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NordVal International Certificate

Issued for:	**BRT hi-sense**
NordVal No:	053
First approval date:	01 March 2019
Renewal date:	01 March 2023
Valid until:	01 March 2025

BRT hi-sense

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 hi-sense 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 hi-sense is according to 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 hi-sense is a 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 3 h 30 min ± 30 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 hi-sense. 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 for Class A. However, two of the tested antiinflammatory substances, one antiparasitic and the polyether-antibiotic Monensin, were found to be presumptive positive (Class B) in 2, 3 and 6 of the 6 samples tested. Thus, when in doubt results should be confirmed.

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. 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, concentrations of veterinary drugs below these limits are accepted. According to this, the BRT hi-sense plates are applicable for the detection of the penicillin compounds: Benzylpenicillin, Ampicillin, Amoxicillin, Cloxacillin, Dicloxacillin, Nafcillin and Oxacillin, for the cephalosporins: Cefapirin, Cefoperazone, Cefazolin, Cefquinome, Ceftiofur and Cefalonium; and for Tylosin, Gentamicin and Neomycin. In addition, for photometric reading only, the method is also applicable for Sulfathiazol and Sulfamethoxypyridazine,

The method was found not to be applicable for the detection of Cefalexin, Erythromysin, Sulfadiazine, Sulfadimethoxin, Sulfamethazine, Sulfadoxin, Chlortetracycline, Tetracycline, Dihydrostreptomycin and Streptomycin at the maximum permitted EU residue level (MRL). Further, for chloramphenicol, the EU minimum required performance limit (MPRL) is 0.3 µg/kg and hence the method is not applicable for this substance (Commission Decision of 13 March 2003 amending Decision 2002/657/EC).

Presumptive results at *CCβ B* level (40 - <65 %, **Table 1**) could be obtained for Cefalexin (photometric reading), Erythromysin, Sulfadiazine, Sulfadimethoxin and Sulfamethazine (photometric reading only), Sulfathiazol (visual reading), Sulfamethoxypyridazine (visual reading), Oxytetracycline (visual reading) and Tetracycline.

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	1	0.6	1	0.75
	Ampicillin	4	1.5	1.25	1.5	1.25
	Amoxicillin	4	1.5	1.25	1.5	1.25
	Cloxacillin	30	10	9	10	9
	Dicloxacillin	30	6	5	6	5
	Nafcillin	30	4	4	4	3
	Oxacillin	30	4	3	4	4
Cephalosporins	Cefalexin	100	*125*	100	*150*	*125*
	Cefapirin	60	3	2.5	3	2.5
	Cefoperazone	50	20	15	20	15
	Cefazolin	50	4	3	4	3
	Cefquinome	20	20	20	20	20
	Ceftiofur	100	10	10	15	10
	Cefalonium	20	6	5	6	5
Macrolides	Erythromycin	40	*80*	40	*80*	40
	Tylosin	50	40	20	40	25
Sulfonamides	Sulfadiazine	100	*200*	60	*200*	60
	Sulfadimethoxin	100	*200*	50	*200*	50
	Sulfamethazine	100	*400*	100	*>400*	*150*
	Sulfathiazol	100	60	40	*>100*	40
	Sulfadoxin	100	*400*	*150*	*400*	*200*
	Sulfamethoxypyridazine	100	100	40	*>200*	60
Tetracyclines	Chlortetracycline	100	*300*	*150*	*400*	*200*
	Oxytetracycline	100	100	75	*150*	75
	Tetracycline	100	*150*	75	*200*	100
Aminoglyco-sides	Dihydrostreptomycin	200	*300*	*150*	*300*	*150*
	Streptomycin	200	*500*	*250*	*500*	*300*
	Gentamicin	100	40	10	40	30
	Neomycin	1,500	150	60	150	60
Fenicol	Chloramphenicol	-	*4,000*	*2,000*	*4,000*	*2,500*

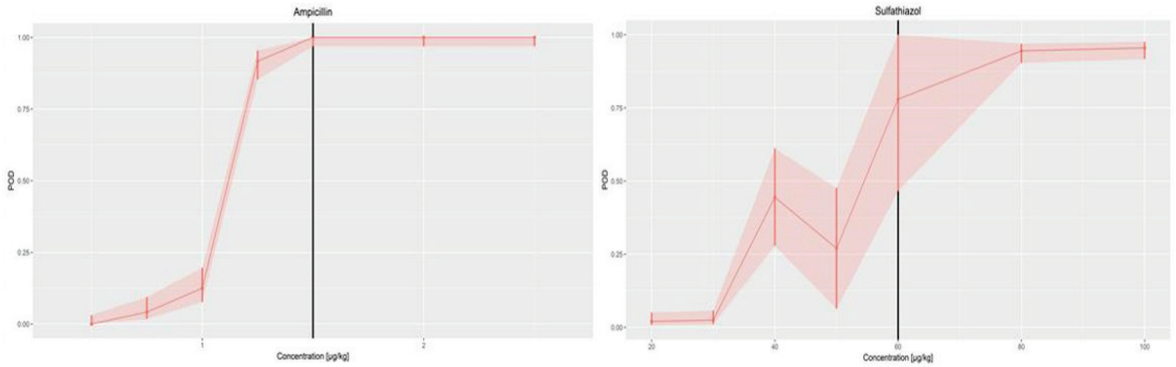
* Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin

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 Sulfathiazol (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 1.25 µg/kg for photometric reading, 43 results equalling 90 % of the results were clearly positive (absorbance level ≥ 65 %). The remaining 5 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. Similarly, for visual reading at 1.25 µg/kg, 93 % of the results were clearly positive (class A), and with 5 presumptive results (Class B), this added up to 100 % of the samples being CCβ B.

At 1.5 µg/kg, both 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 res (pos. class B)	Share of CCβ B	No. of results (negative)
Photo	0.5	48	0	0.00	-	-	0	0.00	48
	0.75	48	2	0.04	-	-	6	0.17	40
	1	48	7	0.15	-	-	23	0.63	18
	1.25	48	43	0.90	-	-	5	1.00	0
	1.5	48	48	1.00	-	-	0	1.00	0
	2	48	48	1.00	-	-	0	1.00	0
	2.5	48	48	1.00	-	-	0	1.00	0
Visual	0.5	72	0	0.00	-	-	0	0.00	72
	0.75	72	3	0.04	-	-	11	0.19	58
	1	72	8	0.11	-	-	50	0.81	14
	1.25	72	67	0.93	-	-	5	1.00	0
	1.5	72	72	1.00	-	-	0	1.00	0
	2	72	72	1.00	-	-	0	1.00	0
	2.5	72	72	1.00	-	-	0	1.00	0
Photo + visual	0.5	120	0	0.00	0.00	0.03	0	-	120
	0.75	120	5	0.04	0.02	0.09	17	-	98
	1	120	15	0.13	0.08	0.20	73	-	32
	1.25	120	110	0.92	0.85	0.95	10	-	0
	1.5	120	120	1.00	0.97	1.00	0	-	0
	2	120	120	1.00	0.97	1.00	0	-	0
	2.5	120	120	1.00	0.97	1.00	0	-	0

In **Table 4**, showing results of the dose-response study for Sulfathiazol, it can be seen that even at the highest concentration tested (100 µg/kg), the ratio of results detected as clearly positive (absorbance level ≥ 65 %; Class A results) was 99 % for photometric reading and 93 % for visual reading.

The sum of the presumptive and clear positive results (positive Class A and positive Class B) adds up to 100 % CCβ B at 40 µg/kg for both photometric and visual reading.

Table 4. Dose-response for Sulfathiazol.

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 res (pos. class B)	Share of CCβ B	No. of results (negative)
Photo	20	80	3	0.04	-	-	26	0.36	51
	30	80	3	0.04	-	-	71	0.93	6
	40	80	42	0.53	-	-	38	1.00	0
	50	80	34	0.43	-	-	46	1.00	0
	60	80	76	0.95	-	-	4	1.00	0
	80	80	80	1.00	-	-	0	1.00	0
	100	80	79	0.99	-	-	1	1.00	0
Visual	20	120	1	0.01	-	-	20	0.18	99
	30	120	2	0.02	-	-	39	0.34	79
	40	120	47	0.39	-	-	73	1.00	0
	50	120	20	0.17	-	-	100	1.00	0
	60	120	80	0.67	-	-	40	1.00	0
	80	120	109	0.91	-	-	11	1.00	0
	100	120	112	0.93	-	-	8	1.00	0
Photo + visual	20	200	4	0.02	0.01	0.05	46	-	150
	30	200	5	0.03	0.01	0.06	110	-	85
	40	200	89	0.45	0.28	0.61	111	-	0
	50	200	54	0.27	0.06	0.48	146	-	0
	60	200	156	0.78	0.47	1.00	44	-	0
	80	200	189	0.95	0.90	0.97	11	-	0
	100	200	191	0.96	0.92	0.98	9	-	0

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

In order to demonstrate that the BRT hi-sense 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 hi-sense. 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, 161 test plates were analysed, including 644 positive control samples and 3,220 samples of negative raw milk, in total giving 3,220 (positive control) and 16,100 (negative milk) readings, respectively, with combined photometric evaluation and visual reading.

The analysis of negative milk samples resulted in 13 false-positive results out of 16,100 results obtained by photometric and visual evaluation, resulting in a false-positive rate of 0.1 %. No false-negative results were obtained when analysing the results of the positive samples.

Table 6. Rates of false-negative and false-positive results of the 3,220 positive controls and 16,100 negative milk samples.

	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 control milk	0	0	0	0	0

For photometric readings, the maximum relative percentage value obtained for negative samples was 39 %, whereas the minimum relative percentage value for positive samples was 90 %. These values demonstrate that with the chosen thresholds for photometric reading (65 % Class A; 40 % Class B, **Table 1**) the false interpretation of positive samples can be avoided, whereas false interpretation of negative samples may occur rarely.

INTERLABORATORY STUDY

The BRT hi-sense was validated in an international proficiency test. 61 laboratories belonging to 55 companies originating from 10 countries, participated in the examination of BRT hi-sense 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 5**) giving a total of 915 results.

Table 7 The antibiotics analysed by 61 laboratories and the percentage of correct and false results.

Antibiotic	**MRL (µg/kg)**	**No. samples**	**BRT hi-sense**	
			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	99.8	0.2

The false-positive results (0.2 %) were reported by a single participant. No false-negative results were reported.

CONCLUSION

The BRT hi-sense (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 hi-sense.

Validation Report
BRT hi-sense

1) Introduction

The BRT hi-sense (AiM – Analytik in Milch GmbH, www.aimbavaria.com) is a highly sensitive 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 hi-sense (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 as well as other supplements and additives, making the test more sensitive than the original BRT. 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, the BRT hi-sense is distinguished by an exceptionally high detection capability for inhibitors. Substances of most relevant classes are detected below MRL level and beta-lactams in particular – for which the test germ possesses a naturally high sensitivity – are detected at very low levels.
The use of antibiotics in the field of farming is more and more within the focus of the public as consumers ask for healthy and residue-free food. Basis for the production of such dairy products is high-quality ex-farm milk, free from residues. The BRT hi-sense was developed in response to the growing demand for tests capable of the detection of anti-infectives at very sensitive levels. It is produced according to Commission Decision 91/180/EEC and § 64 LFGB (German Food, Feed and Consumer Goods Code, Methods L 01.00-11 and L 01.01-5).

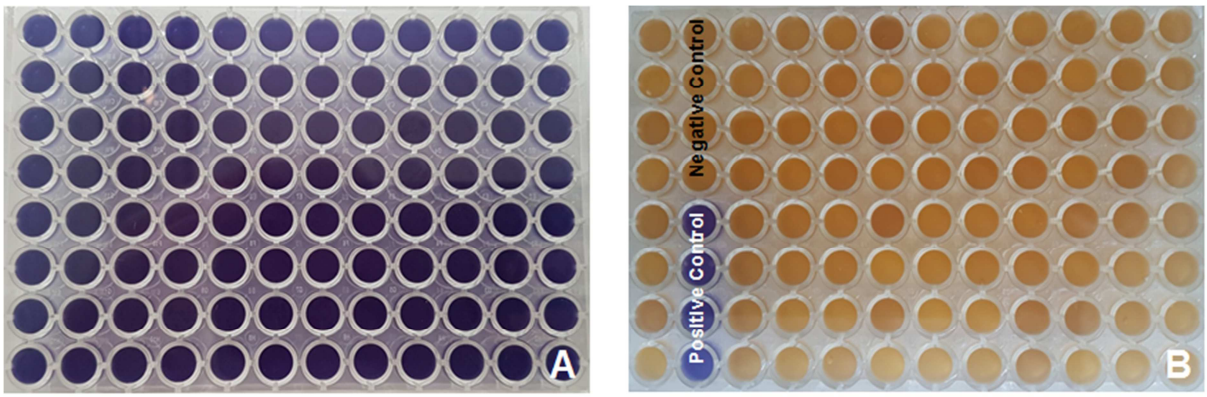


Figure 1. BRT hi-sense test plates before (A) and after (B) incubation

Within the framework of this validation, the BRT hi-sense 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 (3 hrs 30 ± 30 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%

64,400 data results were obtained from the evaluation of 161 BRT hi-sense 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 hi-sense plate batches provided for the validation

Batch	Range of Batch Numbers
A	70701203 - 70705645
B	70901061 - 70905373
C	71203201 - 71208367
D	70401004 - 70402315
E	71201207 - 71203904
F	71601311 - 71603483
G	73001003 - 73003175

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 hi-sense.

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 °C 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 hi-sense.

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

The high sensitivity of the BRT hi-sense in general and in particular for beta-lactams is reflected in the detection limits obtained for the different groups of antibiotics. All of the 14 beta-lactams were detected at or below MRL with both methods of interpretation (CCß A and CCß B) except for Cefalexin, for which only CCß B was identified below MRL, whereas CCß A was - with 125 µg/kg - slightly above MRL (100 µg/kg). The detection limits for some beta-lactams were determined far below MRL. Benzylpenicillin, e. g., obtained a CCß A of 1 µg/kg, which is only ¼ of the MRL (4 µg/kg) and Cefapirin (CCß A 3 µg/kg) was found positive even 20 x below MRL (60 µg/kg).

At least one substance each of all other classes of antibiotics forming part of this validation was classified at or below MRL with both CCß A and CCß B. All macrolides, sulfonamides and tetracylines conformed with the regulatory limit with CCß B with the exception of Sulfadoxin and Chlortetracycline.

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 2,000 µg/kg (CCß B) or 4,000 µg/kg (CCß A). The detection limits for the BRT hi-sense established with the reference method (photometric evaluation) are reported in Table 6, detection limits established with visual reading are reported in Annex Table 1.

In conclusion, antibiotics of all classes analyzed in this validation, comprising the most important compounds used in Germany for the treatment of dairy cows, are detected below EU MRL – especially beta-lactams far below MRL in some cases. Therefore, the BRT hi-sense complies with the requirement of detecting anti-infectives at very low levels and satisfies the public demand.

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	1	0.6
	Ampicillin	4	1.5	1.25
	Amoxicillin	4	1.5	1.25
	Cloxacillin	30	10	9
	Dicloxacillin	30	6	5
	Nafcillin	30	4	4
	Oxacillin	30	4	3
Cephalosporins	Cefalexin	100	125	100
	Cefapirin	60	3	2.5
	Cefoperazone	50	20	15
	Cefazolin	50	4	3
	Cefquinome	20	20	20
	Ceftiofur	100	10	10
	Cefalonium	20	6	5
Macrolides	Erythromycin	40	80	40
	Tylosin	50	40	20
Sulfonamides	Sulfadiazine	100	200	60
	Sulfadimethoxin	100	200	50
	Sulfamethazine	100	400	100
	Sulfathiazol	100	60	40
	Sulfadoxin	100	400	150
	Sulfamethoxypyridazine	100	100	40
Tetracyclines	Chlortetracycline	100	300	150
	Oxytetracycline	100	100	75
	Tetracycline	100	150	75
Aminoglycosides	Dihydrostreptomycin	200	300	150
	Streptomycin	200	500	250
	Gentamicin	100	40	10
	Neomycin	1,500	150	60
Fenicol	Chloramphenicol	-	4,000	2,000

5) Dose-Response Curves

Materials and Methods

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).

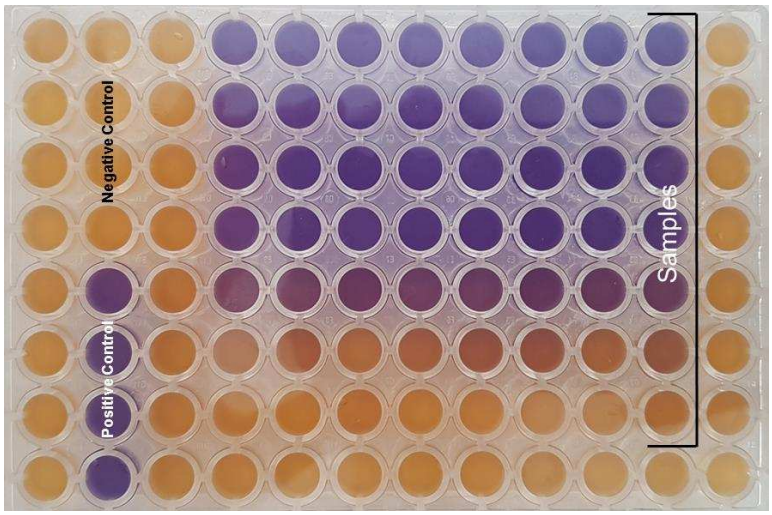


Figure 2. Incubated BRT hi-sense plate inoculated with four positive and four negative controls as well as raw milk samples spiked with 7 different concentrations of an antibiotic substance

Results and Discussion

Figure 3 and Annex Figure 1 depict dose-response curves of all substances included in the validation of the BRT hi-sense. 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 Amoxicillin, e. g., the positive response rate was 2% at 1 µg/kg, 67% at 1.25 µg/kg already and 99% of samples were detected positive at 1.5 µg/kg. Substances like the sulfonamides and macrolides displayed more consistent curve increments, probably due to their bacteriostatic character. Tylosin obtained 6% positive results at 15 µg/kg and ca. 22% respectively 42% at 20 µg/kg and 25 µg/kg. At 30 µg/kg the positive response was 74%, CCß A was established at 40 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.

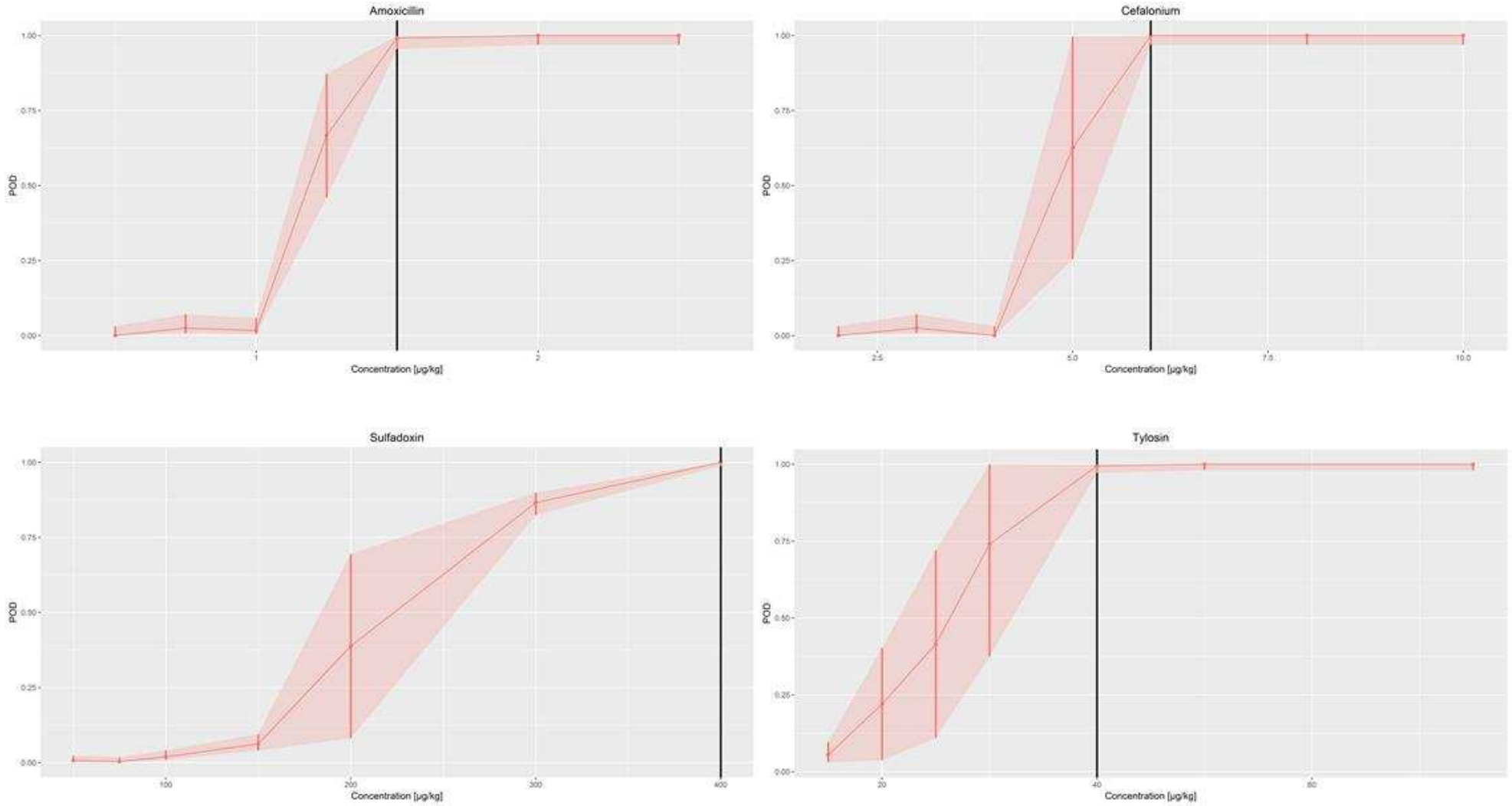


Figure 3. Dose-response curves of the bactericidal antibiotics Amoxicillin and Cefalonium and of the bacteriostatic substances Sulfadoxin and Tylosin.
Red line = dose-response curve; red shade = CI; Black line = CCß A (photometric reading);

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 hi-sense. 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).

Results and Discussion

Highly concentrated samples of Flunixin, Metamizole, Prednisolone, Triclabendazole, Deltamethrin and Monensin did not appear positive when interpreted in comparison with the positive control (class A, Table 7). Furthermore, no positive class B (comparison with negative control) results were obtained for Metamizol and Triclabendazol. However, false-positive class B results were observed with Deltamethrin (1 out of 6 replicates), Flunixin (2/6 replicates) and Prednisolone (3/6 replicates). When analyzing milk samples spiked with Monensin, all 6 replicates generated positive class B responses.

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 and the positive class B results in the selectivity study, false-positive results after the treatment of a cow with Monensin are unlikely due to its pharmacokinetic properties. The same is valid for Deltamethrin, Flunixin and Prednisolone as none of these compounds are excreted via the milk. Nevertheless, accidental direct pollution of bulk milk with these drugs should be avoided.

Principally, the observed results signify a high specificity of the BRT hi-sense for the detection of antibiotics opposed to other classes of veterinary drugs on interpretation according to class A. Positive results obtained with class B should always be confirmed with a second measurement in order to exclude non-antibacterial substances.

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	2/6
	NSAID	Metamizol	50	5,000	0/6	0/6
	Glucocorticoid	Prednisolon	6	600	0/6	3/6
Antiparasitics	Antihelminthic	Triclabendazol	10	1,000	0/6	0/6
	Ectoparasite	Deltamethrin	20	2,000	0/6	1/6
Ketosis Treatment	Polyether-Antibiotic	Monensin	2	200	0/6	6/6

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 hi-sense 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 Sulfathiazol, Sulfamethoxypyridazine and Streptomycin, realized p-value = 1 - for Sulfathiazol, Sulfamethoxypyridazine and Streptomycin 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	1	1	1	**Sulfonamides**	Sulfadiazine	100	200	1	1
	Ampicillin	4	1.5	1	1		Sulfadimethoxin	100	200	1	1
	Amoxicillin	4	1.5	1	1		Sulfamethazine	100	400	1	1
	Cloxacillin	30	10	1	1		Sulfathiazol	100	60	0.06	1
	Dicloxacillin	30	6	1	1		Sulfadoxin	100	400	1	1
	Nafcillin	30	4	1	1		Sulfamethoxypyridazine	100	100	0.06	0.25
	Oxacillin	30	4	1	1	**Tetracyclines**	Chlortetracycline	100	300	1	1
Cephalosporines	Cefalexin	100	125	1	1		Oxytetracycline	100	100	1	1
	Cefapirin	60	3	1	1		Tetracycline	100	150	1	1
	Cefoperazone	50	20	1	1	**Aminoglycosides**	Dihydrostreptomycin	200	300	1	1
	Cefazolin	50	4	1	1		Streptomycin	200	500	0.11	0.11
	Cefquinome	20	20	1	1		Gentamicin	100	40	1	1
	Ceftiofur	100	10	1	1		Neomycin	1,500	150	1	1
	Cefalonium	20	6	1	1	**Fenicols**	Chloramphenicol	-	4,000	1	1
Macrolides	Erythromycin	40	80	1	1						
	Tylosin	50	40	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 hi-sense 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, 161 test plates were analyzed, including 644 positive control samples and 3,220 samples of negative raw milk in total. Thus, 3,220 (positive control) respectively 16,100 (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	161	644	1,288	1,932	3,220
Negative Milk		3,220	6,440	9,660	16,100

Results and Discussion

When analyzing the results of the negative milk samples, 13 false-positive results out of 16,100 results were observed by photometric and visual evaluation, generating a false-positive rate of 0.1%. These false-positive results were probably caused by carry-over of spiked samples inoculated in adjacent cavities.

No false-negative results were obtained when analyzing the results of the positive samples, indicating a false-negative rate of 0%.

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	0.1	0.1	0.1	0.1	0

99.9% of the results obtained with negative milk generated photometric values <40%, only 0.1% of the negative milk results were false-positive with >40%. The maximum relative percentage value (Section 2) obtained with photometric reading for valid negative measurements was 39%. The minimum relative percentage value for positive samples was 90% (Table 11). These numbers 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 mostly be avoided.

Table 11. Minimum and maximum photometric percentage values of valid results (99.9%) obtained with two photometric instruments

Type of Milk Sample	No. Results ELISA Readers	Photometric Values Min/Max [%]
Positive Control	1,288	90
Negative Milk	6,440	39

In conclusion, the relative absence of false-negative and false-positive results imply that the validity of positive as well as negative results obtained for raw milk samples by analysis with the BRT hi-sense 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 hi-sense 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 MRL Screening Test). 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 hi-sense. 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 hi-sense 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 hi-sense 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 hi-sense 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 hi-sense, too.
The reported results of the interlaboratory study of the BRT hi-sense 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, 915 results were reported for the BRT hi-sense by the interlaboratory study participants, 99.8% of these results were correct, with the only exception of 5 inhibitor-free samples, which were evaluated false-positive by one participant, probably due to a too short incubation time. No false-negative results were observed (Figure 4).
This high rate of correct results obtained in different laboratories signifies once more that the BRT hi-sense is suitable for routine analyses as all positive and nearly all negative samples were identified properly and all examined substances were detected at MRL level.

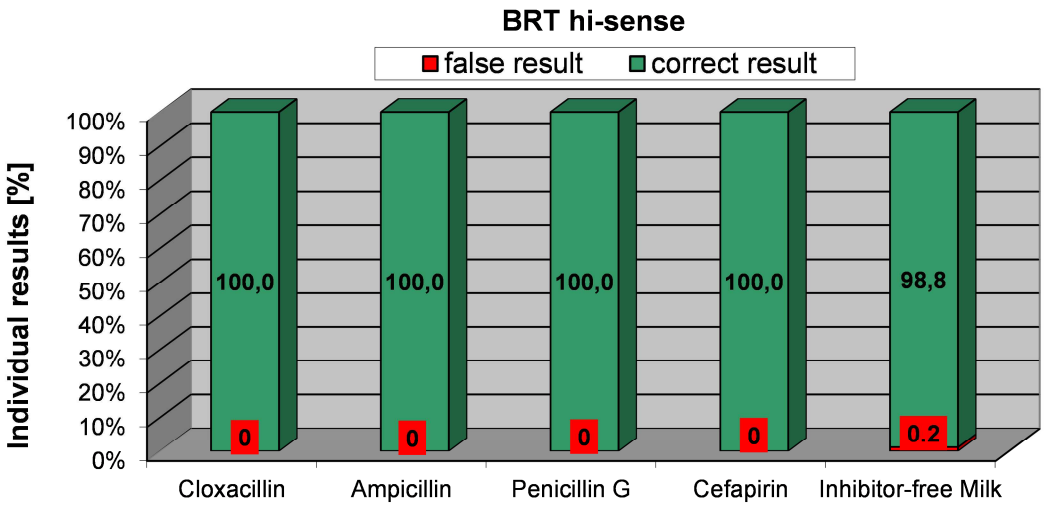


Figure 4. Rate of correct and false results (%) obtained by the interlaboratory study within the framework of the validation of the BRT hi-sense

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 hi-sense. 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%).

Compared with other tests evaluated in the context of the 10th proficiency test, the BRT hi-sense demonstrated an excellent performance, as an extremely low number of false results were observed within the interlaboratory study.

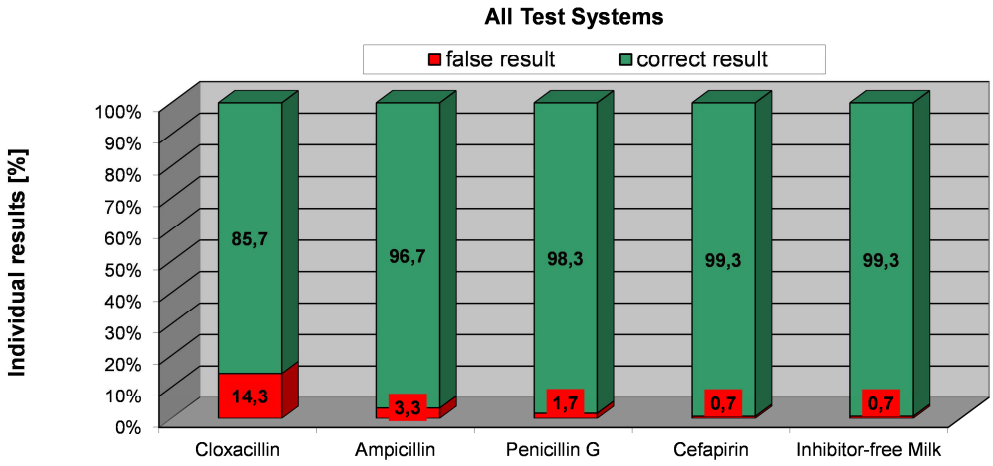


Figure 5. Rates of correct and false results (%) of all test systems (microbiological and receptor tests, 10th proficiency test)

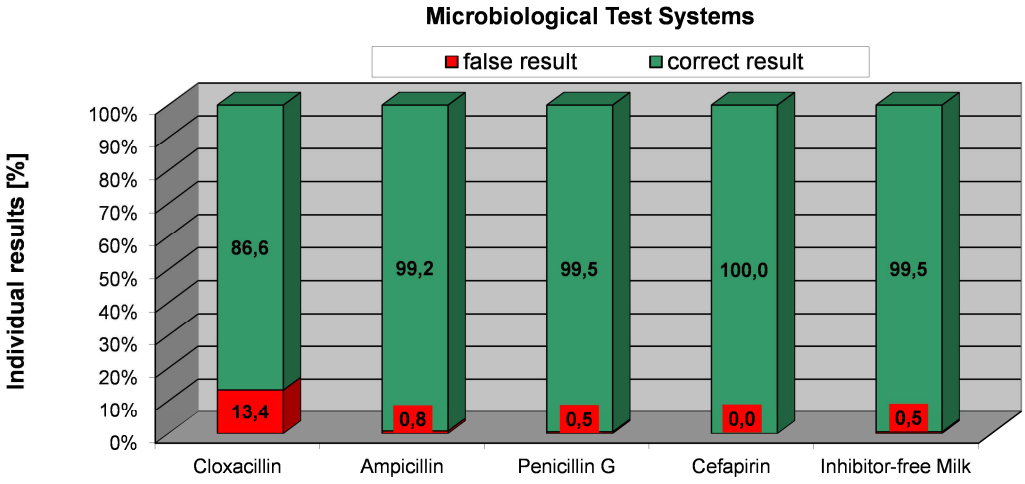


Figure 6. Rates of correct and false results (%) of microbiological test systems (10th proficiency test)

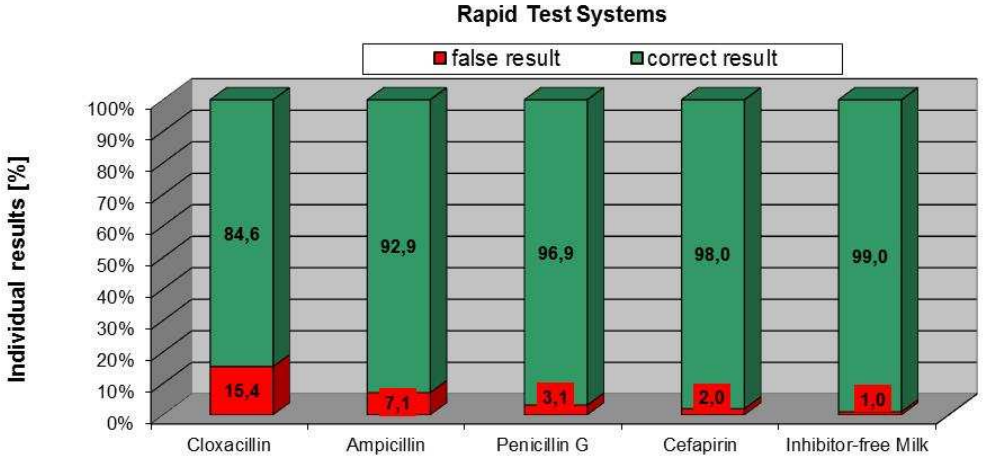


Figure 7. Rates of correct and false results (%) of receptor test systems (10th proficiency test)

11) Conclusions

The BRT hi-sense is capable of the sensitive detection of all beta-lactams and of at least one substance each of all other classes of antibiotics forming part of this validation at or below MRL with both CCß A and CCß B. In total, 25 out of 30 antibiotic compounds investigated in this study were detected 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. The BRT hi-sense displays a high selectivity for antibiotic residues, marker substances of other veterinary classes were not detected at high concentrations when interpreted in comparison with the positive control (class A). On comparison with the negative control false-positive results can occur, even though unlikely as result of a cow´s treatment. Positive results should be confirmed with a second measurement. 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 extremely low false-positive and false-negative rates 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 hi-sense in an international interlaboratory study (99.8% correct results) and in comparison with other inhibitor tests used by laboratories participating in an international proficiency test, which was organized in parallel, the BRT hi-sense proves to be fit for routine laboratory use.

12) References

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German Federal Office of Consumer Protection and Food Safety
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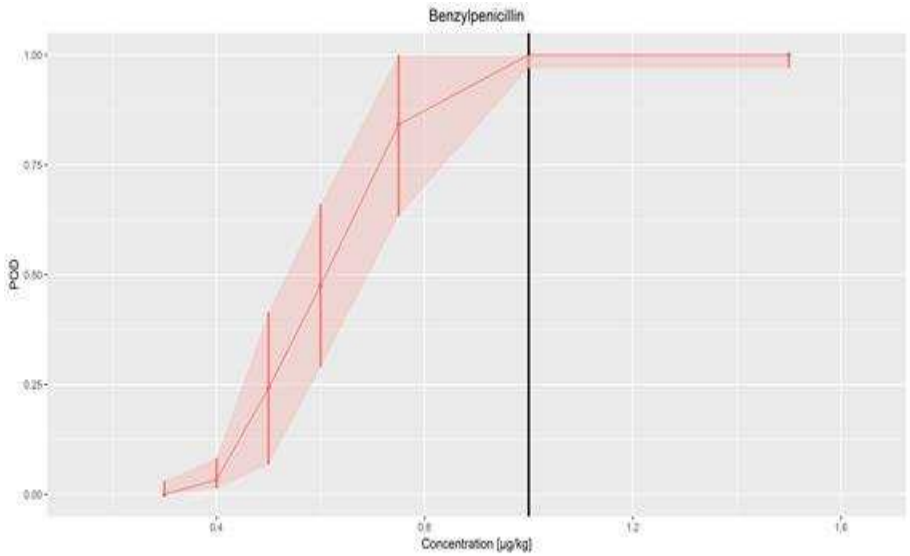
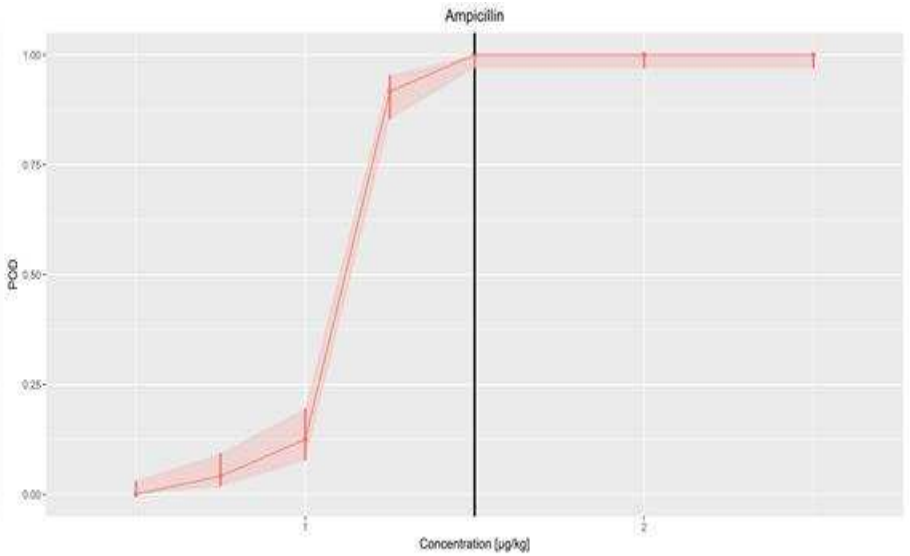
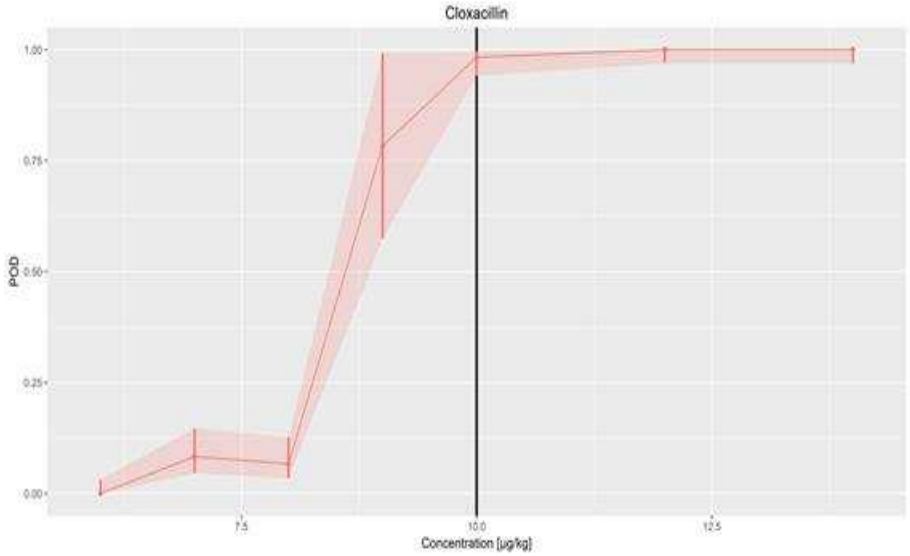
ANNEX

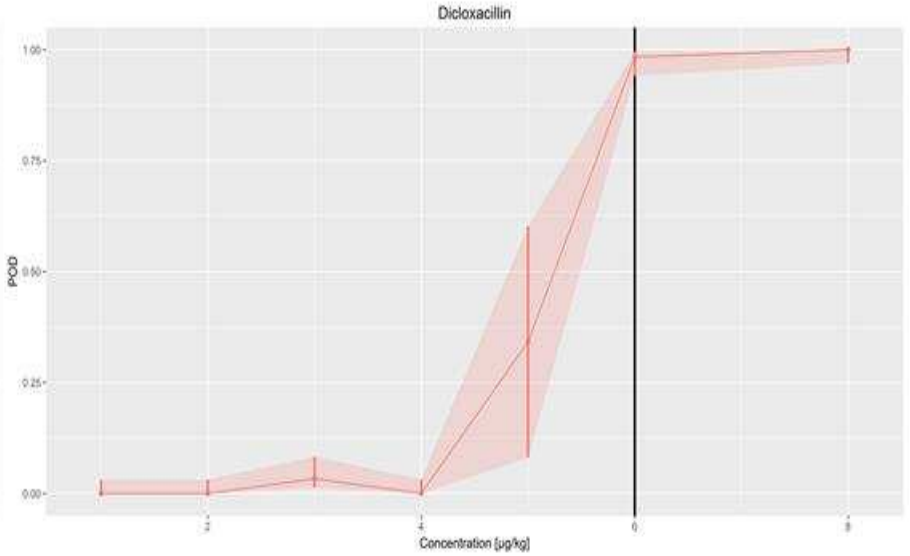
Annex Table1. Established detection limits (visual reading) compared with the EU MRL levels. Marked in red are the substances exceeding the EU MRLs.

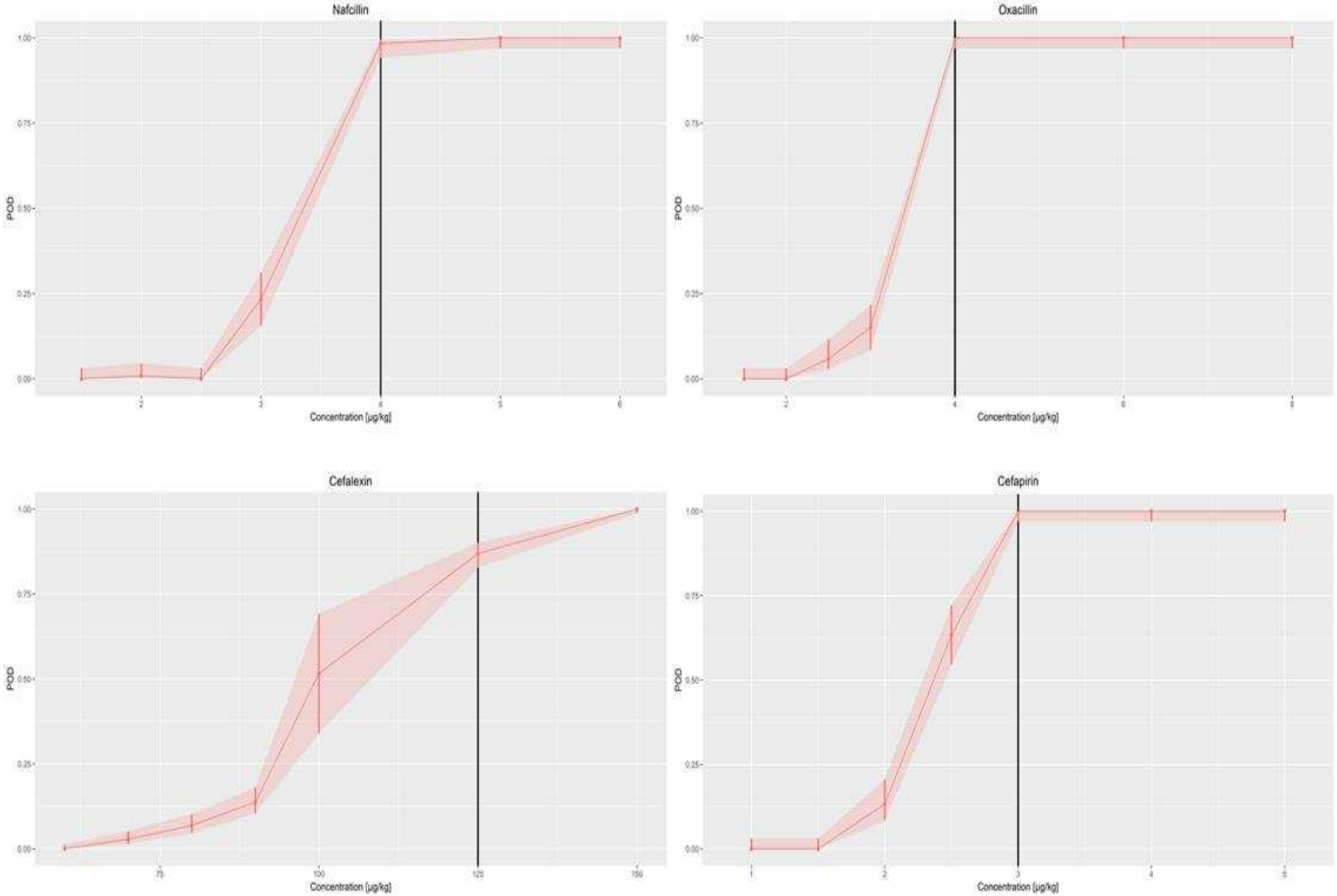
Group of Antibiotics	Substance	MRL EU [µg/kg]	CCß A [µg/kg]	CCß B [µg/kg]
Penicillins	Benzylpenicillin	4	1	0.75
	Ampicillin	4	1.5	1.25
	Amoxicillin	4	1.5	1.25
	Cloxacillin	30	10	9
	Dicloxacillin	30	6	5
	Nafcillin	30	4	3
	Oxacillin	30	4	4
Cephalosporins	Cefalexin	100	150	125
	Cefapirin	60	3	2.5
	Cefoperazone	50	20	15
	Cefazolin	50	4	3
	Cefquinome	20	20	20
	Ceftiofur	100	15	10
	Cefalonium	20	6	5
Macrolides	Erythromycin	40	80	40
	Tylosin	50	40	25
Sulfonamides	Sulfadiazine	100	200	60
	Sulfadimethoxin	100	200	50
	Sulfamethazine	100	>400	150
	Sulfathiazol	100	>100	40
	Sulfadoxin	100	400	200
	Sulfamethoxypyridazine	100	>200	60
Tetracyclines	Chlortetracycline	100	400	200
	Oxytetracycline	100	150	75
	Tetracycline	100	200	100
Aminoglycosides	Dihydrostreptomycin	200	300	150
	Streptomycin	200	500	300
	Gentamicin	100	40	30
	Neomycin	1,500	150	60
Fenicol	Chloramphenicol	-	4,000	2,500

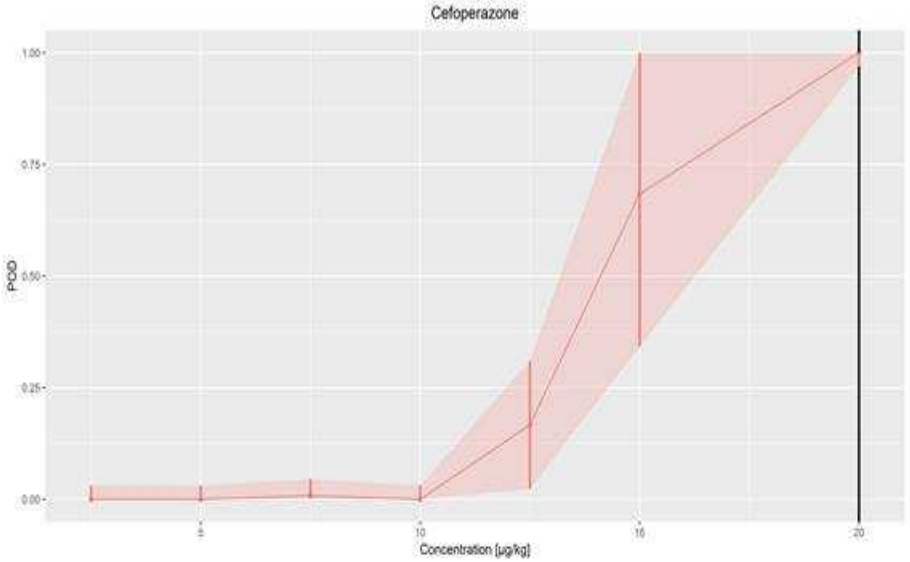

Benzylpenicillin
POD
Concentration [µg/kg]

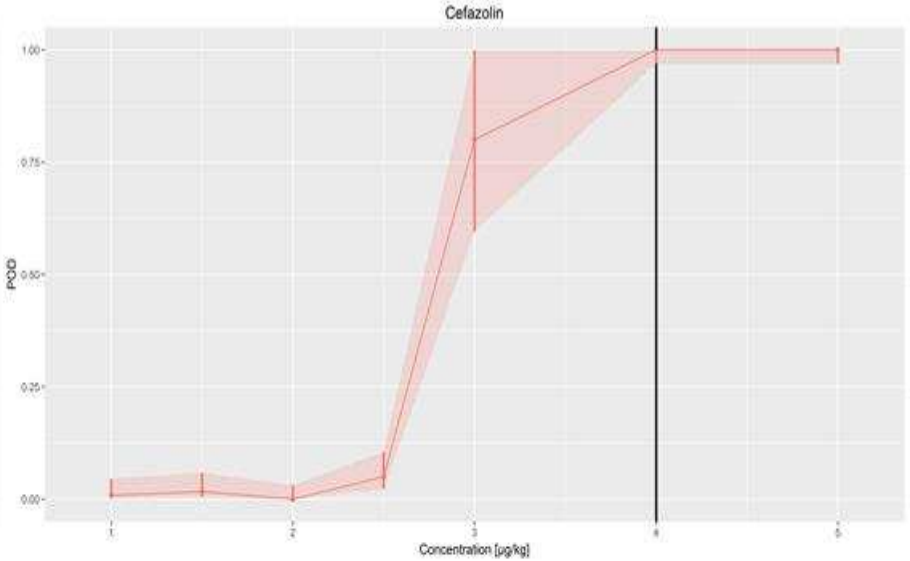

Ampicillin
POD
Concentration [µg/kg]

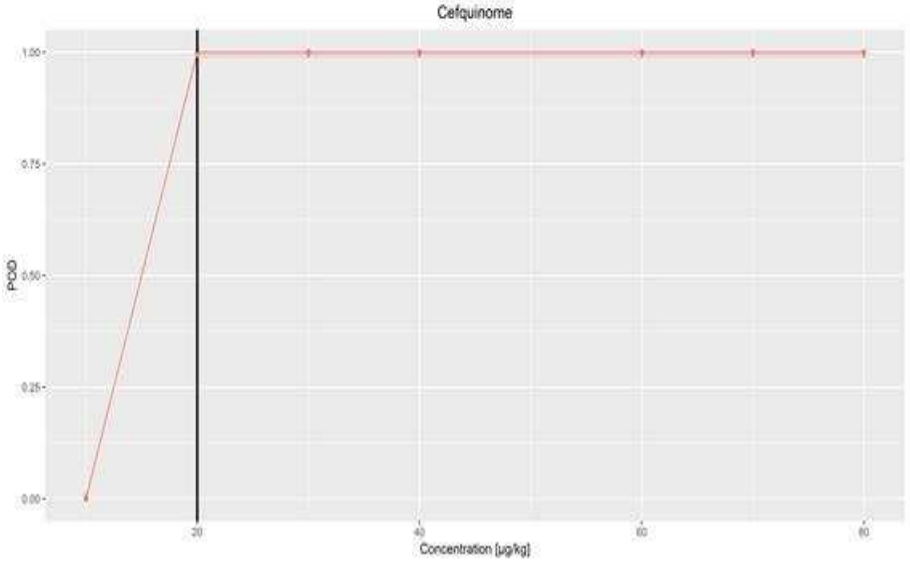

Cloxacillin
POD
Concentration [µg/kg]

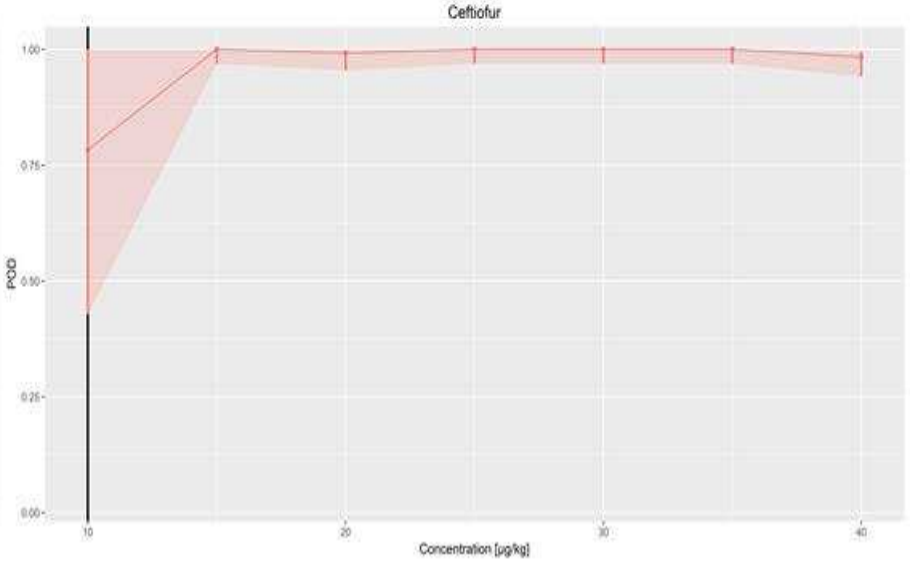

Dicloxacillin
POD
Concentration [µg/kg]

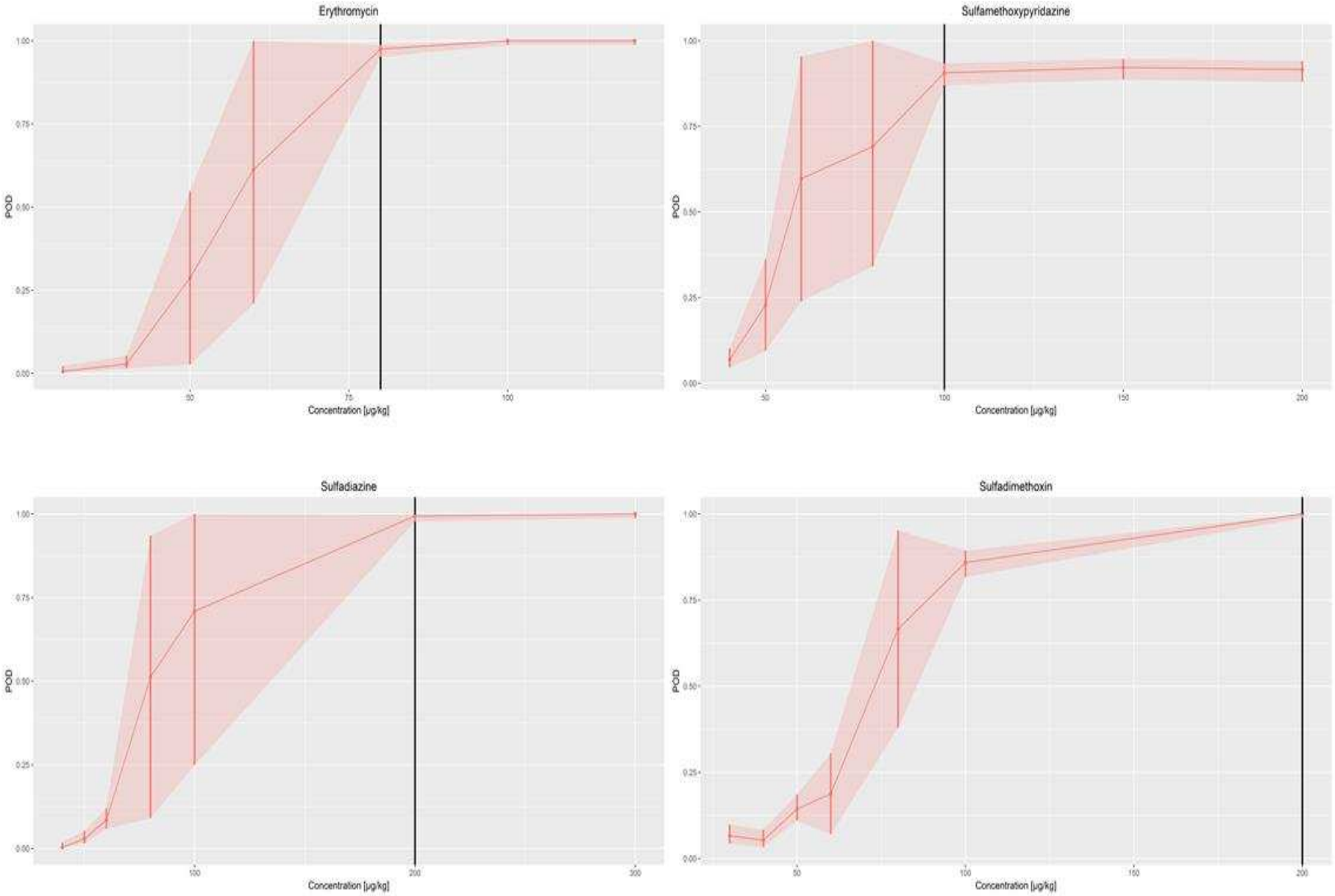

Nafcillin
Oxacillin
Cefalexin
Cefapirin
POD
Concentration [µg/kg]

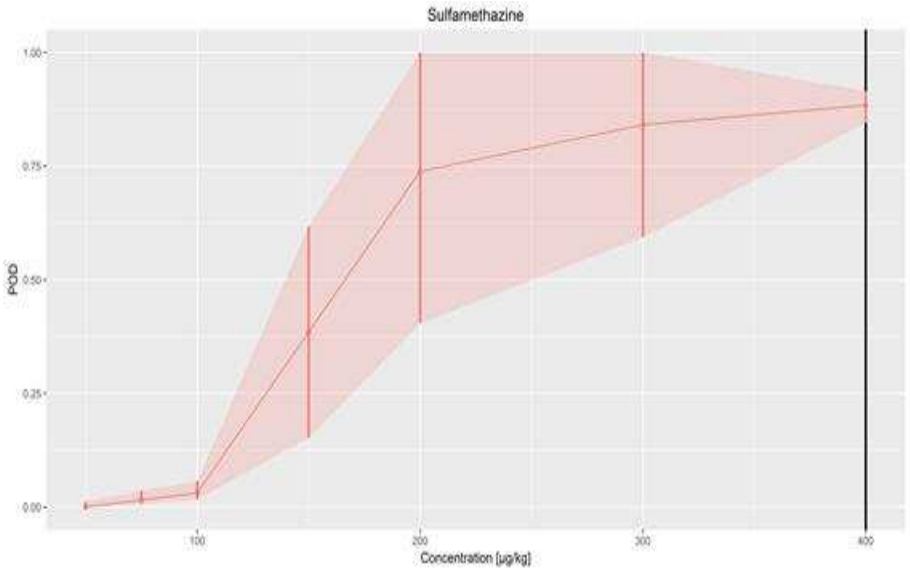

Cefoperazone
POD
Concentration [µg/kg]

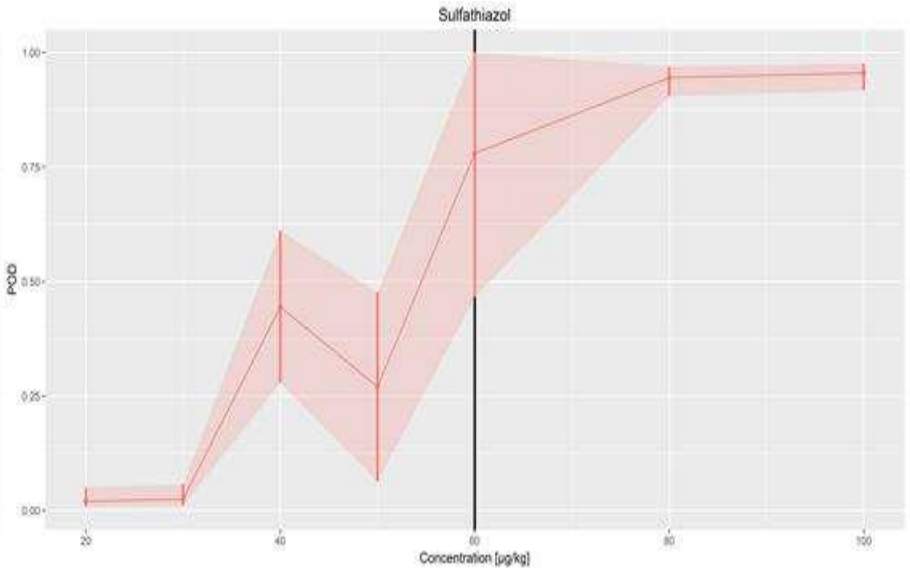

Cefazolin
POD
Concentration [µg/kg]

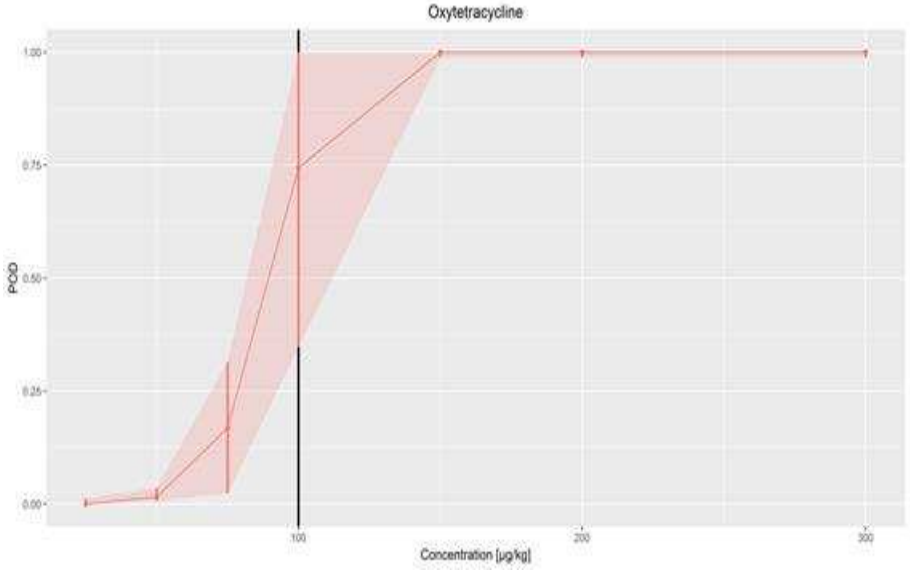

Cefquinome
POD
Concentration [µg/kg]

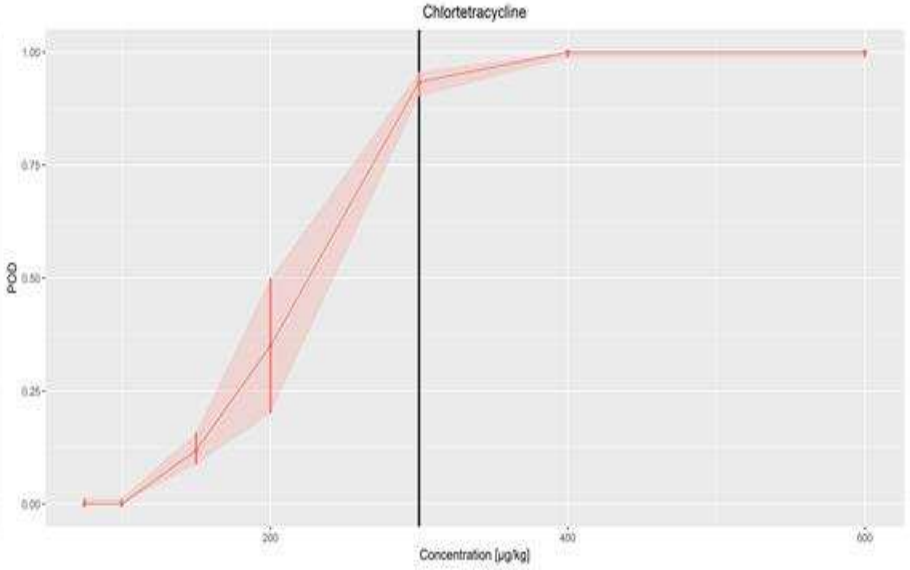

Ceftiofur
POD
Concentration [µg/kg]

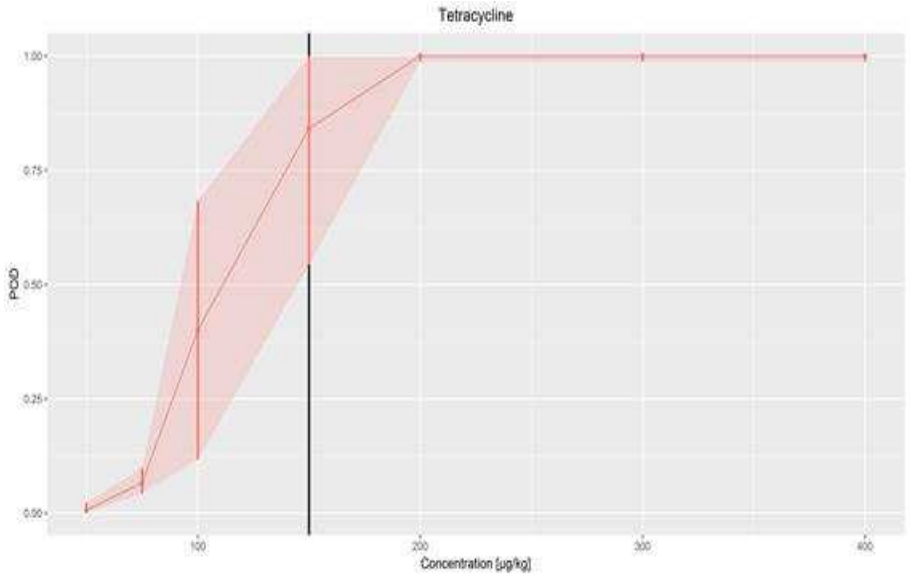

Erythromycin
Sulfamethoxypyridazine
Sulfadiazine
Sulfadimethoxin
POD
Concentration [µg/kg]

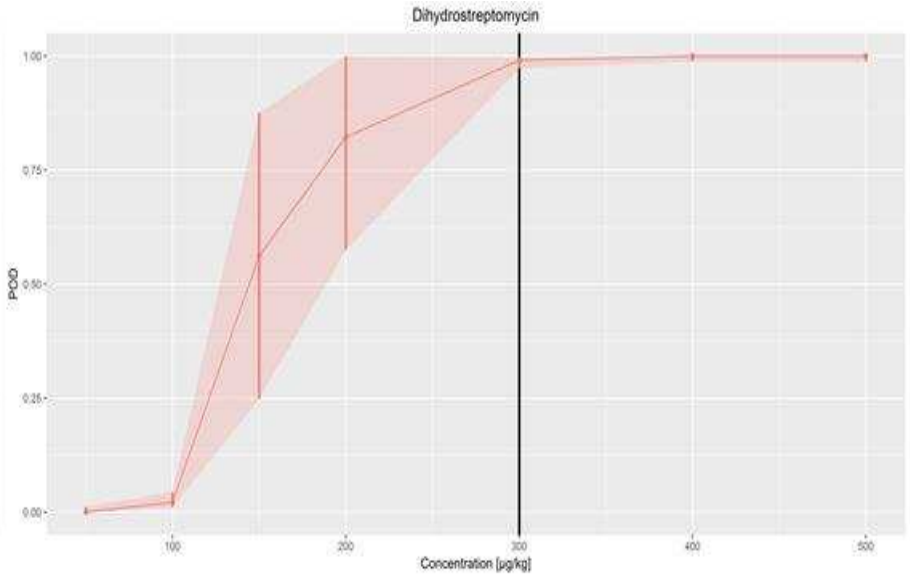

Sulfamethazine
POD
1.00
0.75
0.50
0.25
0.00
100
200
300
400
Concentration [µg/kg]

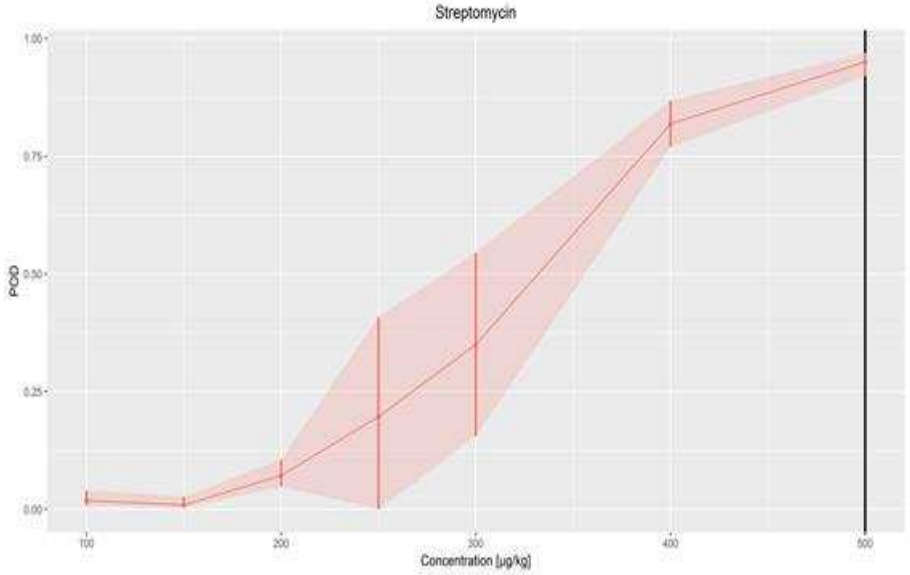

Sulfathiazol
POD
1.00
0.75
0.50
0.25
0.00
20
40
60
80
100
Concentration [µg/kg]

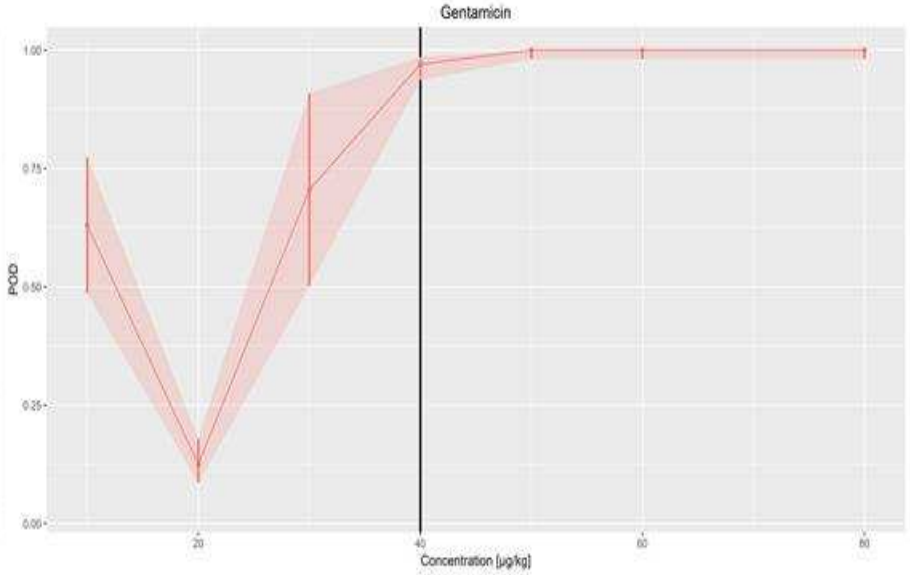

Oxytetracycline
POD
1.00
0.75
0.50
0.25
0.00
100
200
300
Concentration [µg/kg]

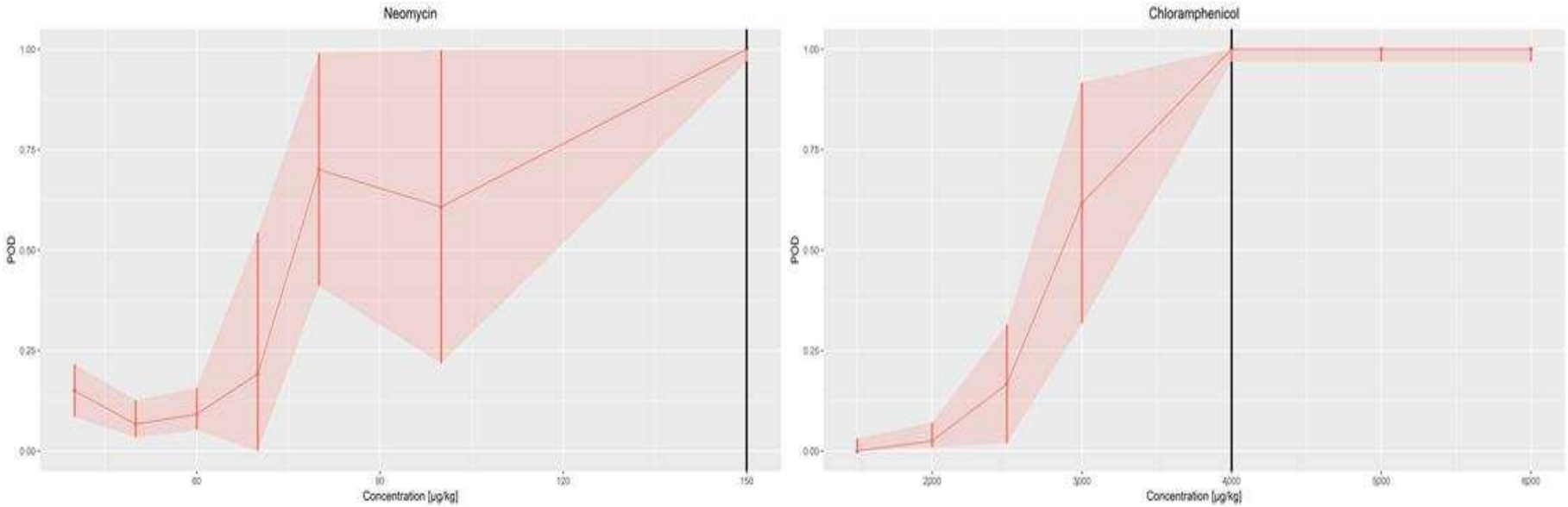

Chlortetracycline
POD
1.00
0.75
0.50
0.25
0.00
200
400
600
Concentration [µg/kg]


Tetracycline
POD
Concentration [µg/kg]


Dihydrostreptomycin
POD
Concentration [µg/kg]


Streptomycin
POD
Concentration [µg/kg]


Gentamicin
POD
Concentration [µg/kg]



Annex Figure 1. Dose-response curves of antibiotic substances included in the validation of the BRT hi-sense
Red line = dose-response curve; red shade = CI; Black line = CCß A (photometric reading);

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	0.3	48	0	0.00	-	-	2	0.04	46
		0.4	48	2	0.04	-	-	30	0.67	16
		0.5	48	18	0.38	-	-	14	0.67	16
		0.6	48	28	0.58	-	-	19	0.98	1
		0.75	48	45	0.94	-	-	3	1.00	0
		1	48	48	1.00	-	-	0	1.00	0
		1.5	48	48	1.00	-	-	0	1.00	0
	Visual	0.3	72	0	0.00	-	-	2	0.03	70
		0.4	72	2	0.03	-	-	31	0.46	39
		0.5	72	11	0.15	-	-	36	0.65	25
		0.6	72	29	0.40	-	-	39	0.94	4
		0.75	72	56	0.78	-	-	16	1.00	0
		1	72	72	1.00	-	-	0	1.00	0
		1.5	72	72	1.00	-	-	0	1.00	0
	Photometric + Visual	0.3	120	0	0.00	0.00	0.03	4	-	116
		0.4	120	4	0.03	0.01	0.08	61	-	55
		0.5	120	29	0.24	0.07	0.42	50	-	41
		0.6	120	57	0.48	0.29	0.66	58	-	5
		0.75	120	101	0.84	0.63	1.00	19	-	0
		1	120	120	1.00	0.97	1.00	0	-	0
		1.5	120	120	1.00	0.97	1.00	0	-	0
Ampicillin	Photometric	0.5	48	0	0.00	-	-	0	0.00	48
		0.75	48	2	0.04	-	-	6	0.17	40
		1	48	7	0.15	-	-	23	0.63	18
		1.25	48	43	0.90	-	-	5	1.00	0
		1.5	48	48	1.00	-	-	0	1.00	0
		2	48	48	1.00	-	-	0	1.00	0
		2.5	48	48	1.00	-	-	0	1.00	0
	Visual	0.5	72	0	0.00	-	-	0	0.00	72
		0.75	72	3	0.04	-	-	11	0.19	58
		1	72	8	0.11	-	-	50	0.81	14
		1.25	72	67	0.93	-	-	5	1.00	0
		1.5	72	72	1.00	-	-	0	1.00	0
		2	72	72	1.00	-	-	0	1.00	0
		2.5	72	72	1.00	-	-	0	1.00	0
	Photometric + Visual	0.5	120	0	0.00	0.00	0.03	0	-	120
		0.75	120	5	0.04	0.02	0.09	17	-	98
		1	120	15	0.13	0.08	0.20	73	-	32
		1.25	120	110	0.92	0.85	0.95	10	-	0
		1.5	120	120	1.00	0.97	1.00	0	-	0
		2	120	120	1.00	0.97	1.00	0	-	0
		2.5	120	120	1.00	0.97	1.00	0	-	0
Amoxicillin	Photometric	0.5	48	0	0.00	-	-	0	0.00	48
		0.75	48	2	0.04	-	-	8	0.21	38
		1	48	0	0.00	-	-	31	0.65	17
		1.25	48	32	0.67	-	-	16	1.00	0
		1.5	48	48	1.00	-	-	0	1.00	0
		2	48	48	1.00	-	-	0	1.00	0
		2.5	48	48	1.00	-	-	0	1.00	0
	Visual	0.5	72	0	0.00	-	-	0	0.00	72
		0.75	72	1	0.01	-	-	8	0.13	63
		1	72	2	0.03	-	-	37	0.54	33
		1.25	72	48	0.67	-	-	24	1.00	0
		1.5	72	71	0.99	-	-	1	1.00	0
		2	72	72	1.00	-	-	0	1.00	0
		2.5	72	72	1.00	-	-	0	1.00	0
	Photometric + Visual	0.5	120	0	0.00	0.00	0.03	0	-	120
		0.75	120	3	0.03	0.01	0.07	16	-	101
		1	120	2	0.02	0.00	0.06	68	-	50
		1.25	120	80	0.67	0.46	0.87	40	-	0
		1.5	120	119	0.99	0.95	1.00	1	-	0
		2	120	120	1.00	0.97	1.00	0	-	0
		2.5	120	120	1.00	0.97	1.00	0	-	0
Cloxacillin	Photometric	6	48	0	0.00	-	-	15	0.31	33
		7	48	4	0.08	-	-	12	0.33	32
		8	48	0	0.00	-	-	32	0.67	16
		9	48	42	0.88	-	-	6	1.00	0
		10	48	48	1.00	-	-	0	1.00	0
		12	48	48	1.00	-	-	0	1.00	0
		14	48	48	1.00	-	-	0	1.00	0
	Visual	6	72	0	0.00	-	-	5	0.07	67
		7	72	6	0.08	-	-	13	0.26	53
		8	72	8	0.11	-	-	33	0.57	31
		9	72	52	0.72	-	-	20	1.00	0
		10	72	70	0.97	-	-	2	1.00	0
		12	72	72	1.00	-	-	0	1.00	0
		14	72	72	1.00	-	-	0	1.00	0
	Photometric + Visual	6	120	0	0.00	0.00	0.03	20	-	100
		7	120	10	0.08	0.05	0.15	25	-	85
		8	120	8	0.07	0.03	0.13	65	-	47
		9	120	94	0.78	0.57	0.99	26	-	0
		10	120	118	0.98	0.94	1.00	2	-	0
		12	120	120	1.00	0.97	1.00	0	-	0
		14	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)
		1	48	0	0.00	-	-	1	0.02	47
		2	48	0	0.00	-	-	0	0.00	48
		3	48	2	0.04	-	-	0	0.04	46
	Photometric	4	48	0	0.00	-	-	8	0.17	40
		5	48	28	0.58	-	-	20	1.00	0
		6	48	48	1.00	-	-	0	1.00	0
		8	48	48	1.00	-	-	0	1.00	0
		1	72	0	0.00	-	-	0	0.00	72
		2	72	0	0.00	-	-	0	0.00	72
		3	72	2	0.03	-	-	0	0.03	70
Dicloxacillin	Visual	4	72	0	0.00	-	-	3	0.04	69
		5	72	13	0.18	-	-	58	0.99	1
		6	72	70	0.97	-	-	2	1.00	0
		8	72	72	1.00	-	-	0	1.00	0
		1	120	0	0.00	0.00	0.03	1	-	119
		2	120	0	0.00	0.00	0.03	0	-	120
		3	120	4	0.03	0.01	0.08	0	-	116
	Photometric + Visual	4	120	0	0.00	0.00	0.03	11	-	109
		5	120	41	0.34	0.08	0.60	78	-	1
		6	120	118	0.98	0.94	1.00	2	-	0
		8	120	120	1.00	0.97	1.00	0	-	0
		1.5	48	0	0.00	-	-	11	0.23	37
		2	48	0	0.00	-	-	22	0.46	26
		2.5	48	0	0.00	-	-	26	0.54	22
	Photometric	3	48	10	0.21	-	-	26	0.75	12
		4	48	48	1.00	-	-	0	1.00	0
		5	48	48	1.00	-	-	0	1.00	0
		6	48	48	1.00	-	-	0	1.00	0
		1.5	72	0	0.00	-	-	11	0.15	61
		2	72	1	0.01	-	-	27	0.39	44
		2.5	72	0	0.00	-	-	43	0.60	29
Nafcillin	Visual	3	72	18	0.25	-	-	52	0.97	2
		4	72	70	0.97	-	-	2	1.00	0
		5	72	72	1.00	-	-	0	1.00	0
		6	72	72	1.00	-	-	0	1.00	0
		1.5	120	0	0.00	0.00	0.03	22	-	98
		2	120	1	0.01	0.00	0.05	49	-	70
		2.5	120	0	0.00	0.00	0.03	69	-	51
	Photometric + Visual	3	120	28	0.23	0.16	0.31	78	-	14
		4	120	118	0.98	0.94	1.00	2	-	0
		5	120	120	1.00	0.97	1.00	0	-	0
		6	120	120	1.00	0.97	1.00	0	-	0
		1.5	48	0	0.00	-	-	5	0.10	43
		2	48	0	0.00	-	-	2	0.04	46
		2.5	48	4	0.08	-	-	16	0.42	28
	Photometric	3	48	8	0.17	-	-	40	1.00	0
		4	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
		1.5	72	0	0.00	-	-	2	0.03	70
		2	72	0	0.00	-	-	2	0.03	70
		2.5	72	3	0.04	-	-	21	0.33	48
Oxacillin	Visual	3	72	10	0.14	-	-	56	0.92	6
		4	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
		1.5	120	0	0.00	0.00	0.03	7	-	113
		2	120	0	0.00	0.00	0.03	4	-	116
		2.5	120	7	0.06	0.03	0.12	37	-	76
	Photometric + Visual	3	120	18	0.15	0.08	0.22	96	-	6
		4	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
		60	128	0	0.00	-	-	43	0.34	85
		70	128	4	0.03	-	-	49	0.41	75
		80	128	18	0.14	-	-	77	0.74	33
	Photometric	90	128	22	0.17	-	-	92	0.89	14
		100	128	68	0.53	-	-	60	1.00	0
		125	128	128	1.00	-	-	0	1.00	0
		150	128	128	1.00	-	-	0	1.00	0
		60	192	0	0.00	-	-	10	0.05	182
		70	192	5	0.03	-	-	34	0.20	153
		80	192	4	0.02	-	-	91	0.49	97
Cefalexin	Visual	90	192	22	0.11	-	-	113	0.70	57
		100	192	97	0.51	-	-	78	0.91	17
		125	192	150	0.78	-	-	42	1.00	0
		150	192	192	1.00	-	-	0	1.00	0
		60	320	0	0.00	0.00	0.01	53	-	267
		70	320	9	0.03	0.01	0.05	83	-	228
		80	320	22	0.07	0.05	0.10	168	-	130
	Photometric + Visual	90	320	44	0.14	0.10	0.18	205	-	71
		100	320	165	0.52	0.34	0.69	138	-	17
		125	320	278	0.87	0.83	0.90	42	-	0
		150	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)
		1	48	0	0.00	-	-	2	0.04	46
		1.5	48	0	0.00	-	-	4	0.08	44
		2	48	8	0.17	-	-	20	0.58	20
	Photometric	2.5	48	30	0.63	-	-	18	1.00	0
		3	48	48	1.00	-	-	0	1.00	0
		4	48	48	1.00	-	-	0	1.00	0
		5	48	48	1.00	-	-	0	1.00	0
		1	72	0	0.00	-	-	3	0.04	69
		1.5	72	0	0.00	-	-	5	0.07	67
		2	72	8	0.11	-	-	42	0.69	22
Cefapirin	Visual	2.5	72	46	0.64	-	-	26	1.00	0
		3	72	72	1.00	-	-	0	1.00	0
		4	72	72	1.00	-	-	0	1.00	0
		5	72	72	1.00	-	-	0	1.00	0
		1	120	0	0.00	0.00	0.03	5	-	115
		1.5	120	0	0.00	0.00	0.03	9	-	111
		2	120	16	0.13	0.08	0.21	62	-	42
	Photometric + Visual	2.5	120	76	0.63	0.55	0.72	44	-	0
		3	120	120	1.00	0.97	1.00	0	-	0
		4	120	120	1.00	0.97	1.00	0	-	0
		5	120	120	1.00	0.97	1.00	0	-	0
		2.5	48	0	0.00	-	-	0	0.00	48
		5	48	0	0.00	-	-	0	0.00	48
		7.5	48	1	0.02	-	-	1	0.04	46
	Photometric	10	48	0	0.00	-	-	5	0.10	43
		12.5	48	9	0.19	-	-	30	0.81	9
		15	48	40	0.83	-	-	8	1.00	0
		20	48	48	1.00	-	-	0	1.00	0
		2.5	72	0	0.00	-	-	1	0.01	71
		5	72	0	0.00	-	-	0	0.00	72
		7.5	72	0	0.00	-	-	2	0.03	70
Cefoperazone	Visual	10	72	0	0.00	-	-	6	0.08	66
		12.5	72	11	0.15	-	-	40	0.71	21
		15	72	42	0.58	-	-	30	1.00	0
		20	72	72	1.00	-	-	0	1.00	0
		2.5	120	0	0.00	0.00	0.03	1	-	119
		5	120	0	0.00	0.00	0.03	0	-	120
		7.5	120	1	0.01	0.00	0.05	3	-	116
	Photometric + Visual	10	120	0	0.00	0.00	0.03	11	-	109
		12.5	120	20	0.17	0.02	0.31	70	-	30
		15	120	82	0.68	0.34	1.00	38	-	0
		20	120	120	1.00	0.97	1.00	0	-	0
		1	48	0	0.00	-	-	4	0.08	44
		1.5	48	0	0.00	-	-	3	0.06	45
		2	48	0	0.00	-	-	8	0.17	40
	Photometric	2.5	48	3	0.06	-	-	23	0.54	22
		3	48	43	0.90	-	-	5	1.00	0
		4	48	48	1.00	-	-	0	1.00	0
		5	48	48	1.00	-	-	0	1.00	0
		1	72	1	0.01	-	-	4	0.07	67
		1.5	72	2	0.03	-	-	3	0.07	67
		2	72	0	0.00	-	-	10	0.14	62
Cefazolin	Visual	2.5	72	3	0.04	-	-	33	0.50	36
		3	72	53	0.74	-	-	18	0.99	1
		4	72	72	1.00	-	-	0	1.00	0
		5	72	72	1.00	-	-	0	1.00	0
		1	120	1	0.01	0.00	0.05	8	-	111
		1.5	120	2	0.02	0.00	0.06	6	-	112
		2	120	0	0.00	0.00	0.03	18	-	102
	Photometric + Visual	2.5	120	6	0.05	0.02	0.11	56	-	58
		3	120	96	0.80	0.60	1.00	23	-	1
		4	120	120	1.00	0.97	1.00	0	-	0
		5	120	120	1.00	0.97	1.00	0	-	0
		10	128	0	0.00	-	-	18	0.14	110
		20	128	128	1.00	-	-	0	1.00	0
		30	128	128	1.00	-	-	0	1.00	0
	Photometric	40	128	128	1.00	-	-	0	1.00	0
		60	128	128	1.00	-	-	0	1.00	0
		70	128	128	1.00	-	-	0	1.00	0
		80	128	128	1.00	-	-	0	1.00	0
		10	192	0	0.00	-	-	6	0.03	186
		20	192	192	1.00	-	-	0	1.00	0
		30	192	192	1.00	-	-	0	1.00	0
Cefquinome	Visual	40	192	192	1.00	-	-	0	1.00	0
		60	192	192	1.00	-	-	0	1.00	0
		70	192	192	1.00	-	-	0	1.00	0
		80	192	192	1.00	-	-	0	1.00	0
		10	320	0	0.00	0.00	0.01	24	-	296
		20	320	320	1.00	0.99	1.00	0	-	0
		30	320	320	1.00	0.99	1.00	0	-	0
	Photometric + Visual	40	320	320	1.00	0.99	1.00	0	-	0
		60	320	320	1.00	0.99	1.00	0	-	0
		70	320	320	1.00	0.99	1.00	0	-	0
		80	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)
		10	48	48	1.00	-	-	0	1.00	0
		15	48	48	1.00	-	-	0	1.00	0
		20	48	48	1.00	-	-	0	1.00	0
	Photometric	25	48	48	1.00	-	-	0	1.00	0
		30	48	48	1.00	-	-	0	1.00	0
		35	48	48	1.00	-	-	0	1.00	0
		40	48	48	1.00	-	-	0	1.00	0
		10	72	46	0.64	-	-	26	1.00	0
		15	72	72	1.00	-	-	0	1.00	0
		20	72	71	0.99	-	-	1	1.00	0
Ceftiofur	Visual	25	72	72	1.00	-	-	0	1.00	0
		30	72	72	1.00	-	-	0	1.00	0
		35	72	72	1.00	-	-	0	1.00	0
		40	72	70	0.97	-	-	2	1.00	0
		10	120	94	0.78	0.43	1.00	26	-	0
		15	120	120	1.00	0.97	1.00	0	-	0
		20	120	119	0.99	0.95	1.00	1	-	0
	Photometric + Visual	25	120	120	1.00	0.97	1.00	0	-	0
		30	120	120	1.00	0.97	1.00	0	-	0
		35	120	120	1.00	0.97	1.00	0	-	0
		40	120	118	0.98	0.94	1.00	2	-	0
		2	48	0	0.00	-	-	2	0.04	46
		3	48	2	0.04	-	-	2	0.08	44
		4	48	0	0.00	-	-	21	0.44	27
	Photometric	5	48	38	0.79	-	-	10	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	1	0.01	-	-	3	0.06	68
		4	72	0	0.00	-	-	26	0.36	46
Cefalonium	Visual	5	72	37	0.51	-	-	35	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	2	-	118
		3	120	3	0.03	0.01	0.07	5	-	112
		4	120	0	0.00	0.00	0.03	47	-	73
	Photometric + Visual	5	120	75	0.63	0.25	1.00	45	-	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
		30	128	1	0.01	-	-	62	0.49	65
		40	128	5	0.04	-	-	121	0.98	2
		50	128	54	0.42	-	-	74	1.00	0
	Photometric	60	128	106	0.83	-	-	22	1.00	0
		80	128	128	1.00	-	-	0	1.00	0
		100	128	128	1.00	-	-	0	1.00	0
		120	128	128	1.00	-	-	0	1.00	0
		30	192	1	0.01	-	-	40	0.21	151
		40	192	4	0.02	-	-	181	0.96	7
		50	192	38	0.20	-	-	154	1.00	0
Erythromycin	Visual	60	192	90	0.47	-	-	102	1.00	0
		80	192	184	0.96	-	-	8	1.00	0
		100	192	192	1.00	-	-	0	1.00	0
		120	192	192	1.00	-	-	0	1.00	0
		30	320	2	0.01	0.00	0.02	102	-	216
		40	320	9	0.03	0.01	0.05	302	-	9
		50	320	92	0.29	0.03	0.55	228	-	0
	Photometric + Visual	60	320	196	0.61	0.21	1.00	124	-	0
		80	320	312	0.98	0.95	0.99	8	-	0
		100	320	320	1.00	0.99	1.00	0	-	0
		120	320	320	1.00	0.99	1.00	0	-	0
		15	80	6	0.08	-	-	51	0.71	23
		20	80	29	0.36	-	-	49	0.98	2
		25	80	46	0.58	-	-	34	1.00	0
	Photometric	30	80	74	0.93	-	-	6	1.00	0
		40	80	80	1.00	-	-	0	1.00	0
		50	80	80	1.00	-	-	0	1.00	0
		75	80	80	1.00	-	-	0	1.00	0
		15	120	5	0.04	-	-	52	0.48	63
		20	120	15	0.13	-	-	90	0.88	15
		25	120	37	0.31	-	-	83	1.00	0
Tylosin	Visual	30	120	74	0.62	-	-	46	1.00	0
		40	120	119	0.99	-	-	1	1.00	0
		50	120	120	1.00	-	-	0	1.00	0
		75	120	120	1.00	-	-	0	1.00	0
		15	200	11	0.06	0.03	0.10	103	-	86
		20	200	44	0.22	0.04	0.40	139	-	17
		25	200	83	0.42	0.11	0.72	117	-	0
	Photometric + Visual	30	200	148	0.74	0.37	1.00	52	-	0
		40	200	199	1.00	0.97	1.00	1	-	0
		50	200	200	1.00	0.98	1.00	0	-	0
		75	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)
		40	128	0	0.00	-	-	73	0.57	55
		50	128	2	0.02	-	-	106	0.84	20
		60	128	0	0.00	-	-	124	0.97	4
	Photometric	80	128	60	0.47	-	-	68	1.00	0
		100	128	100	0.78	-	-	28	1.00	0
		200	128	128	1.00	-	-	0	1.00	0
		300	128	128	1.00	-	-	0	1.00	0
		40	192	1	0.01	-	-	86	0.45	105
		50	192	7	0.04	-	-	172	0.93	10
		60	192	27	0.14	-	-	165	1.00	0
Sulfadiazine	Visual	80	192	104	0.54	-	-	88	1.00	0
		100	192	127	0.66	-	-	65	1.00	0
		200	192	190	0.99	-	-	0	0.99	0
		300	192	192	1.00	-	-	0	1.00	0
		40	320	1	0.00	0.00	0.02	159	-	160
		50	320	9	0.03	0.01	0.05	278	-	30
		60	320	27	0.08	0.06	0.12	289	-	4
	Photometric + Visual	80	320	164	0.51	0.09	0.94	156	-	0
		100	320	227	0.71	0.25	1.00	93	-	0
		200	320	318	0.99	0.98	1.00	0	-	0
		300	320	320	1.00	0.99	1.00	0	-	0
		30	128	8	0.06	-	-	82	0.70	38
		40	128	7	0.05	-	-	103	0.86	18
		50	128	22	0.17	-	-	104	0.98	2
	Photometric	60	128	34	0.27	-	-	94	1.00	0
		80	128	92	0.72	-	-	36	1.00	0
		100	128	113	0.88	-	-	15	1.00	0
		200	128	128	1.00	-	-	0	1.00	0
		30	192	13	0.07	-	-	67	0.42	112
		40	192	10	0.05	-	-	103	0.59	79
		50	192	24	0.13	-	-	161	0.96	7
Sulfadi-methoxin	Visual	60	192	26	0.14	-	-	166	1.00	0
		80	192	121	0.63	-	-	71	1.00	0
		100	192	162	0.84	-	-	30	1.00	0
		200	192	192	1.00	-	-	0	1.00	0
		30	320	21	0.07	0.04	0.10	149	-	150
		40	320	17	0.05	0.03	0.08	206	-	97
		50	320	46	0.14	0.11	0.19	265	-	9
	Photometric + Visual	60	320	60	0.19	0.07	0.31	260	-	0
		80	320	213	0.67	0.38	0.95	107	-	0
		100	320	275	0.86	0.82	0.89	45	-	0
		200	320	320	1.00	0.99	1.00	0	-	0
		50	128	0	0.00	-	-	64	0.50	64
		75	128	2	0.02	-	-	112	0.89	14
		100	128	8	0.06	-	-	120	1.00	0
	Photometric	150	128	56	0.44	-	-	72	1.00	0
		200	128	100	0.78	-	-	28	1.00	0
		300	128	120	0.94	-	-	8	1.00	0
		400	128	126	0.98	-	-	2	1.00	0
		50	192	0	0.00	-	-	27	0.14	165
		75	192	3	0.02	-	-	69	0.38	120
		100	192	2	0.01	-	-	137	0.72	53
Sulfametha-zine	Visual	150	192	67	0.35	-	-	118	0.96	7
		200	192	136	0.71	-	-	56	1.00	0
		300	192	149	0.78	-	-	43	1.00	0
		400	192	157	0.82	-	-	35	1.00	0
		50	320	0	0.00	0.00	0.01	91	-	229
		75	320	5	0.02	0.01	0.04	181	-	134
		100	320	10	0.03	0.02	0.06	257	-	53
	Photometric + Visual	150	320	123	0.38	0.15	0.62	190	-	7
		200	320	236	0.74	0.40	1.00	84	-	0
		300	320	269	0.84	0.59	1.00	51	-	0
		400	320	283	0.88	0.85	0.92	37	-	0
		20	80	3	0.04	-	-	26	0.36	51
		30	80	3	0.04	-	-	71	0.93	6
		40	80	42	0.53	-	-	38	1.00	0
	Photometric	50	80	34	0.43	-	-	46	1.00	0
		60	80	76	0.95	-	-	4	1.00	0
		80	80	80	1.00	-	-	0	1.00	0
		100	80	79	0.99	-	-	1	1.00	0
		20	120	1	0.01	-	-	20	0.18	99
		30	120	2	0.02	-	-	39	0.34	79
		40	120	47	0.39	-	-	73	1.00	0
Sulfathiazol	Visual	50	120	20	0.17	-	-	100	1.00	0
		60	120	80	0.67	-	-	40	1.00	0
		80	120	109	0.91	-	-	11	1.00	0
		100	120	112	0.93	-	-	8	1.00	0
		20	200	4	0.02	0.01	0.05	46	-	150
		30	200	5	0.03	0.01	0.06	110	-	85
		40	200	89	0.45	0.28	0.61	111	-	0
	Photometric + Visual	50	200	54	0.27	0.06	0.48	146	-	0
		60	200	156	0.78	0.47	1.00	44	-	0
		80	200	189	0.95	0.90	0.97	11	-	0
		100	200	191	0.96	0.92	0.98	9	-	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	-	-	22	0.17	106
		75	128	0	0.00	-	-	53	0.41	75
		100	128	2	0.02	-	-	105	0.84	21
	Photometric	150	128	2	0.02	-	-	124	0.98	2
		200	128	51	0.40	-	-	77	1.00	0
		300	128	108	0.84	-	-	20	1.00	0
		400	128	128	1.00	-	-	0	1.00	0
		50	192	2	0.01	-	-	8	0.05	182
		75	192	1	0.01	-	-	36	0.19	155
		100	192	4	0.02	-	-	93	0.51	95
Sulfadoxin	Visual	150	192	18	0.09	-	-	162	0.94	12
		200	192	73	0.38	-	-	119	1.00	0
		300	192	169	0.88	-	-	23	1.00	0
		400	192	192	1.00	-	-	0	1.00	0
		50	320	2	0.01	0.00	0.02	30	-	288
		75	320	1	0.00	0.00	0.02	89	-	230
		100	320	6	0.02	0.01	0.04	198	-	116
	Photometric + Visual	150	320	20	0.06	0.04	0.09	286	-	14
		200	320	124	0.39	0.08	0.69	196	-	0
		300	320	277	0.87	0.82	0.90	43	-	0
		400	320	320	1.00	0.99	1.00	0	-	0
		40	128	8	0.06	-	-	120	1.00	0
		50	128	36	0.28	-	-	92	1.00	0
		60	128	85	0.66	-	-	43	1.00	0
	Photometric	80	128	95	0.74	-	-	33	1.00	0
		100	128	125	0.98	-	-	3	1.00	0
		150	128	128	1.00	-	-	0	1.00	0
		200	128	128	1.00	-	-	0	1.00	0
		40	192	14	0.07	-	-	103	0.61	75
		50	192	37	0.19	-	-	118	0.81	37
		60	192	106	0.55	-	-	86	1.00	0
Sulfamethoxy-pyridazine	Visual	80	192	126	0.66	-	-	66	1.00	0
		100	192	165	0.86	-	-	27	1.00	0
		150	192	167	0.87	-	-	25	1.00	0
		200	192	165	0.86	-	-	27	1.00	0
		40	320	22	0.07	0.05	0.10	223	-	75
		50	320	73	0.23	0.09	0.36	210	-	37
		60	320	191	0.60	0.24	0.95	129	-	0
	Photometric + Visual	80	320	221	0.69	0.34	1.00	99	-	0
		100	320	290	0.91	0.87	0.93	30	-	0
		150	320	295	0.92	0.89	0.95	25	-	0
		200	320	293	0.92	0.88	0.94	27	-	0
		75	128	0	0.00	-	-	60	0.47	68
		100	128	0	0.00	-	-	65	0.51	63
		150	128	22	0.17	-	-	101	0.96	5
	Photometric	200	128	58	0.45	-	-	70	1.00	0
		300	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
		75	192	0	0.00	-	-	20	0.10	172
		100	192	0	0.00	-	-	44	0.23	148
		150	192	16	0.08	-	-	157	0.90	19
Chlortetra-cycline	Visual	200	192	54	0.28	-	-	138	1.00	0
		300	192	171	0.89	-	-	21	1.00	0
		400	192	192	1.00	-	-	0	1.00	0
		600	192	192	1.00	-	-	0	1.00	0
		75	320	0	0.00	0.00	0.01	80	-	240
		100	320	0	0.00	0.00	0.01	109	-	211
		150	320	38	0.12	0.09	0.16	258	-	24
	Photometric + Visual	200	320	112	0.35	0.20	0.50	208	-	0
		300	320	299	0.93	0.90	0.96	21	-	0
		400	320	320	1.00	0.99	1.00	0	-	0
		600	320	320	1.00	0.99	1.00	0	-	0
		25	128	0	0.00	-	-	10	0.08	118
		50	128	4	0.03	-	-	116	0.94	8
		75	128	33	0.26	-	-	95	1.00	0
	Photometric	100	128	126	0.98	-	-	2	1.00	0
		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
		25	192	0	0.00	-	-	0	0.00	192
		50	192	1	0.01	-	-	58	0.31	133
		75	192	21	0.11	-	-	161	0.95	10
Oxytetra-cycline	Visual	100	192	112	0.58	-	-	80	1.00	0
		150	192	192	1.00	-	-	0	1.00	0
		200	192	192	1.00	-	-	0	1.00	0
		300	192	192	1.00	-	-	0	1.00	0
		25	320	0	0.00	0.00	0.01	10	-	310
		50	320	5	0.02	0.01	0.04	174	-	141
		75	320	54	0.17	0.02	0.31	256	-	10
	Photometric + Visual	100	320	238	0.74	0.35	1.00	82	-	0
		150	320	320	1.00	0.99	1.00	0	-	0
		200	320	320	1.00	0.99	1.00	0	-	0
		300	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	-	-	58	0.45	70
		75	128	8	0.06	-	-	116	0.97	4
		100	128	72	0.56	-	-	56	1.00	0
	Photometric	150	128	126	0.98	-	-	2	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
		50	192	2	0.01	-	-	18	0.10	172
		75	192	13	0.07	-	-	125	0.72	54
		100	192	56	0.29	-	-	134	0.99	2
Tetracycline	Visual	150	192	143	0.74	-	-	49	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
		50	320	2	0.01	0.00	0.02	76	-	242
		75	320	21	0.07	0.04	0.10	241	-	58
		100	320	128	0.40	0.12	0.68	190	-	2
	Photometric + Visual	150	320	269	0.84	0.54	1.00	51	-	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
		50	128	0	0.00	-	-	15	0.12	113
		100	128	0	0.00	-	-	103	0.80	25
		150	128	79	0.62	-	-	48	0.99	1
	Photometric	200	128	115	0.90	-	-	13	1.00	0
		300	128	128	1.00	-	-	0	1.00	0
		400	128	128	1.00	-	-	0	1.00	0
		500	128	128	1.00	-	-	0	1.00	0
		50	192	0	0.00	-	-	2	0.01	190
		100	192	7	0.04	-	-	68	0.39	117
		150	192	101	0.53	-	-	88	0.98	3
Dihydro-streptomycin	Visual	200	192	148	0.77	-	-	44	1.00	0
		300	192	189	0.98	-	-	3	1.00	0
		400	192	192	1.00	-	-	0	1.00	0
		500	192	192	1.00	-	-	0	1.00	0
		50	320	0	0.00	0.00	0.01	17	-	303
		100	320	7	0.02	0.01	0.04	171	-	142
	Photometric + Visual	150	320	180	0.56	0.25	0.88	136	-	4
		200	320	263	0.82	0.58	1.00	57	-	0
		300	320	317	0.99	0.97	1.00	3	-	0
		400	320	320	1.00	0.99	1.00	0	-	0
		500	320	320	1.00	0.99	1.00	0	-	0
		100	128	3	0.02	-	-	21	0.19	104
		150	128	2	0.02	-	-	70	0.56	56
		200	128	8	0.06	-	-	91	0.77	29
	Photometric	250	128	28	0.22	-	-	95	0.96	5
		300	128	49	0.38	-	-	76	0.98	3
		400	128	101	0.79	-	-	27	1.00	0
		500	128	122	0.95	-	-	6	1.00	0
		100	192	3	0.02	-	-	11	0.07	178
		150	192	1	0.01	-	-	62	0.33	129
		200	192	15	0.08	-	-	118	0.69	59
Streptomycin	Visual	250	192	35	0.18	-	-	146	0.94	11
		300	192	63	0.33	-	-	121	0.96	8
		400	192	161	0.84	-	-	30	0.99	1
		500	192	182	0.95	-	-	10	1.00	0
		100	320	6	0.02	0.01	0.04	32	-	282
		150	320	3	0.01	0.00	0.03	132	-	185
		200	320	23	0.07	0.05	0.11	209	-	88
	Photometric + Visual	250	320	63	0.20	0.00	0.41	241	-	16
		300	320	112	0.35	0.16	0.55	197	-	11
		400	320	262	0.82	0.77	0.87	57	-	1
		500	320	304	0.95	0.92	0.97	16	-	0
		10	80	58	0.73	-	-	22	1.00	0
		20	80	14	0.18	-	-	62	0.95	4
		30	80	60	0.75	-	-	20	1.00	0
	Photometric	40	80	80	1.00	-	-	0	1.00	0
		50	80	80	1.00	-	-	0	1.00	0
		60	80	80	1.00	-	-	0	1.00	0
		80	80	80	1.00	-	-	0	1.00	0
		10	120	68	0.57	-	-	47	0.96	5
		20	120	11	0.09	-	-	73	0.70	36
		30	120	81	0.68	-	-	39	1.00	0
Gentamicin	Visual	40	120	114	0.95	-	-	6	1,00	0
		50	120	120	1.00	-	-	0	1.00	0
		60	120	120	1.00	-	-	0	1.00	0
		80	120	120	1.00	-	-	0	1.00	0
		10	200	126	0.63	0.49	0.77	69	-	5
		20	200	25	0.13	0.09	0.18	135	-	40
		30	200	141	0.71	0.50	0.91	59	-	0
	Photometric + Visual	40	200	194	0.97	0.94	0.99	6	-	0
		50	200	200	1.00	0.98	1.00	0	-	0
		60	200	200	1.00	0.98	1.00	0	-	0
		80	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)
Neomycin	Photometric	40	48	8	0.17	-	-	8	0.33	32
		50	48	4	0.08	-	-	37	0.85	7
		60	48	5	0.10	-	-	41	0.96	2
		70	48	5	0.10	-	-	43	1.00	0
		80	48	38	0.79	-	-	10	1.00	0
		100	48	34	0.71	-	-	14	1.00	0
		150	48	48	1.00	-	-	0	1.00	0
	Visual	40	72	10	0.14	-	-	17	0.38	45
		50	72	4	0.06	-	-	58	0.86	10
		60	72	6	0.08	-	-	63	0.96	3
		70	72	18	0.25	-	-	54	1.00	0
		80	72	46	0.64	-	-	26	1.00	0
		100	72	39	0.54	-	-	33	1.00	0
		150	72	72	1.00	-	-	0	1.00	0
	Photometric + Visual	40	120	18	0.15	0.08	0.22	25	-	77
		50	120	8	0.07	0.03	0.13	95	-	17
		60	120	11	0.09	0.05	0.16	104	-	5
		70	120	23	0.19	0.00	0.54	97	-	0
		80	120	84	0.70	0.41	0.99	36	-	0
		100	120	73	0.61	0.22	1.00	47	-	0
		150	120	120	1.00	0.97	1.00	0	-	0
Chlor-amphenicol	Photometric	1,500	48	0	0.00	-	-	41	0.85	7
		2,000	48	3	0.06	-	-	45	1.00	0
		2,500	48	15	0.31	-	-	33	1.00	0
		3,000	48	37	0.77	-	-	11	1.00	0
		4,000	48	48	1.00	-	-	0	1.00	0
		5,000	48	48	1.00	-	-	0	1.00	0
		6,000	48	48	1.00	-	-	0	1.00	0
	Visual	1,500	72	0	0.00	-	-	24	0.33	48
		2,000	72	0	0.00	-	-	67	0.93	5
		2,500	72	5	0.07	-	-	67	1.00	0
		3,000	72	37	0.51	-	-	35	1.00	0
		4,000	72	72	1.00	-	-	0	1.00	0
		5,000	72	72	1.00	-	-	0	1.00	0
		6,000	72	72	1.00	-	-	0	1.00	0
	Photometric + Visual	1,500	120	0	0.00	0.00	0.03	65	-	55
		2,000	120	3	0.03	0.01	0.07	112	-	5
		2,500	120	20	0.17	0.02	0.31	100	-	0
		3,000	120	74	0.62	0.32	0.92	46	-	0
		4,000	120	120	1.00	0.97	1.00	0	-	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

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	1	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	
Ampicillin	1.5	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	
Amoxicillin	1.5	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	
Cloxacillin	10	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	
Dicloxacillin	6	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	4	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	4	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	125	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	
Cefapirin	3	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	20	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	

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	4	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	20	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	
Ceftiofur	10	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	
Cefalonium	6	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	
Erytromycin	80	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	
Tylosin	40	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	
Sulfadiazine	200	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	
Sulfadi-methoxin	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	
Sulfametha-zine	400	1	D	16	0	0	
		1	E	24	0	0	1
		1	F	23	1	0	
		2	D	16	0	0	
		2	E	24	0	0	1
		2	F	23	1	0	
Sulfathiazol	60	1	A	16	0	0	
		1	B	12	4	0	0.06
		1	C	8	0	0	
		2	A	16	0	0	
		2	B	16	0	0	1
		2	C	8	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	400	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	
Sulfamethoxy-pyridazine	100	1	D	24	0	0	
		1	E	24	0	0	0.06
		1	F	14	2	0	
		2	D	24	0	0	
		2	E	24	0	0	0.25
		2	F	15	1	0	
Chlortetra-cycline	300	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	
Oxytetra-cycline	100	1	A	24	0	0	
		1	B	23	1	0	1
		1	C	16	0	0	
		2	A	24	0	0	
		2	B	23	1	0	1
		2	C	16	0	0	
Tetracycline	150	1	A	24	0	0	
		1	B	23	1	0	1
		1	C	16	0	0	
		2	A	24	0	0	
		2	B	23	1	0	1
		2	C	16	0	0	
Dihydro-streptomycin	300	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	
Streptomycin	500	1	A	16	0	0	
		1	B	21	3	0	0.11
		1	C	24	0	0	
		2	A	16	0	0	
		2	B	21	3	0	0.11
		2	C	24	0	0	
Gentamicin	40	1	D	8	0	0	
		1	E	16	0	0	1
		1	F	16	0	0	
		2	D	8	0	0	
		2	E	16	0	0	1
		2	F	16	0	0	
Neomycin	150	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	4,000	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	