NMKL - NordVal International
c/o Institute of Marine Research
P.O. box 1870 Nordnes, N-5817 Bergen, Norway
www.nmkl.org

NordVal International Certificate

Issued for:	**BRT MRL Screening Test**
NordVal No:	052
First approval date:	01 March 2019
Renewal date:	01 March 2023
Valid until:	01 March 2025

BRT MRL Screening Test

Manufactured by:

Analytik in Milch Produktions- und Vertriebs-GmbH
Kaiser-Ludwig-Platz 2
80336 München
Germany

fulfils the validation requirements of the NordVal Validation Protocol 2. The BRT MRL Screening Test is a Brilliant Black Reduction Test for the detection of antibiotic residues.

The method is tested for penicillins, cephalosporins, macrolides, sulfonamides, tetracyclines, aminoglycosides and Chloramphenicol in raw bovine milk on microtiter plates. Detection capability for clear results and presumptive results of photometric and visual reading is stated. In order for the method to be applicable in the EU, the detection capabilities for the substances of interest must be below given EU Maximum Residue Limits (MRL). The detection capabilities and associated MRLs for the substances tested are given in Table 2. Examples of the probability of detection (POD) / dose-response curves are given in the certificate for illustration. POD-curves for all substances are given in the validation report, which is enclosed as an annex.

The performance of the method was tested in a comprehensive validation study carried out at the laboratory of Milchprüfring Bayern e. V. and tested in a large proficiency test.
The production of BRT MRL Screening Test is ISO 9001:2015-accredited by LGA InterCert GmbH.

Date: 01 March 2023

Yours sincerely,

Hilde Skår Norli
Chair of NordVal International

Eystein Oveland
NMKL Executive Director

PRINCIPLES OF THE METHOD

The BRT MRL Screening Test is a modified Brilliant Black Reduction Test (BRT) containing the test bacteria *G. stearothermophilus* var. *calidolactis* C953, the redox indicator brilliant black, nutrients and other supplements. Antibiotic residues present in a sample can inhibit the growth of the test bacteria, thus preventing or decelerating the reduction of the colour indicator brilliant black and the consecutive colour change of the test medium from blue to yellow.

Milk samples (100 µl milk volume), positive and negative controls are added to the plates. Incubation is performed at 65 ℃ for 2 h 30 min ± 15 min until the complete discoloration of the negative control (colour change from blue to yellow) indicates the ideal reading time. Thereafter, milk is rinsed off the cavities for easier detection. The plates can be assessed with 2 different reading methods: visual examination or photometric evaluation using the measuring wavelength of 450 nm and the reference wavelength of 620 nm.

The recorded absorption values of the analysed samples are converted into relative percentage values by setting the average absorption level of the negative controls (yellow colour after incubation) as 0 % and that of the positive controls (blue colour after incubation) as 100 %, the absorption levels of the samples are set in relation to negative (0 %) and positive (100 %) controls.

The conversion formula is as follows: (S-NC)/(PC-NC) x 100 = X %
where

S is the analysed sample´s absorption level
NC is the average of the four negative controls absorption levels
PC is the average of the four positive controls absorption levels
X is the relative percentage value of the analysed sample

Results are referred to as either Class A or Class B as shown in **Table 1**.

Table 1. Relation of reading systems and classes of results

	Classes of results		
Reading system	Positive Class A Clear	Positive Class B Presumptive	Negative
Visual	1	2	0
Photometric	≥ 65%	40% - < 65 %	< 40%

Samples used in the study

High-quality raw ex-farm bulk milk proven free of antibiotic residues was spiked with antibiotics. To verify the correct concentration of the stock solutions and the spiked raw milk samples, serial dilutions of the prepared positive samples were analysed with microbiological inhibitor tests and, when applicable, with receptor tests or analysed by LC-MS/MS.

Batch-to-batch variation
Potential deviations in the detection capabilities of different plate batches were evaluated statistically, and it was shown that there were no statistically significant differences between the batches of plates.

METHOD PERFORMANCE CHARACTERISTICS

Selectivity

Marker substances of commonly used classes of veterinary drugs other than antibiotics were analysed with photometric reading in order to determine the selectivity of the BRT MRL Screening Test. The investigated compounds included the anti-inflammatories Flunixin, Metamizole (NSAIDs) and Prednisolone (glucocorticoid) as well as antiparasitic substances (Triclabendazole and Deltamethrin). Furthermore, the polyether-antibiotic Monensin, used for ketosis treatment in dairy cows, was tested. The substances were spiked at a concentration of 100 x EU MRL and inoculated with 6 replicates. None of the tested substances inhibited the growth of the test germs, leading to negative results (both Class A and Class B). Thus, no false-positive results were observed.

Detection capability (CCβ)

For the determination of the detection capability (CCβ), three different batches of plates were used at all times. The lowest concentration obtaining a minimum of 95 % positive results was considered as detection limit (CCβ). Based on the different interpretation methods, CCβ A and CCβ B (for Class A results: clear; and Class B results: presumptive, respectively - **Table 1**) were established in parallel for each substance. **Table 2** shows the results. The substances exceeding the EU MRLs or having a high detection limit (Chloramphenicol) are marked in red and written in italic.

In the EU legislation maximum residue limits (MRL) are stated for milk, and concentrations of veterinary drugs below these limits are accepted. According to this, the BRT MRL Screening Test plates are applicable for the detection of the penicillin compounds: Benzylpenicillin, Ampicillin, Amoxicillin, Cloxacillin, Dicloxacillin, Nafcillin and Oxacillin, for the cephalosporins: Cefapirin, Cefoperazone, Cefazolin and Cefalonium; and for Neomycin. The method was found not to be applicable for the detection of certain cephalosporins, macrolides, sulfonamides, tetracyclines and aminoglycosides at the maximum permitted EU residue level (MRL). Further, for chloramphenicol, the EU minimum required performance limit (MPRL) is 0.3 ug/kg and hence the method is not applicable for this substance (Commission Decision of 13 March 2003 amending Decision 2002/657/EC).

Some compounds could be detected at CCβ B level (presumptive results; 40 - <65 %, **Table 1**) only. Thus, for both photometric and visual reading presumptive results can be obtained for Ceftiofur, Tylosin, Sulfadiazine, Sulfadimethoxin, Sulfathiazol, Sulfamethoxypyridazine and Gentamicin.

Table 2. Substances tested, EU Maximum Residue Limits (MRL) and detection limits by photometric and visual reading for CCβ A (≥ 65 %) and CCβ B (40-65 %).

Group of antibiotics	Substance	MRL EU* [µg/kg]	CCβ A [µg/kg]	CCβ B [µg/kg]	CCβ A [µg/kg]	CCβ B [µg/kg]
			Photometric		Visual	
Penicillins	Benzylpenicillin	4	2	1.5	2	1.5
	Ampicillin	4	2.5	2	2.5	2.5
	Amoxicillin	4	3	2.5	3	2.5
	Cloxacillin	30	25	18	25	20
	Dicloxacillin	30	12.5	10	12.5	10
	Nafcillin	30	10	8	10	8
	Oxacillin	30	8	8	8	8
Cephalosporins	Cefalexin	100	*300*	*250*	*300*	*250*
	Cefapirin	60	5	5	5	5
	Cefoperazone	50	30	20	30	25
	Cefazolin	50	7	6	7	6
	Cefquinome	20	*300*	*200*	*200*	*200*
	Ceftiofur	100	*150*	100	*150*	100
	Cefalonium	20	12	10	12	10
Macrolides	Erythromycin	40	*80*	*50*	*80*	*60*
	Tylosin	50	*75*	30	*75*	40
Sulfonamides	Sulfadiazine	100	*400*	100	*300*	100
	Sulfadimethoxin	100	*600*	100	*400*	100
	Sulfamethazine	100	*>1,000*	*200*	*>1,000*	*200*
	Sulfathiazol	100	*200*	60	*200*	60
	Sulfadoxin	100	*1,500*	*300*	*400*	*300*
	Sulfamethoxypyridazine	100	*500*	100	*300*	100
Tetracyclines	Chlortetracycline	100	*800*	*400*	*600*	*400*
	Oxytetracycline	100	*400*	*200*	*600*	*300*
	Tetracycline	100	*600*	*300*	*400*	*300*
Aminoglycosides	Dihydrostreptomycin	200	*600*	*400*	*700*	*500*
	Streptomycin	200	*1,000*	*500*	*1,500*	*800*
	Gentamicin	100	*150*	80	*125*	80
	Neomycin	1,500	300	200	300	200
Fenicol	Chloramphenicol	-	*5,000*	*3,500*	*5,000*	*3,500*

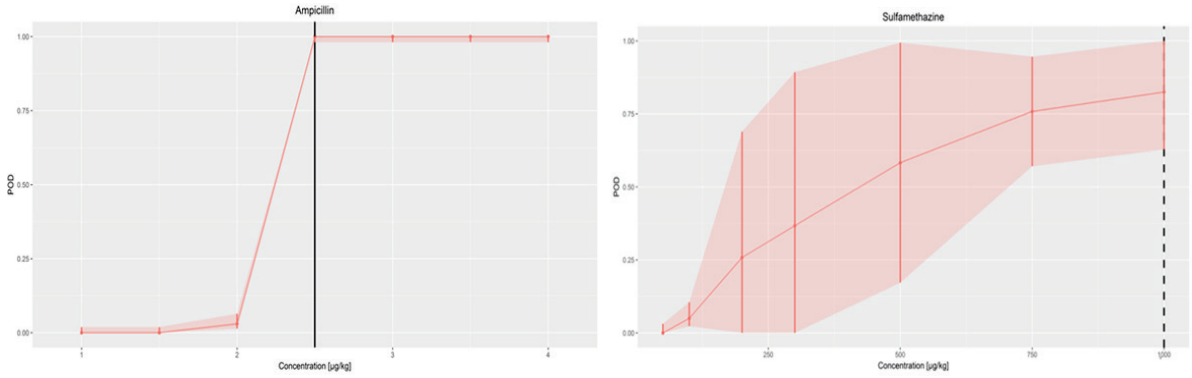
* Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

Probability of detection (POD) / dose-response

Probability of detection was established based on the Class A results with both photometric and visual evaluation (results are presented in the validation report Annex 1, Table 2). For combined photometric and visual readings of the Class A results, lower and upper CIs were calculated (presented in the validation report, Figure 3 and in its annex, Figure 1).

Bactericidal substances like beta-lactams mostly exhibited steeply increasing dose-response curves and narrow CIs (example **Figure 1a**, **Table 3**). In contrast, bacteriostatic substances displayed more consistent curve increments and often showed bigger variations between the two reading methods (example **Figure 1b**, **Table 4**). The width of the confidence interval provides information about the uncertainty of the measurement, as the wider the confidence interval the greater the uncertainty. Compared to photometric reading, the visual interpretation leads to bigger variances in the results and thus to wider CIs.

Figure 1. Dose-response curves for Ampicillin (a) and Sulfamethazine (b), combined results for photometric and visual reading.



In **Table 3**, showing results of the dose-response study for Ampicillin, it can be seen that at 2 µg/kg for photometric reading, two results equalling 3% of the total results, were clearly positive (absorbance level ≥ 65%). The additional 78 results were presumptive positive having absorbance levels of 40% - < 65 %. Adding these results to the clear positives resulted in a total of 100% CCβ B. For visual reading at 2 µg/kg, 4 results were clearly positive (Class A), but 92% of the samples were presumptive (Class B). At 2.5 µg/kg, photometric and visual reading gave 100 % clear positive (Class A) and, thus, the same (100 %) when clear positive and presumptive were combined (CCβ B).

Table 3. Dose-response for Ampicillin

Reading system	Conc µg/kg	No. of total results	No. of results (pos. Class A)	Share of CCβ A	Lower 95 %-CI (CCβ A)	Upper 95 %-CI (CCβ A)	No. of results (pos. Class B)	Share of CCβ B	No. of results (negative)
Photo	1	80	0	0.00	-	-	0	0.00	80
	1.5	80	0	0.00	-	-	6	0.08	74
	2	80	2	0.03	-	-	78	1.00	0
	2.5	80	80	1.00	-	-	0	1.00	0
	3	80	80	1.00	-	-	0	1.00	0
	3.5	80	80	1.00	-	-	0	1.00	0
	4	80	80	1.00	-	-	0	1.00	0
Visual	1	120	0	0.00	-	-	0	0.00	120
	1.5	120	0	0.00	-	-	2	0.02	118
	2	120	4	0.03	-	-	106	0.92	10
	2.5	120	120	1.00	-	-	0	1.00	0
	3	120	120	1.00	-	-	0	1.00	0
	3.5	120	120	1.00	-	-	0	1.00	0
	4	120	120	1.00	-	-	0	1.00	0
Photo + visual	1	200	0	0.00	0.00	0.02	0	-	200
	1.5	200	0	0.00	0.00	0.02	8	-	192
	2	200	6	0.03	0.01	0.06	184	-	10
	2.5	200	200	1.00	0.98	1.00	0	-	0
	3	200	200	1.00	0.98	1.00	0	-	0
	3.5	200	200	1.00	0.98	1.00	0	-	0
	4	200	200	1.00	0.98	1.00	0	-	0

In **Table 4**, showing results of the dose-response study for Sulfamethazine, it can be seen that even at the highest concentration tested (1000 µg/kg), the ratio of results detected as clearly positive (absorbance level ≥ 65 %; Class A results) did not reach more than 65 % for photometric reading and 94 % for visual reading. At 200 µg/kg, for photometric reading, two results, equalling 4 % of the results, were clearly positive (absorbance level ≥ 65 %). The additional 46 results were presumptive positive having absorbance levels between 40 % - < 65 %. Adding this to the clear positives resulted in a total of 100 % CCβ B. 100% CCβ B at 200 µg/kg was also obtained for visual reading with 29 results being clear positive and 43 being presumptive.

Table 4. Dose-response for Sulfamethazine.

Reading system	Conc µg/kg	No. of total results	No. of results (pos. class A)	Share of CCβ A	Lower 95 %-CI (CCβ A)	Upper 95 %-CI (CCβ A)	No. of results (pos. class B)	Share of CCβ B	No. of results (negative)
Photo	50	48	0	0.00	-	-	2	0.04	46
	100	48	1	0.02	-	-	27	0.58	20
	200	48	2	0.04	-	-	46	1.00	0
	300	48	5	0.10	-	-	43	1.00	0
	500	48	11	0.23	-	-	37	1.00	0
	750	48	29	0.60	-	-	19	1.00	0
	1,000	48	31	0.65	-	-	17	1.00	0
Visual	50	72	0	0.00	-	-	11	0.15	61
	100	72	5	0.07	-	-	35	0.56	32
	200	72	29	0.40	-	-	43	1.00	0
	300	72	39	0.54	-	-	33	1.00	0
	500	72	59	0.82	-	-	13	1.00	0
	750	72	62	0.86	-	-	10	1.00	0
	1,000	72	68	0.94	-	-	4	1.00	0
Photo + visual	50	120	0	0.00	0.00	0.03	13	-	107
	100	120	6	0.05	0.02	0.11	62	-	52
	200	120	31	0.26	0.00	0.69	89	-	0
	300	120	44	0.37	0.00	0.89	76	-	0
	500	120	70	0.58	0.17	0.99	50	-	0
	750	120	91	0.76	0.57	0.95	29	-	0
	1,000	120	99	0.83	0.63	1.00	21	-	0

Specificity - Rate of positive results not caused by residues of veterinary drugs

In order to demonstrate that the BRT MRL Screening Test performs properly with a broad range of samples, 704 ex-farm bulk milk samples were examined. Two out of 704 samples (0.28%) were detected positive by the BRT MRL Screening Test. Both samples were confirmed to contain Cloxacillin. Thus, the rate of positive results not caused by residues of veterinary drugs was 0 %, as all positive samples detected were confirmed to contain antibiotic inhibitors (**Table 5**).

Table 5. The specificity tested in 704 samples

	Negative samples		Positive samples			
Total no. samples	No.	Rate	No.	Rate	False positive	Confirmed positive
704	702	99.72 %	2	0.28 %	0 %	100 %

False-negative rate (FN) / False-positive rate (FP)

In this validation, 142 test plates were analysed, including 568 positive control samples and 2,840 samples of negative raw milk, in total giving 2,840 (positive control) and 14,200 (negative milk) readings, respectively, with combined photometric evaluation and visual reading.

No false-positive or false-negative results were observed when analysing the results of the negative milk samples by photometric or visual evaluation.

The maximum relative percentage value obtained with photometric reading for negative samples was 35 %, whereas the minimum relative percentage value for positive samples was 94 %. These values demonstrate that with the chosen thresholds for photometric reading (65 % Class A; 40 % Class B, **Table 1**) the false interpretation of positive as well as negative samples can be avoided.

INTERLABORATORY STUDY

The BRT MRL Screening Test was validated in an international proficiency test. 61 laboratories belonging to 55 companies originating from 10 countries participated in the examination of BRT MRL Screening Test plates provided for the interlaboratory study.
15 randomised and coded lyophilised UHT-milk samples were analysed - 8 samples contained antibiotics at MRL level, 7 samples consisted of inhibitor-free milk (**Table 6**), giving a total of 945 results. No false-positive or false-negative results were observed.

Table 6. The antibiotics analysed by 61 laboratories and the percentage of correct and false results.

Antibiotic	MRL (µg/kg)	No. samples	BRT MRL Screening Test	
			Correct result (%)	False result (%)
Benzylpenicillin	4	2	100	0
Ampicillin	4	2	100	0
Cefapirin	60	2	100	0
Cloxacillin	30	2	100	0
Inhibitor-free milk	-	7	100	0

CONCLUSION

The BRT MRL Screening Test (microtiter plate format) was tested for the detection of antibiotic residues in milk. For use and interpretation of the results the MRLs of the respective countries must be considered. The method is only applicable for substances with detection capability below the MRL or MPRL (where no MRL exists).

ANNEX 1

Validation Report BRT MRL Screening Test.

Validation Report
BRT MRL Screening Test

1) Introduction

The BRT MRL Screening Test (AiM – Analytik in Milch GmbH, www.aimbavaria.com) is a microbiological inhibitor test for the qualitative broad-spectrum detection of antibiotic residues in cow milk. The validation study was carried out at the laboratory of Milchprüfring Bayern e. V. (MPR Bayern, www.mpr-bayern.de), a large raw milk testing laboratory performing 1.8 million inhibitor tests per year, under the conduct of Silvia Orlandini (AEOS) and Christian Baumgartner (MPR Bayern), in accordance with the Commission Decision 2002/657/EC and the CRL Guidelines (Anonymous, 2010).

2) Test Principle, Test Procedure, Reading Methods and Plate Batches

The BRT MRL Screening Test (Figure 1) is a modified Brilliant Black Reduction Test (BRT) containing the test bacteria *G. stearothermophilus* var. *calidolactis* C953, the redox indicator brilliant black, nutrients and other supplements. Antibiotic residues present in a sample can inhibit the growth of the test bacteria, thus preventing or decelerating the reduction of the color indicator brilliant black and the consecutive color change of the test medium from blue to yellow. BRT tests generally detect a broad spectrum of antibiotics and are particularly sensitive to beta-lactams. The BRT MRL Screening Test is distinguished by an increased sensitivity towards certain antibiotic substances compared to the BRT Inhibitor Test.
In Germany, the test is used as a screening method for the detection of anti-infectives in quality control in the dairy industry as well as in monitoring tests and it is part of the official register of inspection procedures according to § 64 LFGB (German Food, Feed and Consumer Goods Code, L 01.00-11). Furthermore, it is produced according to Commission Decision 91/180/EEC.

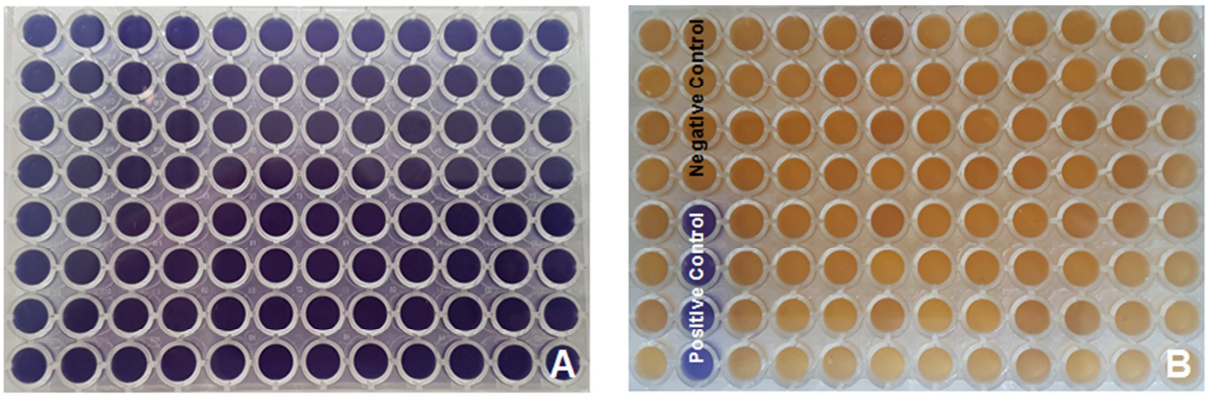


Figure 1. BRT MRL Screening Test plates before (A) and after (B) incubation

Within the framework of this validation, the BRT MRL Screening Test was evaluated in microtiter plate format. The plates were stored refrigerated (6 - 10 °C) until use. Additional to the samples (100 µl milk volume), each plate contained four positive (raw milk spiked with 4 µg/kg Penicillin G, remaining blue after incubation) and four negative controls (inhibitor-free raw milk, turning yellow after incubation) in order to enable a correct evaluation. According to the manufacturer´s instructions, the plates

were incubated at 65 ℃ in a temperature-surveilled water bath until the complete discoloration of the negative control (color change from blue to yellow, Figure 1) indicated the ideal reading time (2 hrs 30 ± 15 min). Thereafter, the milk was rinsed off the cavities and the plates were assessed with 2 different reading methods: Visual examination performed by 3 technical assistants trained particularly for this purpose and photometric evaluation, using 2 instruments (ELISA reader (Multiskan Ascent V1.24, Thermo Labsystems)).
The photometric measurements were evaluated conforming to the relativized absorption method described by Beer and Suhren (1993). Accordingly, the measuring wavelength of 450 nm and the reference wavelength of 620 nm were chosen for reading. The recorded absorption values of the analyzed samples were converted into relative percentage values by setting the average absorption level of the negative controls (yellow color after incubation) as 0% and that of the positive controls (blue color after incubation) as 100%, other absorption levels (samples) were set in relation to negative (0%) and positive (100%) control.
The conversion formula is as follows:

$$(S\text{-}NC)/(PC\text{-}NC) \times 100 = X\ \%$$

where
S is the analyzed sample´s absorption level
NC is the average of the four negative controls absorption levels
PC is the average of the four positive controls absorption levels
X relative percentage value of the analyzed sample

The photometric evaluation was regarded as reference method in this validation study as it provides objective, comparable and documented results and is commonly used by large laboratories.
The interpretation of the samples reading results was carried out in two different ways, in compliance with the method L 01.01-5 (§ 64 LFGB, inspection tests for milk quality payment) all samples exhibiting at least the color of the positive control respectively exceeding the threshold value of 65% (photometric evaluation) were interpreted as positive (indicated as class A) as well as all samples displaying a color which was clearly different from the negative control or exceeding the threshold value of 40% (indicated as class B), according to L 01.00-11 (§ 64 LFGB, German Food, Feed and Consumer Goods Code, MRL Screening test). All samples gaining <40% by photometric evaluation or appeared as yellow as the negative control were categorized as negative (Table 1).
For statistical purposes, the “quantitative” relative percentage photometric results were converted to the same format as the “qualitative” visual data. Thus, the number 1 was assigned to any photometric percentage value of ≥65 %, whereas results with percentages in the range of 40% - <65 % were referred to with 2 and negative results with 0, equaling <40 % (Table 1).

Table 1. Relation of reading systems and classes of results

	Classes of Results		
Reading System	Positive Class A	Positive Class B	Negative
Visual (V)	1	2	0
ELISA (E)	≥65%	40% - <65%	<40%

56,798 data results were obtained from the evaluation of 142 BRT MRL Screening Test plates and treated statistically using "R" software (Version 3.5.0 (2018-04-23)). The confidence interval (CI) was calculated according to the AOAC approach for qualitative data.

For the validation, 7 batches of plates (Table 2) were provided by the manufacturer.

Table 2. BRT MRL Screening Test plate batches provided for the validation

Batch	Range of Batch Numbers
A	31201018 - 31202886
B	31301015 - 31303866
C	31602068 - 31616386
D	31101233 - 31101837
E	31301596 - 31315814
F	31705769 - 31708043
G	32901085 - 32902143

3) Raw Milk Samples

A large quantity of high quality raw ex-farm bulk milk was collected, analyzed for milk quality and components (Table 3) and proven to be free of antibiotic residues by analysis with highly sensitive microbial inhibitor tests (BRT hi-sense and BRT ultra-sense, AiM GmbH, Munich, Germany) and receptor tests (BetaStar® 100, Neogen Corporation, Lansing, USA; Charm® MRL Beta-lactam Test, Charm Sciences Inc., Lawrence, USA; SNAP® Beta-Lactam ST-Test, IDEXX GmbH, Ludwigsburg, Germany). Additionally, the raw milk was tested with the AiM Penase Test (AiM GmbH, Munich, Germany) and proven to be free of penicillinase. Thereafter, the raw milk was aliquoted, frozen and stored until use. For the establishment of the rate of positive results not caused by residues of veterinary drugs, 704 ex-farm bulk milk samples, originating from routine milk quality payment testing, were analyzed with the BRT MRL Screening Test.

Table 3. Analysis results of the raw milk batch used for the validation samples

Type of Milk	FC [g/100 ml][a]	PC [g/100 ml][b]	pH	SCC/ml[c]	CFU/ml[d]
Blank raw Milk	4.17	3.51	6.65	77,000	5,000

[a] Fat content; [b] Protein content; [c] Somatic cell count; [d] Colony forming units

For the preparation of positive samples, blank raw cow milk was defrosted, spiked with a highly concentrated stock solution to obtain the desired level of antibiotic residue and frozen again. When required, the milk samples were defrosted overnight at 6 - 8 ℃ and used the next day. To verify the co rrect concentration of the stock solutions and the spiked raw milk samples, serial dilutions of the prepared positive samples were analyzed with microbiological inhibitor and - if available for a certain substance - receptor tests, then the obtained results were compared with the detection limits of the individual tests. No receptor tests were available for Erythromycin, Tylosin, Neomycin and Gentamicin, Therefore the correct concentrations of the individual stock solutions were verified with LC - MS/MS analysis.
The approach of using a single batch of raw milk as base for the preparation of the spiked milk samples enhances the comparability of results obtained on different validation days and thus objectifies the assessment of the validation results, as irregularities are not attributable to deviating milk qualities. Ex-farm tank milk was chosen as basic matrix for the validation as this is the target product of the BRT MRL Screening Test.

4) Detection Capability

Materials and Methods

Involved in the validation study were 30 antibiotic compounds (Table 6), the concentrations of the samples to analyze were chosen according to the manufacturer´s specification, the choice of increment from concentration to concentration depended on the spiked standards´ concentrations (Table 4) as well as on practical aspects as two classes of results had to be considered, leading to two different detection limits (CCß A and CCß B).

Table 4. Correlation of concentration and increment of the spiked raw milk samples

Concentration [µg/kg]	Increment [µg/kg]
1-10	1
11-20	2
21-50	5
51-100	10
101-250	25
251-500	50
501-1,000	100
1,000-5,000	500

Correlated with the proximity to the respective EU Maximum Residue Limit (MRL) for antibiotic residues in milk, the standards were measured with 20, 40 or 60 replicates (Table 5).

Table 5. Number of replicates depending on the proximity to the respective MRL

Closeness to MRL	No. of Replicates
≤0.5 MRL	20
>0.5 MRL and <0.9 MRL	40
≥0.9 MRL and ≤ MRL	60
> MRL	20

For the determination of the detection capability three different batches of plates were used at all times. The lowest concentration obtaining a minimum of 95% positive results was considered as detection limit (CCß). Based on the different interpretation methods, CCß A and CCß B (Section 2) were established in parallel for each substance. The detection limits determined with photometric evaluation were considered as reference values.

Results and Discussion

Table 6. Established detection limits (photometric reading) compared with the EU MRL levels. Marked in red are the substances exceeding the EU MRLs.

Group of Antibiotics	Substance	MRL EU [µg/kg]	CCß A [µg/kg]	CCß B [µg/kg]
Penicillins	Benzylpenicillin	4	2	1.5
	Ampicillin	4	2.5	2
	Amoxicillin	4	3	2.5
	Cloxacillin	30	25	18
	Dicloxacillin	30	12.5	10
	Nafcillin	30	10	8
	Oxacillin	30	8	8
Cephalosporins	Cefalexin	100	300	250
	Cefapirin	60	5	5
	Cefoperazone	50	30	20
	Cefazolin	50	7	6
	Cefquinome	20	300	200
	Ceftiofur	100	150	100
	Cefalonium	20	12	10
Macrolides	Erythromycin	40	80	50
	Tylosin	50	75	30
Sulfonamides	Sulfadiazine	100	400	100
	Sulfadimethoxin	100	600	100
	Sulfamethazine	100	>1,000	200
	Sulfathiazol	100	200	60
	Sulfadoxin	100	1,500	300
	Sulfamethoxypyridazine	100	500	100
Tetracyclines	Chlortetracycline	100	800	400
	Oxytetracycline	100	400	200
	Tetracycline	100	600	300
Aminoglycosides	Dihydrostreptomycin	200	600	400
	Streptomycin	200	1,000	500
	Gentamicin	100	150	80
	Neomycin	1,500	300	200
Fenicol	Chloramphenicol	-	5,000	3,500

The particular sensitivity of *G. stearothermophilus* for beta-lactams and especially for penicillins is reflected in the detection limits of the different groups of antibiotics. All penicillins were detected below MRL as well as 5 out of 7 cephalosporins (Ceftiofur only CCß B). Out of the groups of the macrolides, sulfonamides and aminoglycosides Tylosin, Sulfadiazine, Sulfadimethoxin, Sulfathiazol, Sulfamethoxypyridazine and Gentamicin conformed with the regulatory limits with CCß B, but not with CCß A, whereas for Neomycin CCß A and CCß B were defined to be below MRL. The detection limits of other tested substances exceeded the respective MRLs. CCß A could not be determined for Sulfamethazine, as the positive response was below 65% relative absorption at the highest concentrations tested.
Chloramphenicol, for which no MRL is established – it is prohibited for use in food producing animals (Commission Regulation (EU) No 37/2010) – can be tested positive at 3,500 µg/kg (CCß B) or 5,000 µg/kg (CCß A). The detection limits for the BRT MRL Screening Test established with the reference method (photometric evaluation) are reported in Table 6, detection limits established with visual reading are reported in the Annex.
In conclusion, the most important antibiotics used in Germany for the treatment of dairy cows (penicillins and cephalosporins), are detected predominantly below EU MRL. A broad range of other inhibitors can be identified as well, however, mostly in concentrations exceeding the regulatory limit.

5) Dose-Response Curves

Materials and Methods

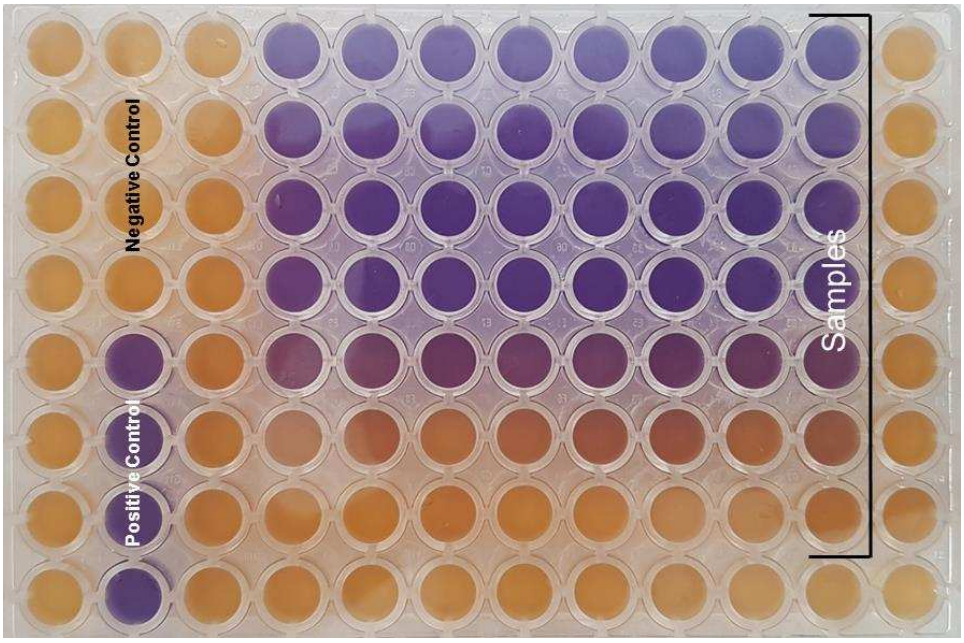


Figure 2. Incubated BRT MRL Screening Test plate inoculated with four positive and four negative controls as well as raw milk samples spiked with 7 different concentrations of an antibiotic substance

Dose-response curves were established for all antibiotics analyzed in the validation based on the class A results (Section 2) obtained within the framework of the detection capability study with both photometric and visual evaluation. For this purpose, 7 samples containing increasing concentrations were examined for each substance, with the aim of identifying the concentrations resulting in approximately 25%, 50%, 75% and 100% positive rates and to determine the highest concentration with 0% positive results. Furthermore, lower and upper CIs were calculated for the class A results under consideration of both reading systems and included in the dose-response curves (Figure 2 and Annex Table 2).

Results and Discussion

Figure 3 and Annex Figure 1 depict dose-response curves of all substances included in the validation of the BRT MRL Screening Test. The response rates generated with the respective concentrations of each substance are specified in Annex Table 2. It was not always possible to obtain dose-response curves entirely corresponding to the requirements of 0%, 25%, 50%, 75% and 100% positive results. Bactericidal substances like beta-lactams mostly exhibited steeply increasing dose-response curves. For Benzylpenicillin, e. g., the positive response rate was 1% at 1 µg/kg, 78% at 1.5 µg/kg already and 100% of samples were detected positive at 2 µg/kg. Substances like the sulfonamides and macrolides displayed more consistent curve increments, probably due to their bacteriostatic character. Sulfadoxin obtained 18% positive results at 200 µg/kg, 43% at 300 µg/kg, 60% respectively 78% at 400 µg/kg and 600 µg/kg. At 800 µg/kg and 1,000 µg/kg, the positive responses were >95%, CCß A was established at 1,500 µg/kg.
Principally, the confidence interval is narrow at the concentrations of the CCß and at concentrations close to 0% of positive results. Bactericidal substances tend to exhibit narrow CIs also at concentrations in between 0% and CCß, which indicates that most results of samples with different concentrations are interpreted in the same way with the different reading systems (photometric and visual) and individual readers.
In contrast, bacteriostatic substances often show bigger variations in the results at the concentrations below the CCß. The bacteriostatic activity causes different degrees of inhibition and consequently of color development, which can be more difficult to interpret by human eye. While the interpretation with photometric reading systems is well standardized, the visual interpretation leads to bigger variances in the results and thus to wider CIs.

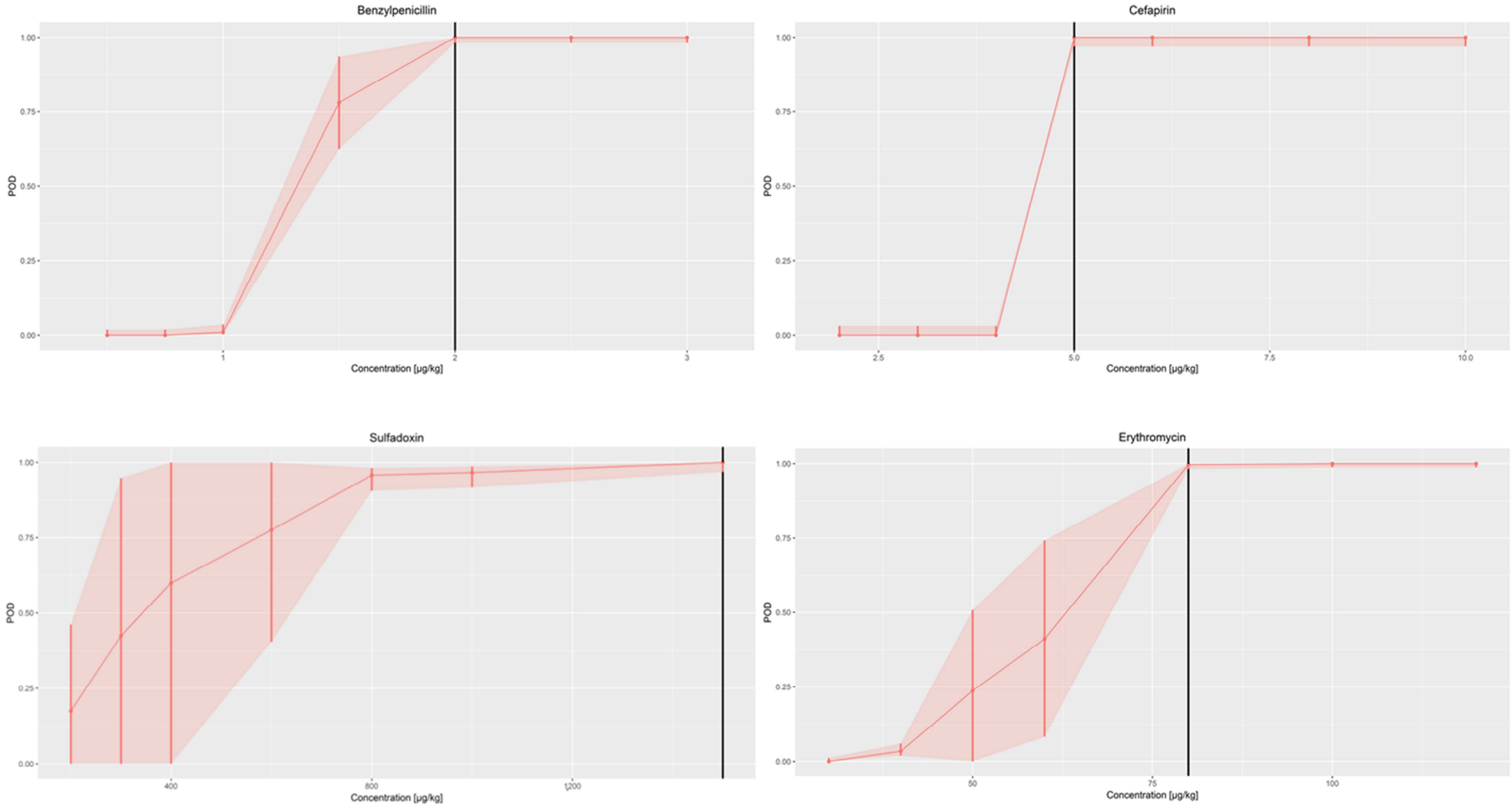


Figure 3. Dose-response curves of the bactericidal antibiotics Benzylpenicillin and Cefapirin and of the bacteriostatic substances Sulfadoxin and Erythromycin.
Red line = dose-response curve; red shade = CI; Black line = CCß A (photometric reading);

6) Selectivity

Materials and Methods

Marker substances of commonly used classes of veterinary drugs other than antibiotics were analyzed with photometric reading in order to determine the selectivity of the BRT MRL Screening Test. The investigated compounds included the anti-inflammatories Flunixin, Metamizole (NSAIDs) and Prednisolone (glucocorticoid) as well as antiparasitic substances (Triclabendazole and Deltamethrin). Furthermore, the polyether-antibiotic Monensin, used for ketosis treatment in dairy cows, was tested. The substances were spiked at a concentration of 100 x EU MRL and inoculated with 6 replicates (Table 7).

Table 7. Selectivity: Concentrations of analyzed substances and test results

					False positive Results	
Use	**Drug Class**	**Substance**	**MRL [µg/kg]**	**Concentration [µg/kg]**	**Class A**	**Class B**
Anti-inflammatory Substances	NSAID	Flunixin	40	4,000	0/6	0/6
	NSAID	Metamizol	50	5,000	0/6	0/6
	Glucocorticoid	Prednisolon	6	600	0/6	0/6
Antiparasitics	Antihelminthic	Triclabendazol	10	1,000	0/6	0/6
	Ectoparasite	Deltamethrin	20	2,000	0/6	0/6
Ketosis Treatment	Polyether-Antibiotic	Monensin	2	200	0/6	1/6

Results and Discussion

Highly concentrated samples of Flunixin, Metamizole, Prednisolone, Triclabendazole, and Deltamethrin did not inhibit the growth of the test germs, leading to negative results. Only 1 false-positive result was obtained for Monensin out of 6 replicates on interpretation in comparison with the negative control (Class B). Compared with the positive control (Class A) all samples appeared negative. Even though Monensin is used for ketosis treatment, it is a bactericidal substance (polyether-antibiotic), which modifies the ruminal flora by inhibiting predominantly gram-positive bacteria, but not gram-negative germs, leading to an improved ruminal metabolism and reduced incidence of ketosis. Monensin is administered locally in the rumen, it is subject to a high first-pass metabolism, residual amounts in the blood circulation are excreted via the bile. Despite the antibiotic mode of action, false-positive results after the treatment of a cow with Monensin are highly unlikely due to its pharmacokinetic properties and the apparently low sensitivity of the BRT MRL Screening Test for this substance. Nevertheless, accidental pollution of the bulk milk with the drug should be avoided.

Principally, the observed results signify a high specificity of the BRT MRL Screening Test for the detection of antibiotic substances opposed to other classes of veterinary drugs.

7) Batch-to-Batch Variability

Materials and Methods

In order to evaluate potential deviations in the detection capabilities of different plate batches statistically, Fisher's exact tests (method: two-sided) were applied at the concentration of the CCß A obtained with photometric reading. Contingency tables were created for the datasets of ELISA reader 1 and ELISA reader 2 to provide a basic picture of the interrelation between the two variables plate batches and number of results (class A) per batch. Due to the duration of the validation study and the limited shelf-life of the BRT MRL Screening Test plates, two sets of plate batches (A, B, C and D, E, F) had to be used.
The Fisher´s test was selected because the test is more precise than Chi square for this number of observations, the null hypothesis is based on the batches independence (the probability of the results is the same for the different batches). The Fisher's exact test was applied only to the analytes for which a CCß A could be determined. If the significance level is $\alpha = 0.05$ and the p-value <0.05, the null hypothesis is rejected, which would mean that there is a probability for batch-to-batch differences concerning the detection capability at the CCß A.

Results and Discussion

The Fisher´s test examinations for the concentrations at CCß A indicate that there are no significant differences in between the detection sensitivities of the different plate batches used in the validation. All substances, except for Gentamicin, Sulfadiazine and Sulfadimethoxin realized p-value = 1 - for Gentamicin, Sulfadiazine and Sulfadimethoxin the p values were $0.05 \leq p < 1$ (Table 8). In addition to the p-values (CCß A), Annex Table 3 comprises the numbers of results per class (1-2-0), plate batch and individual ELISA reader at the concentration of the CCß A.

Table 8. Contingency table created with the Fisher Test for the concentration at CCß A obtained with photometric reading

Group of Antibiotics	Substance	MRL	CCßA [µg/kg]	p Value		Group of Antibiotics	Substance	MRL	CCßA [µg/kg]	p Value	
				ELISA 1	ELISA 2					ELISA 1	ELISA 2
Penicillins	Benzylpenicillin	4	2	1	1	**Sulfonamides**	Sulfadiazine	100	400	0.11	0.11
	Ampicillin	4	2.5	1	1		Sulfadimethoxin	100	600	0.11	0.33
	Amoxicillin	4	3	1	1		Sulfamethazine	100	> 1,000	-	-
	Cloxacillin	30	25	1	1		Sulfathiazol	100	200	1	1
	Dicloxacillin	30	12.5	1	1		Sulfadoxin	100	1,500	1	1
	Nafcillin	30	10	1	1		Sulfamethoxypyridazine	100	500	1	1
	Oxacillin	30	8	1	1	**Tetracyclines**	Chlortetracycline	100	800	1	1
Cephalosporines	Cefalexin	100	300	1	1		Oxytetracycline	100	400	1	1
	Cefapirin	60	5	1	1		Tetracycline	100	600	1	1
	Cefoperazone	50	30	1	1	**Aminoglycosides**	Dihydrostreptomycin	200	600	1	1
	Cefazolin	50	7	1	1		Streptomycin	200	1,000	1	1
	Cefquinome	20	300	1	1		Gentamicin	100	150	0.11	0.33
	Ceftiofur	100	150	1	1		Neomycin	1,500	300	1	1
	Cefalonium	20	12	1	1	**Fenicols**	Chloramphenicol	-	5,000	1	1
Macrolides	Erythromycin	40	80	1	1						
	Tylosin	50	75	1	1						

Significance levels: Low: p <0.05; Medium: p <0.01; High: p <0.001

8) False-Positive and False-Negative Rate

Materials and Methods

With each BRT MRL Screening Test plate used during the validation study, 4 positive and negative control samples (Section 2) as well as additional 16 negative raw milk samples, adding up to 20 negative milk samples, were inoculated. By means of these samples, the rates of false-positive and false-negative results were established.
Within the framework of this validation, 142 test plates were analyzed, including 568 positive control samples and 2,840 samples of negative raw milk in total. Thus, 2,840 (positive control) respectively 14,200 (negative milk) results were obtained with photometric evaluation (2 readers) and visual reading (3 technicians, Table 9).

Table 9. Numbers of positive and negative samples and obtained results used for the establishment of the false-negative and false-positive rates

Type of Milk Sample	No. Plates	No. Samples	No. Results ELISA Readers	No. Results Visual	Total No. Results
Positive Control	142	568	1,136	1,704	2,840
Negative Milk		2,840	5,680	8,520	14,200

Results and Discussion

No false-positive or false-negative results were observed when analyzing the results of the negative milk samples and positive control samples by photometric evaluation or visual reading, leading to false-positive and false-negative rates of 0% (Table 10).

Table 10. Rates of false-negative and false-positive results of all applied reading methods and readers

	Rate of false Results [%]				
Type of Milk Sample	ELISA 1	ELISA 2	Visual 1	Visual 2	Visual 3
Positive Control	0	0	0	0	0
Negative Milk	0	0	0	0	0

The maximum relative percentage value (Section 2) obtained with photometric reading for negative samples was 35%, whereas the minimum relative percentage value for positive samples was 94% (Table 11). These values demonstrate that with the chosen thresholds for photometric reading (65% class A; 40% class B; Table 1) the false interpretation of positive as well as negative samples can be avoided.

Table 11. Minimum and maximum photometric percentage results obtained with two photometric instruments

Type of Milk Sample	No. Results ELISA Readers	Photometric Values Min/Max [%]
Positive Control	1,136	94
Negative Milk	5,680	35

In conclusion, the absence of false-negative and false-positive results indicates that the validity of positive as well as negative results obtained for raw milk samples by analysis with the BRT MRL Screening Test is very high.

9) Rate of Positive Results not caused by Residues of Veterinary Drugs

Materials and Methods

In order to demonstrate that the BRT MRL Screening Test performs properly with a broad range of samples, the rate of positive results not caused by residues of veterinary drugs was established by analyzing 704 ex-farm bulk milk samples, originating from routine inhibitor analysis (milk quality payment testing at MPR Bayern). In order to verify the correct performance of the test all samples were examined in parallel on two different microbiological inhibitor tests (BRT Inhibitor Test and BRT hi-sense). To confirm detected inhibitors, screening-positive samples were tested on the BRT Inhibitor Test, then evaluated with receptor tests (BetaStar® 100, Neogen Corporation, Lansing, USA; Charm MRL Beta-lactam 1-Minute Test, Charm Sciences Inc., Lawrence, USA; SNAP Beta-Lactam ST Plus, IDEXX GmbH, Ludwigsburg, Germany) and identified and quantified by analysis with the biosensor MCR-3 (GWK Präzisionstechnik GmbH, Munich, Germany). The MCR-3 is an antibody-based rapid micro-array chip reader, which is capable of the simultaneous detection and quantification of 13 antibiotic substances. Furthermore, confirmed inhibitor-positive samples were quantified by LC-MS/MS analysis.

Results and Discussion

2 out of 704 samples (0.28%, Table 12) were detected positive by the BRT MRL Screening Test. Both results were confirmed positive by evaluation with other inhibitor tests and receptor tests, the causative substance was identified as Cloxacillin by MCR-3- as well as LC-MS/MS-analysis. Cloxacillin was present in the samples at 64.8 µg/kg respectively 50.6 µg/kg. Thus, the rate of positive results not caused by residues of veterinary drugs was 0%, as all positive samples detected were confirmed to contain antibiotic inhibitors. The correct analysis of routine samples demonstrates the robust performance of the BRT MRL Screening Test with a broad range of samples and it´s applicability for real-life laboratory use.

Table 12. Routine samples analysis results

	Negative Samples		Positive Samples			
Total No. Samples	No.	Rate	No.	Rate	False positive	Confirmed positive
704	702	99.72%	2	0.28%	0%	100%

10) Participation in an International Interlaboratory Study and Comparability

Materials and Methods

The BRT MRL Screening Test was validated in an international interlaboratory study in order to demonstrate its robust performance and suitability for real-life laboratory applications. This interlaboratory study was conducted in parallel with the international 10th proficiency test for inhibitors, organized by the QSE GmbH. 61 laboratories belonging to 55 companies - originating from 10 countries - ,out of 148 laboratories taking part in the 10th proficiency test, assisted with the examination of provided BRT MRL Screening Test plates for the interlaboratory study as a part of the validation.

Within the framework of the 10th proficiency test, 15 randomized and coded lyophilized UHT-milk samples were analyzed - 8 samples contained antibiotics, 7 samples consisted of inhibitor-free milk (Table 13). The antibiotics Penicillin G, Cloxacillin, Ampicillin and Cefapirin, which are often used for treatment of lactating cows, had to be detected at MRL level. These proficiency test sets were used for the interlaboratory study of the BRT MRL Screening Test, too.

The reported results of the interlaboratory study of the BRT MRL Screening Test and the 10th proficiency test were evaluated in parallel and compared in order to assess the performance of the validated test in correlation with other commonly used inhibitor tests (both microbiological and receptor tests).

Table 13. Composition of the proficiency test sets

Substance	Concentration [µg/kg]	No. Samples
Benzylpenicillin	4	2
Ampicillin	4	2
Cefapirin	60	2
Cloxacillin	30	2
-	-	7

Results and Discussion

In total, 945 results were reported for the BRT MRL Screening Test by the interlaboratory study participants, 100% of these results were correct. No false-positive or false-negative results were observed (Figure 4).

This high rate of correct results obtained in different laboratories signifies once more that the BRT MRL Screening Test is suitable for routine analyses as all positive and negative samples were identified properly and all examined substances were detected at MRL level.

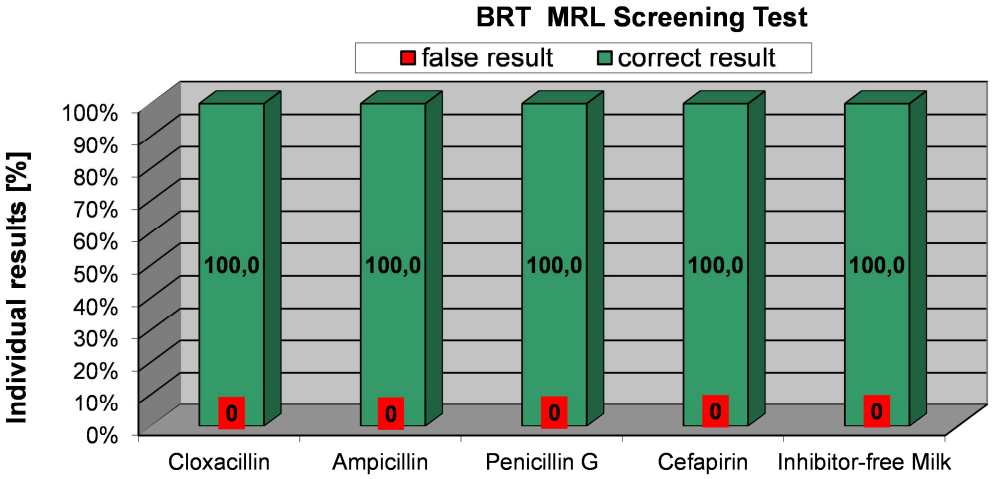


Figure 4. Rate of correct and false results (%) obtained by the interlaboratory study within the framework of the validation of the BRT MRL Screening Test

As part of a comparability study, the results obtained in the framework of the 10th proficiency test were contrasted with the results of the interlaboratory study of the BRT MRL Screening Test. 2,513 results for inhibitor-free milk and 2,872 results for inhibitor-positive samples were forwarded by the 10th proficiency test participants in total. The participating laboratories indicated if microbiological test systems or receptor tests had been used for the examination of the samples. Taking into account both types of test systems, 0.7% of the inhibitor-free milk samples were detected false-positive (Figure 5). Only 0.5% of the samples analyzed with microbiological test systems were reported false-positive (Figure 6), compared with 1.0% of the samples examined with receptor tests (Figure 7). Regarding the inhibitor-positive samples, 5% in total were identified as false-negative. Especially Cloxacillin (14.3%) and Ampicillin (3.3%), but also a few samples of Benzylpenicillin (1.7%) and Cefapirin (0.7%) were not identified correctly (Figure 5). The false-negative rate was higher for receptor test systems (6.9%) than for microbiological test systems (3.7%).

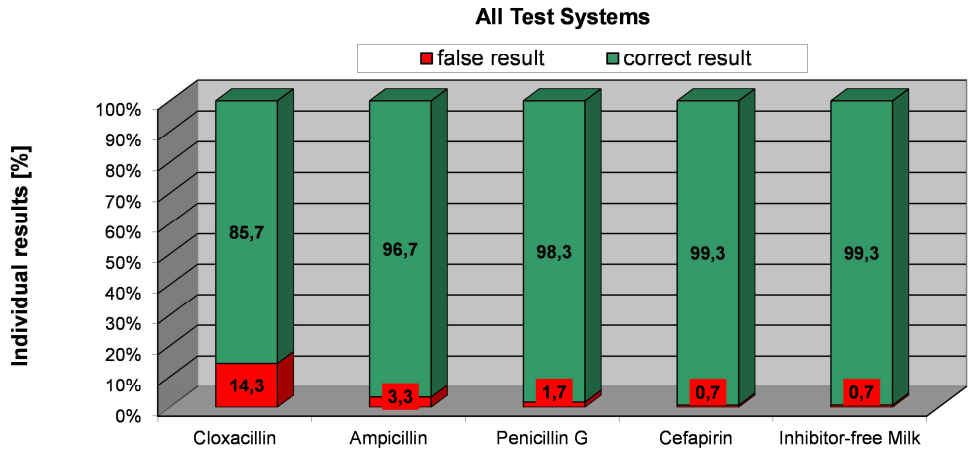


Figure 5. Rates of correct and false results (%) of all test systems (microbiological and receptor tests, 10th proficiency test)

Compared with other tests evaluated in the context of the 10th proficiency test, the BRT MRL Screening Test demonstrated an excellent performance, as no false results were observed within the interlaboratory study.

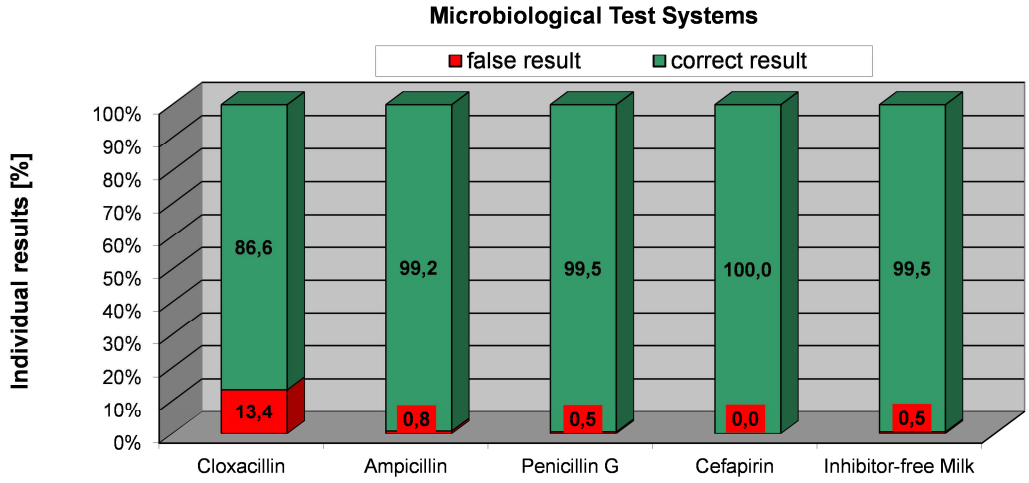


Figure 6. Rates of correct and false results (%) of microbiological test systems (10th proficiency test)

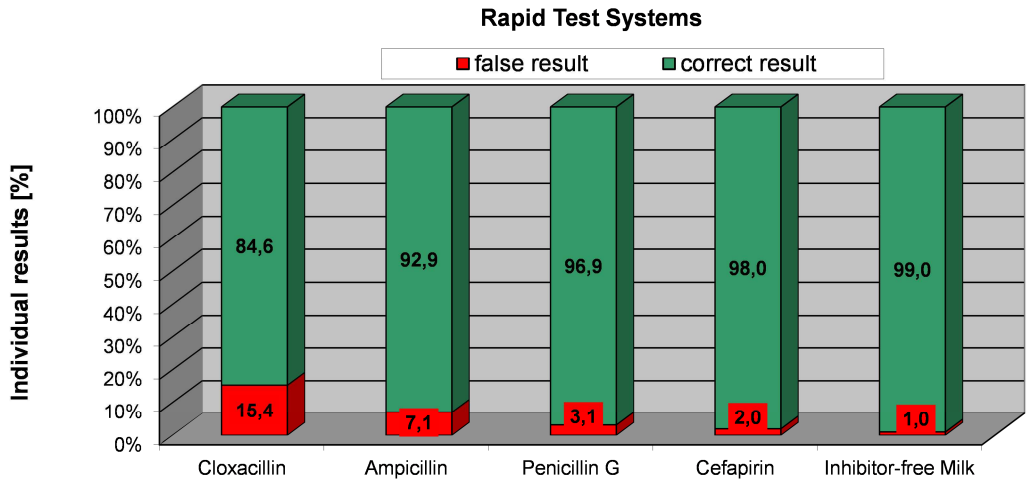


Figure 7. Rates of correct and false results (%) of receptor test systems (10th proficiency test)

11) Conclusions

The BRT MRL Screening Test is capable of the detection of all of the 7 penicillins and 5 out of 7 cephalosporins as well as 6 out of 16 compounds belonging to other antibiotic groups investigated in this study at or below MRL level – depending on the interpretation method. This means that the most important antibiotic compounds used in Germany for the treatment of dairy cows are detected predominantly below MRL. A broad range of other inhibitors can be identified above MRL as well. The BRT MRL Screening Test displays a high selectivity for antibiotic residues, marker substances of other veterinary classes were not detected at high concentrations. The Batch-to-Batch-Variability proved to be low, no significant differences were observed for positive result rates obtained with different plate batches. The validity of obtained results is high as no false-positive or false-negative results were observed in the analysis of positive and negative control samples. With the correct analysis of a broad range of routine milk quality payment samples, the good performance of the BRT MRL Screening Test in an international interlaboratory study (100% correct results) and in comparison with other inhibitor tests used by laboratories participating in an international proficiency test, which was organized in parallel, it could be demonstrated that the test is fit for routine laboratory use.

12) References

COMMISSION DECISION C1 14 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (notified under document number C(2002) 3044) (Text with EEA relevance) (2002/657/EC)
(OJ L 221, 17.8.2002, p. 8)

COMMUNITY REFERENCE LABORATORIES RESIDUES (CRLs) 20/1/2010 - Guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer)

ISO 13969 IDF 183 Milk and Milk products - Guidelines for a standardized description of microbial inhibitor tests

ISO 18330 IDF 188 Milk and milk products - Guidelines for the standardized description of immunoassays or receptor assays for the detection of antimicrobial residues

German Federal Office of Consumer Protection and Food Safety
Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB (vormals $35 LMBG) Band 1 (L), L 01.01-5: Nachweis von Hemmstoffen in Sammelmilch, Agar-Diffusions-Verfahren (Brillantschwarz-Reduktionstest), Januar 2012

German Federal Office of Consumer Protection and Food Safety
Amtliche Sammlung von Untersuchungsverfahren nach § 64 LFGB (vormals $35 LMBG) Band 1 (L), L 01.00-11: Suchverfahren auf das Vorhandensein von Antiinfektiva in Milch, Agar-Diffusions-Verfahren mit Bacillus stearothermophilus (Brillantschwarz-Reduktionstest), Februar 1996

BEER, P. and G. SUHREN (1993): Approach for an inhibitor test kit validation program, IDF-Workshop on Residues of Antibiotics and Other Antimicrobial Inhibitors in Raw and Heat-Treated Milk, Copenhagen, collected abstracts

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

AOAC Binary Data Interlaboratory Study Workbook Version 2.3

QSE GmbH, 2017: 10th proficiency test for inhibitors, Final report

13) Acknowledgements

Authors and responsible persons:
Dr. Christian Baumgartner (Milchprüfring Bayern e. V.) and Dr. Silvia Orlandini (AEOS)
The authors acknowledge the manufacturer´s staff for providing all the necessary equipment and the initial training in the preliminary phase of the validation study as well as the staff of MPR Bayern, who were involved in the validation study, Christine Habel and Kerstin Karl.

Accredited laboratory according to ISO/IEC 17025

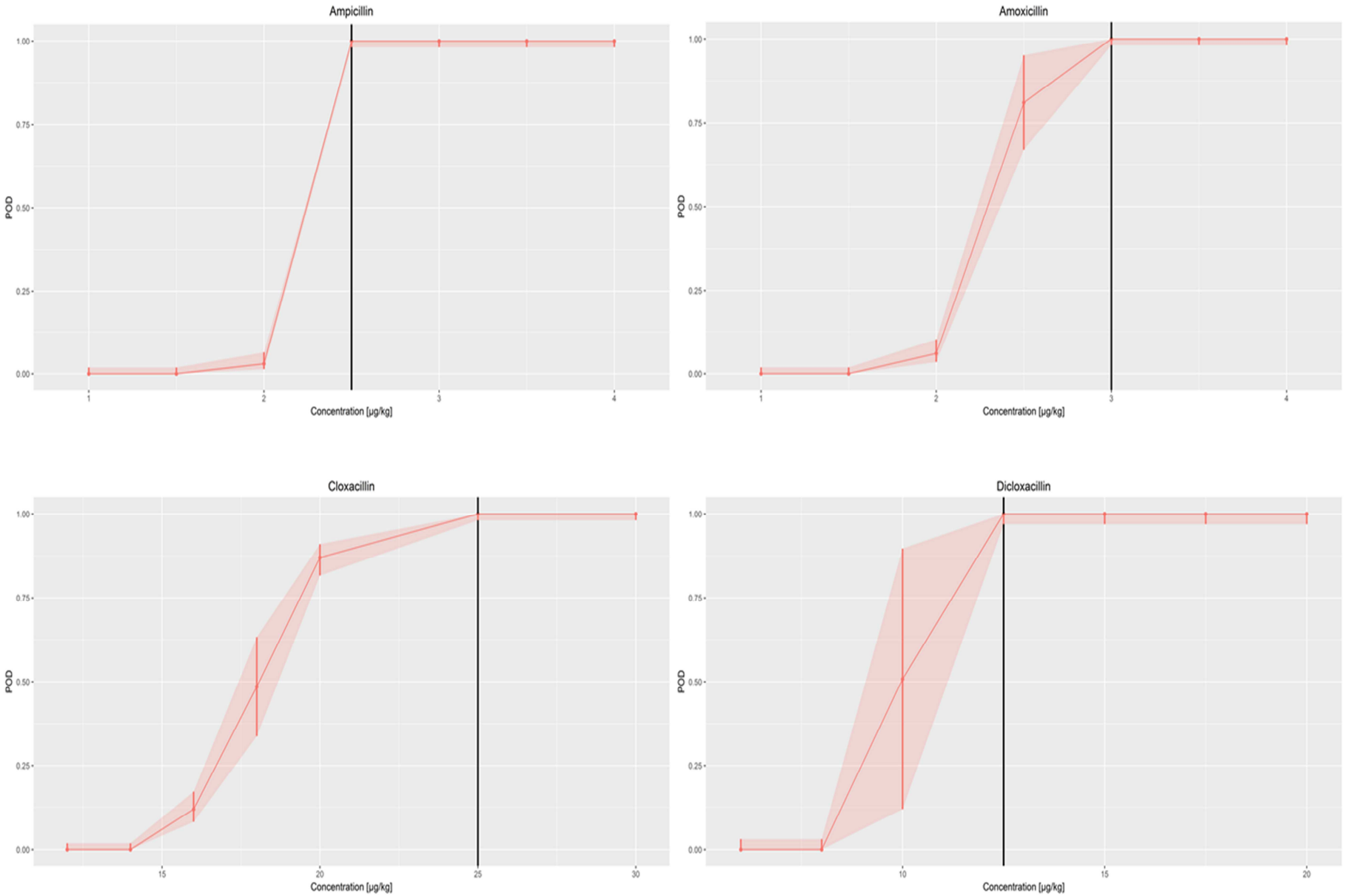
Hochstatt 2, 85283 Wolnzach, Germany
Tel: +49 8442 210, Fax: +49 8444 5210
E-Mail: validation@mpr-bayern.de

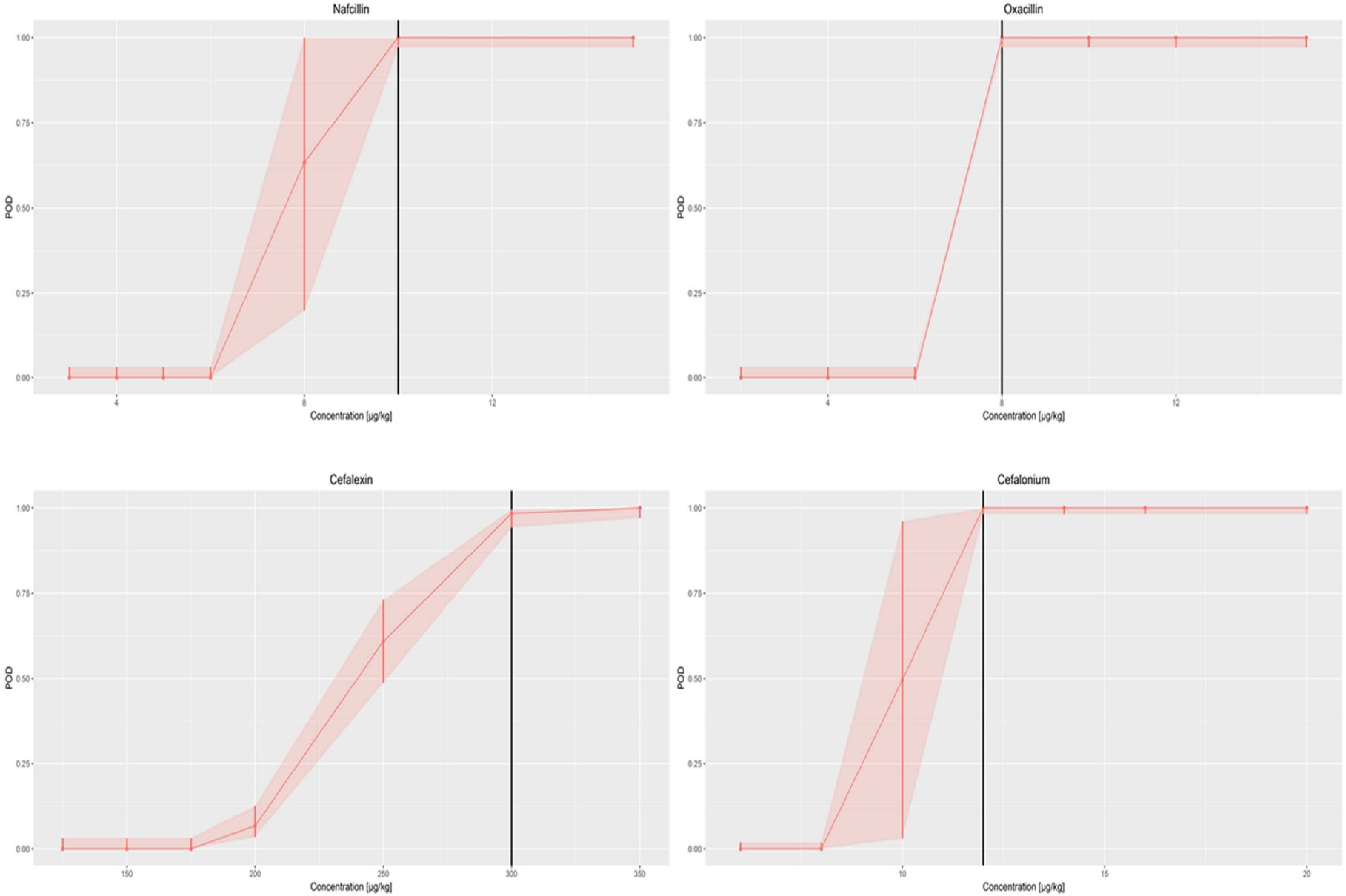
Date of publication: 09.11.2018

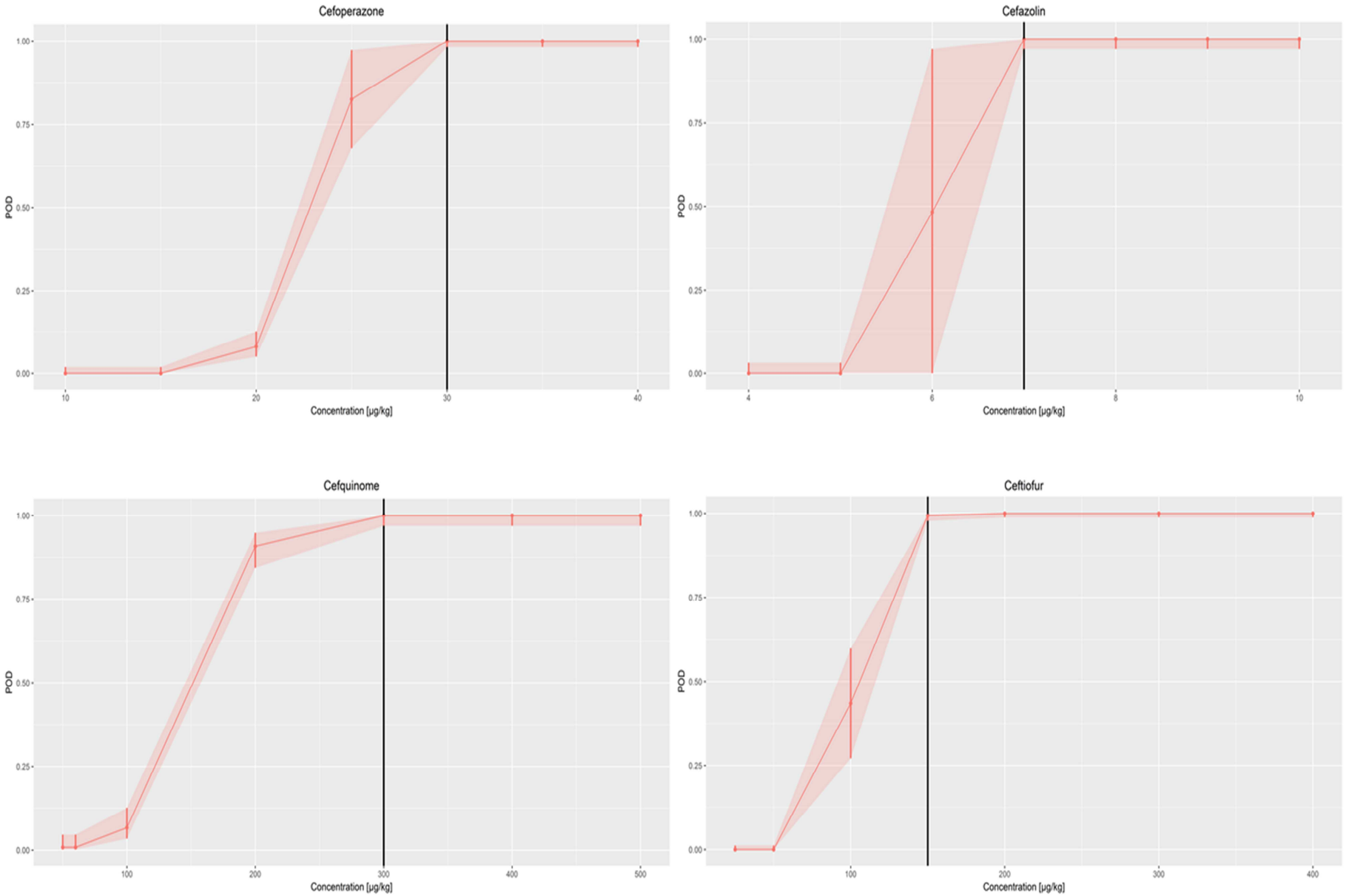
ANNEX

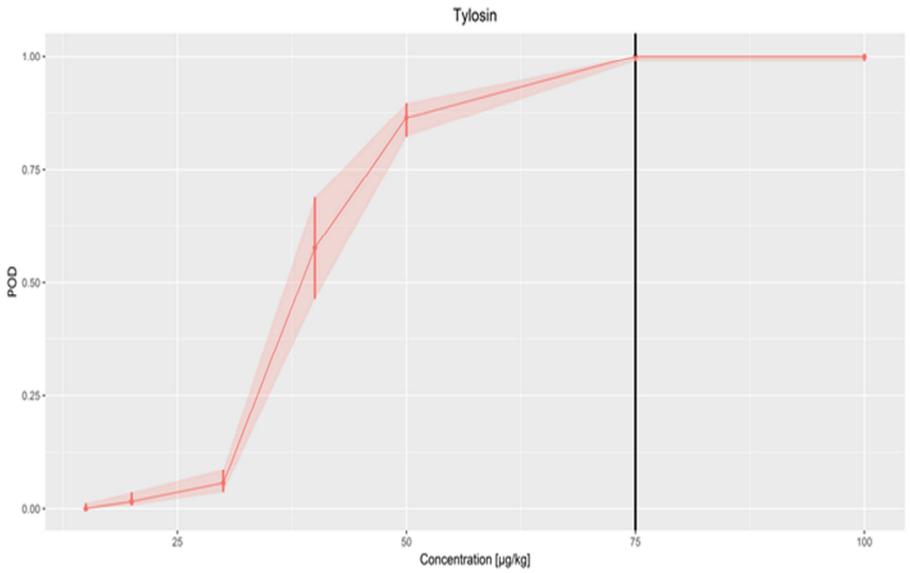
Annex Table1. Established detection limits (visual reading) compared with the EU MRL levels. Marked in red are the substances exceeding the EU MRLs.

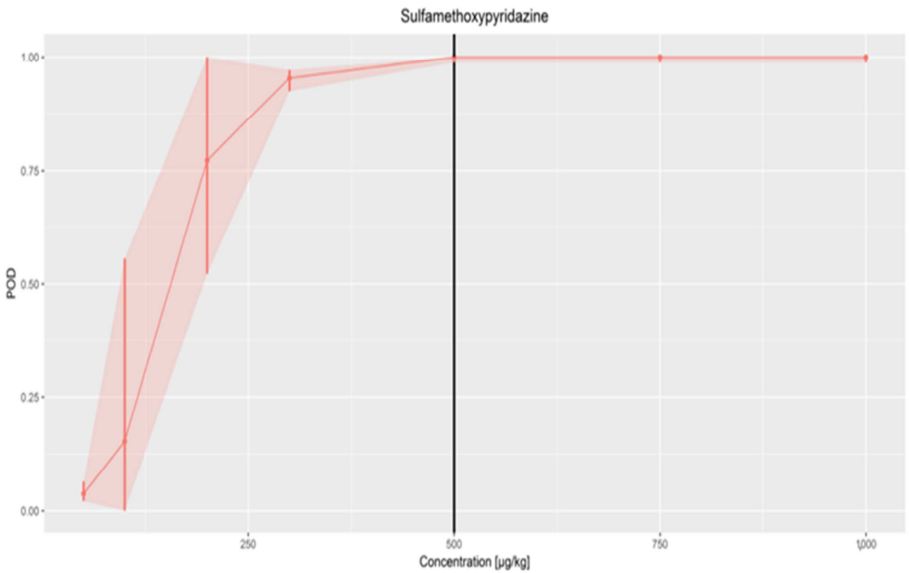
Group of Antibiotics	Substance	MRL EU [µg/kg]	CCß A [µg/kg]	CCß B [µg/kg]
Penicillins	Benzylpenicillin	4	2	1.5
	Ampicillin	4	2.5	2.5
	Amoxicillin	4	3	2.5
	Cloxacillin	30	25	20
	Dicloxacillin	30	12.5	10
	Nafcillin	30	10	8
	Oxacillin	30	8	8
Cephalosporins	Cefalexin	100	300	250
	Cefapirin	60	5	5
	Cefoperazone	50	30	25
	Cefazolin	50	7	6
	Cefquinome	20	200	200
	Ceftiofur	100	150	100
	Cefalonium	20	12	10
Macrolides	Erythromycin	40	80	60
	Tylosin	50	75	40
Sulfonamides	Sulfadiazine	100	300	100
	Sulfadimethoxin	100	400	100
	Sulfamethazine	100	>1,000	200
	Sulfathiazol	100	200	60
	Sulfadoxin	100	400	300
	Sulfamethoxypyridazine	100	300	100
Tetracyclines	Chlortetracycline	100	600	400
	Oxytetracycline	100	600	300
	Tetracycline	100	400	300
Aminoglycosides	Dihydrostreptomycin	200	700	500
	Streptomycin	200	1,500	800
	Gentamicin	100	125	80
	Neomycin	1,500	300	200
Fenicol	Chloramphenicol	-	5,000	3,500

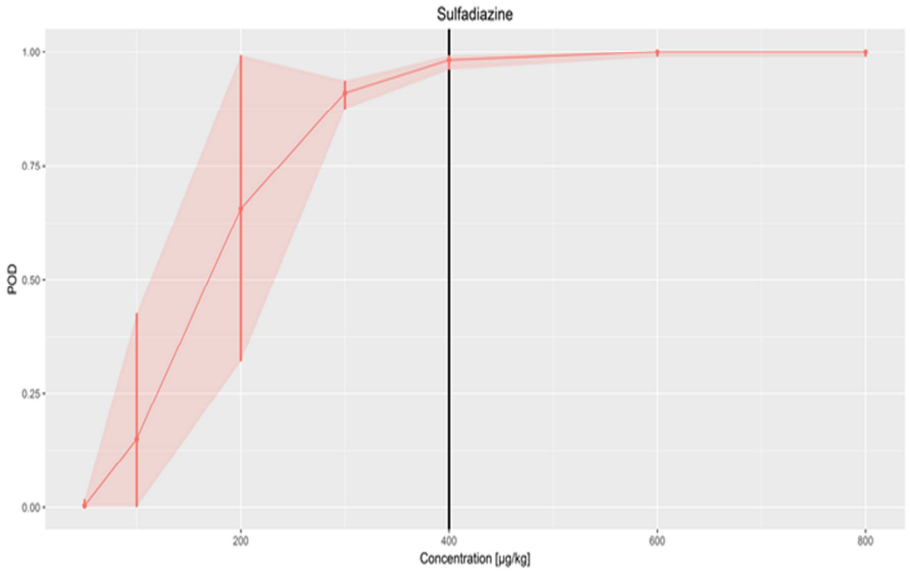

Ampicillin
Amoxicillin
Cloxacillin
Dicloxacillin
POD
Concentration [µg/kg]
1.00
0.75
0.50
0.25
0.00

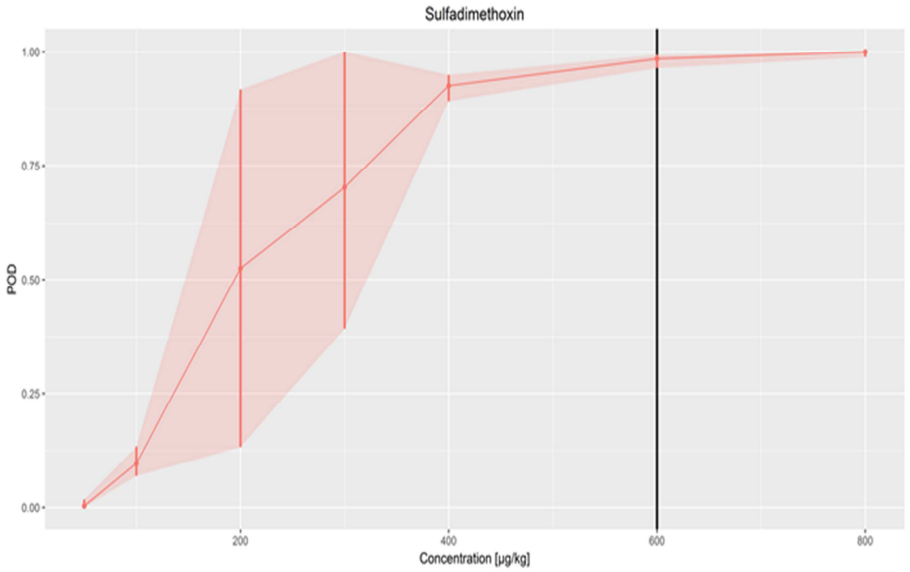

Nafcillin
Oxacillin
Cefalexin
Cefalonium
POD
Concentration [µg/kg]
1.00
0.75
0.50
0.25
0.00
4
8
12
150
200
250
300
350
10
15
20

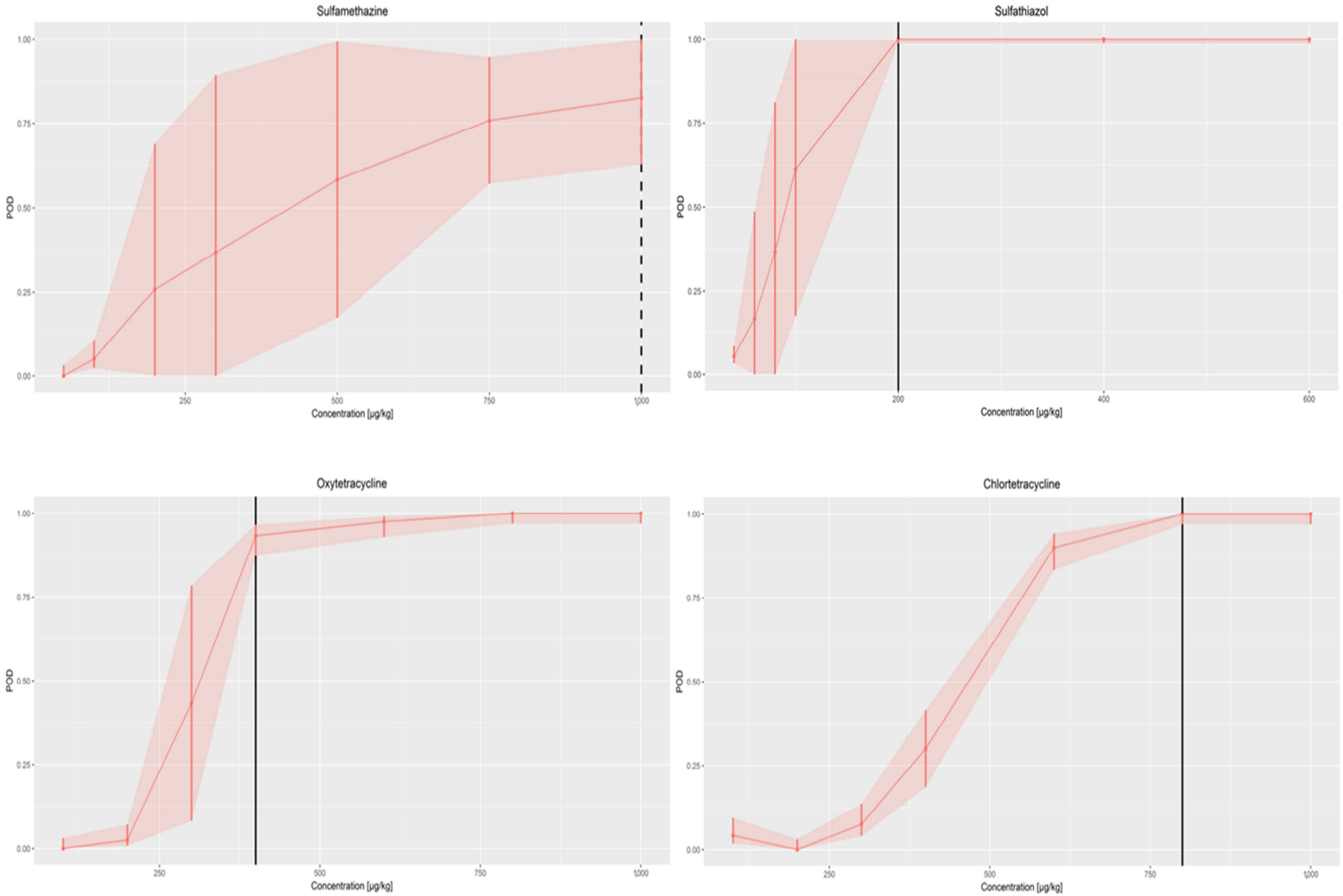

Cefoperazone
POD
1.00
0.75
0.50
0.25
0.00
10
20
30
40
Concentration [µg/kg]
Cefazolin
POD
1.00
0.75
0.50
0.25
0.00
4
6
8
10
Concentration [µg/kg]
Cefquinome
POD
1.00
0.75
0.50
0.25
0.00
100
200
300
400
500
Concentration [µg/kg]
Ceftiofur
POD
1.00
0.75
0.50
0.25
0.00
100
200
300
400
Concentration [µg/kg]

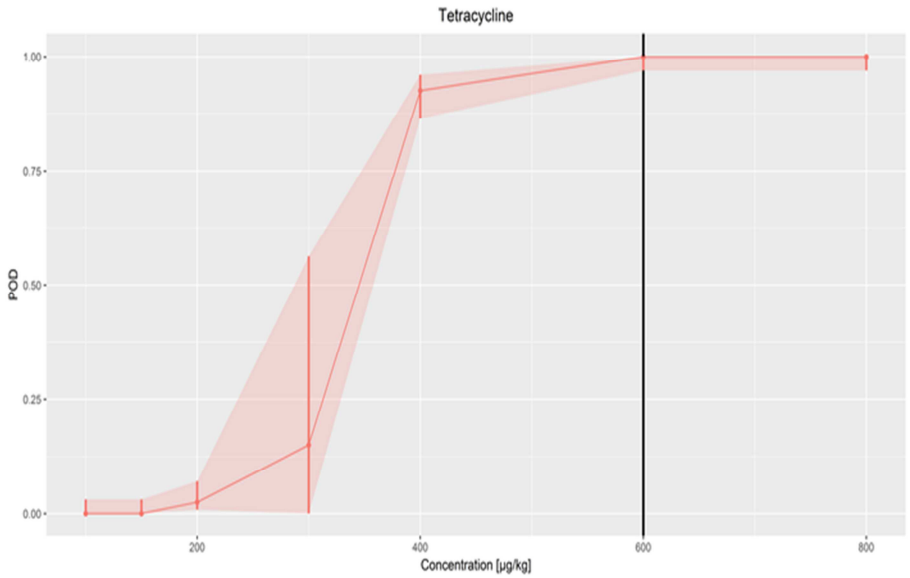

Tylosin
POD
1.00
0.75
0.50
0.25
0.00
25
50
75
100
Concentration [µg/kg]

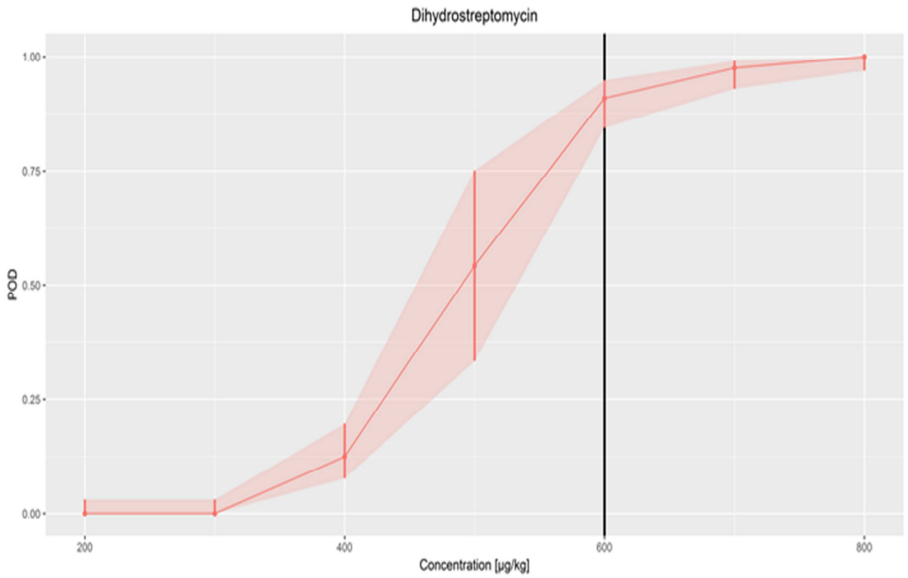

Sulfamethoxypyridazine
POD
1.00
0.75
0.50
0.25
0.00
250
500
750
1000
Concentration [µg/kg]

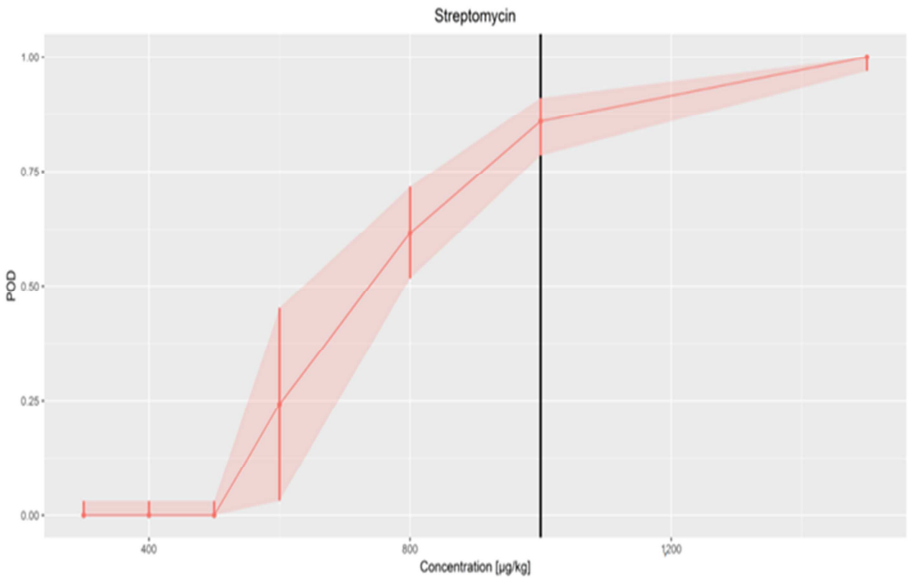

Sulfadiazine
POD
1.00
0.75
0.50
0.25
0.00
200
400
600
800
Concentration [µg/kg]

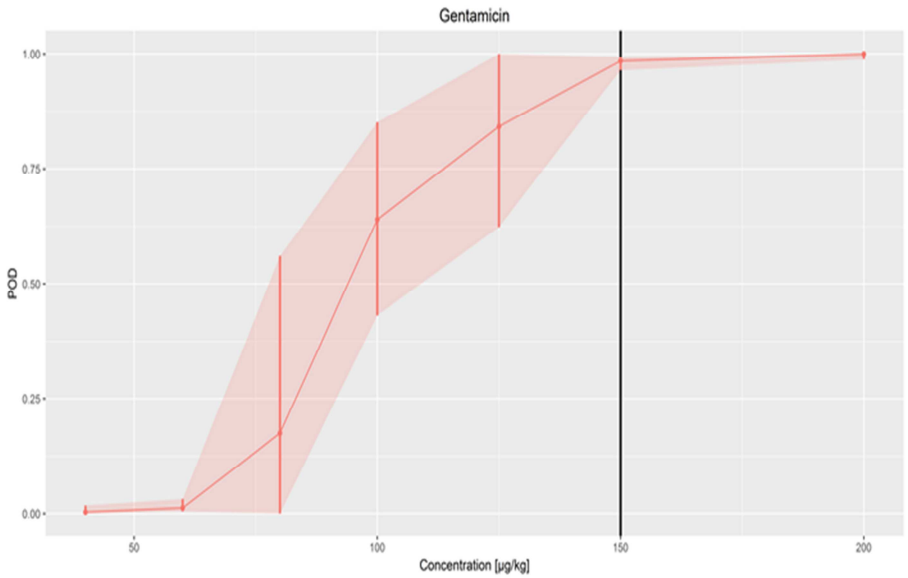

Sulfadimethoxin
POD
1.00
0.75
0.50
0.25
0.00
200
400
600
800
Concentration [µg/kg]

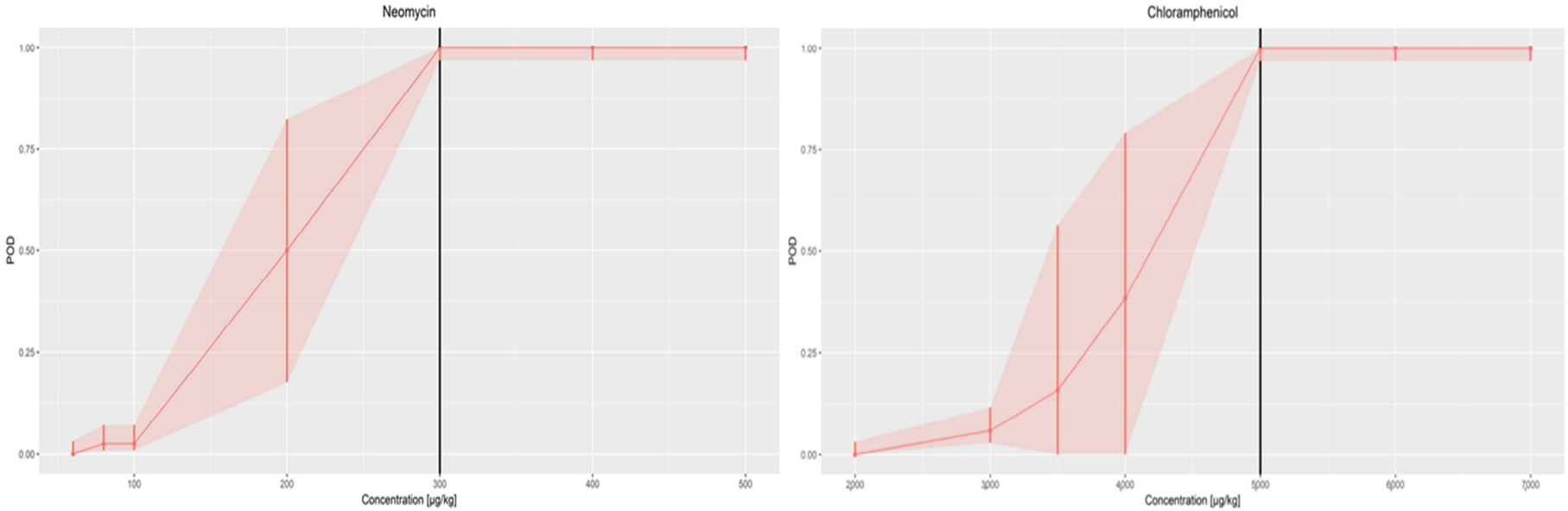

Sulfamethazine
Sulfathiazol
Oxytetracycline
Chlortetracycline
POD
Concentration [µg/kg]
0.00
0.25
0.50
0.75
1.00
250
500
750
1000
200
400
600


Tetracycline
POD
1.00
0.75
0.50
0.25
0.00
200
400
600
800
Concentration [µg/kg]


Dihydrostreptomycin
POD
1.00
0.75
0.50
0.25
0.00
200
400
600
800
Concentration [µg/kg]


Streptomycin
POD
1.00
0.75
0.50
0.25
0.00
400
800
1200
Concentration [µg/kg]


Gentamicin
POD
1.00
0.75
0.50
0.25
0.00
50
100
150
200
Concentration [µg/kg]



Annex Figure 1. Dose-response curves of antibiotic substances included in the validation of the BRT MRL Screening Test
Red line = dose-response curve; red shade = CI; Black line = CCß A (photometric reading); Dotted line = highest concentration analyzed

Annex Table 2. Numbers and percentages of results per concentrations of samples for each class of results (1-2-0) and both reading systems (photometric and visual) separately as well as joint for both reading systems including the CI for class A results.

Substance	Reading System	Concentration	No. of total Results	No. of Results (pos. Class A)	Percentage of CCß A	Lower 95%-CI (CCß A)	Upper 95%-CI (CCß A)	No. of Results (pos. Class B)	Percentage of CCß B	No. of Results (negative)
Benzyl-penicillin	Photometric	0.5	80	0	0.00	-	-	1	0.01	79
		0.75	80	0	0.00	-	-	2	0.03	78
		1	80	0	0.00	-	-	17	0.21	63
		1.5	80	58	0.73	-	-	22	1.00	0
		2	80	80	1.00	-	-	0	1.00	0
		2.5	80	80	1.00	-	-	0	1.00	0
		3	80	80	1.00	-	-	0	1.00	0
	Visual	0.5	120	0	0.00	-	-	0	0.00	120
		0.75	120	0	0.00	-	-	5	0.04	115
		1	120	2	0.02	-	-	19	0.18	99
		1.5	120	98	0.82	-	-	22	1.00	0
		2	120	120	1.00	-	-	0	1.00	0
		2.5	120	120	1.00	-	-	0	1.00	0
		3	119	119	1.00	-	-	0	1.00	0
	Photometric + Visual	0.5	200	0	0.00	0.00	0.02	1	-	199
		0.75	200	0	0.00	0.00	0.02	7	-	193
		1	200	2	0.01	0.00	0.04	36	-	162
		1.5	200	156	0.78	0.62	0.94	44	-	0
		2	200	200	1.00	0.98	1.00	0	-	0
		2.5	200	200	1.00	0.98	1.00	0	-	0
		3	199	199	1.00	0.98	1.00	0	-	0
Ampicillin	Photometric	1	80	0	0.00	-	-	0	0.00	80
		1.5	80	0	0.00	-	-	6	0.08	74
		2	80	2	0.03	-	-	78	1.00	0
		2.5	80	80	1.00	-	-	0	1.00	0
		3	80	80	1.00	-	-	0	1.00	0
		3.5	80	80	1.00	-	-	0	1.00	0
		4	80	80	1.00	-	-	0	1.00	0
	Visual	1	120	0	0.00	-	-	0	0.00	120
		1.5	120	0	0.00	-	-	2	0.02	118
		2	120	4	0.03	-	-	106	0.92	10
		2.5	120	120	1.00	-	-	0	1.00	0
		3	120	120	1.00	-	-	0	1.00	0
		3.5	120	120	1.00	-	-	0	1.00	0
		4	120	120	1.00	-	-	0	1.00	0
	Photometric + Visual	1	200	0	0.00	0.00	0.02	0	-	200
		1.5	200	0	0.00	0.00	0.02	8	-	192
		2	200	6	0.03	0.01	0.06	184	-	10
		2.5	200	200	1.00	0.98	1.00	0	-	0
		3	200	200	1.00	0.98	1.00	0	-	0
		3.5	200	200	1.00	0.98	1.00	0	-	0
		4	200	200	1.00	0.98	1.00	0	-	0
Amoxicillin	Photometric	1	80	0	0.00	-	-	0	0.00	80
		1.5	80	0	0.00	-	-	0	0.00	80
		2	80	0	0.00	-	-	68	0.85	12
		2.5	80	68	0.85	-	-	12	1.00	0
		3	80	80	1.00	-	-	0	1.00	0
		3.5	80	80	1.00	-	-	0	1.00	0
		4	80	80	1.00	-	-	0	1.00	0
	Visual	1	120	0	0.00	-	-	1	0.01	119
		1.5	120	0	0.00	-	-	8	0.07	112
		2	120	12	0.10	-	-	79	0.76	29
		2.5	120	94	0.78	-	-	26	1.00	0
		3	120	120	1.00	-	-	0	1.00	0
		3.5	120	120	1.00	-	-	0	1.00	0
		4	120	120	1.00	-	-	0	1.00	0
	Photometric + Visual	1	200	0	0.00	0.00	0.02	1	-	199
		1.5	200	0	0.00	0.00	0.02	8	-	192
		2	200	12	0.06	0.03	0.10	147	-	41
		2.5	200	162	0.81	0.67	0.95	38	-	0
		3	200	200	1.00	0.98	1.00	0	-	0
		3.5	200	200	1.00	0.98	1.00	0	-	0
		4	200	200	1.00	0.98	1.00	0	-	0
Cloxacillin	Photometric	12	80	0	0.00	-	-	0	0.00	80
		14	80	0	0.00	-	-	0	0.00	80
		16	80	13	0.16	-	-	44	0.71	23
		18	80	48	0.60	-	-	32	1.00	0
		20	80	72	0.90	-	-	8	1.00	0
		25	80	80	1.00	-	-	0	1.00	0
		30	80	80	1.00	-	-	0	1.00	0
	Visual	12	120	0	0.00	-	-	0	0.00	120
		14	120	0	0.00	-	-	0	0.00	120
		16	120	11	0.09	-	-	48	0.49	61
		18	120	49	0.41	-	-	47	0.80	24
		20	120	102	0.85	-	-	18	1.00	0
		25	120	120	1.00	-	-	0	1.00	0
		30	120	120	1.00	-	-	0	1.00	0
	Photometric + Visual	12	200	0	0.00	0.00	0.02	0	-	200
		14	200	0	0.00	0.00	0.02	0	-	200
		16	200	24	0.12	0.08	0.17	92	-	84
		18	200	97	0.49	0.34	0.63	79	-	24
		20	200	174	0.87	0.82	0.91	26	-	0
		25	200	200	1.00	0.98	1.00	0	-	0
		30	200	200	1.00	0.98	1.00	0	-	0

Continuation Annex Table 2

Substance	Reading System	Concentration	No. of total Results	No. of Results (pos. Class A)	Percentage of CCß A	Lower 95%-CI (CCß A)	Upper 95%-CI (CCß A)	No. of Results (pos. Class B)	Percentage of CCß B	No. of Results (negative)
		2	48	0	0.00	-	-	0	0.00	48
		3	48	0	0.00	-	-	16	0.33	32
		4	48	0	0.00	-	-	8	0.17	40
	Photometric	5	48	48	1.00	-	-	0	1.00	0
		6	48	48	1.00	-	-	0	1.00	0
		8	48	48	1.00	-	-	0	1.00	0
		10	48	48	1.00	-	-	0	1.00	0
		2	72	0	0.00	-	-	0	0.00	72
		3	72	0	0.00	-	-	8	0.11	64
		4	72	0	0.00	-	-	5	0.07	67
Cefapirin	Visual	5	72	72	1.00	-	-	0	1.00	0
		6	72	72	1.00	-	-	0	1.00	0
		8	72	72	1.00	-	-	0	1.00	0
		10	72	72	1.00	-	-	0	1.00	0
		2	120	0	0.00	0.00	0.03	0	-	120
		3	120	0	0.00	0.00	0.03	24	-	96
		4	120	0	0.00	0.00	0.03	13	-	107
	Photometric + Visual	5	120	120	1.00	0.97	1.00	0	-	0
		6	120	120	1.00	0.97	1.00	0	-	0
		8	120	120	1.00	0.97	1.00	0	-	0
		10	120	120	1.00	0.97	1.00	0	-	0
		10	80	0	0.00	-	-	0	0.00	80
		15	80	0	0.00	-	-	6	0.08	74
		20	80	2	0.03	-	-	76	0.98	2
	Photometric	25	80	68	0.85	-	-	12	1.00	0
		30	80	80	1.00	-	-	0	1.00	0
		35	80	80	1.00	-	-	0	1.00	0
		40	80	80	1.00	-	-	0	1.00	0
		10	120	0	0.00	-	-	0	0.00	120
		15	120	0	0.00	-	-	7	0.06	113
		20	120	14	0.12	-	-	97	0.93	9
Cefoperazone	Visual	25	120	97	0.81	-	-	23	1.00	0
		30	120	120	1.00	-	-	0	1.00	0
		35	120	120	1.00	-	-	0	1.00	0
		40	120	120	1.00	-	-	0	1.00	0
		10	200	0	0.00	0.00	0.02	0	-	200
		15	200	0	0.00	0.00	0.02	13	-	187
		20	200	16	0.08	0.05	0.13	173	-	11
	Photometric + Visual	25	200	165	0.83	0.68	0.97	35	-	0
		30	200	200	1.00	0.98	1.00	0	-	0
		35	200	200	1.00	0.98	1.00	0	-	0
		40	200	200	1.00	0.98	1.00	0	-	0
		4	48	0	0.00	-	-	3	0.06	45
		5	48	0	0.00	-	-	40	0.83	8
		6	48	30	0.63	-	-	18	1.00	0
	Photometric	7	48	48	1.00	-	-	0	1.00	0
		8	48	48	1.00	-	-	0	1.00	0
		9	48	48	1.00	-	-	0	1.00	0
		10	48	48	1.00	-	-	0	1.00	0
		4	72	0	0.00	-	-	5	0.07	67
		5	72	0	0.00	-	-	51	0.71	21
		6	72	28	0.39	-	-	44	1.00	0
Cefazolin	Visual	7	72	72	1.00	-	-	0	1.00	0
		8	72	72	1.00	-	-	0	1.00	0
		9	72	72	1.00	-	-	0	1.00	0
		10	72	72	1.00	-	-	0	1.00	0
		4	120	0	0.00	0.00	0.03	8	-	112
		5	120	0	0.00	0.00	0.03	91	-	29
		6	120	58	0.48	0.00	0.97	62	-	0
	Photometric + Visual	7	120	120	1.00	0.97	1.00	0	-	0
		8	120	120	1.00	0.97	1.00	0	-	0
		9	120	120	1.00	0.97	1.00	0	-	0
		10	120	120	1.00	0.97	1.00	0	-	0
		50	48	0	0.00	-	-	16	0.33	32
		60	48	0	0.00	-	-	16	0.33	32
		100	48	0	0.00	-	-	36	0.75	12
	Photometric	200	48	40	0.83	-	-	8	1.00	0
		300	48	48	1.00	-	-	0	1.00	0
		400	48	48	1.00	-	-	0	1.00	0
		500	48	48	1.00	-	-	0	1.00	0
		50	72	1	0.01	-	-	20	0.29	51
		60	72	1	0.01	-	-	26	0.38	45
		100	72	8	0.11	-	-	54	0.86	10
Cefquinome	Visual	200	72	69	0.96	-	-	3	1.00	0
		300	72	72	1.00	-	-	0	1.00	0
		400	72	72	1.00	-	-	0	1.00	0
		500	72	72	1.00	-	-	0	1.00	0
		50	120	1	0.01	0.00	0.05	36	-	83
		60	120	1	0.01	0.00	0.05	42	-	77
		100	120	8	0.07	0.03	0.13	90	-	22
	Photometric + Visual	200	120	109	0.91	0.84	0.95	11	-	0
		300	120	120	1.00	0.97	1.00	0	-	0
		400	120	120	1.00	0.97	1.00	0	-	0
		500	120	120	1.00	0.97	1.00	0	-	0

Continuation Annex Table 2

Substance	Reading System	Concentration	No. of total Results	No. of Results (pos. Class A)	Percentage of CCß A	Lower 95%-CI (CCß A)	Upper 95%-CI (CCß A)	No. of Results (pos. Class B)	Percentage of CCß B	No. of Results (negative)
		25	128	0	0.00	-	-	0	0.00	128
		50	128	0	0.00	-	-	35	0.27	93
		100	128	42	0.33	-	-	86	1.00	0
	Photometric	150	128	128	1.00	-	-	0	1.00	0
		200	128	128	1.00	-	-	0	1.00	0
		300	128	128	1.00	-	-	0	1.00	0
		400	128	128	1.00	-	-	0	1.00	0
		25	192	0	0.00	-	-	0	0.00	192
		50	192	0	0.00	-	-	21	0.11	171
		100	192	97	0.51	-	-	91	0.98	4
Ceftiofur	Visual	150	192	190	0.99	-	-	2	1.00	0
		200	192	192	1.00	-	-	0	1.00	0
		300	192	192	1.00	-	-	0	1.00	0
		400	192	192	1.00	-	-	0	1.00	0
		25	320	0	0.00	0.00	0.01	0	-	320
		50	320	0	0.00	0.00	0.01	56	-	264
		100	320	139	0.43	0.27	0.60	177	-	4
	Photometric + Visual	150	320	318	0.99	0.98	1.00	2	-	0
		200	320	320	1.00	0.99	1.00	0	-	0
		300	320	320	1.00	0.99	1.00	0	-	0
		400	320	320	1.00	0.99	1.00	0	-	0
		6	80	0	0.00	-	-	0	0.00	80
		8	80	0	0.00	-	-	50	0.63	30
		10	80	56	0.70	-	-	24	1.00	0
	Photometric	12	80	80	1.00	-	-	0	1.00	0
		14	80	80	1.00	-	-	0	1.00	0
		16	80	80	1.00	-	-	0	1.00	0
		20	80	80	1.00	-	-	0	1.00	0
		6	120	0	0.00	-	-	0	0.00	120
		8	120	0	0.00	-	-	33	0.28	87
		10	120	43	0.36	-	-	77	1.00	0
Cefalonium	Visual	12	120	120	1.00	-	-	0	1.00	0
		14	120	120	1.00	-	-	0	1.00	0
		16	120	120	1.00	-	-	0	1.00	0
		20	120	120	1.00	-	-	0	1.00	0
		6	200	0	0.00	0.00	0.02	0	-	200
		8	200	0	0.00	0.00	0.02	83	-	117
		10	200	99	0.50	0.03	0.96	101	-	0
	Photometric + Visual	12	200	200	1.00	0.98	1.00	0	-	0
		14	200	200	1.00	0.98	1.00	0	-	0
		16	200	200	1.00	0.98	1.00	0	-	0
		20	200	200	1.00	0.98	1.00	0	-	0
		30	128	0	0.00	-	-	45	0.35	83
		40	128	2	0.02	-	-	117	0.93	9
		50	128	16	0.13	-	-	112	1.00	0
	Photometric	60	128	41	0.32	-	-	87	1.00	0
		80	128	127	0.99	-	-	1	1.00	0
		100	128	128	1.00	-	-	0	1.00	0
		120	128	128	1.00	-	-	0	1.00	0
		30	192	0	0.00	-	-	21	0.11	171
		40	192	9	0.05	-	-	86	0.49	97
		50	192	60	0.31	-	-	106	0.86	26
Erythromycin	Visual	60	192	91	0.47	-	-	99	0.99	2
		80	192	192	1.00	-	-	0	1.00	0
		100	192	192	1.00	-	-	0	1.00	0
		120	192	192	1.00	-	-	0	1.00	0
		30	320	0	0.00	0.00	0.01	66	-	254
		40	320	11	0.03	0.02	0.06	203	-	106
		50	320	76	0.24	0.00	0.51	218	-	26
	Photometric + Visual	60	320	132	0.41	0.08	0.74	186	-	2
		80	320	319	1.00	0.98	1.00	1	-	0
		100	320	320	1.00	0.99	1.00	0	-	0
		120	320	320	1.00	0.99	1.00	0	-	0
		15	128	0	0.00	-	-	2	0.02	126
		20	128	2	0.02	-	-	49	0.40	77
		30	128	7	0.05	-	-	116	0.96	5
	Photometric	40	128	77	0.60	-	-	51	1.00	0
		50	128	116	0.91	-	-	12	1.00	0
		75	128	128	1.00	-	-	0	1.00	0
		100	128	128	1.00	-	-	0	1.00	0
		15	192	0	0.00	-	-	9	0.05	183
		20	192	3	0.02	-	-	29	0.17	160
		30	192	11	0.06	-	-	103	0.59	78
Tylosin	Visual	40	192	107	0.56	-	-	82	0.98	3
		50	192	160	0.83	-	-	32	1.00	0
		75	192	192	1.00	-	-	0	1.00	0
		100	192	192	1.00	-	-	0	1.00	0
		15	320	0	0.00	0.00	0.01	11	-	309
		20	320	5	0.02	0.01	0.04	78	-	237
		30	320	18	0.06	0.04	0.09	219	-	83
	Photometric + Visual	40	320	184	0.58	0.46	0.69	133	-	3
		50	320	276	0.86	0.82	0.90	44	-	0
		75	320	320	1.00	0.99	1.00	0	-	0
		100	320	320	1.00	0.99	1.00	0	-	0

Continuation Annex Table 2

Substance	Reading System	Concentration	No. of total Results	No. of Results (pos. Class A)	Percentage of CCß A	Lower 95%-CI (CCß A)	Upper 95%-CI (CCß A)	No. of Results (pos. Class B)	Percentage of CCß B	No. of Results (negative)
		50	128	0	0.00	-	-	36	0.28	92
		100	128	3	0.02	-	-	123	0.98	2
		200	128	50	0.39	-	-	78	1.00	0
	Photometric	300	128	100	0.78	-	-	28	1.00	0
		400	128	122	0.95	-	-	6	1.00	0
		600	128	128	1.00	-	-	0	1.00	0
		800	128	128	1.00	-	-	0	1.00	0
		50	192	1	0.01	-	-	66	0.35	125
		100	192	45	0.23	-	-	146	0.99	1
		200	192	160	0.83	-	-	32	1.00	0
Sulfadiazine	Visual	300	192	191	0.99	-	-	1	1.00	0
		400	192	192	1.00	-	-	0	1.00	0
		600	192	192	1.00	-	-	0	1.00	0
		800	192	192	1.00	-	-	0	1.00	0
		50	320	1	0.00	0.00	0.02	102	-	217
		100	320	48	0.15	0.00	0.43	269	-	3
		200	320	210	0.66	0.32	0.99	110	-	0
	Photometric + Visual	300	320	291	0.91	0.87	0.94	29	-	0
		400	320	314	0.98	0.96	0.99	6	-	0
		600	320	320	1.00	0.99	1.00	0	-	0
		800	320	320	1.00	0.99	1.00	0	-	0
		50	128	0	0.00	-	-	30	0.23	98
		100	128	0	0.00	-	-	126	0.98	2
		200	128	43	0.34	-	-	85	1.00	0
	Photometric	300	128	66	0.52	-	-	62	1.00	0
		400	128	104	0.81	-	-	24	1.00	0
		600	128	123	0.96	-	-	5	1.00	0
		800	128	128	1.00	-	-	0	1.00	0
		50	192	1	0.01	-	-	43	0.23	148
		100	192	31	0.16	-	-	152	0.95	9
		200	192	125	0.65	-	-	67	1.00	0
Sulfadi-methoxin	Visual	300	192	159	0.83	-	-	33	1.00	0
		400	192	192	1.00	-	-	0	1.00	0
		600	192	192	1.00	-	-	0	1.00	0
		800	192	192	1.00	-	-	0	1.00	0
		50	320	1	0.00	0.00	0.02	73	-	246
		100	320	31	0.10	0.07	0.13	278	-	11
		200	320	168	0.53	0.13	0.92	152	-	0
	Photometric + Visual	300	320	225	0.70	0.39	1.00	95	-	0
		400	320	296	0.93	0.89	0.95	24	-	0
		600	320	315	0.98	0.96	0.99	5	-	0
		800	320	320	1.00	0.99	1.00	0	-	0
		50	48	0	0.00	-	-	2	0.04	46
		100	48	1	0.02	-	-	27	0.58	20
		200	48	2	0.04	-	-	46	1.00	0
	Photometric	300	48	5	0.10	-	-	43	1.00	0
		500	48	11	0.23	-	-	37	1.00	0
		750	48	29	0.60	-	-	19	1.00	0
		1,000	48	31	0.65	-	-	17	1.00	0
		50	72	0	0.00	-	-	11	0.15	61
		100	72	5	0.07	-	-	35	0.56	32
		200	72	29	0.40	-	-	43	1.00	0
Sulfametha-zine	Visual	300	72	39	0.54	-	-	33	1.00	0
		500	72	59	0.82	-	-	13	1.00	0
		750	72	62	0.86	-	-	10	1.00	0
		1,000	72	68	0.94	-	-	4	1.00	0
		50	120	0	0.00	0.00	0.03	13	-	107
		100	120	6	0.05	0.02	0.11	62	-	52
		200	120	31	0.26	0.00	0.69	89	-	0
	Photometric + Visual	300	120	44	0.37	0.00	0.89	76	-	0
		500	120	70	0.58	0.17	0.99	50	-	0
		750	120	91	0.76	0.57	0.95	29	-	0
		1,000	120	99	0.83	0.63	1.00	21	-	0
		40	128	0	0.00	-	-	103	0.80	25
		60	128	1	0.01	-	-	127	1.00	0
		80	128	24	0.19	-	-	104	1.00	0
	Photometric	100	128	40	0.31	-	-	88	1.00	0
		200	128	128	1.00	-	-	0	1.00	0
		400	128	128	1.00	-	-	0	1.00	0
		600	128	128	1.00	-	-	0	1.00	0
		40	192	17	0.09	-	-	77	0.49	98
		60	192	52	0.27	-	-	137	0.98	3
		80	191	93	0.49	-	-	98	1.00	0
Sulfathiazol	Visual	100	192	156	0.81	-	-	36	1.00	0
		200	192	192	1.00	-	-	0	1.00	0
		400	192	192	1.00	-	-	0	1.00	0
		600	192	192	1.00	-	-	0	1.00	0
		40	320	17	0.05	0.03	0.08	180	-	123
		60	320	53	0.17	0.00	0.49	264	-	3
		80	319	117	0.37	0.00	0.81	202	-	0
	Photometric + Visual	100	320	196	0.61	0.17	1.00	124	-	0
		200	320	320	1.00	0.99	1.00	0	-	0
		400	320	320	1.00	0.99	1.00	0	-	0
		600	320	320	1.00	0.99	1.00	0	-	0

Continuation Annex Table 2

Substance	Reading System	Concentration	No. of total Results	No. of Results (pos. Class A)	Percentage of CCß A	Lower 95%-CI (CCß A)	Upper 95%-CI (CCß A)	No. of Results (pos. Class B)	Percentage of CCß B	No. of Results (negative)
		200	48	0	0.00	-	-	45	0.94	3
		300	48	0	0.00	-	-	48	1.00	0
		400	48	0	0.00	-	-	48	1.00	0
	Photometric	600	48	21	0.44	-	-	27	1.00	0
		800	48	43	0.90	-	-	5	1.00	0
		1,000	48	44	0.92	-	-	4	1.00	0
		1,500	48	48	1.00	-	-	0	1.00	0
		200	72	21	0.29	-	-	45	0.92	6
		300	72	51	0.71	-	-	21	1.00	0
		400	72	72	1.00	-	-	0	1.00	0
Sulfadoxin	Visual	600	72	72	1.00	-	-	0	1.00	0
		800	72	72	1.00	-	-	0	1.00	0
		1,000	72	72	1.00	-	-	0	1.00	0
		1,500	72	72	1.00	-	-	0	1.00	0
		200	120	21	0.18	0.00	0.46	90	-	9
		300	120	51	0.43	0.00	0.95	69	-	0
		400	120	72	0.60	0.00	1.00	48	-	0
	Photometric + Visual	600	120	93	0.78	0.41	1.00	27	-	0
		800	120	115	0.96	0.91	0.98	5	-	0
		1,000	120	116	0.97	0.92	0.99	4	-	0
		1,500	120	120	1.00	0.97	1.00	0	-	0
		50	128	0	0.00	-	-	107	0.84	21
		100	128	0	0.00	-	-	128	1.00	0
		200	128	78	0.61	-	-	50	1.00	0
	Photometric	300	128	113	0.88	-	-	15	1.00	0
		500	128	128	1.00	-	-	0	1.00	0
		750	128	128	1.00	-	-	0	1.00	0
		1,000	128	128	1.00	-	-	0	1.00	0
		50	192	12	0.06	-	-	128	0.73	52
		100	192	49	0.26	-	-	143	1.00	0
		200	192	169	0.88	-	-	23	1.00	0
Sulfamethoxy-pyridazine	Visual	300	192	192	1.00	-	-	0	1.00	0
		500	192	192	1.00	-	-	0	1.00	0
		750	192	192	1.00	-	-	0	1.00	0
		1,000	192	192	1.00	-	-	0	1.00	0
		50	320	12	0.04	0.02	0.06	235	-	73
		100	320	49	0.15	0.00	0.56	271	-	0
		200	320	247	0.77	0.52	1.00	73	-	0
	Photometric + Visual	300	320	305	0.95	0.92	0.97	15	-	0
		500	320	320	1.00	0.99	1.00	0	-	0
		750	320	320	1.00	0.99	1.00	0	-	0
		1,000	320	320	1.00	0.99	1.00	0	-	0
		100	48	1	0.02	-	-	14	0.31	33
		200	48	0	0.00	-	-	32	0.67	16
		300	48	0	0.00	-	-	45	0.94	3
	Photometric	400	48	11	0.23	-	-	37	1.00	0
		600	48	38	0.79	-	-	10	1.00	0
		800	48	48	1.00	-	-	0	1.00	0
		1,000	48	48	1.00	-	-	0	1.00	0
		100	72	4	0.06	-	-	18	0.31	50
		200	72	0	0.00	-	-	46	0.64	26
		300	72	9	0.13	-	-	56	0.90	7
Chlortetra-cycline	Visual	400	72	25	0.35	-	-	47	1.00	0
		600	72	70	0.97	-	-	2	1.00	0
		800	72	72	1.00	-	-	0	1.00	0
		1,000	72	72	1.00	-	-	0	1.00	0
		100	120	5	0.04	0.02	0.09	32	-	83
		200	120	0	0.00	0.00	0.03	78	-	42
		300	120	9	0.08	0.04	0.14	101	-	10
	Photometric + Visual	400	120	36	0.30	0.19	0.42	84	-	0
		600	120	108	0.90	0.83	0.94	12	-	0
		800	120	120	1.00	0.97	1.00	0	-	0
		1,000	120	120	1.00	0.97	1.00	0	-	0
		100	48	0	0.00	-	-	0	0.00	48
		200	48	0	0.00	-	-	48	1.00	0
		300	48	25	0.52	-	-	23	1.00	0
	Photometric	400	48	48	1.00	-	-	0	1.00	0
		600	48	48	1.00	-	-	0	1.00	0
		800	48	48	1.00	-	-	0	1.00	0
		1,000	48	48	1.00	-	-	0	1.00	0
		100	72	0	0.00	-	-	2	0.03	70
		200	72	3	0.04	-	-	45	0.67	24
		300	72	27	0.38	-	-	42	0.96	3
Oxytetra-cycline	Visual	400	72	64	0.89	-	-	8	1.00	0
		600	72	69	0.96	-	-	3	1.00	0
		800	72	72	1.00	-	-	0	1.00	0
		1,000	72	72	1.00	-	-	0	1.00	0
		100	120	0	0.00	0.00	0.03	2	-	118
		200	120	3	0.03	0.01	0.07	93	-	24
		300	120	52	0.43	0.08	0.79	65	-	3
	Photometric + Visual	400	120	112	0.93	0.87	0.97	8	-	0
		600	120	117	0.98	0.93	0.99	3	-	0
		800	120	120	1.00	0.97	1.00	0	-	0
		1,000	120	120	1.00	0.97	1.00	0	-	0

Continuation Annex Table 2

Substance	Reading System	Concentration	No. of total Results	No. of Results (pos. Class A)	Percentage of CCß A	Lower 95%-CI (CCß A)	Upper 95%-CI (CCß A)	No. of Results (pos. Class B)	Percentage of CCß B	No. of Results (negative)
		100	48	0	0.00	-	-	0	0.00	48
		150	48	0	0.00	-	-	0	0.00	48
		200	48	0	0.00	-	-	23	0.48	25
	Photometric	300	48	0	0.00	-	-	48	1.00	0
		400	48	39	0.81	-	-	9	1.00	0
		600	48	48	1.00	-	-	0	1.00	0
		800	48	48	1.00	-	-	0	1.00	0
		100	72	0	0.00	-	-	10	0.14	62
		150	72	0	0.00	-	-	30	0.42	42
		200	72	3	0.04	-	-	53	0.78	16
Tetracycline	Visual	300	72	18	0.25	-	-	54	1.00	0
		400	72	72	1.00	-	-	0	1.00	0
		600	72	72	1.00	-	-	0	1.00	0
		800	72	72	1.00	-	-	0	1.00	0
		100	120	0	0.00	0.00	0.03	10	-	110
		150	120	0	0.00	0.00	0.03	30	-	90
		200	120	3	0.03	0.01	0.07	76	-	41
	Photometric + Visual	300	120	18	0.15	0.00	0.56	102	-	0
		400	120	111	0.93	0.86	0.96	9	-	0
		600	120	120	1.00	0.97	1.00	0	-	0
		800	120	120	1.00	0.97	1.00	0	-	0
		200	48	0	0.00	-	-	0	0.00	48
		300	48	0	0.00	-	-	42	0.88	6
		400	48	4	0.08	-	-	44	1.00	0
	Photometric	500	48	22	0.46	-	-	26	1.00	0
		600	48	46	0.96	-	-	2	1.00	0
		700	48	48	1.00	-	-	0	1.00	0
		800	48	48	1.00	-	-	0	1.00	0
		200	72	0	0.00	-	-	3	0.04	69
		300	72	0	0.00	-	-	26	0.36	46
		400	72	11	0.15	-	-	51	0.86	10
Dihydro-streptomycin	Visual	500	72	43	0.60	-	-	28	0.99	1
		600	72	63	0.88	-	-	9	1.00	0
		700	72	69	0.96	-	-	3	1.00	0
		800	72	72	1.00	-	-	0	1.00	0
		200	120	0	0.00	0.00	0.03	3	-	117
		300	120	0	0.00	0.00	0.03	68	-	52
		400	120	15	0.13	0.08	0.20	95	-	10
	Photometric + Visual	500	120	65	0.54	0.33	0.75	54	-	1
		600	120	109	0.91	0.84	0.95	11	-	0
		700	120	117	0.98	0.93	0.99	3	-	0
		800	120	120	1.00	0.97	1.00	0	-	0
		300	48	0	0.00	-	-	11	0.23	37
		400	48	0	0.00	-	-	28	0.58	20
		500	48	0	0.00	-	-	46	0.96	2
	Photometric	600	48	14	0.29	-	-	34	1.00	0
		800	48	32	0.67	-	-	16	1.00	0
		1,000	48	47	0.98	-	-	1	1.00	0
		1,500	48	48	1.00	-	-	0	1.00	0
		300	72	0	0.00	-	-	5	0.07	67
		400	72	0	0.00	-	-	16	0.22	56
		500	72	0	0.00	-	-	42	0.58	30
Streptomycin	Visual	600	72	15	0.21	-	-	41	0.78	16
		800	72	42	0.58	-	-	28	0.97	2
		1,000	72	56	0.78	-	-	16	1.00	0
		1,500	72	72	1.00	-	-	0	1.00	0
		300	120	0	0.00	0.00	0.03	16	-	104
		400	120	0	0.00	0.00	0.03	44	-	76
		500	120	0	0.00	0.00	0.03	88	-	32
	Photometric + Visual	600	120	29	0.24	0.03	0.45	75	-	16
		800	120	74	0.62	0.52	0.72	44	-	2
		1,000	120	103	0.86	0.78	0.91	17	-	0
		1,500	120	120	1.00	0.97	1.00	0	-	0
		40	128	0	0.00	-	-	18	0.14	110
		60	128	0	0.00	-	-	104	0.81	24
		80	128	7	0.05	-	-	120	0.99	1
	Photometric	100	128	68	0.53	-	-	60	1.00	0
		125	128	83	0.65	-	-	45	1.00	0
		150	128	123	0.96	-	-	5	1.00	0
		200	128	128	1.00	-	-	0	1.00	0
		40	192	1	0.01	-	-	11	0.06	180
		60	192	4	0.02	-	-	134	0.72	54
		80	192	49	0.26	-	-	141	0.99	2
Gentamicin	Visual	100	192	137	0.71	-	-	55	1.00	0
		125	192	186	0.97	-	-	6	1.00	0
		150	192	192	1.00	-	-	0	1.00	0
		200	192	192	1.00	-	-	0	1.00	0
		40	320	1	0.00	0.00	0.02	29	-	290
		60	320	4	0.01	0.00	0.03	238	-	78
		80	320	56	0.18	0.00	0.56	261	-	3
	Photometric + Visual	100	320	205	0.64	0.43	0.85	115	-	0
		125	320	269	0.84	0.62	1.00	51	-	0
		150	320	315	0.98	0.96	0.99	5	-	0
		200	320	320	1.00	0.99	1.00	0	-	0

Continuation Annex Table 2

Substance	Reading System	Concentration	No. of total Results	No. of Results (pos. Class A)	Percentage of CCß A	Lower 95%-CI (CCß A)	Upper 95%-CI (CCß A)	No. of Results (pos. Class B)	Percentage of CCß B	No. of Results (negative)
Neomycin	Photometric	60	48	0	0.00	-	-	2	0.04	46
		80	48	0	0.00	-	-	26	0.54	22
		100	48	0	0.00	-	-	13	0.27	35
		200	48	19	0.40	-	-	29	1.00	0
		300	48	48	1.00	-	-	0	1.00	0
		400	48	48	1.00	-	-	0	1.00	0
		500	48	48	1.00	-	-	0	1.00	0
	Visual	60	72	0	0.00	-	-	7	0.10	65
		80	72	3	0.04	-	-	24	0.38	45
		100	72	3	0.04	-	-	30	0.46	39
		200	72	41	0.57	-	-	31	1.00	0
		300	72	72	1.00	-	-	0	1.00	0
		400	72	72	1.00	-	-	0	1.00	0
		500	72	72	1.00	-	-	0	1.00	0
	Photometric + Visual	60	120	0	0.00	0.00	0.03	9	-	111
		80	120	3	0.03	0.01	0.07	50	-	67
		100	120	3	0.03	0.01	0.07	43	-	74
		200	120	60	0.50	0.18	0.82	60	-	0
		300	120	120	1.00	0.97	1.00	0	-	0
		400	120	120	1.00	0.97	1.00	0	-	0
		500	120	120	1.00	0.97	1.00	0	-	0
Chlor-amphenicol	Photometric	2,000	48	0	0.00	-	-	13	0.27	35
		3,000	48	0	0.00	-	-	43	0.90	5
		3,500	48	1	0.02	-	-	47	1.00	0
		4,000	48	8	0.17	-	-	40	1.00	0
		5,000	48	48	1.00	-	-	0	1.00	0
		6,000	48	48	1.00	-	-	0	1.00	0
		7,000	48	48	1.00	-	-	0	1.00	0
	Visual	2,000	72	0	0.00	-	-	32	0.44	40
		3,000	72	7	0.10	-	-	53	0.83	12
		3,500	72	18	0.25	-	-	54	1.00	0
		4,000	72	38	0.53	-	-	34	1.00	0
		5,000	72	72	1.00	-	-	0	1.00	0
		6,000	72	72	1.00	-	-	0	1.00	0
		7,000	72	72	1.00	-	-	0	1.00	0
	Photometric + Visual	2,000	120	0	0.00	0.00	0.03	45	-	75
		3,000	120	7	0.06	0.03	0.12	96	-	17
		3,500	120	19	0.16	0.00	0.56	101	-	0
		4,000	120	46	0.38	0.00	0.79	74	-	0
		5,000	120	120	1.00	0.97	1.00	0	-	0
		6,000	120	120	1.00	0.97	1.00	0	-	0
		7,000	120	120	1.00	0.97	1.00	0	-	0

Annex Table 3. Contingency table created with the Fisher Test for the concentration at CCß A obtained with photometric reading, including the numbers of results of the different classes of results (1-2-0) for the different plate batches and ELISA readers

Substance	Concentration CCß A	No. of ELISA Reader	Batch	No. of Results (pos. Class A)	No. of Results (pos. Class B)	No. of Results (negative)	p-Value Fisher´s Exact Test (CCß A)
Benzyl-penicillin	2	1	A	16	0	0	
		1	B	16	0	0	1
		1	C	8	0	0	
		2	A	16	0	0	
		2	B	16	0	0	1
		2	C	8	0	0	
Ampicillin	2.5	1	A	16	0	0	
		1	B	8	0	0	1
		1	C	16	0	0	
		2	A	16	0	0	
		2	B	8	0	0	1
		2	C	16	0	0	
Amoxicillin	3	1	A	16	0	0	
		1	B	8	0	0	1
		1	C	16	0	0	
		2	A	16	0	0	
		2	B	8	0	0	1
		2	C	16	0	0	
Cloxacillin	25	1	D	16	0	0	
		1	E	16	0	0	1
		1	F	8	0	0	
		2	D	16	0	0	
		2	E	16	0	0	1
		2	F	8	0	0	
Dicloxacillin	12.5	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Nafcillin	10	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Oxacillin	8	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Cefalexin	300	1	D	8	0	0	
		1	E	8	0	0	1
		1	F	8	0	0	
		2	D	8	0	0	
		2	E	8	0	0	1
		2	F	8	0	0	
Cefapirin	5	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Cefoperazone	30	1	A	16	0	0	
		1	B	8	0	0	1
		1	C	16	0	0	
		2	A	16	0	0	
		2	B	8	0	0	1
		2	C	16	0	0	

Continuation Annex Table 3

Substance	Concentration CCß A	No. of ELISA Reader	Batch	No. of Results (pos. Class A)	No. of Results (pos. Class B)	No. of Results (negative)	p-Value Fisher´s Exact Test (CCß A)
Cefazolin	7	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Cefquinome	300	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Ceftiofur	150	1	A	24	0	0	
		1	B	16	0	0	1
		1	C	24	0	0	
		2	A	24	0	0	
		2	B	16	0	0	1
		2	C	24	0	0	
Cefalonium	12	1	A	16	0	0	
		1	B	8	0	0	1
		1	C	16	0	0	
		2	A	16	0	0	
		2	B	8	0	0	1
		2	C	16	0	0	
Erythromycin	80	1	A	24	0	0	
		1	B	23	1	0	1
		1	C	16	0	0	
		2	A	24	0	0	
		2	B	24	0	0	1
		2	C	16	0	0	
Tylosin	75	1	D	16	0	0	
		1	E	24	0	0	1
		1	F	24	0	0	
		2	D	16	0	0	
		2	E	24	0	0	1
		2	F	24	0	0	
Sulfadiazine	400	1	A	24	0	0	
		1	B	16	0	0	0.11
		1	C	21	3	0	
		2	A	24	0	0	
		2	B	16	0	0	0.11
		2	C	21	3	0	
Sulfadi-methoxin	600	1	A	24	0	0	
		1	B	21	3	0	0.11
		1	C	16	0	0	
		2	A	24	0	0	
		2	B	22	2	0	0.33
		2	C	16	0	0	
Sulfamethazine	NA	1	A	7	1	0	
		1	B	5	3	0	-
		1	C	3	5	0	
		2	A	7	1	0	
		2	B	6	2	0	-
		2	C	3	5	0	
Sulfathiazol	200	1	A	16	0	0	
		1	B	24	0	0	1
		1	C	24	0	0	
		2	A	16	0	0	
		2	B	24	0	0	1
		2	C	24	0	0	

Continuation Annex Table 3

Substance	Concentration CCß A	No. of ELISA Reader	Batch	No. of Results (pos. Class A)	No. of Results (pos. Class B)	No. of Results (negative)	p-Value Fisher´s Exact Test (CCß A)
Sulfadoxin	1,500	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Sulfamethoxy-pyridazine	500	1	A	16	0	0	
		1	B	24	0	0	1
		1	C	24	0	0	
		2	A	16	0	0	
		2	B	24	0	0	1
		2	C	24	0	0	
Chlortetra-cycline	800	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Oxytetracycline	400	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Tetracycline	600	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Dihydro-streptomycin	600	1	D	8	0	0	
		1	E	8	0	0	1
		1	F	7	1	0	
		2	D	8	0	0	
		2	E	8	0	0	1
		2	F	7	1	0	
Streptomycin	1,000	1	D	7	1	0	
		1	E	8	0	0	1
		1	F	8	0	0	
		2	D	8	0	0	
		2	E	8	0	0	1
		2	F	8	0	0	
Gentamicin	150	1	A	24	0	0	
		1	B	21	3	0	0.11
		1	C	16	0	0	
		2	A	24	0	0	
		2	B	22	2	0	0.33
		2	C	16	0	0	
Neomycin	300	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	
Chlor-amphenicol	5,000	1	A	8	0	0	
		1	B	8	0	0	1
		1	C	8	0	0	
		2	A	8	0	0	
		2	B	8	0	0	1
		2	C	8	0	0	