating laboratories received a detailed method protocol, samples to be analysed and electronic forms to report the results of the analysis. In total each participant received 172 samples to be analysed, including samples for an identification study and corresponding blanks. All participants were asked to evaluate the feed samples for the presence or absence of the banned antibiotics. This method is a qualitative method in nature, therefore only three responses were allowed: 1 positive (substance present), 2 negative (not present), 3 no results. Laboratories reported no results when they were not able to report (in time).

Additional collaborative study.

After analysing the results of the first collaborative study round, an additional collaborative study proved to be necessary for avoparcin, containing eight feed materials. Each participant received 20 extra samples including four blind replicates of each spiked feed material and one sample of each blank feed.

Statistics

All results for this method were of qualitative kind, therefore the usual procedures for quantitative results were not applicable. In order to be able to assess the method performance, the number of correct positives, correct negatives, false positives and false negatives were calculated in relation to the compound, its concentration and the different feed matrices (Vincent et al, 2007).

To be able to describe the capability of the method, the accuracy, the sensitivity and the specificity were calculated according to following equations (Vincent et al, 2007; Langton et al. 2002):

$$\text{Accuracy AC} = (\text{PA} + \text{NA}) / (\text{PA} + \text{ND} + \text{PD} + \text{NA}) * 100\%$$

PA = positive agreements (correctly identified positives)

NA = negative agreements (correctly identified negatives)

ND = negative deviations (false negatives)

PD = positive deviations (false positives)

$$\text{Sensitivity SE} = (\text{PA} / (\text{PA} + \text{ND})) * 100\%$$

$$\text{Specificity SP} = (\text{NA} / (\text{PD} + \text{NA})) * 100\%$$

The sensitivity (SE) is the ability of the method to classify a positive sample as positive, whereas the specificity (SP) is the ability to classify a negative sample as negative.

False negative(s) are defined as samples that are truly non-compliant, even though a compliant measurement has been obtained. False positives(s) are defined as samples that are truly compliant, even though a non-compliant measurement has been obtained.

A 100% score for accuracy means that all positive samples were correctly classified as positive and all negative samples were correctly classified as negative. For all scores below 100%, indicating that not all samples were classified correctly, it was investigated whether this was caused by false negatives or false positive samples. The limit of detection for the different antibiotics was set at the lowest concentration at which 95% of the samples were still identified as positive. This means that the sensitivity (SE) has to be equal to or above 95% (European Community, 2002; Vincent et al, 2007;).

Test materials

Four different animal feed samples were used for sample preparation in this study: pig, poultry, cattle and calf feed. The different blank feeds were produced in bulk quantities for the SIMBAG FEED project and stored at -20°C until use. They were defrosted shortly before use and distributed in bottles in 10 g portions by IRMM (Belgium). Spiked samples were prepared by RIKILT. Pure antibiotic standards were used for the preparation of the spiked feed samples. Stock solutions were prepared according to Table I, and made freshly on the day of use. Samples were prepared by adding a known amount of stock solution to each 10 g feed portion to obtain 1, 2, 3 and 5 mg kg⁻¹ (end concentration in feed). After addition of the antibiotic solution the feed was mixed and stored at -20°C.

Stability tests

Prior to the preparation of the collaborative study samples, a stability study was performed. In this study 3 mg kg⁻¹ spikes of avoparcin, tylosin and spiramycin en 4 mg kg⁻¹ spikes of virginiamycin and zinc bacitracin were prepared in 10 gram portions. The samples were stored at -20, 4, 20 and 30°C for one month. Sample transport was mimicked by storing part of the samples at 4, 20 or 30°C for two days, followed by storage at -20°C. The samples were considered as stable when the concentration in the stored samples was higher or equal to 95% compared to the concentration in freshly prepared samples (tested using the official EU microbiological quantification methods, including freshly prepared spikes and calibration curves (European Community, 1970, 1972, 1981, 1984a,b)). Sufficient stability was obtained for tylosin, avoparcin and virginiamycin in all tested conditions. Zinc bacitracin should be stored at 4°C and spiramycin at -20°C. As a result the participants were advised to keep the antibiotics frozen under all conditions. Combined with a limited (three-four weeks) time to perform the analysis, stability was guaranteed.

Method protocol

The overall purpose of the developed method was to screen animal feed for the presence of the five banned antibiotics. The detail protocol is described on the SIMBAG FEED website (van Egmond, 2005a) and will be published (in preparation). In short, the feed samples (10 g) are extracted with a mixture (475/25/500) of acetone, hydrochloric-acid (HCl) and water (20 ml) for 15 minutes followed by further extraction for another 15 minutes by addition of 10 ml phosphate buffer pH 6.5. The supernatant is adjusted to pH 6.0-6.5 and dispensed into wells in three different agar media. The agars differ in inoculated bacterial strains and pH (see table II for details). After incubation, presence of antibiotics is shown by the formation of zones of inhibition. An indication of the group of antibiotics present is possible by observing with which micro-organisms zones of inhibition are produced.

Single laboratory validation of the method

Single laboratory validation of the method included sensitivity (SE), specificity (SP), interference and ruggedness. The detailed results of this in-house validation are described on the SIMBAG FEED website (de Jong et al, 2005) and will be published (in preparation). The method showed satisfactory performance for tylosin, avoparcin, spiramycin at 1 mg/kg (SE>95%) and for virginiamycin and zinc bacitracin at 2 mg/kg (SE >95%). Most antibiotics used as veterinary medicine (e.g. tetracyclines, quinolones, other macrolides, β -lactams, aminoglycosides) showed interference, which can be recognized by the inclusion of three additional test plates. Avilamycin, flavomycin, ionophores, benzimidazolen, ivermectine, ronidazole, sulphonamides, maduramycin, colistin and, spectinomycin showed no interference.

Transferability

Satisfactory transferability of the method was confirmed by two other laboratories, being able to produce comparable sensitivity results. The detailed results of transferability tests will be published (in preparation).

Results and discussion

Training phase

The overall purpose of the study was to assess the fitness-for-purpose of the developed microbiological screening method for the screening of avoparcin, zinc bacitracin, spiramycin, tylosin and virginiamycin in feedingstuffs at the target level of 1 mg kg⁻¹. A training period was organised to get the participating laboratories acquainted with the developed method. In total 20 laboratories volunteered to participate in the training period of which 18 laboratories reported results (see table III). At the end of the training period all eighteen labs were selected for the analysis of tylosin/spiramycin/virginiamycin and avoparcin. Two labs were disqualified for the analysis of zinc bacitracin, due to lack of sensitivity of the bio-assay. Some of the remaining laboratories did not implement the method fully satisfactory or did not reported results of the training period (intermediate classification). The “intermediate” laboratories were invited for participation in the collaborative study with the restriction that results should be in accordance with those of the other laboratories, else their results would not be used in the calculations.

Collaborative study

In total 17 out of 18 laboratories reported results of the collaborative study (see table III). Two of these 17 laboratories (lab's 2 and 16), did not report results for tylosin/spiramycin/virginiamycin due to growth problems of the *M.luteus* ATCC 9341. Three (out of the remaining four) intermediate laboratories did not show improvements

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4 for tylosin/spiramycin/virginiamycin (lab's 12, 14, 17). These laboratories did not
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6 succeed in producing stable bio-assay systems (sensitivity or growth problems of the
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8 strains). One intermediate laboratory (lab. 6) reporting results in the training study that
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10 could only be explained by occurrence of sample-exchange, reported satisfactory
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12 results in de collaborative study
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17 For zinc bacitracin, 15 laboratories reported results. Three (out of four) intermediate
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19 laboratories (Lab's 2, 7, 8) were not able to show improvements in their results and
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21 were therefore excluded from the study. These three laboratories had problems with
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23 positive blanks and unstable bio-assay systems.
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28 For avoparcin, two laboratories (lab's 1 and 17) incubated part of the plates at wrong
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30 temperature and these data were excluded from the results. Four laboratories showed
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32 intermediate results for avoparcin after the training period. One of these four (lab. 2)
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34 was not able to improve their results and was excluded from the collaborative study.
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36 This laboratory had problems with positive blanks and an unstable bio-assay system.
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41 In more detail: for the tylosin/ spiramycin/ virginiamycin group, 288 samples for each
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43 antibiotic were taken into account in the statistical evaluation originating from 12
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45 laboratories. For zinc bacitracin, 288 samples were evaluated from 12 laboratories and
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47 for avoparcin 537 results were evaluated from 15 laboratories, including the samples
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49 from the extra collaborative study.
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54 All data obtained from laboratories which successfully applied the method in the
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56 training period were used, except the avoparcin data originating from the incorrect
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58 incubation temperature. It is noticeable that one laboratory (lab. 2) was not able to
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60 produce acceptable results for all three groups of antibiotics

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4 Table IV, V and VI show detailed information on the performance of the method for
5 each single compound and the influence of the matrix on the detection of
6 tylosin/virginiamycin/spiramycin, zinc bacitracin and avoparcin. The statistical
7 calculations are reported in Table VII. For tylosin/virginiamycin/spiramycin the number
8 of false positives was rather low with only 2%. At the target limit of detection of 1 mg kg⁻¹
9 the number of false negatives was respectively 3%, 0%, 6% (out of 96 results). The
10 sensitivity (SE) shows acceptable values for detecting 1 mg kg⁻¹ with respect to tylosin
11 and virginiamycin. For spiramycin the SE values are just slightly below the required
12 95%, this is mainly caused by the results of cattle feed at this concentration.
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26 For zinc bacitracin the number of false positives was rather high (11%). Most false
27 positives originated from one laboratory (lab. 1). Excluding those results reduced the
28 number of false positives to 5%. The target level of 1 mg kg⁻¹ could not be reached in
29 any type of feed. The limit of detection lies between 3 and 5 mg kg⁻¹, as the required
30 95% positive samples is only achieved at 5 mg kg⁻¹ level. At 3 mg kg⁻¹ level it depends
31 on the type of feed, poultry feed for instance gave 100% correct results while in pig
32 feed this was much more difficult. The detection of zinc bacitracin is less sensitive than
33 was expected based on results obtained from the in-house validation and
34 transferability-test to two other laboratories. One of the reasons for this could be the
35 strain used. The *M.luteus* ATCC 10240 strain used for the zinc bacitracin detection is
36 not as robust as the *M.luteus* ATCC 9341 strain used for
37 tylosin/virginiamycin/spiramycin. The amount of *M.luteus* ATCC 10240 cells added to
38 the agar is essential for a good sensitivity and laboratories using non-controlled inocula
39 might observe large variations of sensitivity in time. This could be one of the reasons
40 for the lack of sensitivity in some laboratories.
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4 After analyzing the results of the collaborative study an additional collaborative study
5 for avoparcin proved to be necessary. This was due to the fact that it was not possible
6 to calculate the limit of detection of the method for the different types of feed. Most
7 laboratories (79%) (unexpectedly) found a false negative result for 1 mg kg⁻¹ in calf
8 feed, whereas in cattle feed this concentration was positive for all participants. This
9 result for calf feed (milk replacer) was not in accordance with the results obtained in the
10 single laboratory validation and transferability studies where 1 mg/kg was good
11 detectable.
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24 It was decided to organise an additional collaborative study with three different levels of
25 avoparcin in calf feed (1, 3, 5 mg kg⁻¹) and a pig feed spiked with 1 mg kg⁻¹ avoparcin.
26 With the results of this additional study the limit of detection in calf feed could be
27 determined and it was proved that 1 mg kg⁻¹ avoparcin was detectable in other feeds
28 (SE = 96%). The number of false positives was only 2%. For calf feed, (milk replacer),
29 the number of false positives was 4%.
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39 The limit of detection for calf feed (milk replacer) is somewhat higher, viz. between 3
40 and 5 mg kg⁻¹. The number of false negatives (in the second study) is 12% (1 mg kg⁻¹),
41 7% (3 mg kg⁻¹) and 0% (5 mg kg⁻¹). This is at the 1 mg kg⁻¹ avoparcin level significantly
42 lower than the observed 79% in the first study
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50 Most possibly the storage conditions of the calf feed has influenced the results
51 negatively. The calf feed from the first collaborative study was stored in 10 gram
52 portions at -18°C for almost a year, while the calf feed for the second collaborative
53 study was stored in bulk quantities at - 18°C and distributed shortly before use in 10
54 gram portions. The cause of this interference is not yet known but might be due to
55 some physical or chemical changes in the feed during storage in contact with air. In
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4 practice these storage conditions are not regular (feed is normally stored for a
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6 maximum of three months) and therefore it was decided to only use the results of the
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8 second collaborative study for calf feed.
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10 11 12 13 *Discrimination study.*

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15 A study was set up to assess the discrimination properties of the method by the
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17 different laboratories. Therefore 20 unknown samples were sent to the participants to
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19 be analysed for antibiotics. In total 14 laboratories participated in this study of which 2
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21 laboratories had difficulties in differentiating between tylosin/virginiamycin/spiramycin
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23 and zinc bacitracin/avoparcin. Most laboratories were able to detect the presence of
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25 tylosin/ virginiamycin/spiramycin (inhibition of *M.luteus* ATCC 9341) (see table VIII).
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27 Distinction between zinc bacitracin and avoparcin turned out to be difficult. Samples
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29 containing 2-3 mg kg⁻¹ zinc bacitracin were often classified as blanks. This is in
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31 accordance with the fact that the sensitivity of the method for zinc bacitracin lies around
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33 3-5 mg kg⁻¹. All together, a first distinction between groups of blanks,
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35 tylosin/virginiamycin/spiramycin and zinc bacitracin/avoparcin proved to be possible.
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41 **Conclusions**

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43 In summary, this method showed good performance in detecting
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45 tylosin/virginiamycin/spiramycin at the target concentration of 1 mg kg⁻¹. Results were
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47 similar or even better in the case of spiramycin compared to the TLC chromatography
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49 and bio-autography described by Vincent et al. (2007). For avoparcin, the level of
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51 sensitivity depended on the type of feed. In general, the target concentration of 1 mg
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53 kg⁻¹ was reached for feed. When looking at milk replacers in particular, the level of
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55 detection lies between 3 and 5 mg kg⁻¹. Detection of zinc bacitracin proved to be more
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57 difficult. The target level of sensitivity could not be reached, but lies around 3-5 mg kg⁻¹.
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59 Overall, the performance characteristics of this method prove that the method is
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4 suitable for inclusion in the control strategy as a screening method for the presence of
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6 the three groups of compounds, Moreover, the method allows to discriminate between
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8 the presence of tylosin/virginiamycin/spiramycin on the one hand and zinc
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10 bacitracin/avoparcin on the other hand providing valuable information for confirmatory
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12 analysis. This definitely has added value in decreasing the number of samples for
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14 confirmatory analysis resulting in lower costs.
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17 18 19 20 **Acknowledgments**

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