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Proficiency test for antibiotics in beef

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Summary

The proficiency test for antibiotics in beef was organized by Rikilt - Institute of Food Safety and in accordance with ISO/IEC Guide 43-1 and 43-2 and ILAC-G13. The quantitative and confirmatory part was carried out under accreditation (Dutch Accreditation Board, ILAC-G13).

For this proficiency study, three test materials were prepared:
- a blank beef material;
- a beef material containing flumequine aimed at 280 µg/kg;
- a beef material containing lincomycin aimed at 120 µg/kg and spectinomycin aimed at 230 µg/kg.

The materials containing antibiotics were all prepared by spiking blank beef materials. During homogeneity testing, all materials proved to be sufficient homogenous for proficiency testing. The stability test demonstrated that no statistically significant loss of any of the compounds occurred during the timescale of the proficiency test.

The laboratories were asked to first carry out a screening analysis followed by a quantitative confirmatory analysis for the compounds found suspected. Twenty-six laboratories subscribed for participation in the proficiency study. Within the timeframe of the stability study all laboratories submitted results: 23 laboratories managed to submit results for the screening analysis and 19 for the quantitative confirmatory part.

Seven laboratories did not detect any antibiotics using their screening methodology. Nine laboratories characterized the samples correctly (compliant or suspect) based on the screening analysis and only three laboratories indicated the correct compound groups for all materials. It is stated that these three laboratories carried out several parallel LC-MS/MS methods as the screening approach.

An overview of the screening analysis results is presented in Table 1. A result is considered to be a false negative result if a compound present in the sample is not detected although it is included in the method. If each compound is considered separately, the false negative rate of the screening analyses is 53%. For microbiological methods the overall false negative rate is 73%, for biochemical methods this is 50% and for instrumental analysis this is 22%. The false positive rate is 7% which is due to microbiological screening methods only.

Table 1. Overview of correct, false negative and false positive results for microbiolocical, biochemical and instrumental methods.

Material	A	B	C	
Compounds		Flumequine	Lincomycin	Spectinomycin
No. of methods applied*		23	24	17
Correct results		15	9	6
Microbiology methods		4	4	2**
Biochemical methods		2	-	-
Instrumental methods		9	5	4
False negatives		8	15	11
Microbiology methods		7	10	10
Biochemical methods		-	2	-
Instrumental methods		1	3	1
False positives	3	1[#]	-	-

** Because some laboratories applied several different methods and some laboratories do not have all compounds relevant for this proficiency test included in their method, this number is different from the number of laboratories.*

*** Only growth inhibition was reported (no antibiotic group specified).*

[#] *Found suspect for containing macrolides*

Regarding the applied methods it is concluded that:

- the applied microbiological screening methods showed 73% of false negative results;
- many combination of screening tests are used to cover the broad range of antibiotic groups;
- the Nouws Antibiotic Test is the only microbiological test that correctly assigned material A negative and material B and C suspect.
- microbiological and biochemical methods do not seem capable of detecting spectinomycin at a relevant level.

Seventeen laboratories carried out a quantitative confirmatory analysis for quinolones, seven for macrolides and four for aminoglycosides. No false negatives occurred during the confirmatory analysis. One laboratory detected 25 µg/kg sulfamethazin in one of the samples which is considered as a false positive result. For the quantitive confirmatory analysis of flumequine and lincomycin all laboratories obtained satisfactory z-scores. No statistical evaluation is carried out for spectinomycin because only four results are reported.

Based on the results of this proficiency test it is concluded that:

- a huge effort is needed to improve the effectiveness and efficiency for the screening of veterinary drugs in muscle samples;
- a huge effort is needed to decrease the false negative rate of microbiological screening methods;
- for effectively applying instrumental screening methods (LC-MS/MS or LC-ToF MS) effort is needed to include a wider range of compounds;
- the performance of all laboratories regarding the quantitative analysis of flumequine and lincomycin in beef is satisfactory.

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1 Introduction

Proficiency testing is conducted to provide laboratories with a powerful tool to evaluate and demonstrate the reliability of the data that is produced. Next to validation and accreditation, proficiency testing is an important requirement of the EU Additional Measures Directive 93/99/EEC [1] and is demanded by ISO 17025:2005 [2].

No internationally focused broad range proficiency studies regarding the analysis of antibiotics in bovine muscle that focused on the screening analysis and the quantitative and confirmatory aspect were organized during the last years: an inter-laboratory quality control that combines screening and confirmatory methods of this type was lacking. Therefore, Rikilt decided to organize a proficiency study regarding this subject.

The aim of this proficiency study was to give laboratories the possibility to evaluate or demonstrate their competence for the analysis of antibiotics in bovine tissues, including the screening analysis. This study also provided an evaluation of the methods applied for screening and quantitative and confirmatory analysis of antibiotics in beef.

This proficiency study was conducted in accordance with guidelines ISO/IEC 43-1 [3], ISO/IEC 43-2 [4] and ILAC-G13 [5]. The preparation of the materials, including the suitability testing of the materials and the evaluation of the quantitative and confirmatory results were carried out under accreditation by Rikilt - Institute of Food Safety.

2 Test materials

This proficiency study focused on flumequine (a quinolone) and the combination of lincomycin (a lincosamide, closely related to macrolides) and spectinomycin (an aminoglycoside). The maximum residue limits (MRLs) for these compounds in beef are presented in Table 2.

Table 2. MRL in bovine muscle of the compounds included in the proficiency test [6]

Compound	MRL in beef (µg/kg)
Flumequine	200
Lincomycin	100
Spectinomycin	300

2.1 Sample preparation

One blank material (A), one material (B) containing flumequine (FLU) and one material (C) containing a combination of lincomycin (LMC) and spectinomycin (SMC). Material B and C were prepared by adding methanolic solutions of the selected compounds to blank bovine muscle. We aimed at the levels as presented in Table 3. Each of the materials was homogenized under cryogenic conditions according to in-house standard operating procedures.

Table 3. Target amount of antibiotics in the proficiency test materials

Material code	Target amount (µg/kg)		
	FLU	LMC	SMC
A	-	-	-
B	280	-	-
C	-	120	230

2.2 Sample identification

The materials were stored in polypropylene containers containing at least 75 gram of sample, yielding a total of 42 containers of material A and 98 containers of material B and C. The samples were randomly coded with a code from AB1/2009/MUSCLE/001 through 238.

For homogeneity and stability testing, 20 randomly selected containers of material B and C were assigned. For each laboratory a sample set was prepared consisting of one randomly selected sample of material A, B and C. The codes of the samples belonging to each sample set are presented in Annex 1.

2.3 Homogeneity study

The homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories [7] and ISO/DIS 13528 [8], taking into account the insights discussed by Thompson [9] regarding the Horwitz equation. With this procedure the between-sample standard deviation (s_s) is compared with the target standard deviation derived from the Horwitz equation (σ_H , §4.2.3). A material is considered adequately homogeneous if $s_s \leq 0.3\sigma_H$.

Ten containers of materials B were analyzed in duplicate for flumequine and ten containers of sample C were analyzed in duplicate for lincomycin and spectinomycin to determine the homogeneity of the materials. The results of the homogeneity study and their statistical evaluation are presented in Annex 2a through c. All materials demonstrated to be sufficiently homogeneous for use in the proficiency study. The amounts determined during the homogeneity study are presented in table 3.

No extensive homogeneity study was carried out for material A. The homogeneity of these materials is not relevant because the results of these materials will not be evaluated in a quantitative way. Furthermore, it is assumed that the homogeneity of material A is comparable to the homogeneity of the other materials because all materials are homogenized in the same way. Nevertheless, three randomly selected samples of material A were analyzed for quinolones, macrolides and aminoglycosides. None of these antibiotics were detected. It was concluded that material A is suitable to use as blank materials in the proficiency study.

2.4 Participants

Twenty-six laboratories subscribed for participation in the proficiency study antibiotics in beef of which 25 are situated within Europe. Twenty-three laboratories indicated to carry out a screening analysis. Twenty-two laboratories indicated to have a quantitative confirmatory method operational for quinolones, 17 for macrolides and 11 for aminoglycoside.

2.5 Sample distribution

Each of the participating laboratories received a randomly assigned laboratory code (1 through 26). The sample sets with the corresponding number, consisting of three coded samples (Annex 1) were sent to the participating laboratories at May 27th, 2009. The sample sets were packed in an insulating box containing dry ice or cool packs and were dispatched to the participants immediately by courier. One laboratory (laboratory 16) reported that the samples were not sufficiently frozen at arrival. This laboratory indicated to only carry out a screening analysis and no quantitative analysis and therefore it was decided that the samples were still suited for the laboratory's goal. All other laboratories confirmed the receipt of the samples in good condition (frozen). The samples were accompanied by a letter (Annex 3) describing the requested analyses, an acknowledgement of receipt form and a results form.

The laboratories were asked to store the samples until analysis according to their own laboratory's procedure. A single analysis of each sample was requested, resulting in one results for materials A, B and C. The deadline for sending in results was August 1st 2009, allowing the participants at least 9 weeks for analysis.

2.6 Stability

Just after preparation of the materials six randomly selected samples of each material were stored at <-70 °C. It is assumed that the antibiotics included in this proficiency test are stable at these storage conditions. The remaining samples were stored at -20 °C. Of these, six at random selected samples were subjected to a thaw-freeze cycles to verify if thawing and freezing samples, as is likely to occur when a screening and confirmatory analysis is carried out, does not affect the stability.

At May 6th two sets of six samples were selected and stored at <-70°C. In the morning of July 29th two sets of six samples were selected from the samples stored at -20°C and thawed. After four hours at room temperature these samples were again stored at -20°C. On August 11th, eleven days after the deadline of the proficiency test, six samples that had been stored at -20°C, six samples that were subjected to a thaw-freeze cycle and six samples that had been stored at <-70°C were analyzed for flumequine, lincomycin and spectinomycin. For each set of samples, the average of the results and the standard deviation was calculated.

First it was determined if a consequential instability occurred [7, 8]. A consequential instability occurs when the average value of the samples stored at -20°C or the samples subjected to the thaw-freeze cycle is more than $0.3\sigma_H$ below the average value of the samples stored at <-70 °C. If so, the instability has a significant influence on the calculated z-scores. Second, it was determined if a statistically significant instability occurred using a Students t-test [8]. The results and statistical evaluation of the stability test are presented in Annex 4.

For flumequine no consequential nor a statisitical significant difference was observed between the samples stored at <-70°C and the samples stored at -20°C. The samples subjected to a thaw-freeze cycle resulted in an average that is higher than the average of the samples stored at <-70°C. Because this difference is not statistical significant, it is attributed to a random variation in the analytical method.

For lincomycin no consequential nor a statisitical significant difference was observed between the samples stored at <-70°C, the samples stored at -20°C and the samples that were subjected to a thaw-freeze cycle. The samples are considered sufficiently stable.

For spectinomycin no consequential nor a statisitical significant difference was observed between the samples stored at <-70°C and the samples subjected to a thaw-freeze cycle. The samples stored at -20°C resulted in an average that is below the average of the samples stored at <-70°C. This results in a consequential difference, however this difference is not statistically significant. Surprisingly, the samples subjected to a thaw-freeze cycle result in better stability than the samples stored at -20°C. Therefore it is assumed that the instability is a result of random error of the applied analytical method.

3 Applied methods of analysis

The participating laboratories applied biological, biochemical or instrumental methods or a combination of these methods for screening analysis. An overview of applied screening methods is presented in Annex 5. Eleven laboratories applied a plate test ranging from four to six plates among which two laboratories applied the Nouws Antibiotic Test (NAT). Three laboratories applied the Premi-Test (one preceding a solvent extraction) either or not in combination with other microbiological, biochemical or instrumental methods. Three laboratories applied biochemical methods among which the Charm II methodology. Eleven laboratories applied an instrumental method (LC-MS/MS, LC-ToF MS, LC-FLD or LC-DAD) for the screening analysis.

Eighteen laboratories carried out one or more confirmatory analyses. The substance groups for which a confirmatory analysis was carried out were either selected based on the screening results or carried out as an additional control. An overview of quantitative confirmatory methods applied and the compounds included in the methods is presented in Annex 6a.

For the quantitative and confirmatory analysis of flumequine in beef several different methods are applied. An overview of the applied confirmatory analyses is presented in Annex 6b. For the analysis of flumequine in beef tissue many different extraction solvents or mixtures of solvents were used. For the sample clean up also several different techniques were applied: five laboratories applied solid phase extraction using either the reversed phase or ion exchange principle. Two laboratories use liquid-liquid extraction to clean up their raw extract. The other laboratories filter their extract before injection or inject the raw extract without further clean-up. Three main detection techniques were applied for the quantitative analysis of flumequine in beef: five laboratories applied LC combined with fluorescence (FLD) and/or diode array detection (DAD), nine laboratories used MS/MS as the detection technique and one laboratory applied ToF/MS.

Of the participants that used mass spectrometric detection, nine used an internal standard for the quantification of flumequine. The internal standards used are:

- Cincophen;
- Norfloxacin;
- Norfloxacin-d_5;
- Ciprofloxacin-d_8;
- $^{13}C_3$-flumequine;
- Carbendazim-d_4.

The methods applied for the analysis of lincomycin and spectinomycin are not presented, because only a limited number of laboratories reported information regarding these methods..

4 Statistical evaluation

The evaluation of the screening and quantitative analysis are carried out separately. The screening analysis is evaluated in a qualitative way resulting in a false negative and false positive rate [10]. The statistical evaluation of the quantitative part of the study was carried out according to the International Harmonized Protocol for the Proficiency Testing of Analytical Laboratories [7], elaborated by ISO, IUPAC and AOAC and ISO/DIS 13528 [8] in combination with the insights published by the Analytical Methods Committee [11, 12] regarding robust statistics.

4.1 Screening analysis

First, all laboratories were evaluated separately regarding the screening results in which the number of false positives and false negatives is determined for each laboratory. The number of false positives is the number of samples in which growth inhibition was detected although no antibiotic was present. A result is assigned as false negative if an antibiotic present is not detected although it is included in the method. It is stated that some microbial methods are not able to distinguish between different antibiotic group but only detect growth inhibition in general. In case material C, containing lincomycin and spectinomycin, was reported negative this is considered a false negative result for both compounds. In case growth inhibition was detected in this material, it was assumed correct for both lincomycin and spectinomycin.
After the individual evaluation of the laboratories an overall evaluation was carried out. In this the overall false positive and false negative rate was calculated for all laboratories that send in results for the screening analysis [10]. Next it was studied if any relation exists between false negatives occurring and applied screening methods.

4.2 Quantitative analysis

For the evaluation of the quantitative results the assigend value, the uncertainty of the assigend value, a target standard deviation and z-scores were calculated. For the materials for which less than seven laboratories reported quantitative results, the data is not statistically evaluated.

4.2.1 Calculation of the assigned value

The assigned value (X) was determined using robust statistics [8,11,12]. The advantage of robust statistics is that all values are taken into account: outlying observations are retained, but given less weight. Furthermore, it is not expected to receive normally distributed data in a proficiency test. When using robust statistics, the data does not have to be normally distributed in contrast to conventional outlier elimination methods.
The robust mean of the reported results of all participants, calculated from an iterative process that starts at the median of the reported results using a cut-off value depending on the number of results, was used as the assigned value [8,11]. The assigned value is therefore a consensus value.

4.2.2 Calculation of the uncertainty of the assigned value

The uncertainty of the assigned value is calculated to determine the influence of this uncertainty on the evaluation of the laboratories. A high uncertainty of the assigned value will lead to a high uncertainty of the calculated participants z_a-scores. If the uncertainty of the assigned value and thus the uncertainty of the z_a-score is high, the evaluation could indicate unsatisfactory method performance without any cause within the laboratory. In other words, illegitimate conclusions could be drawn regarding the performance of the participating laboratories from the calculated z_a-scores if the uncertainty of the assigned value is not taken into account.
The uncertainty of the assigned value (the robust mean) is calculated from the estimate of the standard deviation of the assigned value and the number of values used for the calculation of the assigned value:

$$u = \frac{\hat{\sigma}}{\sqrt{n}}$$

where:
u = uncertainty of the assigned value;
n = number of values used to calculate the assigned value;
$\hat{\sigma}$ = The estimate of the standard deviation of the assigned value resulting from robust statistics.

According to ISO/DIS 13528 [8] the uncertainty of the assigned value (u) is negligible and therefore does not have to be included in the statistical evaluation if:

$$u \leq 0{,}3\sigma_p$$

where:
u = The uncertainty of the assigned value;
σ_p = target standard deviation (§ 4.3).

In case the uncertainty of the assigned value does not comply with this criterion, the uncertainty of the assigned value should be taken into account when evaluating the performance of the participants regarding the accuracy (§ 4.4).

4.2.3 Calculation of the target standard deviation

According to Commission Decision 2002/657/EC [13], the coefficient of variation for the repeated analysis of a reference or fortified material under reproducibility conditions, shall not exceed the level calculated by the Horwitz equation. The Horwitz equation, $\sigma_H = 0.02c^{0.8495}$, presents a useful and widespread applied relation between the expected standard deviation of a singular analysis result under reproducibility conditions, σ_H and the concentration, c (g/g). It expresses inter-laboratory precision expected in inter-laboratory trials. Therefore, this relation is suitable for calculating the target standard deviation, σ_p in proficiency tests.

Thompson [7] demonstrated that the Horwitz equation is not applicable to the lower concentration range (<120 µg/kg) as well as to the higher concentration range (>138 g/kg). Therefore a complementary model is suggested:

For analyte concentrations <120 µg/kg:
$\sigma_H = 0.22c$

For analyte concentrations >138 g/kg:
$\sigma_H = 0.01c^{0.5}$

where:
σ_H = expected standard deviation in inter-laboratory trials;
c = concentration of the analyte (g/g).

The target standard deviation (σ_p) of flumequine was determined using the regular Horwitz equation. In this calculation c = the assigned value (X) expressed in g/g and $\sigma_H = \sigma_p$.

4.2.4 Performance characteristics with regard to the accuracy

For illustrating the performance of the participating laboratories with regard to the accuracy a z_a-score is calculated. For the evaluation of the performance of the laboratories, the Guidelines of ISO/IEC Guide 43-1 [3] and ISO/DIS 13528 [8] are applied. According to these guidelines z_a-scores are classified as presented in Table 4.

Table 4: Classification of z_a-scores

$\|z\| \leq 2$	Satisfactory
$2 < \|z\| < 3$	Questionable
$\|z\| \geq 3$	Unsatisfactory

If the calculated uncertainty of the assigned value complies with the criterion mentioned in § 4.2.2, the uncertainty is negligible. In this case the accuracy z-score is calculated from:

$$z_a = \frac{\bar{x} - X}{\sigma_p}$$

where:
z_a = accuracy z-score;
$\bar{x}$ = the average result of the laboratory;
X = assigned value;
σ_p = target standard deviation.

However, if the uncertainty of the assigned value does not comply with the criterion mentioned in § 4.2, it could influence the evaluation of the laboratories. Therefore in this case, the uncertainty is taken into account by calculating the accuracy z-score [8]:

$$z'_a = \frac{\bar{x} - X}{\sqrt{\sigma_p^2 + u^2}}$$

where:
z'_a = accuracy z-score taking into account the uncertainty of the assigned value;
$\bar{x}$ = the average result of the laboratory;
X = assigned value;
σ_p = target standard deviation;
u = uncertainty of the assigned value.

If a consequential instability of the proficiency test materials exists, this can influence the evaluation of the laboratory performance. Therefore, in that case the consequential instability should be taken into account when calculating z-scores. If this is done, the absolute value of the z-scores will decrease. In this proficiency study no correction in z-scores was made for possible consequential instabilities, because all z-scores obtained indicated satisfactory performance already.

5 Results and discussion

Twenty-six laboratories subscribed for the participation in the proficiency test for antibiotics in beef. All laboratories managed to report their results within the timeframe of the study. Of these 23 laboratories carried out a screening analysis and 19 carried out at least one confirmatory analysis (Table 5). For laboratories that carried out a screening and a confirmatory analysis the choice of the applied confirmatory analysis was either based on the screening analysis results or many different confirmatory analysis were applied independent of the screening analysis results.

Table 5. Amount of laboratories that reported results for each analysis.

Analysis	Compound	No. of labs that reported a result
Screening		23
Quantitative / confirmatory	Total	19
	Flumequine	17
	Lincomycin	7
	Spectinomycin	4

5.1 Evaluation of the screening analysis

In the ideal case each laboratory that carried out a screening analysis would find the sample of material A compliant, the sample of material B and C suspect (respectively for quinolones and lincomycin/spectinomycin). The actual screening results are presented in Annex 7a.

In this proficiency test for the screening analysis five false positive results (7%) and 34 false negative results (53%) occurred. For flumequine of the 22 labs that have flumequine included in their method 14 reported material B as a suspect sample for either flumequine, quinolones or a growth inhibitor (32% false negative). For lincomycin of the 20 labs that have lincomycin included in their method nine reported material C as a suspect sample for either lincomycin, macrolides or a growth inhibitor (55% false negatives). For spectinomycin of the 17 labs that have spectinomycin included in their method six reported material C as a suspect sample for either spectinomycin, aminoglycosides or a growth inhibitor (65% false negatives). It is stated that some laboratories applied multiple methods for the screening analysis.

The false negative rate (not detected but included in the method) for the microbiological methods is 73%, for biochemical methods 50% and for instrumental screening methods 22%.

For flumequine it stands out that the false negative results mainly occurred for laboratories that relied on a microbiological screening method. It can be concluded that an *E. coli* plate at pH=8 is not suited for the screening of bovine muscle on the presence of flumequine at relevant levels. The Nouws Antibiotic Test (NAT, 5-plate test) correctly indicated the presence of quinolones in material B. Also one of the

laboratories (lab 6) reported a suspect Premi-test result for material B, but in this case also the blank material was reported suspect.

Lincomycin in material C is only detected by instrumental methods, the microbiological NAT as applied by laboratory 4 and 7 and the Premi-test after solvent extraction as applied by lab 10. Also laboratory 6 reported the presence of a growth inhibitor in the sample of material C using the Premi-test but this laboratory also assigned material A as suspect (false positive). Other laboratories using the Premi-test did not find material C suspect. Also the EU four plate test appears incapable of detecting the antibiotic combination in material C. The same accounts for the biochemical Charm II test, although it should respond to macrolides and lincosamines.
It is concluded that only instrumental methods, the NAT and the Premi-test after solvent extraction are suited for the screening analysis of lincomycin at relevant levels.

Spectinomycin is only detected by instrumental screening methods. Laboratory 6 reported the presence of a growth inhibitor in the sample of material C using the Premi-test but this laboratory also reported growth inhibition for material A and other laboratories using the Premi-test did not find material C suspect. The biochemical Charm II test did not detect spectinomycin, because it responds to streptomycins only. It is concluded that none of the applied microbiological or biochemical methods is able to detect spectinomycin at the level chosen in this proficiency test.

Using instrumental screening methods four false negative results were reported. One was obtained using a targeted method (lab 18, LC-DAD and LC-FLD) and three using non-targeted analysis (lab 19 and 21, LC-ToF/MS). For screening analysis using targeted methods it is of importance that all relevant compounds are included. Flumequine was not included by one laboratory, lincomycin by three laboratories and spiramycin by two laboratories that carried out an instrumental screening analysis for the corresponding antibiotic group.

5.2 Evaluation of the quantitative analysis

Nineteen laboratories carried out one or more quantitative confirmatory analyses. An overview of the compounds found in the samples by the participating laboratories is presented in Annex 8a. Annex 8b gives an overview of false positive results that occurred for the quantitative analysis. One laboratory (Lab 1) reported sulfamethazine with an amount of 25 µg/kg in the sample belonging to material C. This is considered as a false positive result. No false negative results occurred.

Nineteen laboratories carried out a quantitative confirmatory analysis for flumequine. All of these laboratories confirmed the presence of flumequine and reported a quantitative result (Annex 9). The lowest value reported is 209.3 µg/kg and the highest value is 342 µg/kg. The assigned value of flumequine is 267.2 µg/kg with an uncertainty of 7.6 µg/kg. The uncertainty of the assigned value does not exceeds $0.3\sigma_p$ (§4.2), therefore the uncertainty of the assigned value is not taken into account in the evaluation of the laboratories. The z_a-scores obtained by each laboratory were calculated (Annex 9, a graphical representation of the z_a-scores is included). With respect to the accuracy all results are satisfactory.

Seven laboratories carried out a quantitative confirmatory analysis for lincomycin. All of these laboratories confirmed the presence of lincomycin and reported a quantitative result (Annex 10). The lowest value reported is 90 µg/kg and the highest value is 118 µg/kg. The assigned value of lincomycin is 111.7 µg/kg with an uncertainty of 2.5 µg/kg. The uncertainty of the assigned value does exceeds $0.3\sigma_p$ (§4.2), therefore the uncertainty of the assigned value is taken into account in the evaluation of the laboratories. The z'_a-scores obtained by each laboratory were calculated (Annex 10, a graphical representation of the z'_a-scores is included). With respect to the accuracy all results are satisfactory.

Four laboratories carried out a quantitative/confirmatory analysis for spectinomycin. All of these laboratories confirmed the presence of spectinomycin and reported a quantitative result. The lowest value reported is 128 µg/kg and the highest value is 390 µg/kg. Because only four results are reported, no statistical evaluation is carried out.

6 Conclusions

All of the 26 participating laboratories reported results for the proficiency study of antibiotics in beef. In this three laboratories showed optimal performance by detecting all compounds, the absence of false positives and false negatives and a correct quantification of flumequine and lincomycin.

For the screening analysis a high percentage (53%) of false negatives occurred. For the microbiological methods the total false negative rate is 73%, for biochemical tests this is 50% and for instrumental methods this is 22%. The high false negative rate for microbiological and biochemical methods is mainly due to the use of *E. coli* plates at pH=8 for the screening of quinolones giving a negative result for flumequine and to the use of microbiological and biochemical methods that are not able to detect lincomycin and/or spectinomycin.

For the quantitative and confirmatory analysis 17 laboratories reported results for flumequine, seven for lincomycin and four for spectinomycin. For flumequine and lincomycin a statistical evaluation was carried out. For spectinomycin the number of results is too low to draw statistically significant conclusions. For flumequin all laboratories obtained a z_a-score between -1.08 and 1.47 meaning that the performance for the quantitative analysis of flumequine is satisfactory for all laboratories. For lincomycin all laboratories obtained a z'_a-score between -0.88 and 0.26 meaning that the performance for the quantitative analysis of lincomycin is satisfactory for all laboratories.

Based on the results of this proficiency test it is concluded that:

- a huge effort is needed to improve the effectiveness and efficiency for the analyses of unknown samples;
- a huge effort is needed to decrease the false negative rate of mainly microbiological screening methods;
- for effectively applying instrumental screening methods (LC-MS/MS or LC-ToF MS) an effort is needed to include a much wider range of compounds;
- the laboratories performance regarding the quantitative analysis of flumequine and lincomycin in beef is satisfactory.

7 References

1 Council directive 93/99/EEC of 29 October 1993 on the subject of additional measures concerning the official control of foodstuffs. Official Journal L 290, 24/11/1993, 0014 - 0017.

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Annex 1 Codification of the samples

Sample set no.	Material A*	Material B*	Material C*
1	032	145	160
2	078	004	200
3	175	083	197
4	043	105	071
5	188	140	183
6	059	082	177
7	185	161	228
8	041	023	135
9	132	052	235
10	214	172	084
11	147	158	211
12	053	182	226
13	002	232	065
14	215	116	019
15	204	238	067
16	144	031	122
17	077	162	164
18	184	006	206
19	060	213	141
20	010	236	186
21	088	119	180
22	094	165	221
23	223	069	093
24	014	035	202
25	174	027	210
26	229	133	201

** all sample codes start with AB1/2009/MUSCLE/*

Annex 2a Statistical evaluation of homogeneity data of material B for flumequine

	Flumequine (µg/kg)	
Sample No.	Replicate 1	Replicate 2
062	403.4	421.7
086	394.2	388.7
091	441.8	407.1
117	460.9	455.8
150	403.1	398.4
151	396.3	421.6
171	412.4	412.0
178	442.9	367.6
199	439.6	386.4
222	441.6	432.9
Grand mean	415.4	
Cochran's test		
C	0.372	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 75.9	
s_x	20.2	
s_w	20.3	
s_s	14.2	
Critical = 0.3 σ_H	22.8	
s_s < critical?	ACCEPTED	

s_x = standard deviation of the sample averages
s_w = within-sample standard deviation
s_s = between-sample standard deviation

Annex 2b Statistical evaluation of homogeneity data of material C for lincomycin

	Lincomycin (µg/kg)	
Sample No.	Replicate 1	Replicate 2
15	117.6	123.8
42	122.2	117.9
50	118.8	123.5
97	113.2	119.1
98	127.0	121.6
125	122.0	119.5
195	126.5	122.0
218	121.4	118.2
224	121.9	123.7
230	117.8	129.7
Grand mean	121.4	
Cochran's test		
C	0.436	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 26.7	
s_x	2.5	
s_w	4.0	
s_s	0.0	
Critical = 0.3 σ_H	8.0	
s_s < critical?	ACCEPTED	

s_x = standard deviation of the sample averages
s_w = within-sample standard deviation
s_s = between-sample standard deviation

Annex 2c Statistical evaluation of homogeneity data of material C for spectinomycin

	Spectinomycin (µg/kg)	
Sample No.	Replicate 1	Replicate 2
15	195.0	215.2
42	235.5	223.1
50	231.7	199.7
97	229.6	185.9
98	250.2	229.9
125	193.1	238.0
195	231.2	221.8
218	217.4	223.7
224	209.9	188.8
230	209.5	181.9
Grand mean	215.6	
Cochran's test		
C	0.278	
Ccrit	0.602	
C < Ccrit?	NO OUTLIERS	
Target s = σ_H	Horwitz: 43.5	
s_x	14.0	
s_w	19.1	
s_s	3.8	
Critical = 0.3 σ_H	13.0	
s_s < critical?	ACCEPTED	

s_x = standard deviation of the sample averages
s_w = within-sample standard deviation
s_s = between-sample standard deviation

Annex 3 Instruction letter

RIKILT
INSTITUTE OF FOOD SAFETY
WAGENINGEN UR

DATE
11 May 2009

SUBJECT
Proficiency study for antibiotics in beef
Pr. Nr72.036.01

ENCLOSURE(S)
2

OUR REFERENCE
09/RIK0337
est

HANDLED BY
B.J.A. Berendsen

DIRECT (TELEPHONE) LINE
+31 317 48 03 14

E-MAIL
bjorn.berendsen@wur.nl

Dear participant,

Thank you very much for your interest in the proficiency study for antibiotics in beef.

Hereby I send you a parcel containing three randomly coded samples. The samples may contain one or more analytes belonging to one ore more of the following groups (in alphabetical order): aminoglycosides, ß-lactams, macrolides, quinolones, sulfonamides and tetracyclins.
Please fill out the accompanied 'acknowledgement of receipt form' and return it immediately upon receipt of the samples, preferably by fax.

Your laboratory code is:

Instructions:

- After arrival store the samples according to your laboratory's procedure.
- Defrost the samples before analysis and homogenize them according to your laboratoy's procedure.
- Please analyze the samples according to the standard protocols of your laboratory. The samples should be treated as if they are routine samples.
- Please make use your own reference standards. Unfortunately RIKILT – Institute of Food Safety, can not supply any of these reference standards.
- Carry out a **single analysis** for each sample. Please confirm the identity of any detected residues of according to 2002/657/EC.
- Each sample consists of at least 75 g. Please contact me if this is not sufficient for a screening, quantitative and confirmatory analysis.
- The results should be reported before the **1st of August 2009**.
- Please use the results form for reporting the results.
- The evaluation will focus on the screening and quantitative analysis.

Please contact me if you have any questions or need any assistance.

Kind regards,

Bjorn Berendsen

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Annex 4 Statistical evaluation of stability data of material M-B

Statistical evaluation for flumequine in material B

Storage temp	-80 °C	-20°C	Thaw - freeze
Time at -20°C (days)	0	97	97
Calculated amounts (µg/kg)	222.4	276.1	296.4
	242.9	251.0	255.0
	230.9	260.5	221.9
	250.2	236.3	266.8
	207.1	202.3	263.2
	239.0	209.2	235.2
Average amount (µg/kg)	232.1	239.2	256.4
n	6	6	6
st. dev (µg/kg)	15.6	29.0	26.1
Difference		+7.19	+24.4
$0.3\sigma_H$	14.06		
Consequential difference?		No	Yes
Diff < $0.3\sigma_H$			
t		0.53	1.96
t crit		2.23	2.23
Statistical difference?		No	No
T < t crit			

Statistical evaluation for lincomycin in material C

Storage temp	-80 °C	-20°C	Thaw - freeze
Time at -20°C (days)	0	97	97
Calculated amounts (µg/kg)	118	125	124
	114	107	113
	119	114	106
	114	107	111
	108	112	104
	107	104	110
Average amount (µg/kg)	113.6	111.4	111.3
n	6	6	6
st. dev (µg/kg)	4.83	7.67	7.05
Difference		-2.17	-2.35
$0.3\sigma_H$	7.43		
Consequential difference?		No	No
Diff < $0.3\sigma_H$			
t		0.59	0.67
t crit		2.23	2.23
Statistical difference?		No	No
T < t crit			

Statistical evaluation for spectinomycin in material C

Storage temp	-80 °C	-20°C	Thaw - freeze
Time at -20°C (days)	0	97	97
Calculated amounts (µg/kg)	241	192	236
	195	209	232
	208	212	222
	239	217	209
	199	171	205
	231	192	186
Average amount (µg/kg)	218.8	198.8	215.0
n	6	6	6
st. dev (µg/kg)	20.6	17.2	18.7
Difference		-20.0	-3.8
$0.3\sigma_H$	12.7		
Consequential difference?		Yes	No
Diff < $0.3\sigma_H$			
t		1.83	0.34
t crit		2.23	2.23
Statistical difference?		No	No
T < t crit			

Annex 5 Overview of the applied screening methods

Lab	Aminoglycosides	Macrolides	Quinolones	ß-lactams	Sulfonamides	Tetracyclins
2	Not tested	LC-MS/MS	Ridascreen (R-Biopharm)	LC-MS/MS	LC-MS/MS	ELISA (TECNA)
3	LC-MS/MS	LC-UV	LC-FLD	LC-UV	LC-UV	LV-UV
4	NAT: B. subtilis BGA, pH=8.5	NAT : K. rhizophila, pH=8.0	NAT : Yersinia ruckeri, pH=6.5	NAT: Kocuria rhizophila, pH=8.0	NAT : B. pumilis, pH=7.0	NAT : B. cereus, pH=6.0
6	Premi-test	Premi-test	Premi-test	Premi-test	Premi-test	Premi-test
7	NAT: B. subtilis BGA, pH=8.5	NAT : K. rhizophila, pH=8.0	NAT : Yersinia ruckeri, pH=6.5	NAT: Kocuria rhizophila, pH=8.0	NAT : B. pumilis, pH=7.0	NAT : B. cereus, pH=6.0
8	EU 4 plate test	EU 4 plate test	E. coli, pH=8	EU 4 plate test	EU 4 plate test	B. cereus, pH=6
9	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
10	Charm II Streptomycins	Solvent extraction + Premi-test Charm II Macrolides	E. coli Fluoroquinolone EIA Flumequine EIA	Solvent extraction + Premi-test ßeta-s.t.a.r.	Solvent extraction + Premi-test Charm II Sulfa drugs	Tetrasensor tissue 20
11	EU 4 plate test	EU 4 plate test	E. coli, pH=8	EU 4 plate test	EU 4 plate test	EU 4 plate test
12	EU 4 plate test	EU 4 plate test	E. coli, pH=8	EU 4 plate test	EU 4 plate test	EU 4 plate test
13	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
14	B. subtilis	K. rhizophila HPLC-MS	HPLC-FLD	Charm	Charm	B. cereus 1178 B. cereus K250 Charm
15	EU 4 plate test	EU 4 plate test	E. coli, pH=8	EU 4 plate test	EU 4 plate test	B. cereus, pH=6
16	EU 4 plate test	EU 4 plate test	E. coli, pH=8	EU 4 plate test	EU 4 plate test LC-MS/MS	B. cereus, pH=6
17	Charm II	Charm II	LC-FLD	Charm II	LC-DAD	Charm II
18	Not tested	LC-MS/MS	LC-DAD/FLD	LC-MS/MS	TLC	Charm II

Lab	Aminoglycosides	Macrolides	Quinolones	ß-lactams	Sulfonamides	Tetracyclins
19	UPLC-ToF MS	UPLC-ToF MS	UPLC-ToF MS	UPLC-ToF MS	UPLC-ToF MS	UPLC-ToF MS
20	B. subtilis, pH=6, 7.4 and 8	B. subtilis, pH=6, 7.4 and 8	B. subtilis, pH=6, 7.4 and 8	B. subtilis, pH=6, 7.4 and 8	B. subtilis, pH=6, 7.4 and 8	B. subtilis, pH=6, 7.4 and 8
21	UPLC-ToF MS	UPLC-ToF MS	UPLC-ToF MS	UPLC-ToF MS	UPLC-ToF MS	UPLC-ToF MS
22	Premi-test LC-MS/MS	Premi-test LC-MS/MS	Premi-test LC-MS/MS	Premi-test	Premi-test	Premi-test
24	Premi-test	Premi-test LC-MS/MS	Premi-test LC-MS/MS	Premi-test	Premi-test	Premi-test
25	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
26	EU 4 plate test + B. stearothermophilus	EU 4 plate test + B. stearothermophilus	E. coli, pH=8	EU 4 plate test + B. stearothermophilus	EU 4 plate test + B. stearothermophilus	EU 4 plate test + B. stearothermophilus

Kocuria rhizophila = *Micrococcus luteus* ATCC 9341

The EU 4 plate test comprises *B. subtilis* at pH 6, 7.2 or 7.4 (+ trimethoprim) and pH 8, and *K. rhizophila*

Annex 6a Overview of compounds included in the quantitative methods applied

Lab	Aminoglycosides	Macrolides	Quinolones	ß-lactams	Sulfonamides	Tetracyclins
1		Spiramycin, erythromycin, josamycin	**Flumequine**, enrofloxacin, marbofloxacin, danofloxacin, oxolinic acid	Amoxicillin, trimethoprim, ampicillin, penicillin G, penicillin V, oxicillin, cloxacillin, dicloxacillin	sulfadiazine, sulfathiazole, sulfapyridine, sulfamreazine, sulfachloropyridazine, sulfamethazine, sulfamethizole, sulfmethoxypyridizine, sulfamonomethoxine, mulfamethoxazole, sulfadoxine, sulfisoxazole, sulfadimethoxine, sulfaquinoxaline	
2	Gentamycin, neomycin, dihydrostreptomycin, streptomycin, kanamycin	* Tylosin	**Flumequine**, marbofloxacin, norfloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid	* Amoxycillin, ampicillin, cloxacillin, dicloxacillin, oxacillin, penicillin G, penicillin V	* Sulfadiazine, sulfamerazine, sulfamethazine, sulfadoxine, sulfadimethoxine, sulfamethoxazole, sulfathiazole, sulfamethoxypyridazine, trimethoprim	oxytetracycline, tetracycline, chlortetracycline, doxycycline
3	* Apramycin, dihydrostreptomycin, neomycin, paromomycin, streptomycin	* **Lincomycin**, pirlimycin Spiramycin, tilmicosin, tylosin	**Flumequine**, danofloxacin, enrofloxacin, ciprofloxacin, marbofloxacin, oxolinic acid, nalidixic acid, sarafloxacin	Amoxicillin, ampicillin, penicillin G, cloxacillin, dicloxacillin, nafcillin, oxacillin, penicillin V	Sulfadiazine, sulfachlorpyridazine, sulfadoxine, sulfamerazine, sulfamethazine, sulfamethizole, sulfemethoxazole, sulfathiazole	oxytetracycline, tetracycline, chlortetracycline, doxycycline
4		**Lincomycin**, tulathromycin, spiramycin, tilmicosin, tylosin, erythromycin, josamycin, aivlosin, pirlimycin, tiamulin, valnemulin	**Flumequine**, ciprofloxacin, enrofloxacin			
5					Sulfadiazine, sulfapyridine, sulfamethazine, sulfadimethoxine, sulfamethoxypyridazine	

Annex 6a continued Overview of compounds included in the quantitative methods applied

Lab	Aminoglycosides	Macrolides	Quinolones	ß-lactams	Sulfonamides	Tetracyclins
7		**Lincomycin**, tulathromycin, spiramycin, tilmicosin, tylosin, erythromycin, josamycin, aivlosin, pirlimycin, tiamulin, valnemulin	**Flumequine**, marbofloxacin, norfloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid			
8			**Flumequine**, marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid		* Sulfadiazine, sulfathiazole, sulfapyridine, trimethoprim, sulfamerazine, sulfamethizole, sulfamethazine, sulfamethoxypyridazine, sulfamonomethoxine, sulfachloropyridazine, dapsone, sulfamethoxazole, sulfisoxazole, sulfaquinoxaline, sulfadimethoxine	* Oxytetracycline, tetracycline, chlortetracycline, doxycycline
10			**Flumequine**			
11			**Flumequine**, danofloxacin, difloxacin, enrofloxacin, ciprofloxacin, oxolinic acid	Amoxycillin, ampicillin, penicillin G, penicillin V, cloxacillin, dicloxacillin, nafcillin, oxacillin, caphalexin, cefoperazone, ceftiofur	Sulfadimethoxine, sulfamethazine, sulfamethoxypiridazine, sulfapyridine, sulfathiazole, sulfaquinoxaline, sulfadiazine, sulfamerazine, sulfacetamide	Oxytetracycline, tetracycline, chlortetracycline, doxycycline
13	* **Spectinomycin**, Steptomycin, dihydrostreptomycin, kanamycin, apramycin, paromycin, gentamycin, neomycin	* **Lincomycin**	**Flumequine**, marbofloxacin, norfloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid			
14		Spiramycin, tylosin, erythromycin, tilmicosin, josamycin	**Flumequine**, norfloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid			

Lab	Aminoglycosides	Macrolides	Quinolones	ß-lactams	Sulfonamides	Tetracyclins
17			**Flumequine**, marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid			
19	**Spectinomycin**		**Flumequine**			
20		* **Lincomycin**, erythromycin, spiramycin, josamycin, tylosin	* **Flumequine**, norfloxacin, marbofloxacin, difloxacin, enrofloxacin, oxolinic acid, sarafloxacin	* Amoxycillin, cephalexin, cloxacillin, nafcillin, oxacillin, dicloxacillin, penicillin G, cephazoline, ampicillin, penicillin V, cephapirin	Sulfadiazine, sulfamethizole, sulfathiazole, sulfapyridine, sulfamerazine, sulfachloropyridazine, sulfamoxole, sulfamethazine, sulfamethoxypyridazine, sulfaquinoxaline, sulfamethoxine, sulfamethoxazole	Oxytetracycline, tetracycline, chlortetracycline, doxycycline
21			**Flumequine**, norfloxacin, ciprofloxacin, enrofloxacin, danofloxacin, marbofloxacin, sarafloxacin, difloxacin, cinoxacin, lomefloxacin, ofloxacin, enoxacin, fleroxacin, oxolinic acid, nalidixic acid, pefloxacin methanesulfonate			
22	Spectinomycin	Lincomycin	**Flumequine**	,		
23			**Flumequine**, oxolinic acid		Sulfadiazine, sulfathiazole, sulfamerazine, sulfamethazine, sulfamethoxypyridazine, sulfachloropyridazine, sulfamethoxazole, sulfameter, sulfaquinoxaline, sulfamethoxine	Oxytetracycline, tetracycline, chlortetracycline
25	**Spectinomycin**, streptomycin, dihydrostreptomycin, apramycin, neomycin, gentamycin, paromomycin, kanamycin	* **Lincomycin**, tulathromycin, tylosin, tilmicosin, erythromycin, spiramycin	* **Flumequine**, danofloxacin, difloxacin, enrofloxacin, ciprofloxacin, marbofloxacin, oxolinic acid		* Sulfathiazole, sulfamethoxazole, sulfamerazine, sulfamethoxypyridazine, sulfadiazine, sulfadoxine, sulfadimethoxine, sulfadimidine, sulfanilamide, trimethoprim	* Oxytetracycline, tetracycline, chlortetracycline, doxycycline

Annex 6a continued Overview of compounds included in the quantitative methods applied

Lab	Aminoglycosides	Macrolides	Quinolones	ß-lactams	Sulfonamides	Tetracyclins
26	Streptomycin, dihydrostreptomycin, neomycin, gentamycin	Erythromycin , josamycin, spiramycin, tilmicosin, tylosin, tulathromycin			Sulfachloropyridazine, sulfamethoxazole, sulfadiazine, sulfathiazole, sulfadimethoxine, sulfaquinoxaline, sulfadimidine, sulfamethoxydiazine, sulfadoxine	Oxytetracycline, tetracycline, chlortetracycline, doxycycline

** combined in one method*

Annex 6b Overview of the applied quantitative methods for flumequine

Lab code	Extraction	Sample purification	Internal standard	Detection method
1	60% MeOH + EDTA	Dilution with water, Filtration 0.22 µm	Norfloxacin-d_5	LC-MS/MS
2	Phosphatebuffer pH=7.4	SPE (OASIS HLB), evaporation of solvent,, filtration 0.45 µm		LC-DAD / FLD
3		SPE		LC-FLD
4	Water	Filtration (paper filter)		LC-FLD
7	Water	Ultrafiltration	Cincophen	LC-MS/MS
8	4% TFA in water	SPE (C18), evaporation of solvent	Norfloxacin	UPLC-MS/MS
10	Acetonitril		Cincophen	LC-MS/MS
11	Water	SPE (OASIS-HLB)	Norfloxacin-d_5	LC-MS/MS
13	TCA in water	Filtration	Norfloxacin-d_5	LC-MS/MS
14	Metaphosphoric acid, MeOH, ACN	SPE (OASIS HLB)		LC-FLD
17	EtOH, Acetic acid	Partially evaporation of solvent, add EtOH, phosphoric acid/acetic cid/triethylamin and ACN		LC-FLD
19	Ethanol	SPE (NH_2, PRS), evaporation of solvent		LC-MS/MS
20	70% MeOH + EDTA	Dilute with water		LC-MS/MS
21	ACN, Na_2SO_4	Add DMSO to supernatant, evaporation of ACN, ultracentrifuge	Carbendazim-d_4	UPLC-ToF MS
22	ACN, formic acid	SPE (OASIS MCX)	$^{13}C_3$-flumequine	LC-MS/MS
23	Acetone	Add propanol, evaporate solvent, add chloroform, adjust pH to 5-6, extraction with chloroform, evaporation of solvent		LC-FLD
25	ACN, MCIlvaine buffer	Extraction with ethylacetate, evaportaion of solvent	Ciprofloxacin-d_8	LC-MS/MS

Annex 7a Overview of screening results

Lab	Material A	Material B	Material C
2	-	Quinolones	-
3	-	Flumequine	Lincomycin
4	-	Quinolones	Macrolide
6	Growth inhibitor	Growth inhibitor	Growth inhibitor
7	-	Quinolones	Macrolide
8	Macrolide	Macrolide	-
9	-	-	-
10	-	Flumequine	Growth inhibitor
11	-	-	-
12	-	-	-
13	-	Flumequine	Lincomycin Spectinomycin
14	-	Quinolones	-
15	ß-lactam Macrolide	-	-
16	-	-	-
17	-	Flumequine	-
18	-	-	-
19	-	Flumequine	Spectinomycin
20	-	-	-
21	-	Flumequine	-
22	-	Flumequine	Lincomycin Spectinomycin
24	-	Flumequine	Lincomycin
25	-	Flumequine	Lincomycin Spectinomycin

- = not detected

Annex 7b False positives and false negatives in screening analysis

False positive results

Lab code	Sample code	Material	Suspect for
6	059	A	Growth inhibitor
8	041	A	Macrolides
8	023	B	Macrolides
15	204	A	ß-lactams
15	204	A	Macrolides

False negative results

Lab code	Sample code	Flumequine	Sample code	Lincomycin	Spectinomycin
2			200	X	X*
3			197		X*
4			071		X
7			228		X
8	023	X	135	X	X
9	052	X*	235	X*	X*
11	158	X	211	X	X
12	182	X	226	X	X
14			019	X	X*
15	238	X	067	X	X
16	031	X	122	X	X
17			164	X	X
18	006	X	206	X*	X*
19			141	X*	
20	236	X	186	X	X
21			180	X	X
24			202		X

X = Not detected

* not included in the analysis

Annex 8a Overview of quantitative/confirmatory results

Lab	Material A	Material B	Material C
1		Flumequine	Sulfamethazine
2		Flumequine	
3		Flumequine	Lincomycin
4		Flumequine	Lincomycin
5			
7		Flumequine	Lincomycin
8		Flumequine	
10		Flumequine	
11		Flumequine	
13		Flumequine	Lincomycin, Spectinomycin
14		Flumequine	
17		Flumequine	
19		Flumequine	Spectinomycin
20		Flumequine	Lincomycin
21		Flumequine	
22		Flumequine	Lincomycin Spectinomycin
23		Flumequine	
25		Flumequine	Lincomycin Spectinomycin
26			

Annex 8b False positives and false negatives in quantitative/confirmatory analysis

False positive results

Lab code	Sample code	Material	Compound confirmed
1	160	C	Sulfamethazine

False negative results

None

Annex 9 Results for the analysis of flumequine

Flumequine Assigned value: 267.2 µg/kg Uncertainty of assigned value: 7.6 µg/kg Target standard deviation (Horwitz, Thompson): 52.1 µg/kg		
Lab code	Result (µg/kg)	z_a-score
1	342	1,43
2	266	-0,02
3	285	0,34
4	300,1	0,63
7	320	1,01
8	209,3	-1,11
10	276	0,17
11	269	0,03
13	218	-0,94
14	290	0,44
17	239	-0,54
19	210	-1,10
20	250	-0,33
21	250	-0,33
22	260	-0,14
23	278	0,21
25	276	0,17

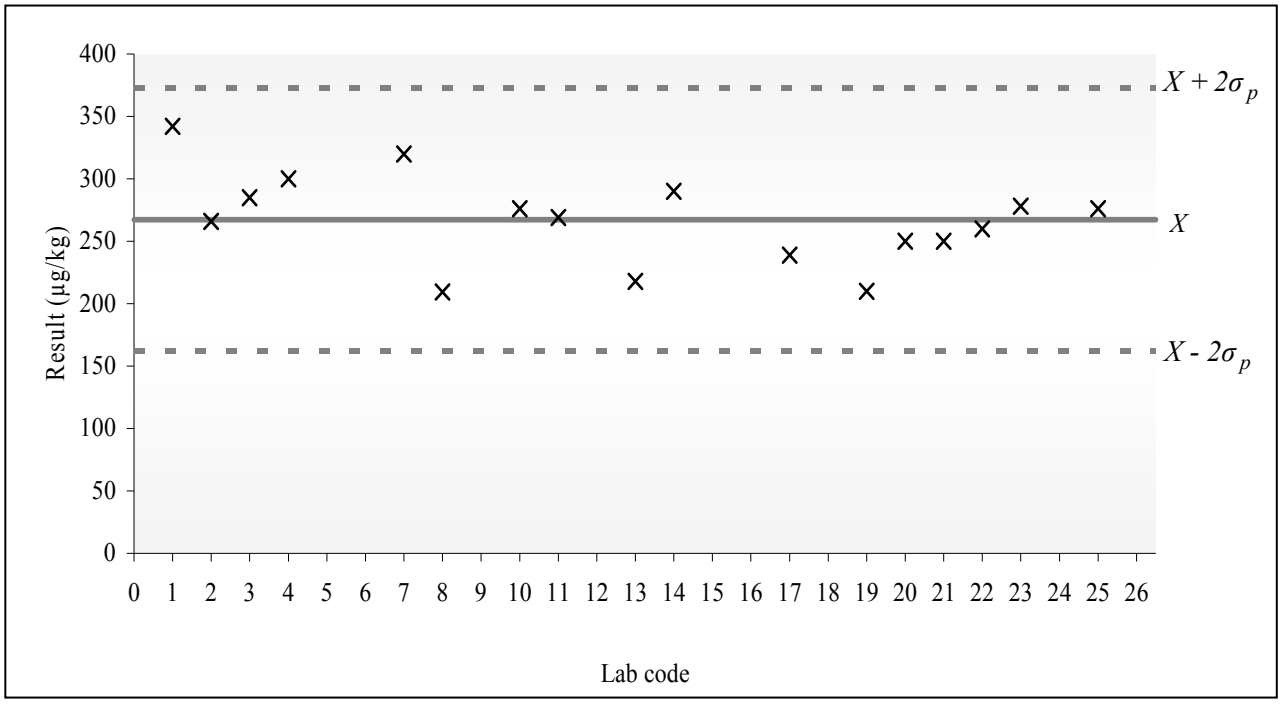

Figure a: Graphical representation of the reported results

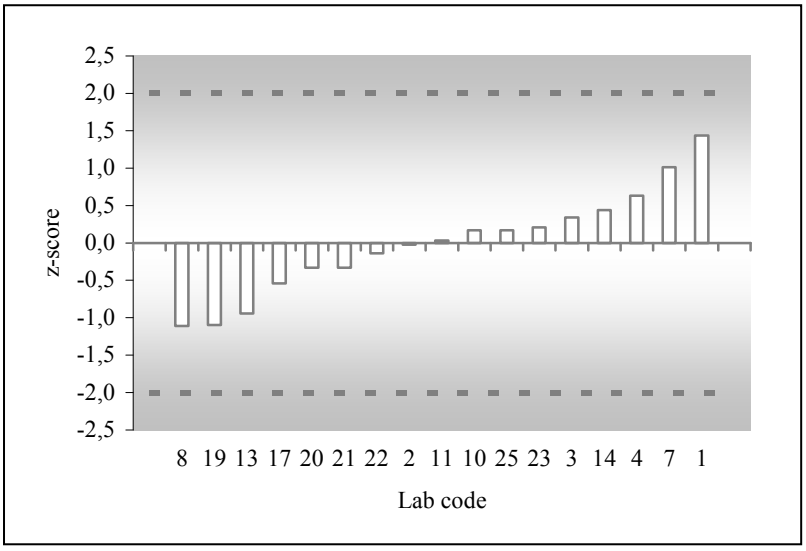

Figure b: Graphical representation of z-scores

Annex 10 Results for the analysis of lincomycin

Lincomycin Assigned value: 111.7 µg/kg Uncertainty of assigned value: 2.46 µg/kg Target standard deviation (Horwitz, Thompson): 24.6 µg/kg		
Lab code	Result (µg/kg)	z'_a-score
3	90	-0,88
4	117,3	0,23
7	114	0,09
13	115	0,14
20	118	0,26
22	104	-0,31
25	105	-0,27

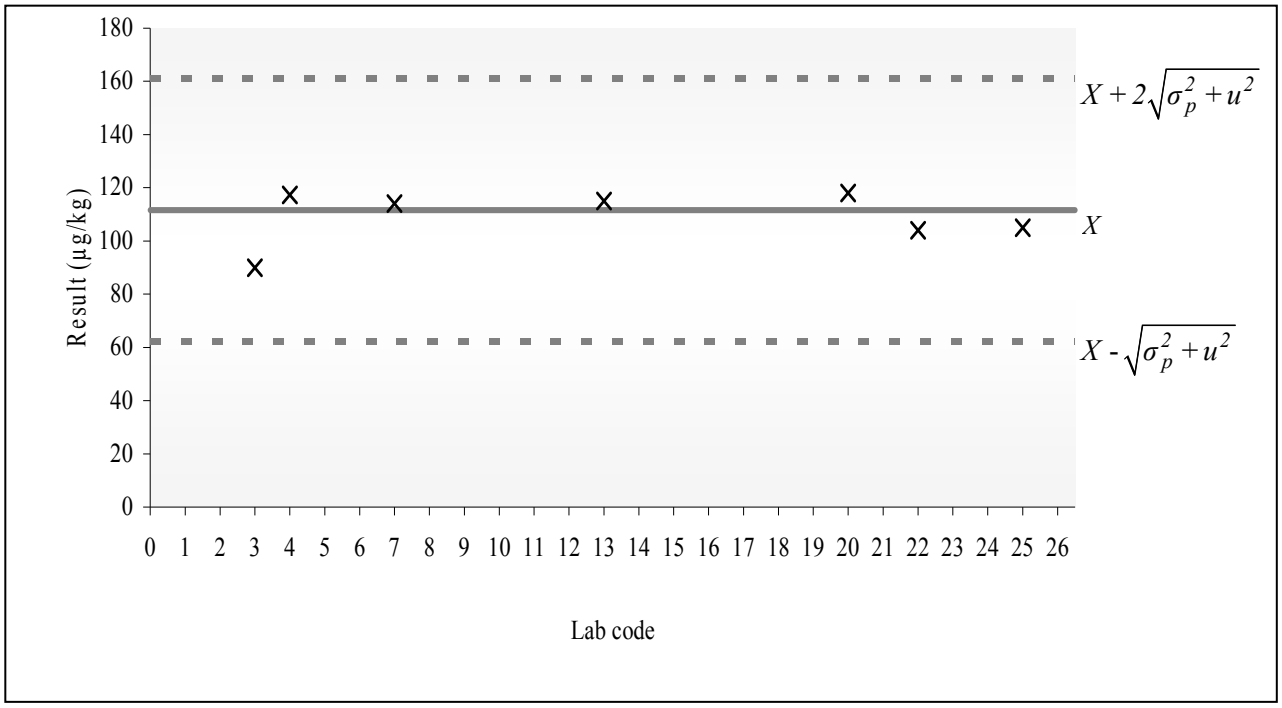

Figure a: Graphical representation of the reported results

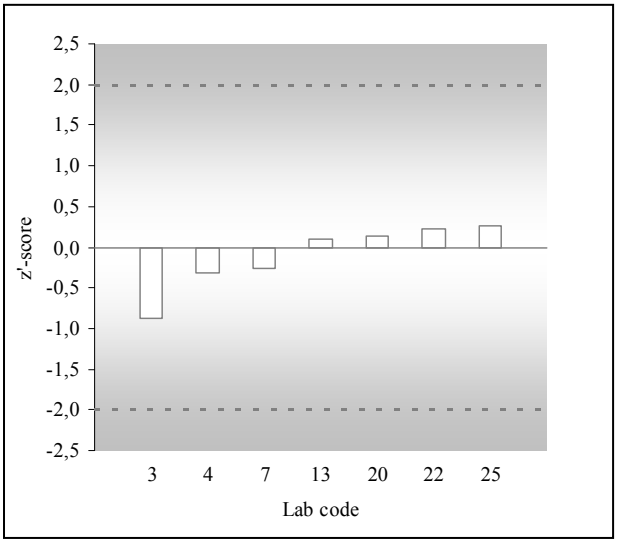

Figure b: Graphical representation of z'-scores

Annex 11 Results for the analysis of spectinomycin

Spectinomycin	
Lab code	Result (µg/kg)
13	163
19	210
22	390
25	128