Hemmstofftestsysteme der AiM GmbH unabhängig zertifiziert

Independent certification of AiM GmbH´s Inhibitor Test Systems

www.aim-bayern.de

Hemmstofftestsysteme der AiM GmbH unabhängig zertifiziert

Die AiM GmbH hat mit der unabhängigen Validierung ihrer Hemmstofftestsysteme und deren Zertifizierung durch NordVal International einen wichtigen Schritt zur internationalen Akzeptanz ihrer Produkte realisiert und gleichzeitig als erster Hersteller seine Tests nach der neuen Norm ISO/TS 16393 und der gerade initiierten Norm ISO/TS 23758 (IDF/RM 251) „Guidelines for the validation of qualitative screening tests for the detection of residues of veterinary drugs in milk and milk products" überprüfen lassen.

Neben der Ermittlung der Nachweisempfindlichkeiten wurden erstmals Standardkurven für alle untersuchten antibiotischen Substanzen bestimmt. Außerdem wurden die Selektivität, die Vergleichbarkeit der Chargen, die Performance mit Routineproben sowie die Falsch-positiv- und die Falsch-negativ-Raten und der Einsatz in einer internationalen Hemmstoff-Eignungsprüfung untersucht. Das umfassende Datenmaterial wurde mittels einer speziell für diese Validierung entwickelten Statistik-Methode ausgewertet und die Ergebnisse anschaulich für jedes Testsystem separat in einem ausführlichen Bericht dargestellt. Für kein anderes Testsystem zum Nachweis von Antibiotika in Milch liegen aktuell derart dichte und aussagekräftige Daten vor, die den Kunden in die Lage versetzen das optimale Testsystem auszuwählen und die Ergebnisse maximal abzusichern.

Ihr Team der AiM GmbH

Independent certification of AiM GmbH´s Inhibitor Test Systems

With the independent validation of the inhibitor test systems and its certification by NordVal International, AiM GmbH implemented an important milestone for the international acceptance of its products. Simultaneously, AiM GmbH is the first manufacturer to have commissioned an evaluation of its test systems according to the new norm ISO/TS 16393 and the just initialized norm ISO/TS 23758 (IDF/RM 251) „Guidelines for the validation of qualitative screening tests for the detection of residues of veterinary drugs in milk and milk products".

In addition to the assessment of the detection sensitivities, dose-response curves for all examined antibiotic substances were determined for the first time. Selectivity, batch-to-batch variability, performance with field samples as well as false-positive and false-negative rate and the performance in an international proficiency test for inhibitors were tested as well. The comprehensive data set obtained was evaluated statistically using a method developed specifically for this validation study and the results were depicted in detailed reports for each test system. At the moment, no other test system for the detection of antibiotic residues in milk is provided with such dense and significant data, which enable the customers to choose the optimal test system and assure the results at a maximum.

Your AiM GmbH Team

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Zertifikate
Certificates

Die vollständigen Zertifikate stehen ab sofort auf der Homepage der AiM GmbH (www.aim-bayern.de) oder direkt bei NordVal International zum Download bereit (https://www.nmkl.org/index.php/en/component/zoo/category/chemical-nordval-certificates?f=1&Itemid=618).

The entire certificates are available for download on the homepage of AiM GmbH (www.aim-bayern.de) as well as directly on the homepage of NordVal International (https://www.nmkl.org/index.php/en/component/zoo/category/chemical-nordval-certificates?f=1&Itemid=618).

Zertifikat – BRT Hemmstofftest

Certificate – BRT Inhibitor Test

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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 Inhibitor Test
NordVal No:	051
First approval date:	01 March 2019
Renewal date:	01 March 2021
Valid until:	01 March 2023

BRT Inhibitor 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 Inhibitor Test is a brilliant black reduction test for 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 Inhibitor Test is ISO 9001:2015-accredited by LGA InterCert GmbH.

Date: 01 February 2021

Yours sincerely,

Hilde Skår Norli
Chair of NordVal International

Eystein Oveland
NMKL Secretary General

Zertifikat – BRT MRL-Suchtest

Certificate – BRT MRL-Screening Test

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Validation Report – page 55

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 2021
Valid until:	01 March 2023

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 February 2021

Yours sincerely,

Hilde Skår Norli
Chair of NordVal International

Eystein Oveland
NMKL Secretary General

Zertifikat – BRT hi-sense

Certificate – BRT hi-sense

Validierungsbericht – Seite 93
Validation Report – page 93

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 hi-sense
NordVal No:	053
First approval date:	01 March 2019
Renewal date:	01 March 2021
Valid until:	01 March 2023

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 February 2021

Yours sincerely,

Hilde Skår Norli
Chair of NordVal International

Eystein Oveland
NMKL Secretary General

Validierungsberichte
Validation Reports

Validierungsbericht
Validation Report

BRT Hemmstofftest
BRT Inhibitor Test

1) Introduction

The BRT Inhibitor 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 Inhibitor Test (Figure 1) 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 color indicator brilliant black and the consecutive color change of the test medium from blue to yellow. The BRT Inhibitor Test detects a broad spectrum of antibiotics, but is especially sensitive to beta-lactams.
The test is designed in particular to satisfy the demands of milk quality regulation in Germany and is used for quality evaluation and payment for tank milk. It is recognized as official inspection method for the detection of inhibitors in tank milk and is part of the official register of inspection procedures according to § 64 LFGB (German Food, Feed and Consumer Goods Code, L 01.01-5). Furthermore, it is produced according to Commission Decision 91/180/EEC.

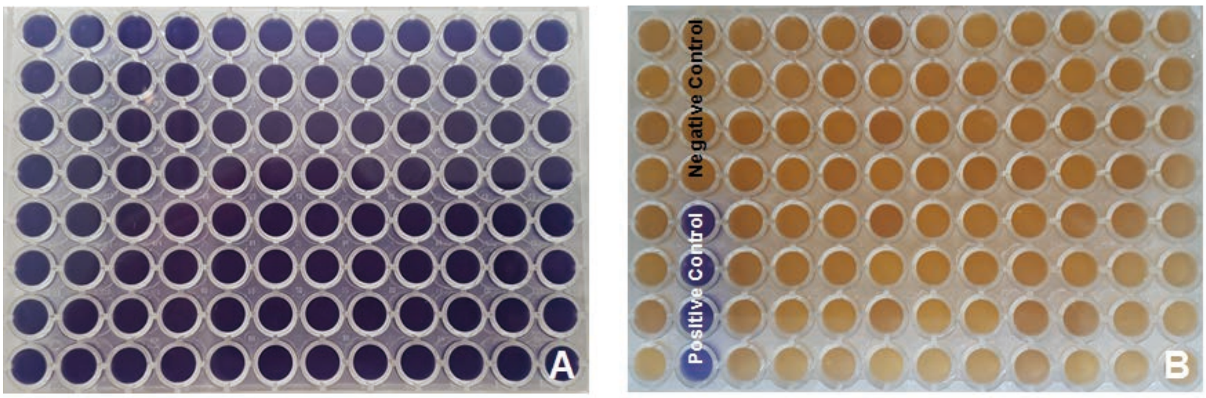

Figure 1. BRT Inhibitor Test plates before (A) and after (B) incubation

Within the framework of this validation, the BRT Inhibitor 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 °C 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 15 ± 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

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

60,340 data results were obtained from the evaluation of 154 BRT Inhibitor 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 Inhibitor Test plate batches provided for the validation

Batch	Range of Batch Numbers
A	20709235 - 20713911
B	20516031 - 20524980
C	20801052 - 20812126
D	21301001 - 21308901
E	21409615 - 21410635
F	21601002 - 21610752
G	22801005 - 22814753

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 Inhibitor 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 °C and used the next day. To verify the correct 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 Inhibitor 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	2.5	2
	Ampicillin	4	3.5	3
	Amoxicillin	4	3	2.5
	Cloxacillin	30	25	20
	Dicloxacillin	30	15	12.5
	Nafcillin	30	15	10
	Oxacillin	30	10	8
Cephalosporins	Cefalexin	100	400	300
	Cefapirin	60	6	5
	Cefoperazone	50	35	25
	Cefazolin	50	9	7
	Cefquinome	20	500	300
	Ceftiofur	100	200	150
	Cefalonium	20	14	12
Macrolides	Erythromycin	40	100	50
	Tylosin	50	75	40
Sulfonamides	Sulfadiazine	100	>800	100
	Sulfadimethoxin	100	>800	200
	Sulfamethazine	100	1,000	300
	Sulfathiazol	100	400	60
	Sulfadoxin	100	>1,500	400
	Sulfamethoxypyridazine	100	500	100
Tetracyclines	Chlortetracycline	100	>1,000	800
	Oxytetracycline	100	800	400
	Tetracycline	100	1,000	600
Aminoglycosides	Dihydrostreptomycin	200	600	400
	Streptomycin	200	1,500	600
	Gentamicin	100	200	100
	Neomycin	1,500	400	200
Fenicol	Chloramphenicol	-	7,000	4,000

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 4 out of 7 cephalosporins. Out of the groups of the macrolides, sulfonamides and aminoglycosides Tylosin, Sulfadiazine, 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. Additionally, for a low number of substances (Sulfadiazine, Sulfadimethoxin, Sulfadoxin and Chlortetracycline) CCß A could not be determined 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 4,000 µg/kg (CCß B) or 7,000 µg/kg (CCß A). The detection limits for the BRT Inhibitor Test 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, 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

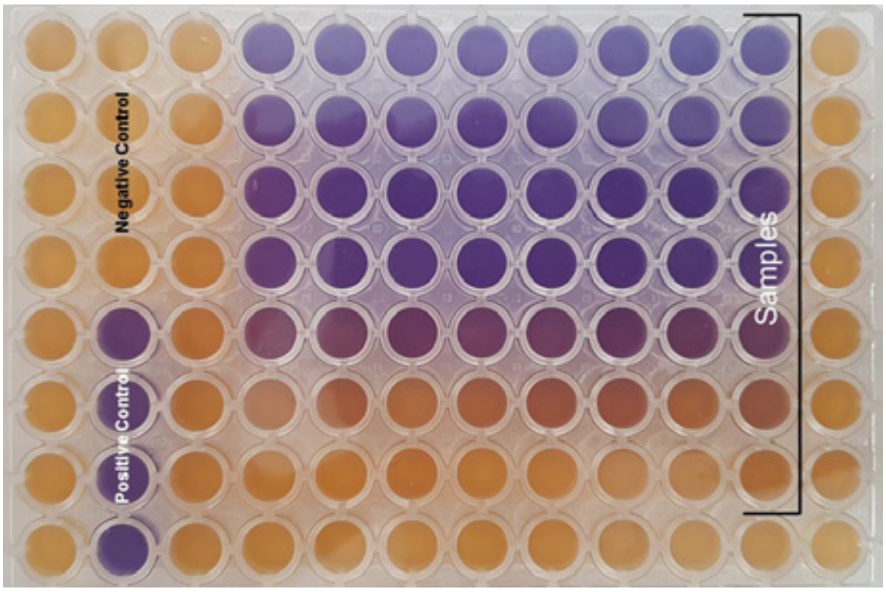

Figure 2. Incubated BRT Inhibitor Test plate inoculated with four positive and four negative controls as well as raw milk samples spiked with 7 different concentrations of an antibiotic substance

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).

Results and Discussion

Figure 3 and Annex Figure 1 depict dose-response curves of all substances included in the validation of the BRT Inhibitor 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 0% at 1.5 µg/kg, 76% at 2 µg/kg already and 100% of samples were detected positive at 2.5 µg/kg. Substances like the sulfonamides and tetracyclines displayed more consistent curve increments, probably due to their bacteriostatic character. Sulfadoxin obtained 0% positive results at 100 µg/kg, 7% at 400 µg/kg, 31% respectively 39% at 600 µg/kg and 800 µg/kg. At 1,000 µg/kg, the positive response was 47% and with the highest concentration analyzed (1,500 µg/kg) 63% - no CCß A was established for this substance.

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.

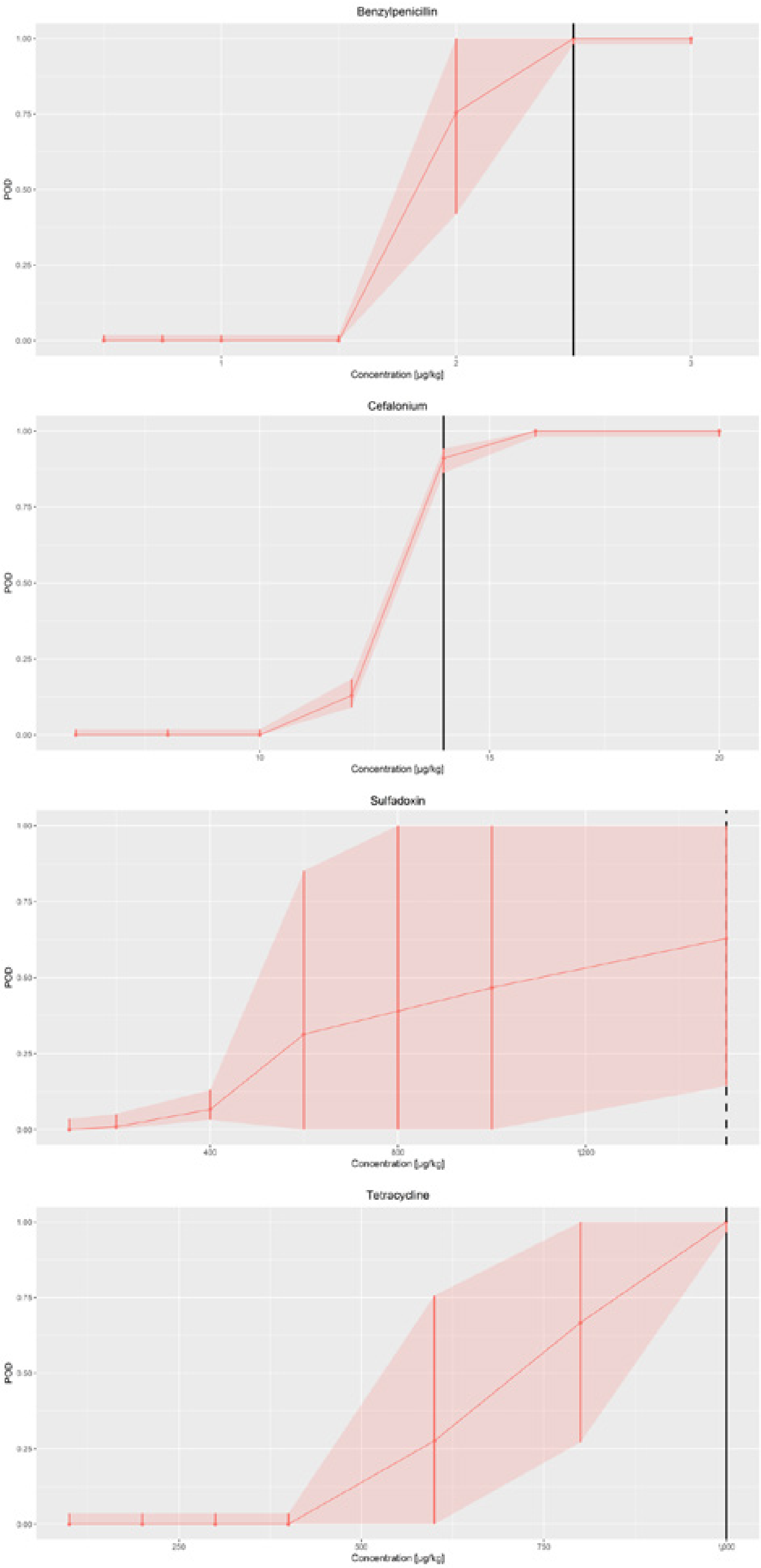

Figure 3. Dose-response curves of the bactericidal antibiotics Benzylpenicillin and Cefalonium and of the bacteriostatic substances Sulfadoxin and Tetracycline.
Red line = dose-response curve; red shade = CI; Black line = CCß A (photometric reading); Dotted line = highest concentration analyzed

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 Inhibitor 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

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

Results and Discussion

Highly concentrated samples of Flunixin, Metamizole, Prednisolone, Triclabendazole, Deltamethrin and Monensin did not inhibit the growth of the test germs, leading to negative results (both class A and class B). Thus, no false positive results were observed, signifying a high specificity of the BRT Inhibitor Test for the detection of antibiotics 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 Inhibitor 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 α = 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. Most substances realized p-value = 1, for Cloxacillin, Oxacillin, Ceftiofur and Sulfathiazol the p values were 0.05 ≤ 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	CCß A [µg/kg]	p value		Group of Antibiotics	Substance	MRL	CCß A [µg/kg]	p value	
				ELISA 1	ELISA 2					ELISA 1	ELISA 2
	Benzylpenicillin	4	2.5	1	1		Sulfadiazine	100	NA	-	-
	Ampicillin	4	3.5	1	1		Sulfadimethoxin	100	NA	-	-
	Amoxicillin	4	3	1	1	**Sulfonamides**	Sulphamethazine	100	1,000	1	1
Penicillins	Cloxacillin	30	25	0.33	1		Sulfathiazol	100	400	1	0.06
	Dicloxacillin	30	15	1	1		Sulfadoxin	100	NA	-	-
	Nafcillin	30	15	1	1		Sulfamethoxypyridazine	100	500	1	1
	Oxacillin	30	10	0.20	1		Chlortetracycline	100	NA	-	-
	Cefalexin	100	400	1	1	**Tetracyclines**	Oxytetracycline	100	800	1	1
	Cefapirin	60	6	1	1		Tetracycline	100	1,000	1	1
	Cefoperazone	50	35	1	1		Dihydrostreptomycin	200	600	1	1
Cephalosporines	Cefazolin	50	9	1	1		Streptomycin	200	1,500	1	1
	Cefquinome	20	500	1	1	**Aminoglycosides**	Gentamicin	100	200	1	1
	Ceftiofur	100	200	0.11	0.11		Neomycin	1,500	400	1	1
	Cefalonium	20	14	1	1	**Fenicols**	Chloramphenicol	-	7,000	1	1
Macrolides	Erythromycin	40	100	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 Inhibitor 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, 154 test plates were analyzed, including 616 positive control samples and 3,080 samples of negative raw milk in total. Thus, 3,080 (positive control) respectively 15,400 (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	154	616	1,232	1,848	3,080
Negative Milk		3,080	6,160	9,240	15,400

Results and Discussion

No false-positive results were observed when analyzing the results of the negative milk samples by photometric evaluation or visual reading, indicating a false-positive rate of 0%.

With photometric evaluation no false-negative result was obtained out of 3,080 results for the positive control samples. Only 1 sample was classified false-negative by 1 out of 3 examining technicians, leading to a false-negative rate of 0.2% for this person (Table 10). As none of the other readers (technicians and ELISA readers) assessed this sample as positive, the negative result is considered as outlier and might have been caused by a typing error.

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	0.2
Negative Milk	0	0	0	0	0

The maximum relative percentage value (Section 2) obtained with photometric reading for negative samples was 33%, whereas the minimum relative percentage value for positive samples was 93% (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	1,232	93
Negative Milk	6,160	33

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 Inhibitor 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 Inhibitor 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 MRL Screening Test and BRT hi-sense). To confirm detected inhibitors, screening-positive samples were tested a second time 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 Inhibitor 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 Inhibitor 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 Inhibitor 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 Inhibitor 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 Inhibitor Test, too.
The reported results of the interlaboratory study of the BRT Inhibitor 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

The participants of the interlaboratory study analyzed the coded samples with the BRT Inhibitor Test, reported the observed results in a supplied evaluation sheet and returned these sheets to the QSE GmbH, where the results were decoded. It was not reported by the laboratories which reading system was used for the examination of the test plates.

Results and Discussion

In total, 945 results were reported for the BRT Inhibitor 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 Inhibitor Test is suitable for routine analyses as all positive and negative samples were identified properly and all examined substances were detected at MRL level.

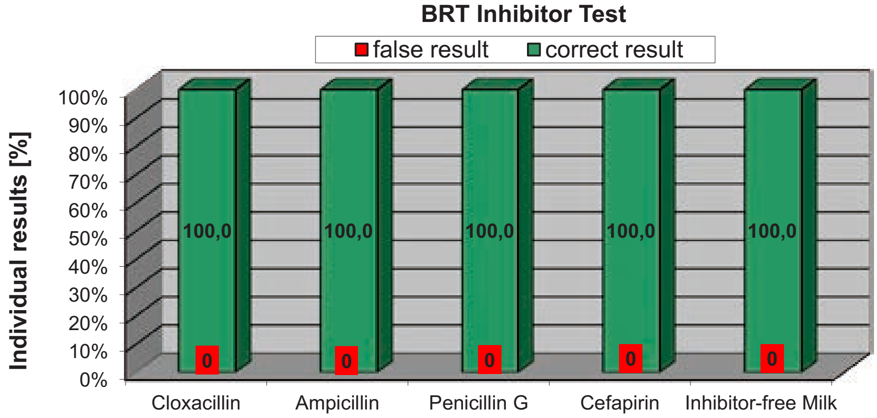

Figure 4. Rates of correct and false results (%) obtained by the interlaboratory study within the framework of the validation of the BRT Inhibitor Test

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 Inhibitor 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%).

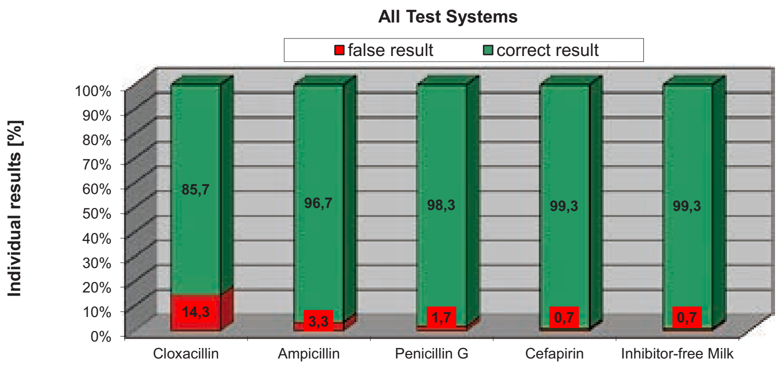

Figure 5. Rates of correct and false results (%) of all test systems (microbiological and receptor tests, 10th proficiency test)

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

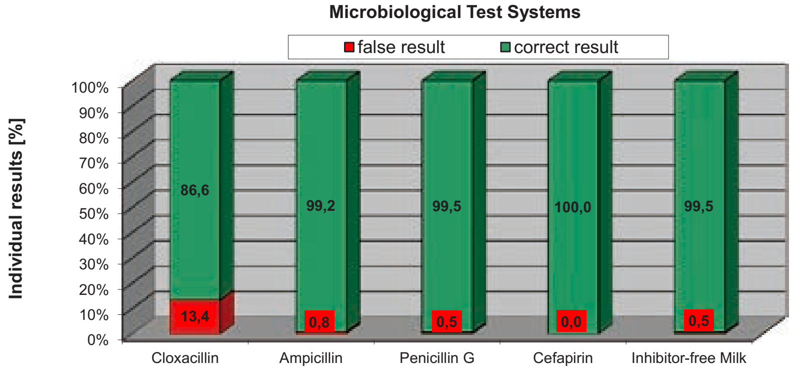

Figure 6. Rates of correct and false results (%) of microbiological test systems (10th proficiency test)

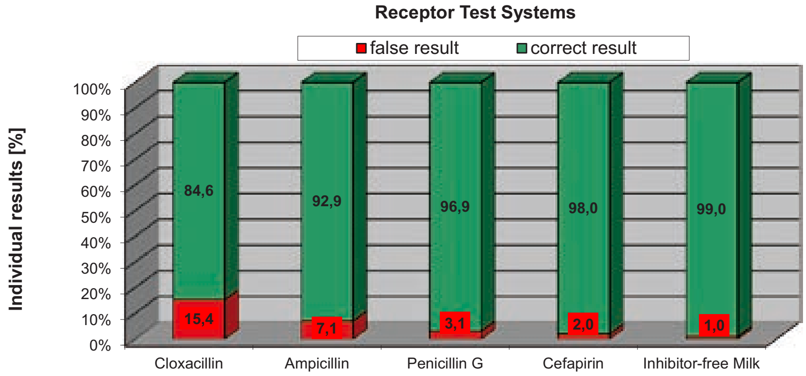

Figure 7. Rates of correct and false results (%) of receptor test systems (10th proficiency test)

11) Conclusions

The BRT Inhibitor Test is capable of the detection of all of the 7 penicillins and 4 out of 7 cephalosporins as well as 6 out of 15 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 Inhibitor 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 and an extremely low rate of 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 Inhibitor 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

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German Federal Office of Consumer Protection and Food Safety
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German Federal Office of Consumer Protection and Food Safety
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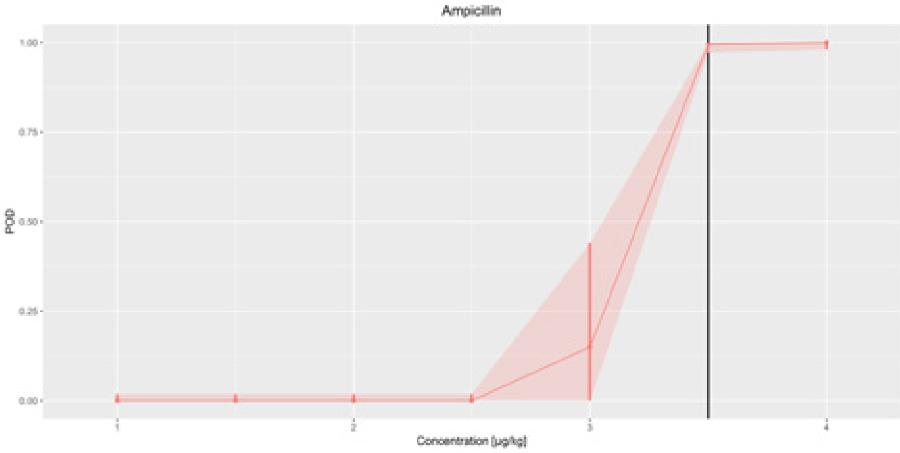
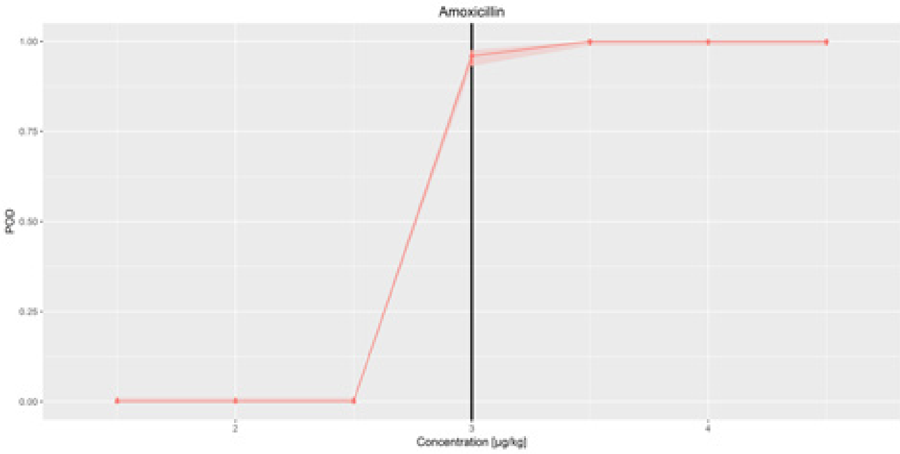
Date of publication: 09.11.2018

ANNEX

Annex Table 1. 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	2.5	2
	Ampicillin	4	3.5	3
	Amoxicillin	4	3.5	3
	Cloxacillin	30	30	25
	Dicloxacillin	30	15	12.5
	Nafcillin	30	15	8
	Oxacillin	30	12	10
Cephalosporins	Cefalexin	100	500	400
	Cefapirin	60	8	5
	Cefoperazone	50	35	25
	Cefazolin	50	9	7
	Cefquinome	20	600	300
	Ceftiofur	100	250	200
	Cefalonium	20	16	12
Macrolides	Erythromycin	40	100	60
	Tylosin	50	100	30
Sulfonamides	Sulfadiazine	100	>800	100
	Sulfadimethoxin	100	>800	200
	Sulfamethazine	100	>1,000	200
	Sulfathiazol	100	400	60
	Sulfadoxin	100	>1,500	400
	Sulfamethoxypyridazine	100	500	100
Tetracyclines	Chlortetracycline	100	>1,000	800
	Oxytetracycline	100	800	400
	Tetracycline	100	1,000	600
Aminoglycosides	Dihydrostreptomycin	200	500	400
	Streptomycin	200	1,500	600
	Gentamicin	100	150	80
	Neomycin	1,500	300	200
Fenicol	Chloramphenicol	-	5,000	3,500


Ampicillin
POD
Concentration [µg/kg]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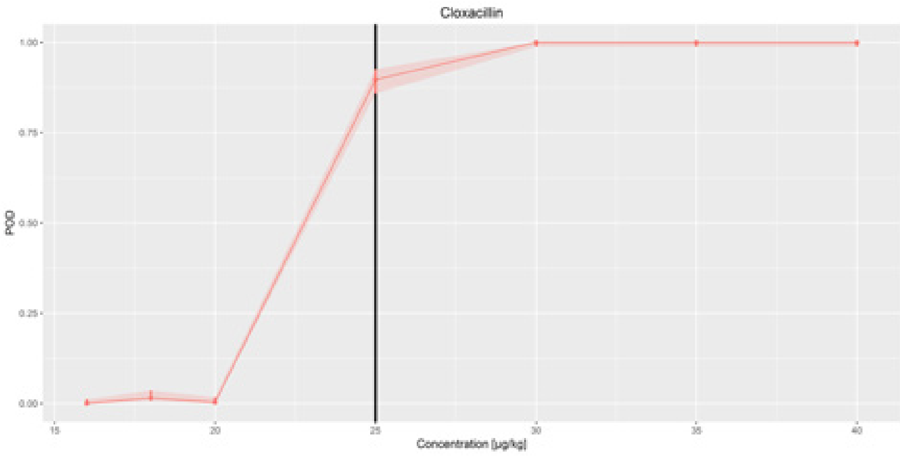
Concentration [µg/kg]


Cloxacillin
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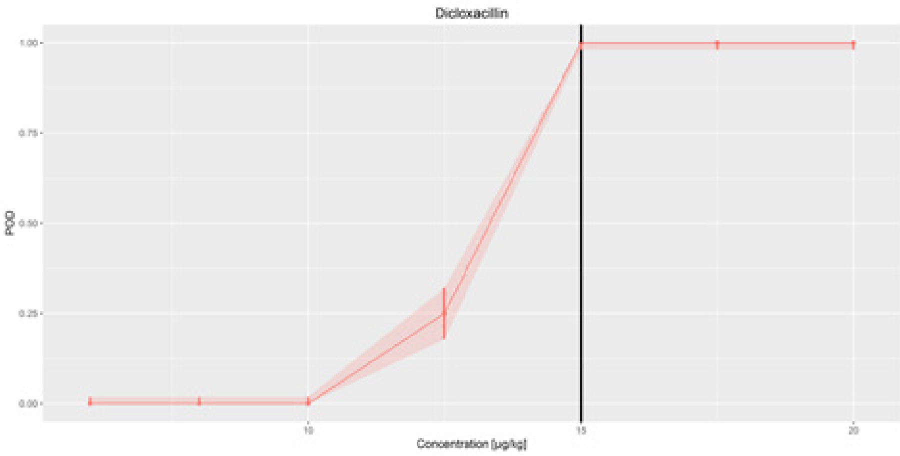
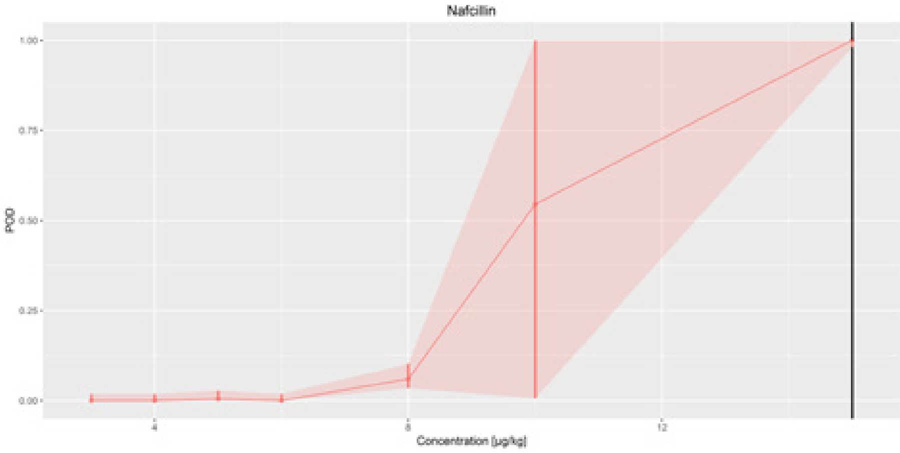
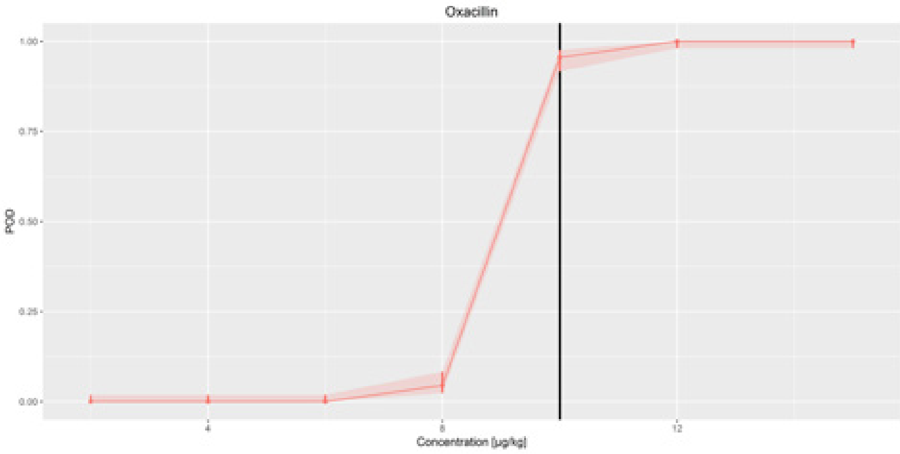

Dicloxacillin
POD
Concentration [µg/kg]


Nafcillin
POD
Concentration [µg/kg]


Oxacillin
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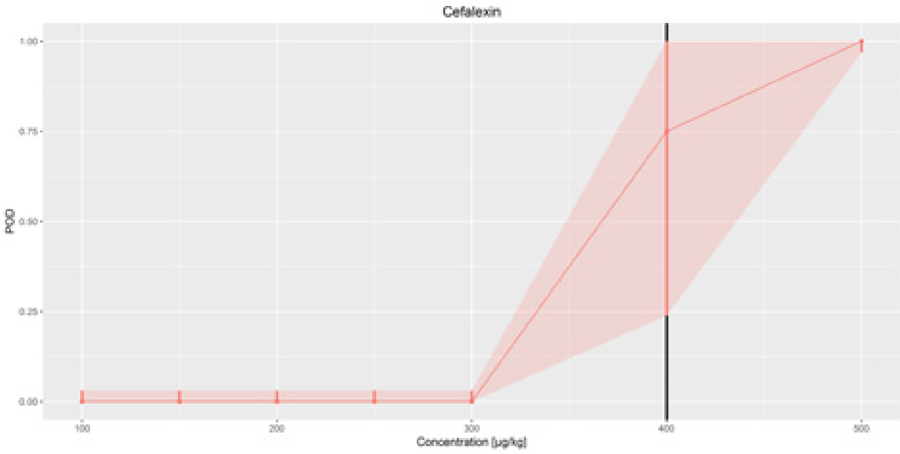
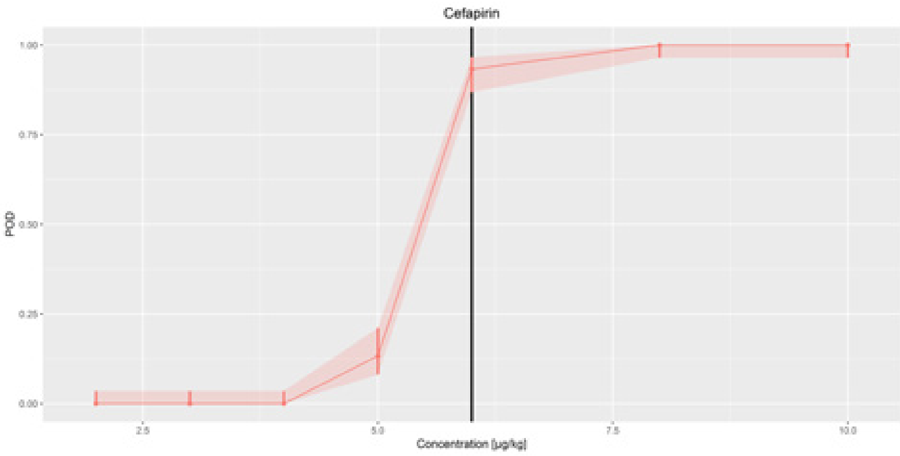
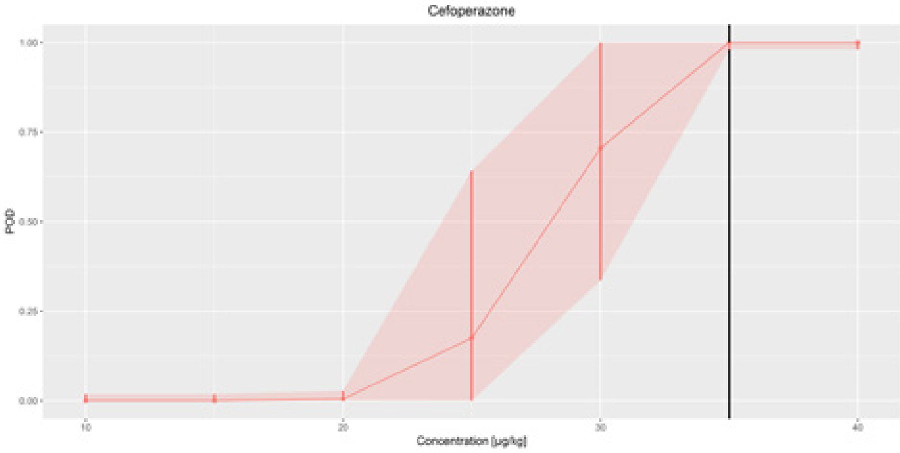
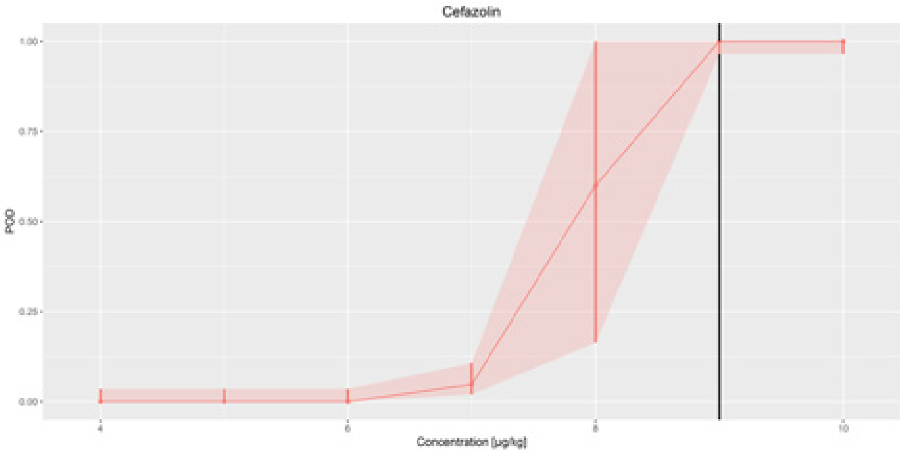
Concentration [µg/kg]


Cefalexin
POD
Concentration [µg/kg]


Cefapirin
POD
Concentration [µg/kg]



Cefoperazone
POD
1.00
0.75
0.50
0.25
0.00
10
20
30
40
Concentration [µg/kg]



Cefazolin
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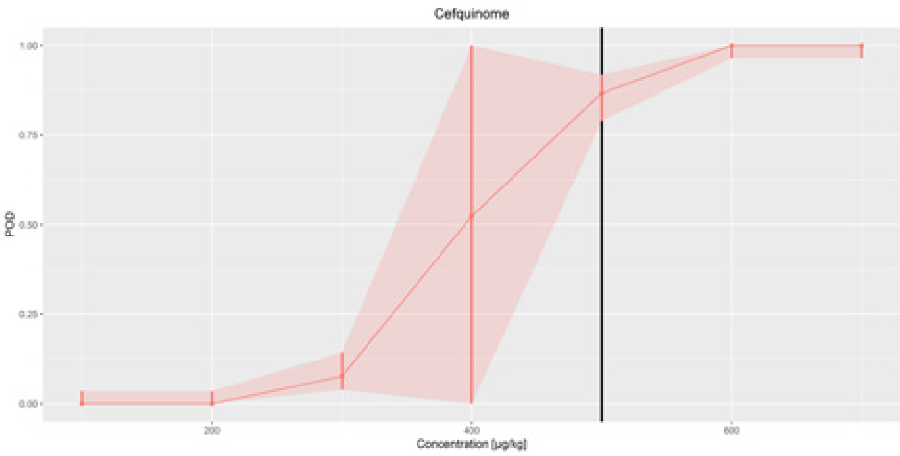
0.00
4
6
8
10
Concentration [µg/kg]



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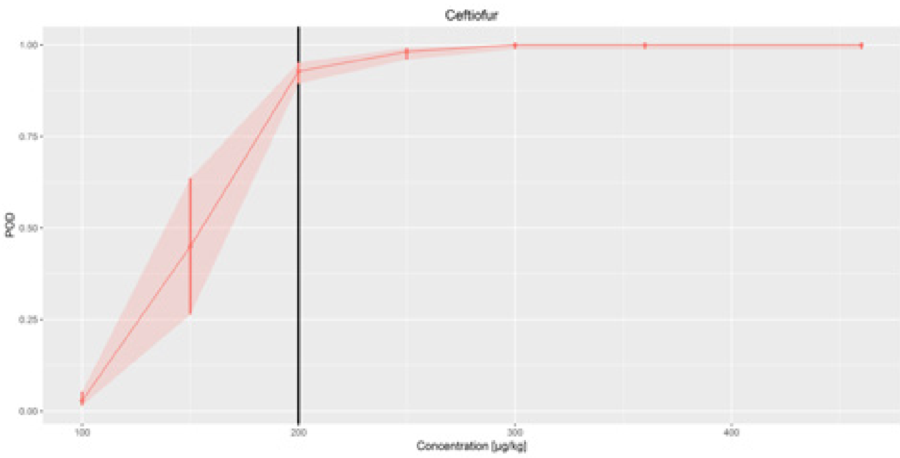
0.25
0.00
200
400
600
Concentration [µg/kg]



Ceftiofur
POD
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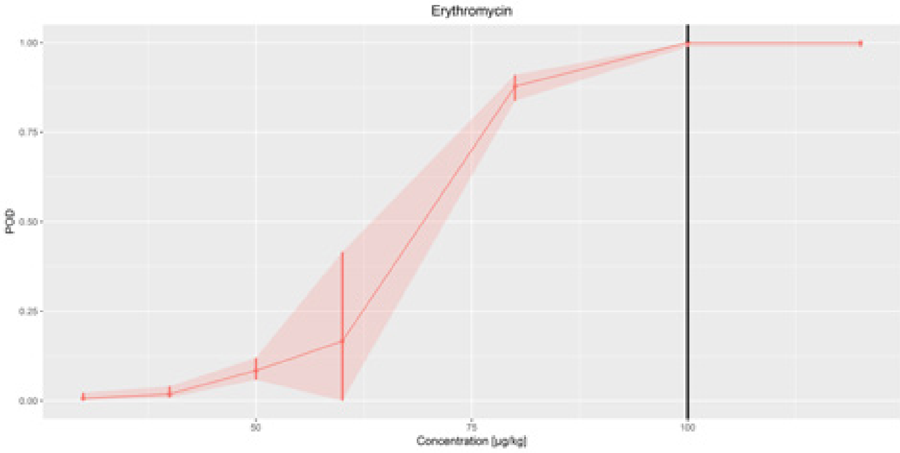
0.75
0.50
0.25
0.00
100
200
300
400
Concentration [µg/kg]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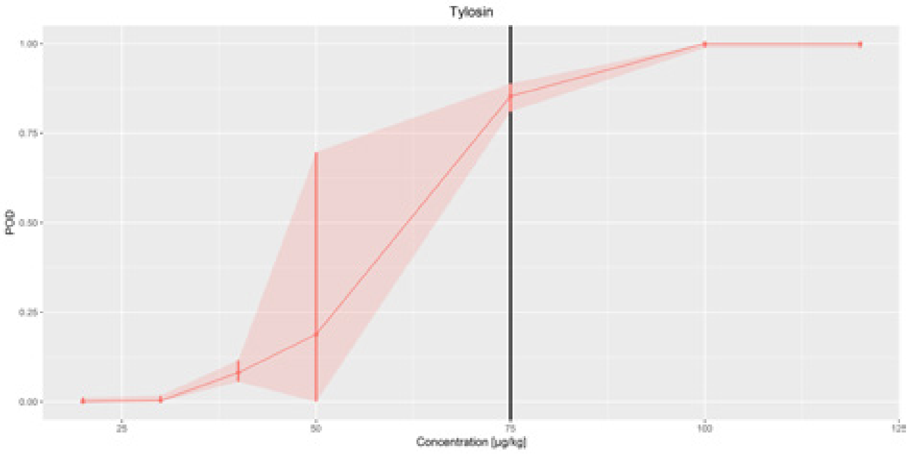
0.00
POD
50
75
100
Concentration [µg/kg]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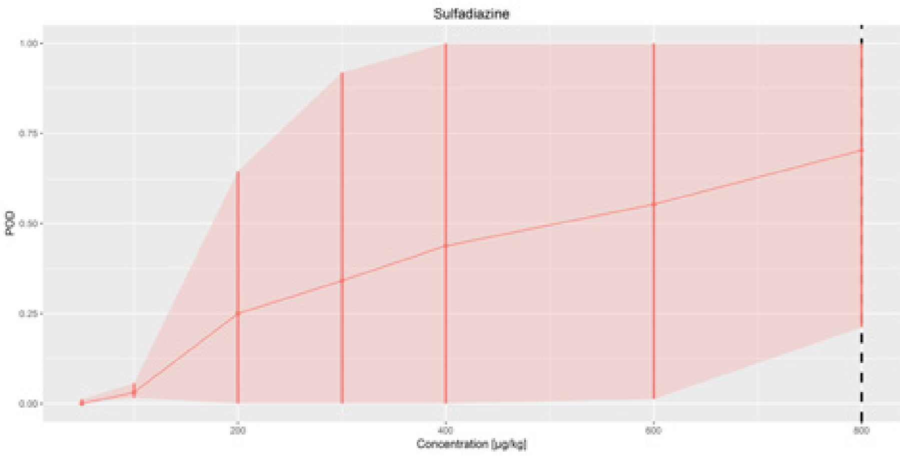
50
75
100
125
Concentration [µg/kg]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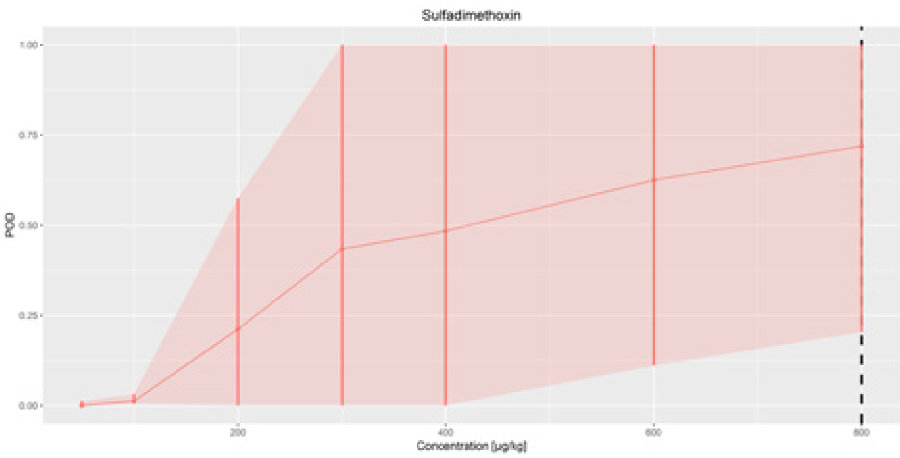
POD
200
400
600
800
Concentration [µg/kg]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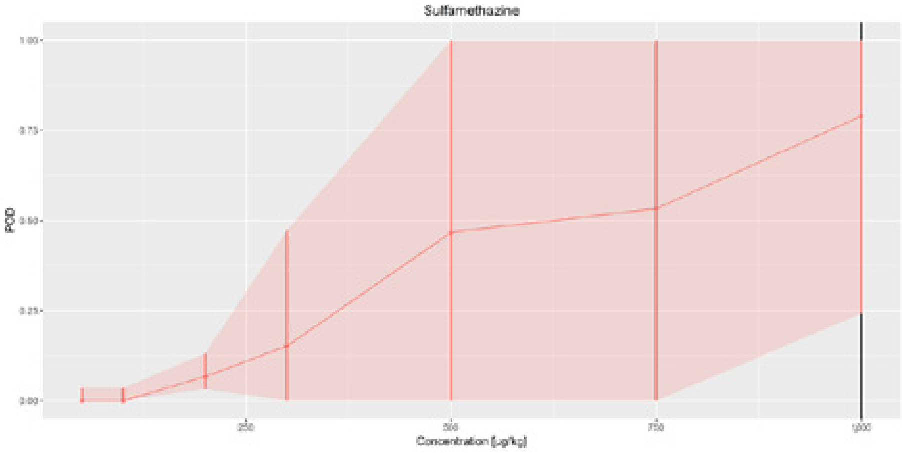
400
600
800
Concentration [µg/kg]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POD
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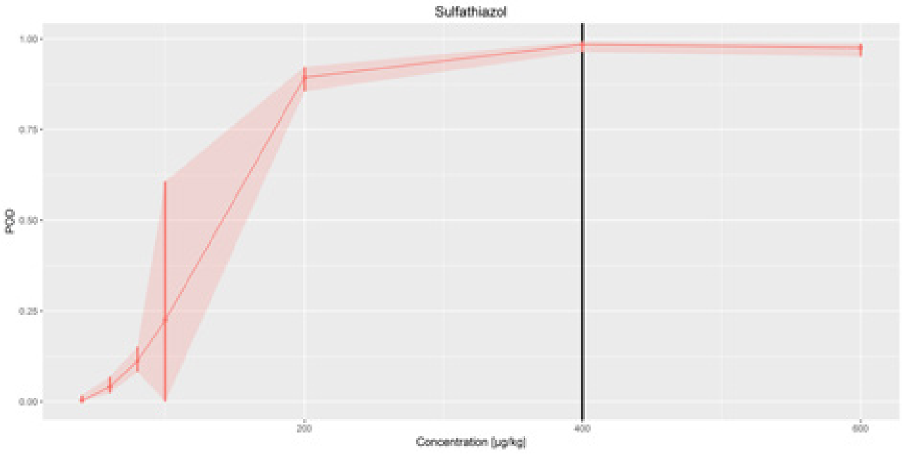
500
750
1,000
Concentration [µg/kg]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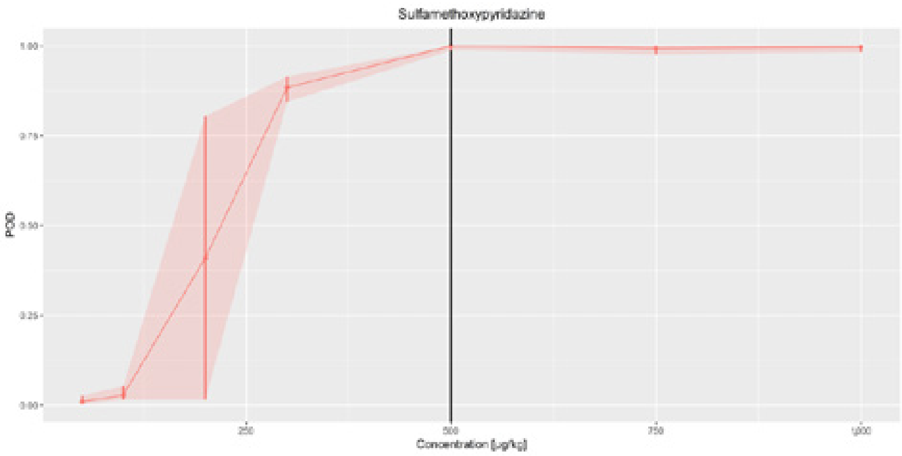
0.25
0.00
POD
200
400
600
Concentration [µg/kg]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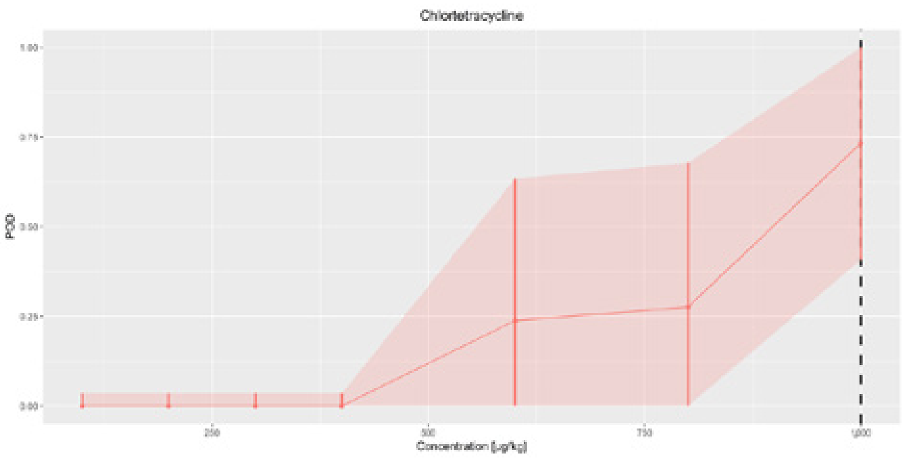
POD
250
500
750
1,000
Concentration [µg/kg]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250
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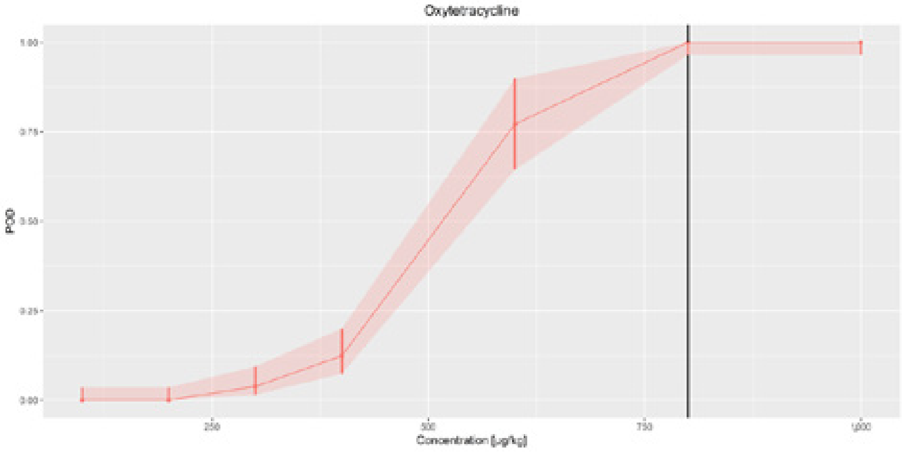
1,000
Concentration [µg/kg]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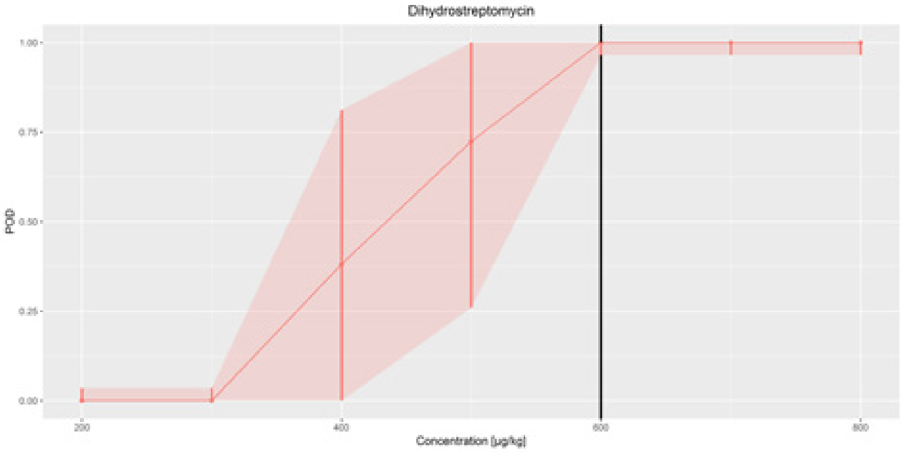
Concentration [µg/kg]


Dihydrostreptomycin
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0.75
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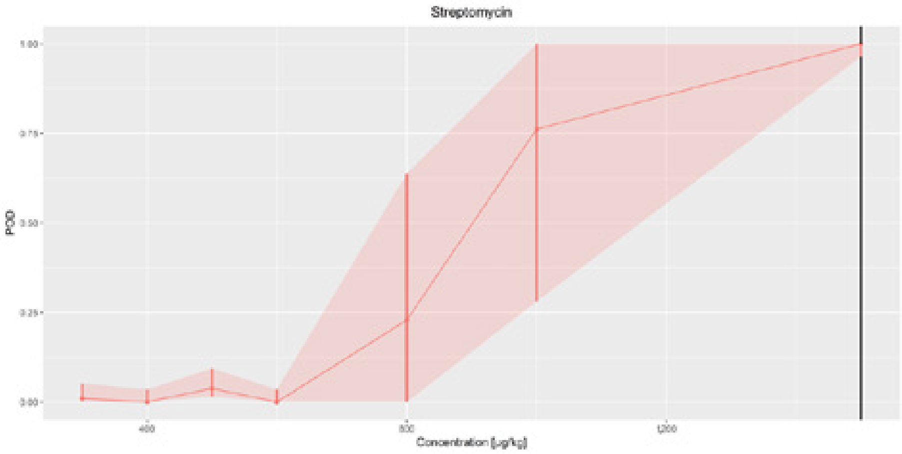
0.25
0.00
POD
200
400
600
800
Concentration [µg/kg]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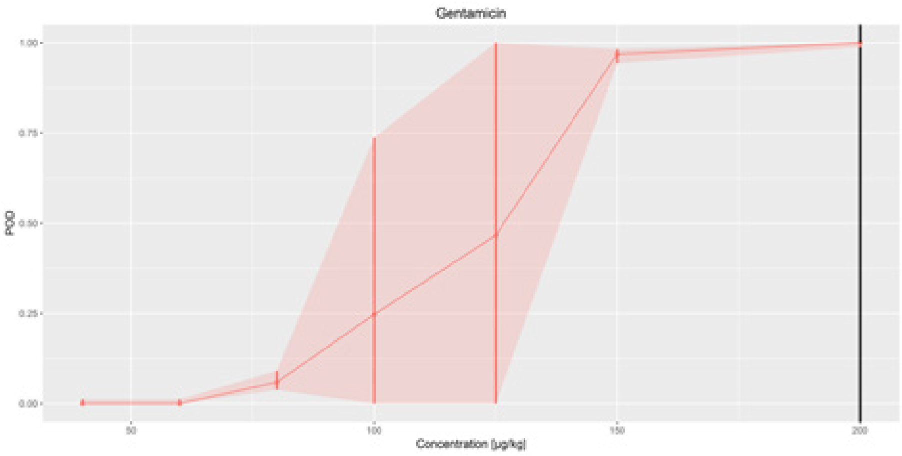
0.00
POD
400
800
1,200
Concentration [µg/kg]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POD
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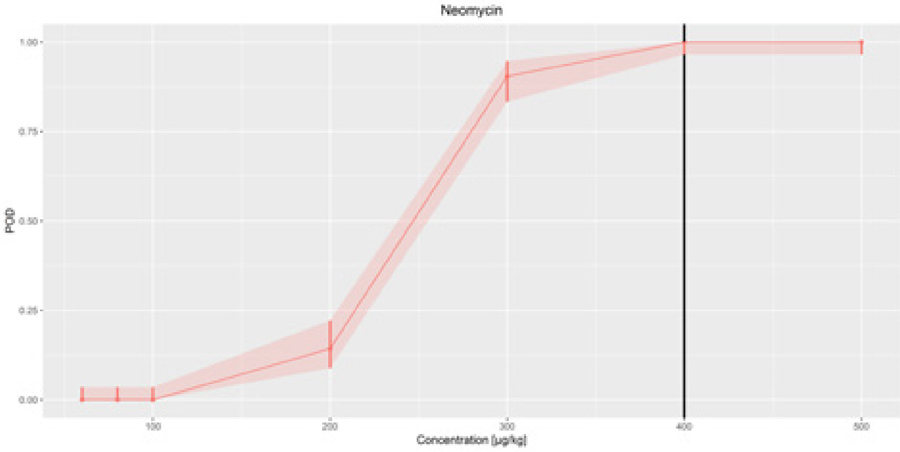
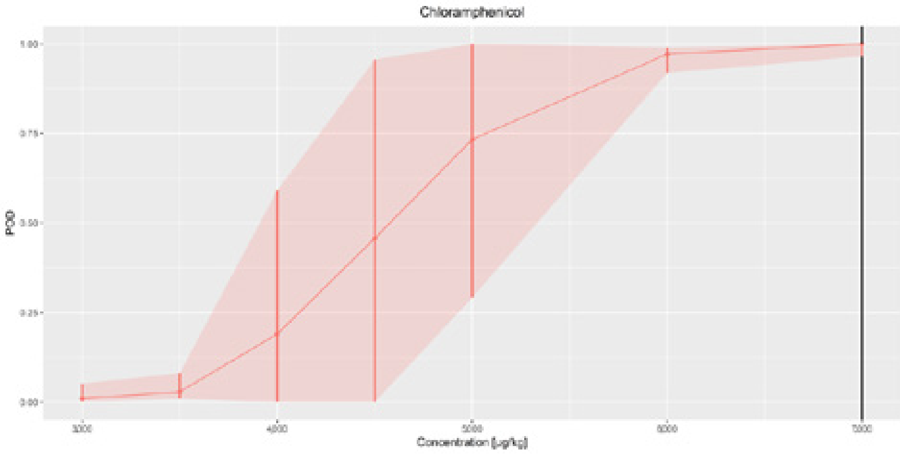
150
200
Concentration [µg/kg]





Annex Figure 1. Dose-response curves of antibiotic substances included in the validation of the BRT Inhibitor 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)
		0.5	80	0	0.00	-	-	0	0.00	80
		0.75	80	0	0.00	-	-	0	0.00	80
		1	80	0	0.00	-	-	0	0.00	80
	Photometric	1.5	80	0	0.00	-	-	26	0.33	54
		2	80	68	0.85	-	-	12	1.00	0
		2.5	80	80	1.00	-	-	0	1.00	0
		3	80	80	1.00	-	-	0	1.00	0
		0.5	120	0	0.00	-	-	2	0.02	118
		0.75	120	0	0.00	-	-	1	0.01	119
		1	120	0	0.00	-	-	6	0.05	114
Benzyl-penicillin	Visual	1.5	120	0	0.00	-	-	60	0.50	60
		2	120	83	0.69	-	-	37	1.00	0
		2.5	120	120	1.00	-	-	0	1.00	0
		3	120	120	1.00	-	-	0	1.00	0
		0.5	200	0	0.00	0.00	0.02	2	-	198
		0.75	200	0	0.00	0.00	0.02	1	-	199
		1	200	0	0.00	0.00	0.02	6	-	194
	Photometric + Visual	1.5	200	0	0.00	0.00	0.02	86	-	114
		2	200	151	0.76	0.42	1.00	49	-	0
		2.5	200	200	1.00	0.98	1.00	0	-	0
		3	200	200	1.00	0.98	1.00	0	-	0
		1	80	0	0.00	-	-	0	0.00	80
		1.5	80	0	0.00	-	-	0	0.00	80
		2	80	0	0.00	-	-	0	0.00	80
	Photometric	2.5	80	0	0.00	-	-	17	0.21	63
		3	80	3	0.04	-	-	77	1.00	0
		3.5	80	80	1.00	-	-	0	1.00	0
		4	80	80	1.00	-	-	0	1.00	0
		1	120	0	0.00	-	-	0	0.00	120
		1.5	120	0	0.00	-	-	0	0.00	120
		2	120	0	0.00	-	-	1	0.01	119
Ampicillin	Visual	2.5	120	0	0.00	-	-	32	0.27	88
		3	120	27	0.23	-	-	90	0.98	3
		3.5	120	119	0.99	-	-	1	1.00	0
		4	120	120	1.00	-	-	0	1.00	0
		1	200	0	0.00	0.00	0.02	0	-	200
		1.5	200	0	0.00	0.00	0.02	0	-	200
		2	200	0	0.00	0.00	0.02	1	-	199
	Photometric + Visual	2.5	200	0	0.00	0.00	0.02	49	-	151
		3	200	30	0.15	0.00	0.44	167	-	3
		3.5	200	199	1.00	0.97	1.00	1	-	0
		4	200	200	1.00	0.98	1.00	0	-	0
		1.5	128	0	0.00	-	-	0	0.00	128
		2	128	0	0.00	-	-	6	0.05	122
		2.5	128	0	0.00	-	-	126	0.98	2
	Photometric	3	128	128	1.00	-	-	0	1.00	0
		3.5	128	128	1.00	-	-	0	1.00	0
		4	128	128	1.00	-	-	0	1.00	0
		4.5	128	128	1.00	-	-	0	1.00	0
		1.5	192	0	0.00	-	-	0	0.00	192
		2	192	0	0.00	-	-	0	0.00	192
		2.5	192	0	0.00	-	-	142	0.74	50
Amoxicillin	Visual	3	192	179	0.93	-	-	13	1.00	0
		3.5	192	192	1.00	-	-	0	1.00	0
		4	192	192	1.00	-	-	0	1.00	0
		4.5	192	192	1.00	-	-	0	1.00	0
		1.5	320	0	0.00	0.00	0.01	0	-	320
		2	320	0	0.00	0.00	0.01	6	-	314
		2.5	320	0	0.00	0.00	0.01	268	-	52
	Photometric + Visual	3	320	307	0.96	0.93	0.98	13	-	0
		3.5	320	320	1.00	0.99	1.00	0	-	0
		4	320	320	1.00	0.99	1.00	0	-	0
		4.5	320	320	1.00	0.99	1.00	0	-	0
		16	128	0	0.00	-	-	0	0.00	128
		18	128	2	0.02	-	-	85	0.68	41
		20	128	0	0.00	-	-	124	0.97	4
	Photometric	25	128	126	0.98	-	-	2	1.00	0
		30	128	128	1.00	-	-	0	1.00	0
		35	128	128	1.00	-	-	0	1.00	0
		40	128	128	1.00	-	-	0	1.00	0
		16	192	0	0.00	-	-	1	0.01	191
		18	192	3	0.02	-	-	42	0.23	147
		20	192	1	0.01	-	-	167	0.88	24
Cloxacillin	Visual	25	192	161	0.84	-	-	31	1.00	0
		30	192	192	1.00	-	-	0	1.00	0
		35	192	192	1.00	-	-	0	1.00	0
		40	192	192	1.00	-	-	0	1.00	0
		16	320	0	0.00	0.00	0.01	1	-	319
		18	320	5	0.02	0.01	0.04	127	-	188
		20	320	1	0.00	0.00	0.02	291	-	28
	Photometric + Visual	25	320	287	0.90	0.86	0.93	33	-	0
		30	320	320	1.00	0.99	1.00	0	-	0
		35	320	320	1.00	0.99	1.00	0	-	0
		40	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)
Dicloxacillin	Photometric	6	80	0	0.00	-	-	0	0.00	80
		8	80	0	0.00	-	-	0	0.00	80
		10	80	0	0.00	-	-	12	0.15	68
		12.5	80	17	0.21	-	-	63	1.00	0
		15	80	80	1.00	-	-	0	1.00	0
		17.5	80	80	1.00	-	-	0	1.00	0
		20	80	80	1.00	-	-	0	1.00	0
	Visual	6	120	0	0.00	-	-	8	0.07	112
		8	120	0	0.00	-	-	10	0.08	110
		10	120	0	0.00	-	-	36	0.30	84
		12.5	120	33	0.28	-	-	86	0.99	1
		15	120	120	1.00	-	-	0	1.00	0
		17.5	120	120	1.00	-	-	0	1.00	0
		20	120	120	1.00	-	-	0	1.00	0
	Photometric + Visual	6	200	0	0.00	0.00	0.02	8	-	192
		8	200	0	0.00	0.00	0.02	10	-	190
		10	200	0	0.00	0.00	0.02	48	-	152
		12.5	200	50	0.25	0.18	0.32	149	-	1
		15	200	200	1.00	0.98	1.00	0	-	0
		17.5	200	200	1.00	0.98	1.00	0	-	0
		20	200	200	1.00	0.98	1.00	0	-	0
Nafcillin	Photometric	3	80	0	0.00	-	-	0	0.00	80
		4	80	0	0.00	-	-	0	0.00	80
		5	80	0	0.00	-	-	1	0.01	79
		6	80	0	0.00	-	-	2	0.03	78
		8	80	0	0.00	-	-	61	0.76	19
		10	80	30	0.38	-	-	50	1.00	0
		15	80	80	1.00	-	-	0	1.00	0
	Visual	3	120	0	0.00	-	-	6	0.05	114
		4	120	0	0.00	-	-	15	0.13	105
		5	120	1	0.01	-	-	34	0.29	85
		6	120	0	0.00	-	-	59	0.49	61
		8	120	12	0.10	-	-	102	0.95	6
		10	120	79	0.66	-	-	41	1.00	0
		15	120	120	1.00	-	-	0	1.00	0
	Photometric + Visual	3	200	0	0.00	0.00	0.02	6	-	194
		4	200	0	0.00	0.00	0.02	15	-	185
		5	200	1	0.01	0.00	0.03	35	-	164
		6	200	0	0.00	0.00	0.02	61	-	139
		8	200	12	0.06	0.03	0.10	163	-	25
		10	200	109	0.55	0.01	1.00	91	-	0
		15	200	200	1.00	0.98	1.00	0	-	0
Oxacillin	Photometric	2	80	0	0.00	-	-	0	0.00	80
		4	80	0	0.00	-	-	0	0.00	80
		6	80	0	0.00	-	-	2	0.03	78
		8	80	0	0.00	-	-	80	1.00	0
		10	80	79	0.99	-	-	1	1.00	0
		12	80	80	1.00	-	-	0	1.00	0
		15	80	80	1.00	-	-	0	1.00	0
	Visual	2	120	0	0.00	-	-	4	0.03	116
		4	120	0	0.00	-	-	3	0.03	117
		6	120	0	0.00	-	-	41	0.34	79
		8	120	9	0.08	-	-	102	0.93	9
		10	120	112	0.93	-	-	8	1.00	0
		12	120	120	1.00	-	-	0	1.00	0
		15	120	120	1.00	-	-	0	1.00	0
	Photometric + Visual	2	200	0	0.00	0.00	0.02	4	-	196
		4	200	0	0.00	0.00	0.02	3	-	197
		6	200	0	0.00	0.00	0.02	43	-	157
		8	200	9	0.05	0.02	0.08	182	-	9
		10	200	191	0.96	0.92	0.98	9	-	0
		12	200	200	1.00	0.98	1.00	0	-	0
		15	200	200	1.00	0.98	1.00	0	-	0
Cefalexin	Photometric	100	48	0	0.00	-	-	0	0.00	48
		150	48	0	0.00	-	-	0	0.00	48
		200	48	0	0.00	-	-	0	0.00	48
		250	48	0	0.00	-	-	0	0.00	48
		300	48	0	0.00	-	-	48	1.00	0
		400	48	48	1.00	-	-	0	1.00	0
		500	48	48	1.00	-	-	0	1.00	0
	Visual	100	72	0	0.00	-	-	1	0.01	71
		150	72	0	0.00	-	-	1	0.01	71
		200	72	0	0.00	-	-	7	0.10	65
		250	72	0	0.00	-	-	10	0.14	62
		300	72	0	0.00	-	-	39	0.54	33
		400	72	42	0.58	-	-	30	1.00	0
		500	72	72	1.00	-	-	0	1.00	0
	Photometric + Visual	100	120	0	0.00	0.00	0.03	1	-	119
		150	120	0	0.00	0.00	0.03	1	-	119
		200	120	0	0.00	0.00	0.03	7	-	113
		250	120	0	0.00	0.00	0.03	10	-	110
		300	120	0	0.00	0.00	0.03	87	-	33
		400	120	90	0.75	0.24	1.00	30	-	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)
		2	42	0	0.00	-	-	0	0.00	42
		3	42	0	0.00	-	-	0	0.00	42
		4	42	0	0.00	-	-	0	0.00	42
	Photometric	5	42	0	0.00	-	-	42	1.00	0
		6	42	42	1.00	-	-	0	1.00	0
		8	42	42	1.00	-	-	0	1.00	0
		10	42	42	1.00	-	-	0	1.00	0
		2	63	0	0.00	-	-	1	0.02	62
		3	63	0	0.00	-	-	9	0.14	54
		4	63	0	0.00	-	-	9	0.14	54
Cefapirin	Visual	5	63	14	0.22	-	-	49	1.00	0
		6	63	56	0.89	-	-	7	1.00	0
		8	63	63	1.00	-	-	0	1.00	0
		10	63	63	1.00	-	-	0	1.00	0
		2	105	0	0.00	0.00	0.04	1	-	104
		3	105	0	0.00	0.00	0.04	9	-	96
		4	105	0	0.00	0.00	0.04	9	-	96
	Photometric + Visual	5	105	14	0.13	0.08	0.21	91	-	0
		6	105	98	0.93	0.87	0.97	7	-	0
		8	105	105	1.00	0.97	1.00	0	-	0
		10	105	105	1.00	0.97	1.00	0	-	0
		10	80	0	0.00	-	-	0	0.00	80
		15	80	0	0.00	-	-	0	0.00	80
		20	80	0	0.00	-	-	11	0.14	69
	Photometric	25	80	0	0.00	-	-	80	1.00	0
		30	80	52	0.65	-	-	28	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	-	-	5	0.04	115
		15	120	0	0.00	-	-	16	0.13	104
		20	120	1	0.01	-	-	74	0.63	45
Cefoperazone	Visual	25	120	35	0.29	-	-	83	0.98	2
		30	120	89	0.74	-	-	31	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	5	-	195
		15	200	0	0.00	0.00	0.02	16	-	184
		20	200	1	0.01	0.00	0.03	85	-	114
	Photometric + Visual	25	200	35	0.18	0.00	0.64	163	-	2
		30	200	141	0.71	0.34	1.00	59	-	0
		35	200	200	1.00	0.98	1.00	0	-	0
		40	200	200	1.00	0.98	1.00	0	-	0
		4	42	0	0.00	-	-	0	0.00	42
		5	42	0	0.00	-	-	0	0.00	42
		6	42	0	0.00	-	-	4	0.10	38
	Photometric	7	42	1	0.02	-	-	41	1.00	0
		8	42	28	0.67	-	-	14	1.00	0
		9	42	42	1.00	-	-	0	1.00	0
		10	42	42	1.00	-	-	0	1.00	0
		4	63	0	0.00	-	-	2	0.03	61
		5	63	0	0.00	-	-	6	0.10	57
		6	63	0	0.00	-	-	16	0.25	47
Cefazolin	Visual	7	63	4	0.06	-	-	58	0.98	1
		8	63	35	0.56	-	-	28	1.00	0
		9	63	63	1.00	-	-	0	1.00	0
		10	63	63	1.00	-	-	0	1.00	0
		4	105	0	0.00	0.00	0.04	2	-	103
		5	105	0	0.00	0.00	0.04	6	-	99
		6	105	0	0.00	0.00	0.04	20	-	85
	Photometric + Visual	7	105	5	0.05	0.02	0.11	99	-	1
		8	105	63	0.60	0.16	1.00	42	-	0
		9	105	105	1.00	0.97	1.00	0	-	0
		10	105	105	1.00	0.97	1.00	0	-	0
		100	42	0	0.00	-	-	0	0.00	42
		200	42	0	0.00	-	-	7	0.17	35
		300	42	0	0.00	-	-	42	1.00	0
	Photometric	400	42	14	0.33	-	-	28	1.00	0
		500	42	42	1.00	-	-	0	1.00	0
		600	42	42	1.00	-	-	0	1.00	0
		700	42	42	1.00	-	-	0	1.00	0
		100	63	0	0.00	-	-	5	0.08	58
		200	63	0	0.00	-	-	40	0.63	23
		300	63	8	0.13	-	-	55	1.00	0
Cefquinome	Visual	400	63	41	0.65	-	-	22	1.00	0
		500	63	49	0.78	-	-	14	1.00	0
		600	63	63	1.00	-	-	0	1.00	0
		700	63	63	1.00	-	-	0	1.00	0
		100	105	0	0.00	0.00	0.04	5	-	100
		200	105	0	0.00	0.00	0.04	47	-	58
		300	105	8	0.08	0.04	0.14	97	-	0
	Photometric + Visual	400	105	55	0.52	0.00	1.00	50	-	0
		500	105	91	0.87	0.79	0.92	14	-	0
		600	105	105	1.00	0.97	1.00	0	-	0
		700	105	105	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)
Ceftiofur	Photometric	100	128	7	0.05	-	-	101	0.84	20
		150	128	64	0.50	-	-	62	0.98	2
		200	128	122	0.95	-	-	6	1.00	0
		250	128	128	1.00	-	-	0	1.00	0
		300	128	128	1.00	-	-	0	1.00	0
		360	128	128	1.00	-	-	0	1.00	0
		460	128	128	1.00	-	-	0	1.00	0
	Visual	100	192	2	0.01	-	-	118	0.63	72
		150	192	80	0.42	-	-	81	0.84	31
		200	192	175	0.91	-	-	17	1.00	0
		250	192	186	0.97	-	-	6	1.00	0
		300	192	192	1.00	-	-	0	1.00	0
		360	192	192	1.00	-	-	0	1.00	0
		460	192	192	1.00	-	-	0	1.00	0
	Photometric + Visual	100	320	9	0.03	0.01	0.05	219	-	92
		150	320	144	0.45	0.26	0.64	143	-	33
		200	320	297	0.93	0.89	0.95	23	-	0
		250	320	314	0.98	0.96	0.99	6	-	0
		300	320	320	1.00	0.99	1.00	0	-	0
		360	320	320	1.00	0.99	1.00	0	-	0
		460	320	320	1.00	0.99	1.00	0	-	0
Cefalonium	Photometric	6	80	0	0.00	-	-	0	0.00	80
		8	80	0	0.00	-	-	0	0.00	80
		10	80	0	0.00	-	-	6	0.08	74
		12	80	1	0.01	-	-	79	1.00	0
		14	80	79	0.99	-	-	1	1.00	0
		16	80	80	1.00	-	-	0	1.00	0
		20	80	80	1.00	-	-	0	1.00	0
	Visual	6	120	0	0.00	-	-	1	0.01	119
		8	120	0	0.00	-	-	10	0.08	110
		10	120	0	0.00	-	-	43	0.36	77
		12	120	25	0.21	-	-	93	0.98	2
		14	120	103	0.86	-	-	17	1.00	0
		16	120	120	1.00	-	-	0	1.00	0
		20	120	120	1.00	-	-	0	1.00	0
	Photometric + Visual	6	200	0	0.00	0.00	0.02	1	-	199
		8	200	0	0.00	0.00	0.02	10	-	190
		10	200	0	0.00	0.00	0.02	49	-	151
		12	200	26	0.13	0.09	0.18	172	-	2
		14	200	182	0.91	0.86	0.94	18	-	0
		16	200	200	1.00	0.98	1.00	0	-	0
		20	200	200	1.00	0.98	1.00	0	-	0
Erythromycin	Photometric	30	128	0	0.00	-	-	32	0.25	96
		40	128	0	0.00	-	-	93	0.73	35
		50	128	5	0.04	-	-	123	1.00	0
		60	128	13	0.10	-	-	115	1.00	0
		80	128	119	0.93	-	-	9	1.00	0
		100	128	128	1.00	-	-	0	1.00	0
		120	128	128	1.00	-	-	0	1.00	0
	Visual	30	192	2	0.01	-	-	30	0.17	160
		40	192	6	0.03	-	-	142	0.77	44
		50	192	22	0.11	-	-	154	0.92	16
		60	192	40	0.21	-	-	151	0.99	1
		80	192	162	0.84	-	-	30	1.00	0
		100	192	192	1.00	-	-	0	1.00	0
		120	192	192	1.00	-	-	0	1.00	0
	Photometric + Visual	30	320	2	0.01	0.00	0.02	62	-	256
		40	320	6	0.02	0.01	0.04	235	-	79
		50	320	27	0.08	0.06	0.12	277	-	16
		60	320	53	0.17	0.00	0.42	266	-	1
		80	320	281	0.88	0.84	0.91	39	-	0
		100	320	320	1.00	0.99	1.00	0	-	0
		120	320	320	1.00	0.99	1.00	0	-	0
Tylosin	Photometric	20	128	0	0.00	-	-	0	0.00	128
		30	128	0	0.00	-	-	106	0.83	22
		40	128	2	0.02	-	-	126	1.00	0
		50	128	1	0.01	-	-	127	1.00	0
		75	128	126	0.98	-	-	2	1.00	0
		100	128	128	1.00	-	-	0	1.00	0
		120	128	128	1.00	-	-	0	1.00	0
	Visual	20	192	0	0.00	-	-	25	0.13	167
		30	192	1	0.01	-	-	189	0.99	2
		40	192	24	0.13	-	-	168	1.00	0
		50	192	59	0.31	-	-	133	1.00	0
		75	192	147	0.77	-	-	45	1.00	0
		100	192	192	1.00	-	-	0	1.00	0
		120	192	192	1.00	-	-	0	1.00	0
	Photometric + Visual	20	320	0	0.00	0.00	0.01	25	-	295
		30	320	1	0.00	0.00	0.02	295	-	24
		40	320	26	0.08	0.06	0.12	294	-	0
		50	320	60	0.19	0.00	0.70	260	-	0
		75	320	273	0.85	0.81	0.89	47	-	0
		100	320	320	1.00	0.99	1.00	0	-	0
		120	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)
Sulfadiazine	Photometric	50	128	0	0.00	-	-	12	0.09	116
		100	128	0	0.00	-	-	123	0.96	5
		200	128	3	0.02	-	-	125	1.00	0
		300	128	0	0.00	-	-	128	1.00	0
		400	128	12	0.09	-	-	116	1.00	0
		600	128	49	0.38	-	-	79	1.00	0
		800	128	95	0.74	-	-	33	1.00	0
	Visual	50	192	0	0.00	-	-	44	0.23	148
		100	192	10	0.05	-	-	176	0.97	6
		200	192	77	0.40	-	-	115	1.00	0
		300	192	109	0.57	-	-	83	1.00	0
		400	192	128	0.67	-	-	64	1.00	0
		600	192	128	0.67	-	-	64	1.00	0
		800	192	130	0.68	-	-	62	1.00	0
	Photometric + Visual	50	320	0	0.00	0.00	0.01	56	-	264
		100	320	10	0.03	0.02	0.06	299	-	11
		200	320	80	0.25	0.00	0.65	240	-	0
		300	320	109	0.34	0.00	0.92	211	-	0
		400	320	140	0.44	0.00	1.00	180	-	0
		600	320	177	0.55	0.01	1.00	143	-	0
		800	320	225	0.70	0.21	1.00	95	-	0
Sulfadi-methoxin	Photometric	50	128	0	0.00	-	-	8	0.06	120
		100	128	0	0.00	-	-	116	0.91	12
		200	128	0	0.00	-	-	128	1.00	0
		300	128	13	0.10	-	-	115	1.00	0
		400	128	27	0.21	-	-	101	1.00	0
		600	128	73	0.57	-	-	55	1.00	0
		800	128	102	0.80	-	-	26	1.00	0
	Visual	50	192	0	0.00	-	-	53	0.28	139
		100	192	4	0.02	-	-	173	0.92	15
		200	192	68	0.35	-	-	122	0.99	2
		300	192	126	0.66	-	-	66	1.00	0
		400	192	128	0.67	-	-	64	1.00	0
		600	192	127	0.66	-	-	65	1.00	0
		800	192	128	0.67	-	-	64	1.00	0
	Photometric + Visual	50	320	0	0.00	0.00	0.01	61	-	259
		100	320	4	0.01	0.00	0.03	289	-	27
		200	320	68	0.21	0.00	0.58	250	-	2
		300	320	139	0.43	0.00	1.00	181	-	0
		400	320	155	0.48	0.00	1.00	165	-	0
		600	320	200	0.63	0.11	1.00	120	-	0
		800	320	230	0.72	0.20	1.00	90	-	0
Sulfametha-zine	Photometric	50	42	0	0.00	-	-	0	0.00	42
		100	42	0	0.00	-	-	8	0.19	34
		200	42	0	0.00	-	-	39	0.93	3
		300	42	0	0.00	-	-	42	1.00	0
		500	42	5	0.12	-	-	37	1.00	0
		750	42	14	0.33	-	-	28	1.00	0
		1,000	42	41	0.98	-	-	1	1.00	0
	Visual	50	63	0	0.00	-	-	6	0.10	57
		100	63	0	0.00	-	-	54	0.86	9
		200	63	7	0.11	-	-	55	0.98	1
		300	63	16	0.25	-	-	47	1.00	0
		500	63	44	0.70	-	-	19	1.00	0
		750	63	42	0.67	-	-	21	1.00	0
		1,000	63	42	0.67	-	-	21	1.00	0
	Photometric + Visual	50	105	0	0.00	0.00	0.04	6	-	99
		100	105	0	0.00	0.00	0.04	62	-	43
		200	105	7	0.07	0.03	0.13	94	-	4
		300	105	16	0.15	0.00	0.47	89	-	0
		500	105	49	0.47	0.00	1.00	56	-	0
		750	105	56	0.53	0.00	1.00	49	-	0
		1,000	105	83	0.79	0.24	1.00	22	-	0
Sulfathiazol	Photometric	40	128	0	0.00	-	-	86	0.67	42
		60	128	0	0.00	-	-	126	0.98	2
		80	128	1	0.01	-	-	127	1.00	0
		100	128	5	0.04	-	-	123	1.00	0
		200	128	106	0.83	-	-	22	1.00	0
		400	128	126	0.98	-	-	2	1.00	0
		600	128	125	0.98	-	-	3	1.00	0
	Visual	40	192	1	0.01	-	-	147	0.77	44
		60	192	13	0.07	-	-	178	0.99	1
		80	192	35	0.18	-	-	157	1.00	0
		100	192	67	0.35	-	-	125	1.00	0
		200	192	180	0.94	-	-	12	1.00	0
		400	192	189	0.98	-	-	3	1.00	0
		600	192	187	0.97	-	-	5	1.00	0
	Photometric + Visual	40	320	1	0.00	0.00	0.02	233	-	86
		60	320	13	0.04	0.02	0.07	304	-	3
		80	320	36	0.11	0.08	0.15	284	-	0
		100	320	72	0.23	0.00	0.61	248	-	0
		200	320	286	0.89	0.86	0.92	34	-	0
		400	320	315	0.98	0.96	0.99	5	-	0
		600	320	312	0.98	0.95	0.99	8	-	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	42	0	0.00	-	-	5	0.12	37
		200	42	0	0.00	-	-	23	0.55	19
		400	42	0	0.00	-	-	42	1.00	0
	Photometric	600	42	0	0.00	-	-	42	1.00	0
		800	42	0	0.00	-	-	42	1.00	0
		1,000	42	9	0.21	-	-	33	1.00	0
		1,500	42	25	0.60	-	-	17	1.00	0
		100	63	0	0.00	-	-	41	0.65	22
		200	63	1	0.02	-	-	56	0.90	6
		400	63	7	0.11	-	-	56	1.00	0
Sulfadoxin	Visual	600	63	33	0.52	-	-	30	1.00	0
		800	63	41	0.65	-	-	22	1.00	0
		1,000	63	40	0.63	-	-	23	1.00	0
		1,500	63	41	0.65	-	-	22	1.00	0
		100	105	0	0.00	0.00	0.04	46	-	59
		200	105	1	0.01	0.00	0.05	79	-	25
	Photometric + Visual	400	105	7	0.07	0.03	0.13	98	-	0
		600	105	33	0.31	0.00	0.85	72	-	0
		800	105	41	0.39	0.00	1.00	64	-	0
		1,000	105	49	0.47	0.00	1.00	56	-	0
		1,500	105	66	0.63	0.14	1.00	39	-	0
		50	128	1	0.01	-	-	99	0.78	28
		100	128	0	0.00	-	-	126	0.98	2
		200	128	24	0.19	-	-	104	1.00	0
	Photometric	300	128	107	0.84	-	-	21	1.00	0
		500	128	128	1.00	-	-	0	1.00	0
		750	128	127	0.99	-	-	0	0.99	1
		1,000	128	127	0.99	-	-	1	1.00	0
		50	192	2	0.01	-	-	119	0.63	71
		100	192	9	0.05	-	-	177	0.97	6
		200	192	107	0.56	-	-	85	1.00	0
Sulfamethoxy-pyridazine	Visual	300	192	176	0.92	-	-	16	1.00	0
		500	192	192	1.00	-	-	0	1.00	0
		750	192	191	0.99	-	-	1	1.00	0
		1,000	192	192	1.00	-	-	0	1.00	0
		50	320	3	0.01	0.00	0.03	218	-	99
		100	320	9	0.03	0.01	0.05	303	-	8
		200	320	131	0.41	0.01	0.80	189	-	0
	Photometric + Visual	300	320	283	0.88	0.84	0.92	37	-	0
		500	320	320	1.00	0.99	1.00	0	-	0
		750	320	318	0.99	0.98	1.00	1	-	1
		1,000	320	319	1.00	0.98	1.00	1	-	0
		100	42	0	0.00	-	-	0	0.00	42
		200	42	0	0.00	-	-	0	0.00	42
		300	42	0	0.00	-	-	4	0.10	38
	Photometric	400	42	0	0.00	-	-	17	0.40	25
		600	42	0	0.00	-	-	32	0.76	10
		800	42	2	0.05	-	-	38	0.95	2
		1,000	42	28	0.67	-	-	14	1.00	0
		100	63	0	0.00	-	-	12	0.19	51
		200	63	0	0.00	-	-	12	0.19	51
		300	63	0	0.00	-	-	23	0.37	40
Chlortetra-cycline	Visual	400	63	0	0.00	-	-	36	0.57	27
		600	63	25	0.40	-	-	31	0.89	7
		800	63	27	0.43	-	-	36	1.00	0
		1,000	63	49	0.78	-	-	14	1.00	0
		100	105	0	0.00	0.00	0.04	12	-	93
		200	105	0	0.00	0.00	0.04	12	-	93
		300	105	0	0.00	0.00	0.04	27	-	78
	Photometric + Visual	400	105	0	0.00	0.00	0.04	53	-	52
		600	105	25	0.24	0.00	0.63	63	-	17
		800	105	29	0.28	0.00	0.68	74	-	2
		1,000	105	77	0.73	0.41	1.00	28	-	0
		100	42	0	0.00	-	-	0	0.00	42
		200	42	0	0.00	-	-	9	0.21	33
		300	42	1	0.02	-	-	28	0.69	13
	Photometric	400	42	0	0.00	-	-	42	1.00	0
		600	42	29	0.69	-	-	13	1.00	0
		800	42	42	1.00	-	-	0	1.00	0
		1,000	42	42	1.00	-	-	0	1.00	0
		100	63	0	0.00	-	-	3	0.05	60
		200	63	0	0.00	-	-	34	0.54	29
		300	63	3	0.05	-	-	51	0.86	9
Oxytetra-cycline	Visual	400	63	13	0.21	-	-	50	1.00	0
		600	63	52	0.83	-	-	11	1.00	0
		800	63	63	1.00	-	-	0	1.00	0
		1,000	63	63	1.00	-	-	0	1.00	0
		100	105	0	0.00	0.00	0.04	3	-	102
		200	105	0	0.00	0.00	0.04	43	-	62
		300	105	4	0.04	0.01	0.09	79	-	22
	Photometric + Visual	400	105	13	0.12	0.07	0.20	92	-	0
		600	105	81	0.77	0.64	0.90	24	-	0
		800	105	105	1.00	0.97	1.00	0	-	0
		1,000	105	105	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)
Tetracycline	Photometric	100	42	0	0.00	-	-	0	0.00	42
		200	42	0	0.00	-	-	0	0.00	42
		300	42	0	0.00	-	-	5	0.12	37
		400	42	0	0.00	-	-	15	0.36	27
		600	42	0	0.00	-	-	42	1.00	0
		800	42	21	0.50	-	-	21	1.00	0
		1,000	42	42	1.00	-	-	0	1.00	0
	Visual	100	63	0	0.00	-	-	6	0.10	57
		200	63	0	0.00	-	-	16	0.25	47
		300	63	0	0.00	-	-	39	0.62	24
		400	63	0	0.00	-	-	49	0.78	14
		600	63	29	0.46	-	-	34	1.00	0
		800	63	49	0.78	-	-	14	1.00	0
		1,000	63	63	1.00	-	-	0	1.00	0
	Photometric + Visual	100	105	0	0.00	0.00	0.04	6	-	99
		200	105	0	0.00	0.00	0.04	16	-	89
		300	105	0	0.00	0.00	0.04	44	-	61
		400	105	0	0.00	0.00	0.04	64	-	41
		600	105	29	0.28	0.00	0.76	76	-	0
		800	105	70	0.67	0.27	1.00	35	-	0
		1,000	105	105	1.00	0.97	1.00	0	-	0
Dihydro-streptomycin	Photometric	200	42	0	0.00	-	-	1	0.02	41
		300	42	0	0.00	-	-	11	0.26	31
		400	42	7	0.17	-	-	35	1.00	0
		500	42	13	0.31	-	-	29	1.00	0
		600	42	42	1.00	-	-	0	1.00	0
		700	42	42	1.00	-	-	0	1.00	0
		800	42	42	1.00	-	-	0	1.00	0
	Visual	200	63	0	0.00	-	-	26	0.41	37
		300	63	0	0.00	-	-	55	0.87	8
		400	63	33	0.52	-	-	30	1.00	0
		500	63	63	1.00	-	-	0	1.00	0
		600	63	63	1.00	-	-	0	1.00	0
		700	63	63	1.00	-	-	0	1.00	0
		800	63	63	1.00	-	-	0	1.00	0
	Photometric + Visual	200	105	0	0.00	0.00	0.04	27	-	78
		300	105	0	0.00	0.00	0.04	66	-	39
		400	105	40	0.38	0.00	0.81	65	-	0
		500	105	76	0.72	0.26	1.00	29	-	0
		600	105	105	1.00	0.97	1.00	0	-	0
		700	105	105	1.00	0.97	1.00	0	-	0
		800	105	105	1.00	0.97	1.00	0	-	0
Streptomycin	Photometric	300	42	0	0.00	-	-	0	0.00	42
		400	42	0	0.00	-	-	2	0.05	40
		500	42	0	0.00	-	-	30	0.71	12
		600	42	0	0.00	-	-	42	1.00	0
		800	42	2	0.05	-	-	40	1.00	0
		1,000	42	36	0.86	-	-	6	1.00	0
		1,500	42	42	1.00	-	-	0	1.00	0
	Visual	300	63	1	0.02	-	-	7	0.13	55
		400	63	0	0.00	-	-	21	0.33	42
		500	63	4	0.06	-	-	54	0.92	5
		600	63	0	0.00	-	-	63	1.00	0
		800	63	22	0.35	-	-	41	1.00	0
		1,000	63	44	0.70	-	-	19	1.00	0
		1,500	63	63	1.00	-	-	0	1.00	0
	Photometric + Visual	300	105	1	0.01	0.00	0.05	7	-	97
		400	105	0	0.00	0.00	0.04	23	-	82
		500	105	4	0.04	0.01	0.09	84	-	17
		600	105	0	0.00	0.00	0.04	105	-	0
		800	105	24	0.23	0.00	0.64	81	-	0
		1,000	105	80	0.76	0.28	1.00	25	-	0
		1,500	105	105	1.00	0.97	1.00	0	-	0
Gentamicin	Photometric	40	128	0	0.00	-	-	1	0.01	127
		60	128	0	0.00	-	-	37	0.29	91
		80	128	0	0.00	-	-	121	0.95	7
		100	128	0	0.00	-	-	128	1.00	0
		125	128	8	0.06	-	-	120	1.00	0
		150	128	118	0.92	-	-	10	1.00	0
		200	128	128	1.00	-	-	0	1.00	0
	Visual	40	192	0	0.00	-	-	31	0.16	161
		60	192	0	0.00	-	-	159	0.83	33
		80	192	19	0.10	-	-	173	1.00	0
		100	192	79	0.41	-	-	113	1.00	0
		125	192	141	0.73	-	-	51	1.00	0
		150	192	192	1.00	-	-	0	1.00	0
		200	192	192	1.00	-	-	0	1.00	0
	Photometric + Visual	40	320	0	0.00	0.00	0.01	32	-	288
		60	320	0	0.00	0.00	0.01	196	-	124
		80	320	19	0.06	0.04	0.09	294	-	7
		100	320	79	0.25	0.00	0.74	241	-	0
		125	320	149	0.47	0.00	1.00	171	-	0
		150	320	310	0.97	0.94	0.98	10	-	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	42	0	0.00	-	-	0	0.00	42
		80	42	0	0.00	-	-	2	0.05	40
		100	42	0	0.00	-	-	1	0.02	41
		200	42	0	0.00	-	-	42	1.00	0
		300	42	32	0.76	-	-	10	1.00	0
		400	42	42	1.00	-	-	0	1.00	0
		500	42	42	1.00	-	-	0	1.00	0
	Visual	60	63	0	0.00	-	-	9	0.14	54
		80	63	0	0.00	-	-	34	0.54	29
		100	63	0	0.00	-	-	24	0.38	39
		200	63	15	0.24	-	-	48	1.00	0
		300	63	63	1.00	-	-	0	1.00	0
		400	63	63	1.00	-	-	0	1.00	0
		500	63	63	1.00	-	-	0	1.00	0
	Photometric + Visual	60	105	0	0.00	0.00	0.04	9	-	96
		80	105	0	0.00	0.00	0.04	36	-	69
		100	105	0	0.00	0.00	0.04	25	-	80
		200	105	15	0.14	0.09	0.22	90	-	0
		300	105	95	0.90	0.83	0.95	10	-	0
		400	105	105	1.00	0.97	1.00	0	-	0
		500	105	105	1.00	0.97	1.00	0	-	0
Chlor-amphenicol	Photometric	3,000	42	0	0.00	-	-	17	0.40	25
		3,500	42	0	0.00	-	-	29	0.69	13
		4,000	42	0	0.00	-	-	42	1.00	0
		4,500	42	2	0.05	-	-	40	1.00	0
		5,000	42	14	0.33	-	-	28	1.00	0
		6,000	42	39	0.93	-	-	3	1.00	0
		7,000	42	42	1.00	-	-	0	1.00	0
	Visual	3,000	63	1	0.02	-	-	37	0.60	25
		3,500	63	3	0.05	-	-	60	1.00	0
		4,000	63	20	0.32	-	-	43	1.00	0
		4,500	63	46	0.73	-	-	17	1.00	0
		5,000	63	63	1.00	-	-	0	1.00	0
		6,000	63	63	1.00	-	-	0	1.00	0
		7,000	63	63	1.00	-	-	0	1.00	0
	Photometric + Visual	3,000	105	1	0.01	0.00	0.05	54	-	50
		3,500	105	3	0.03	0.01	0.08	89	-	13
		4,000	105	20	0.19	0.00	0.59	85	-	0
		4,500	105	48	0.46	0.00	0.96	57	-	0
		5,000	105	77	0.73	0.29	1.00	28	-	0
		6,000	105	102	0.97	0.92	0.99	3	-	0
		7,000	105	105	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	2.5	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	3.5	1	A	8	0	0	
		1	B	16	0	0	1
		1	C	16	0	0	
		2	A	8	0	0	
		2	B	16	0	0	1
		2	C	16	0	0	
Amoxicillin	3	1	D	24	0	0	
		1	E	16	0	0	1
		1	F	24	0	0	
		2	D	24	0	0	
		2	E	16	0	0	1
		2	F	24	0	0	
Cloxacillin	25	1	D	16	0	0	
		1	E	22	2	0	0.33
		1	F	24	0	0	
		2	D	16	0	0	
		2	E	24	0	0	1
		2	F	24	0	0	
Dicloxacillin	15	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	
Nafcillin	15	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	
Oxacillin	10	1	A	16	0	0	
		1	B	7	1	0	0.2
		1	C	16	0	0	
		2	A	16	0	0	
		2	B	8	0	0	1
		2	C	16	0	0	
Cefalexin	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	
Cefapirin	6	1	A	7	0	0	
		1	B	7	0	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	
Cefoperazone	35	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	9	1	A	7	0	0	
		1	B	7	0	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	
Cefquinome	500	1	A	7	0	0	
		1	B	7	0	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	
Ceftiofur	200	1	D	21	3	0	
		1	E	24	0	0	0.11
		1	F	16	0	0	
		2	D	21	3	0	
		2	E	24	0	0	0.11
		2	F	16	0	0	
Cefalonium	14	1	A	8	0	0	
		1	B	16	0	0	1
		1	C	15	1	0	
		2	A	8	0	0	
		2	B	16	0	0	1
		2	C	16	0	0	
Erythromycin	100	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	75	1	A	16	0	0	
		1	B	24	0	0	1
		1	C	23	1	0	
		2	A	16	0	0	
		2	B	24	0	0	1
		2	C	23	1	0	
Sulfadiazine	NA	1	A	22	2	0	
		1	B	18	6	0	-
		1	C	5	11	0	
		2	A	23	1	0	
		2	B	20	4	0	-
		2	C	7	9	0	
Sulfadi-methoxin	NA	1	A	8	16	0	
		1	B	16	0	0	-
		1	C	23	1	0	
		2	A	15	9	0	
		2	B	16	0	0	-
		2	C	24	0	0	
Sulfamethazine	1,000	1	A	7	0	0	
		1	B	6	1	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	
Sulfathiazol	400	1	A	16	0	0	
		1	B	24	0	0	1
		1	C	24	0	0	
		2	A	14	2	0	
		2	B	24	0	0	0.06
		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	NA	1	A	1	6	0	
		1	B	7	0	0	-
		1	C	4	3	0	
		2	A	3	4	0	
		2	B	6	1	0	-
		2	C	4	3	0	
Sulfamethoxy-pyridazine	500	1	A	24	0	0	
		1	B	24	0	0	1
		1	C	16	0	0	
		2	A	24	0	0	
		2	B	24	0	0	1
		2	C	16	0	0	
Chlortetra-cycline	NA	1	A	7	0	0	
		1	B	0	7	0	-
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	0	7	0	-
		2	C	7	0	0	
Oxytetracycline	800	1	A	7	0	0	
		1	B	7	0	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	
Tetracycline	1,000	1	A	7	0	0	
		1	B	7	0	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	
Dihydro-streptomycin	600	1	A	7	0	0	
		1	B	7	0	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	
Streptomycin	1,500	1	A	7	0	0	
		1	B	7	0	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	
Gentamicin	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	
Neomycin	400	1	A	7	0	0	
		1	B	7	0	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	
Chlor-amphenicol	7,000	1	A	7	0	0	
		1	B	7	0	0	1
		1	C	7	0	0	
		2	A	7	0	0	
		2	B	7	0	0	1
		2	C	7	0	0	

Validierungsbericht
Validation Report

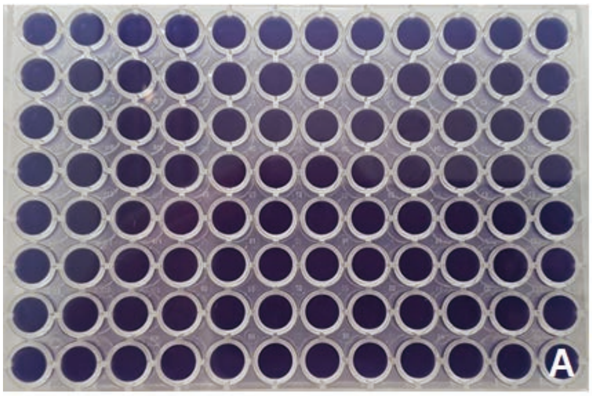
BRT MRL-Suchtest
BRT MRL-Screening Test

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.



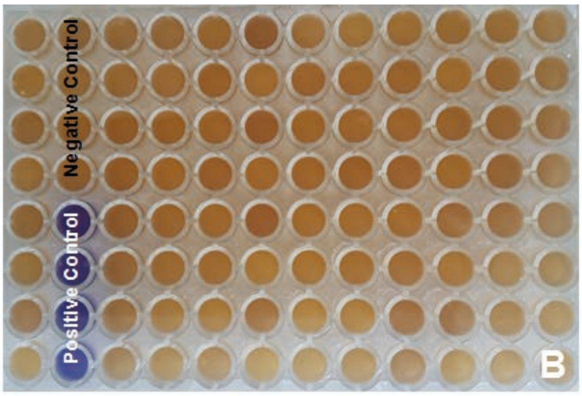


Figure 1. BRT MRL-Screening Test plates before (A) and after (B) incubation

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 °C 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:

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

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

Reading System	Classes of Results: 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 °C and used the next day. To verify the correct 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.

<u>Results and Discussion</u>

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

<u>Materials and Methods</u>

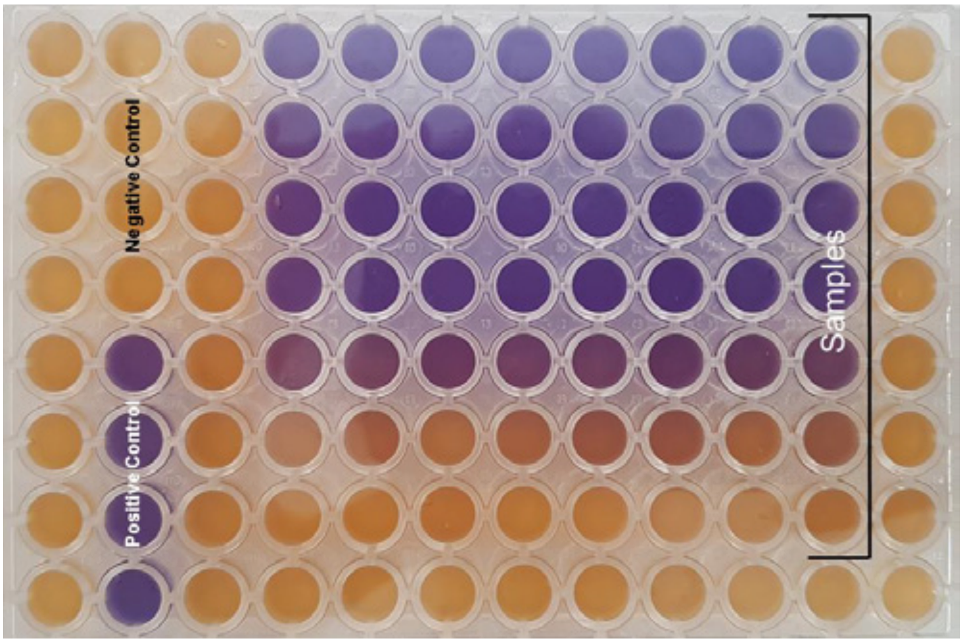


Figure 2. Incubated BRT MRL-Screening Test plate inoculated with four positive and four negative controls as well as raw milk samples spiked with 7 different concentrations of an antibiotic substance

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.

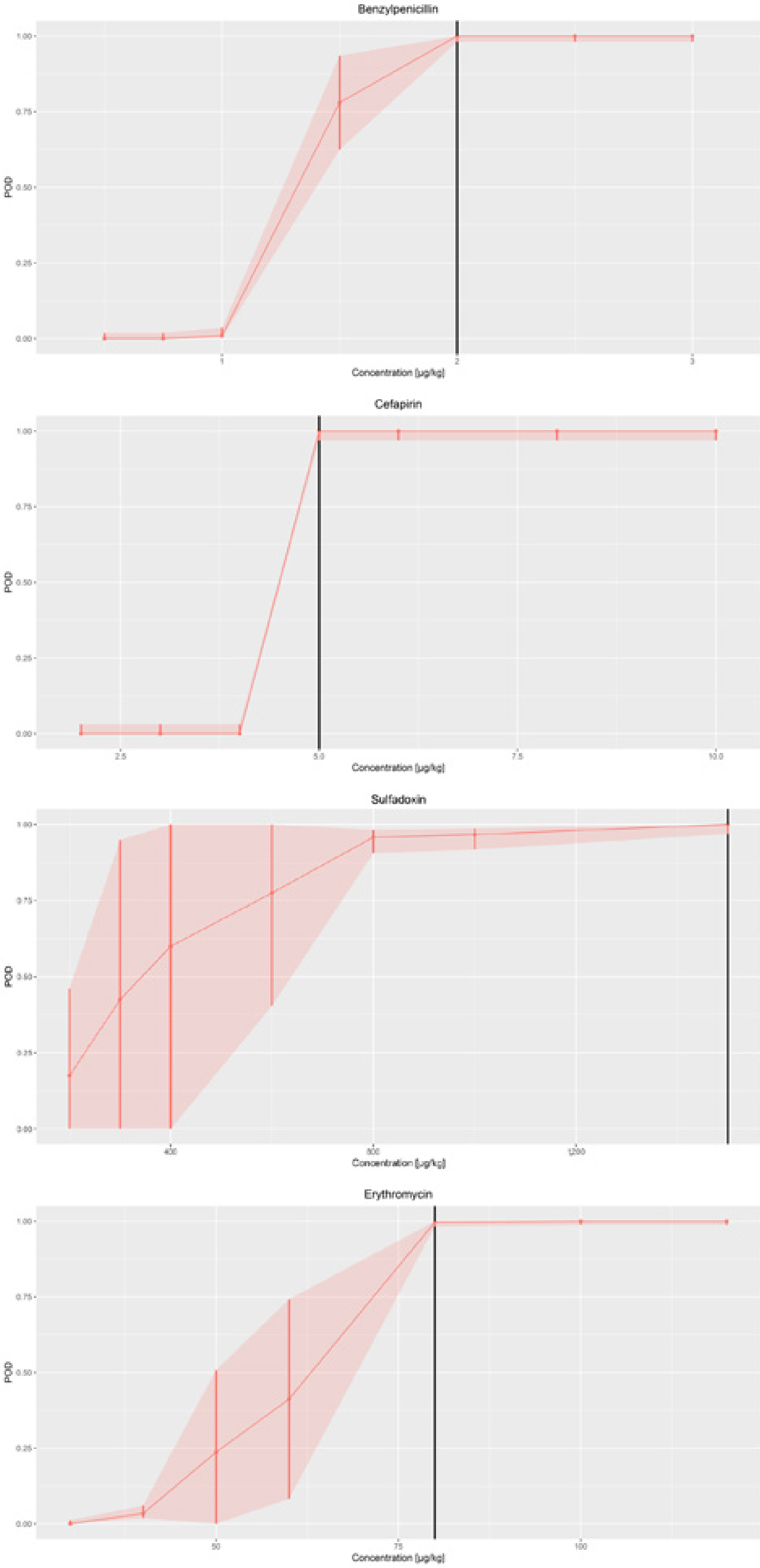


Figure 3. Dose-response curves of the bactericidal antibiotics Benzylpenicillin and Cefapirin and of the bacteriostatic substances Sulfadoxin and Erythromycin.
Red line = dose-response curve; red shade = CI; Black line = CCß A (photometric reading);

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 α = 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 ≤ 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.

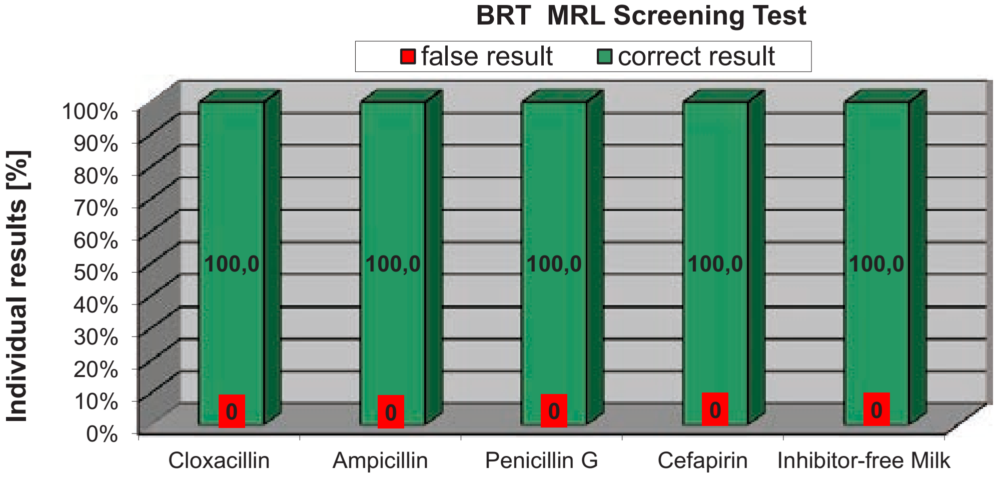


Figure 4. Rate of correct and false results (%) obtained by the interlaboratory study within the framework of the validation of the BRT MRL-Screening Test

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%).

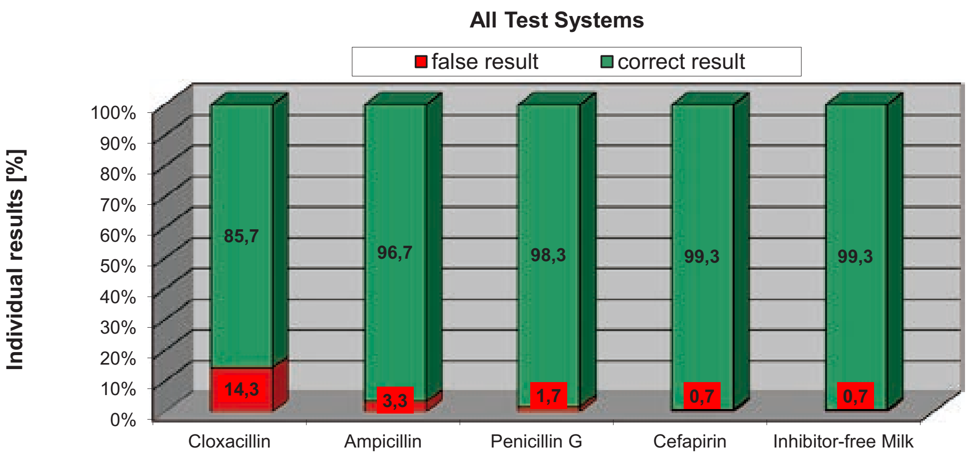


Figure 5. Rates of correct and false results (%) of all test systems (microbiological and receptor tests, 10th proficiency test)

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.

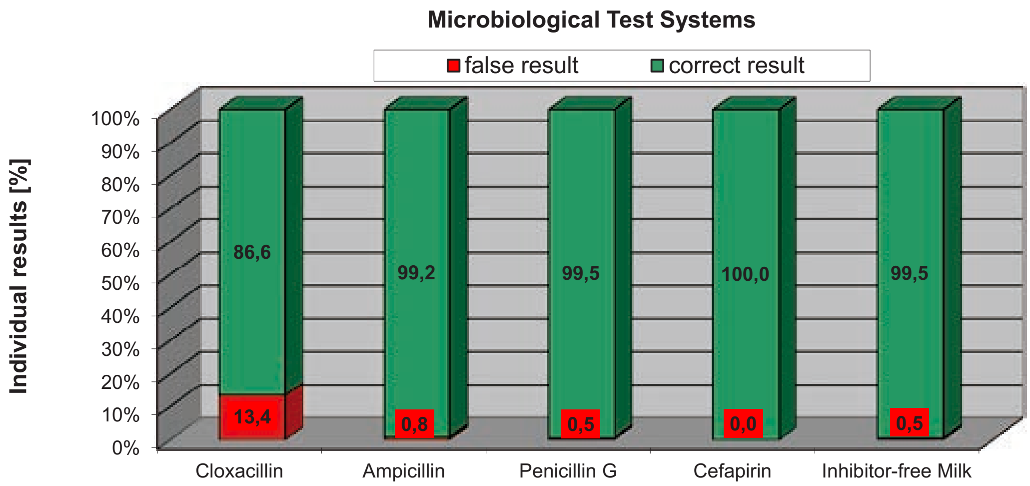


Figure 6. Rates of correct and false results (%) of microbiological test systems (10th proficiency test)

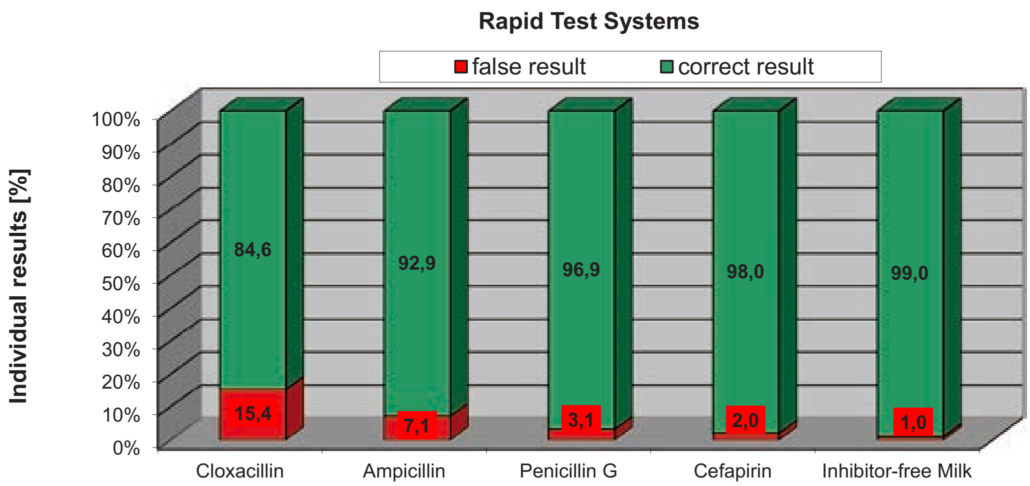


Figure 7. Rates of correct and false results (%) of receptor test systems (10th proficiency test)

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

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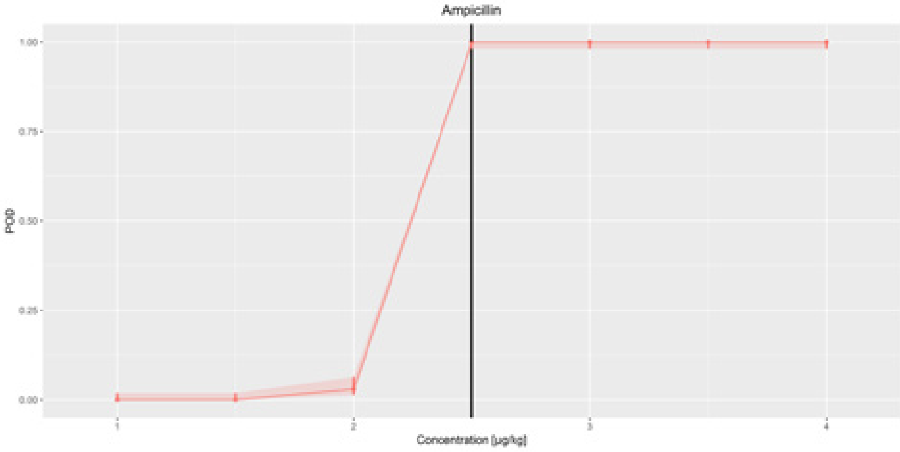
Date of publication: 09.11.2018

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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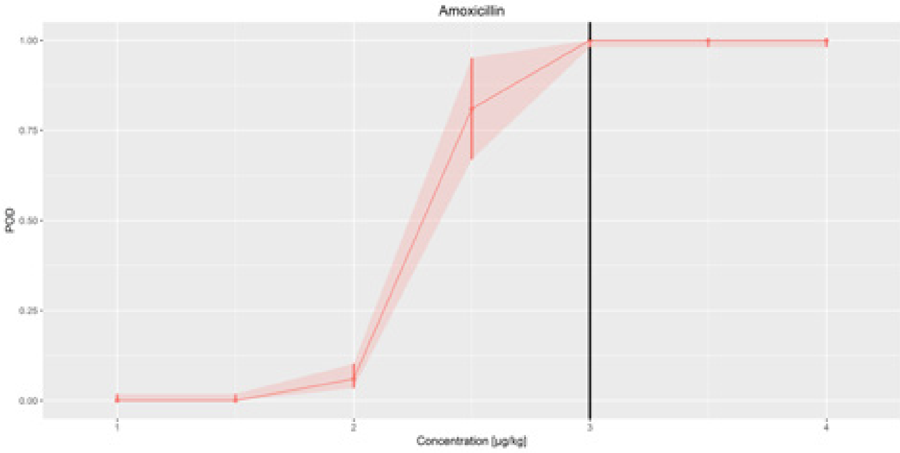
0.00
POD
1
2
3
4
Concentration [µg/kg]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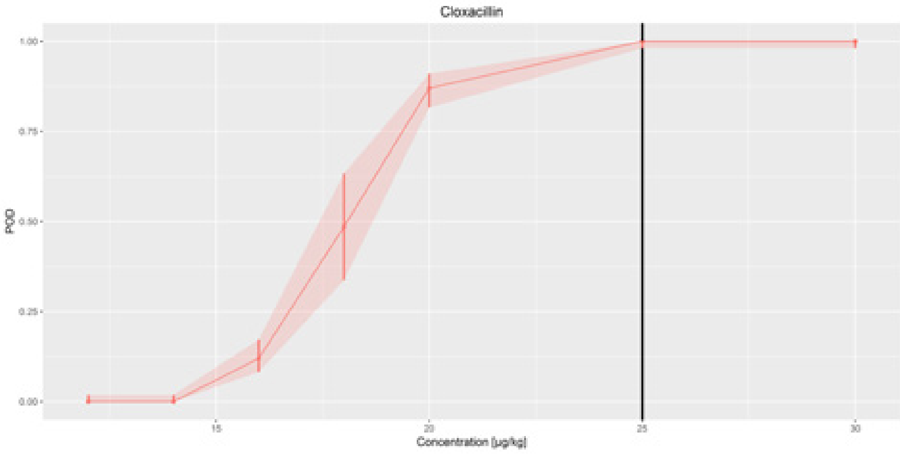
3
4
Concentration [µg/kg]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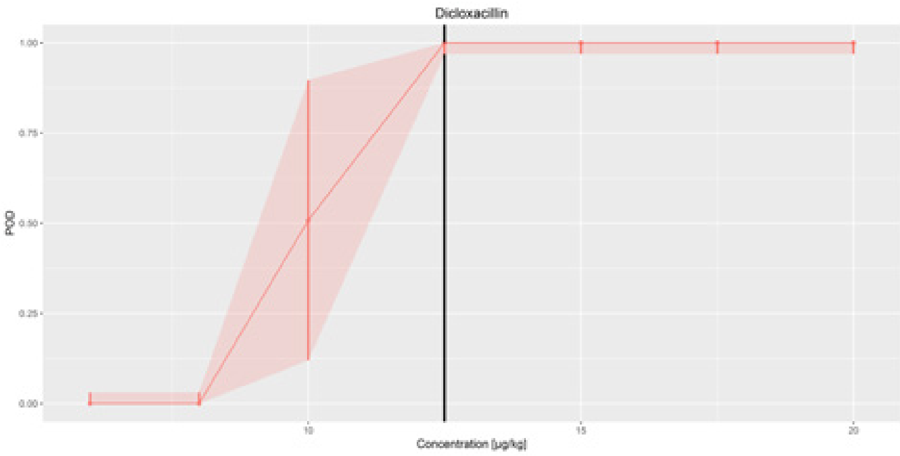
0.25
0.00
POD
15
20
25
30
Concentration [µg/kg]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0.00
POD
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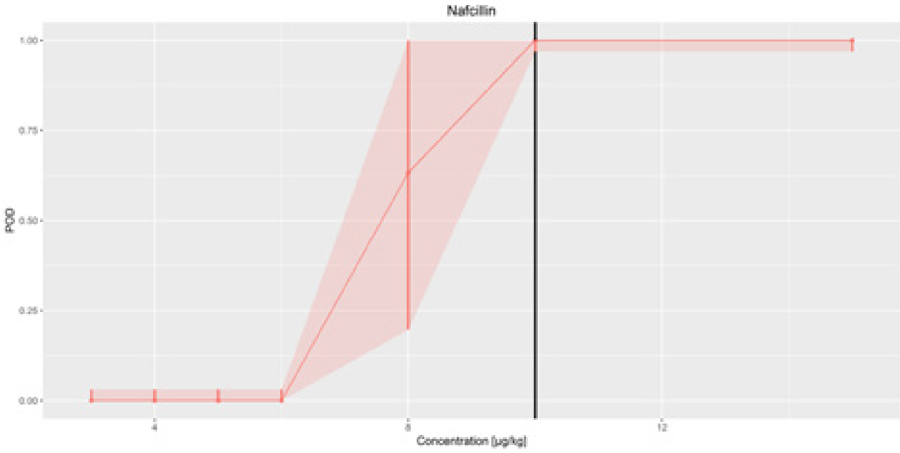
15
20
Concentration [µg/kg]



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POD
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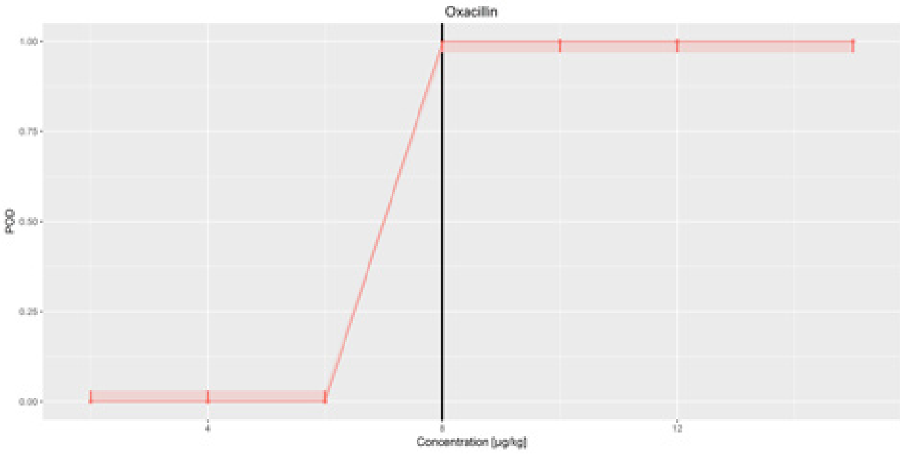
8
12
Concentration [µg/kg]



Oxacillin
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0.25
0.00
POD
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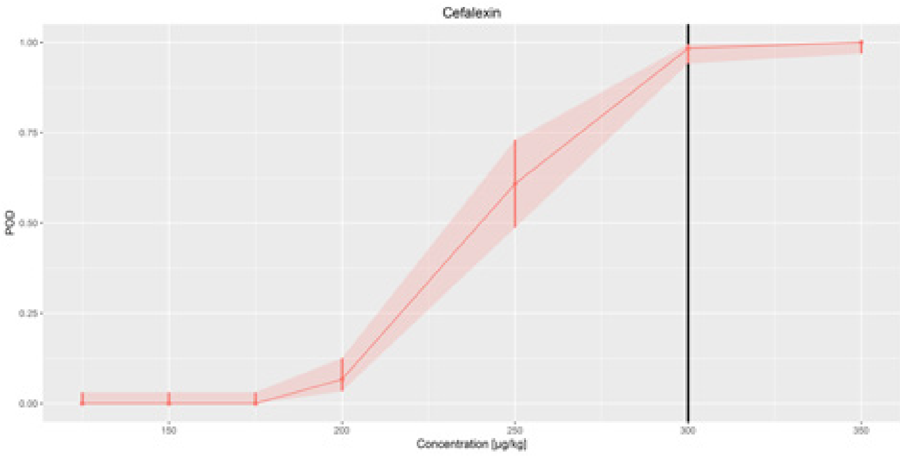
8
12
Concentration [µg/kg]



Cefalexin
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POD
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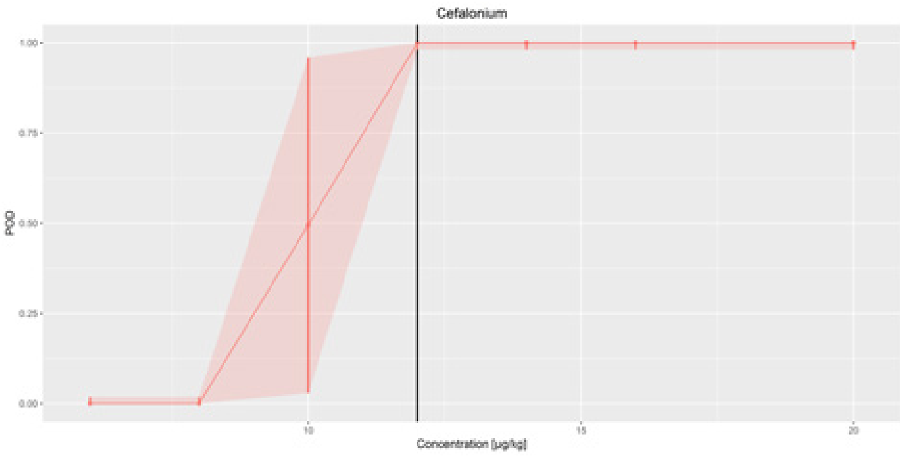
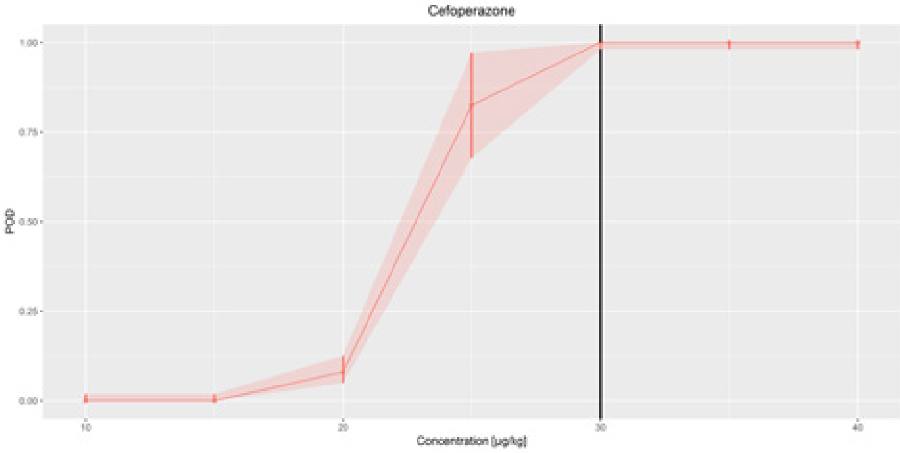
200
250
300
350
Concentration [µg/kg]



Cefalonium
1.00
0.75
0.50
0.25
0.00
POD
10
15
20
Concentration [µg/kg]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POD
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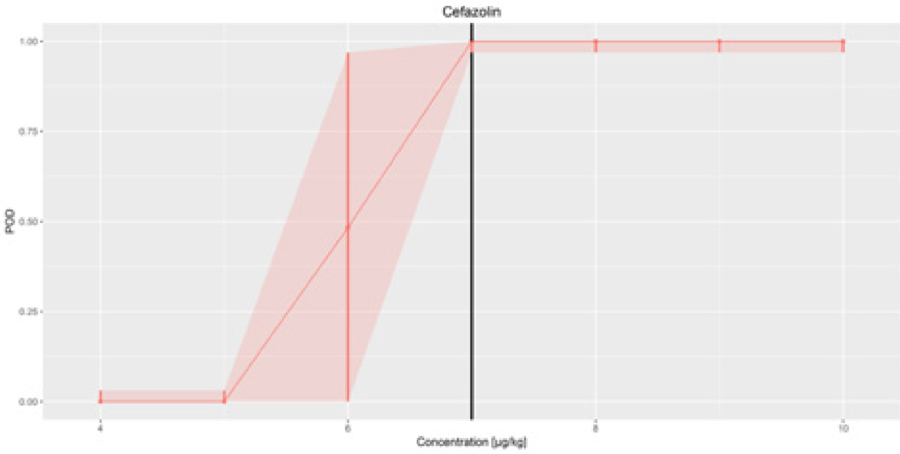
20
30
40
Concentration [µg/kg]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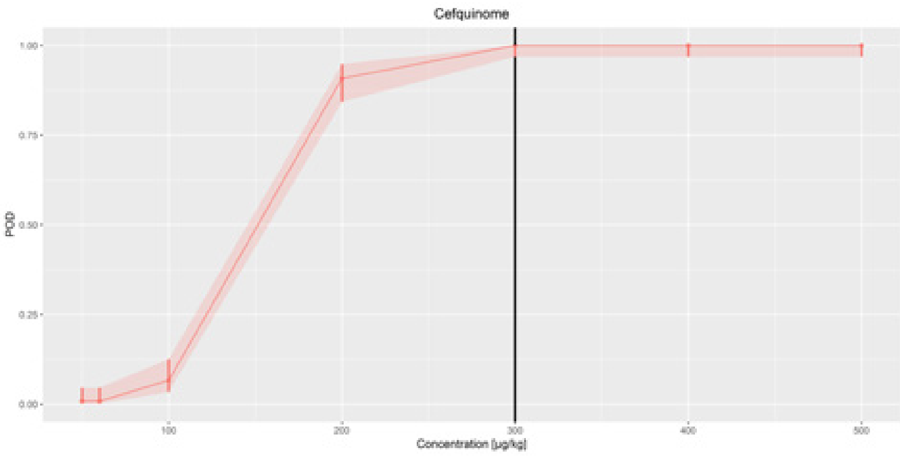
Concentration [µg/kg]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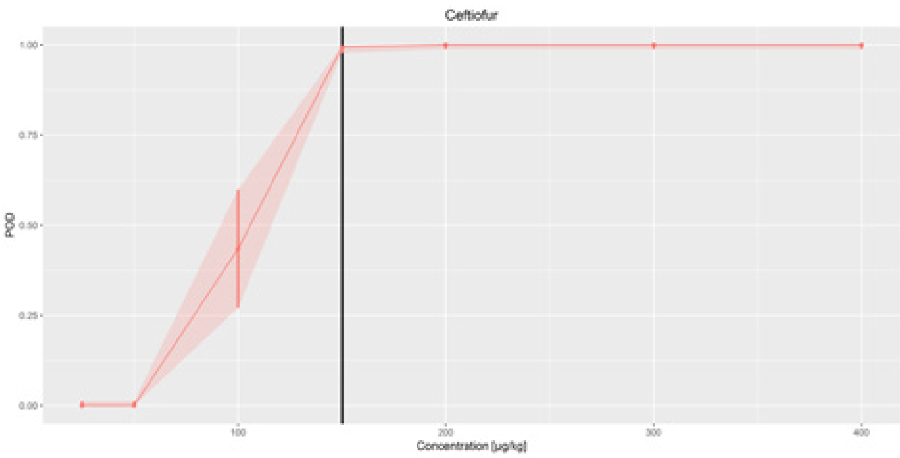
Concentration [µg/kg]


Ceftiofur
POD
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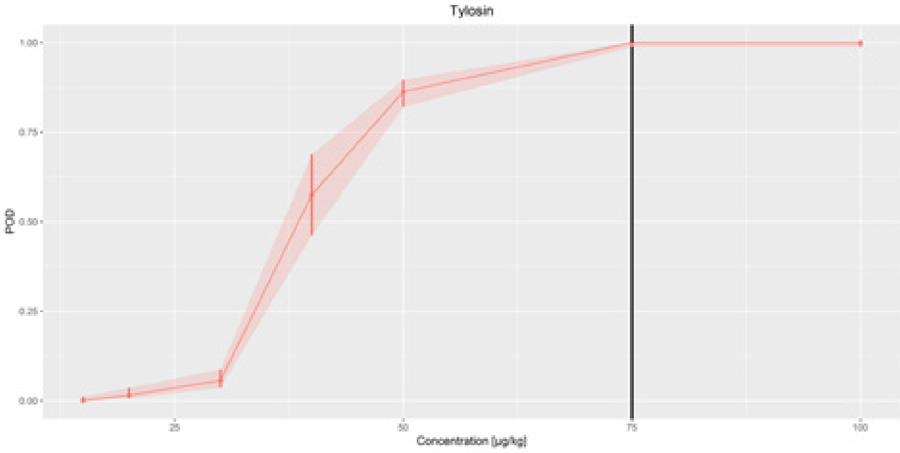
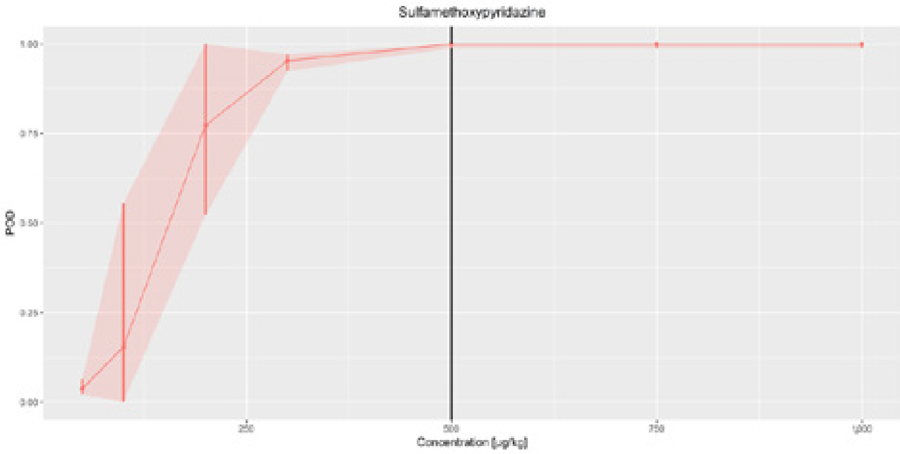
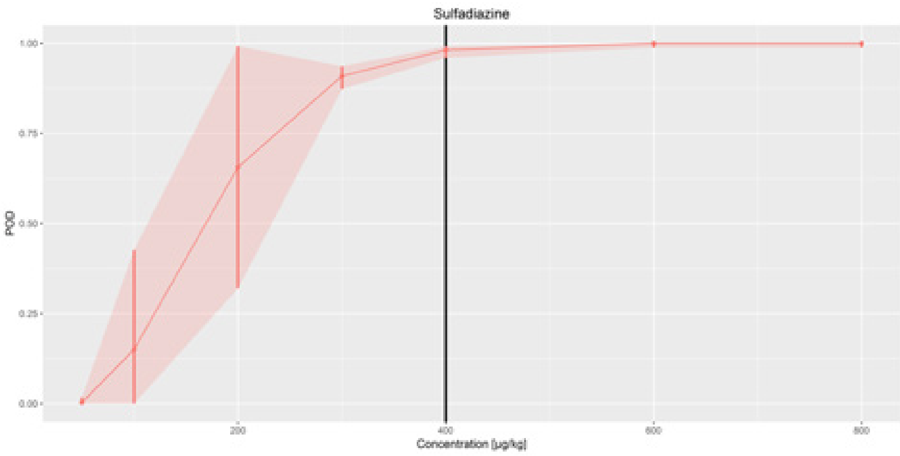
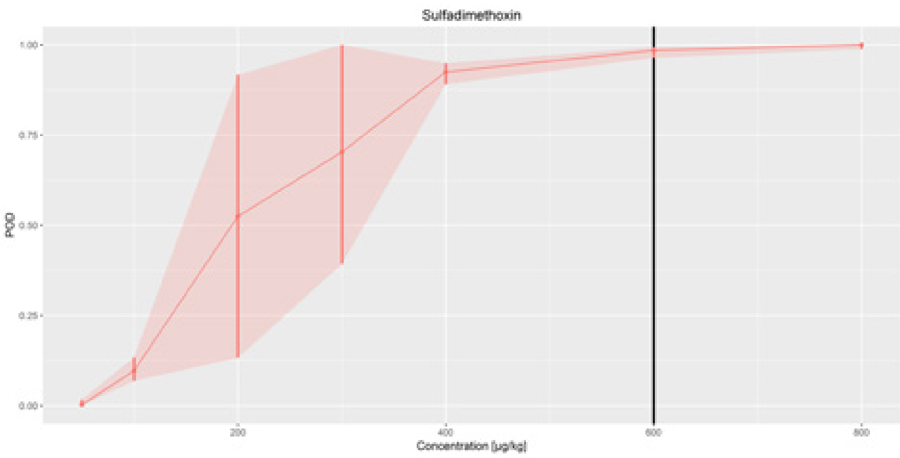
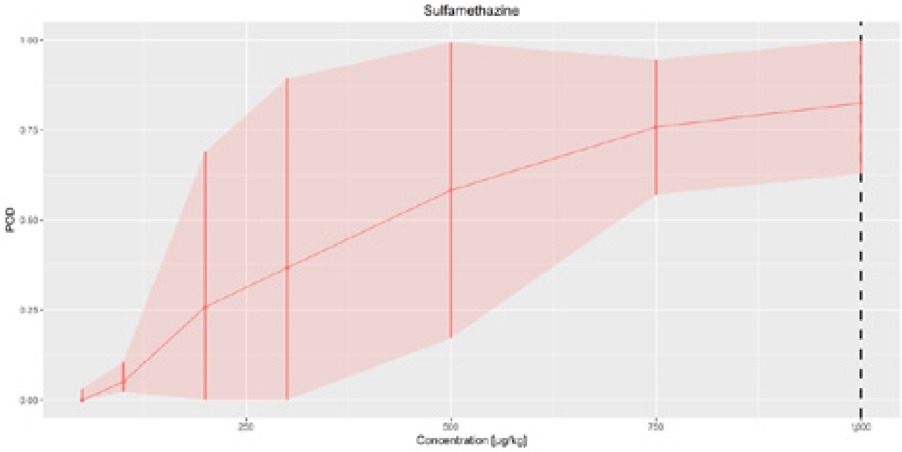
0.50
0.25
0.00
100
200
300
400
Concentration [µg/kg]

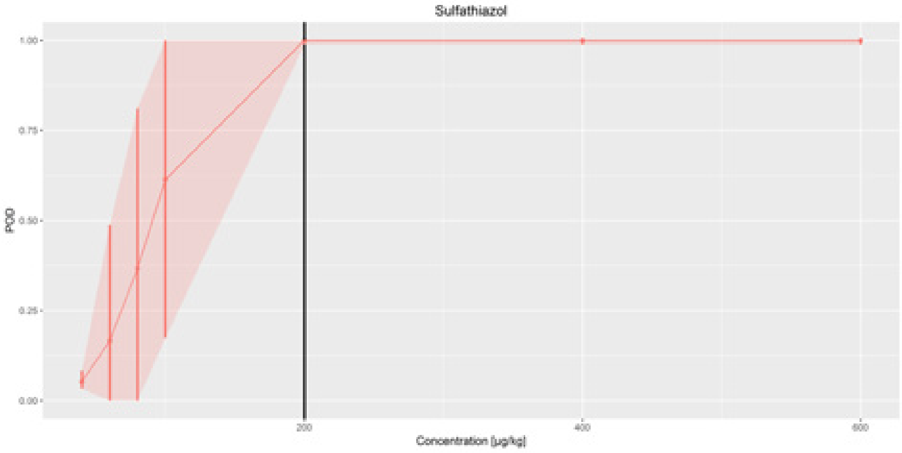

Tylosin
POD
1.00
0.75
0.50
0.25
0.00
25
50
75
100
Concentration [µg/kg]

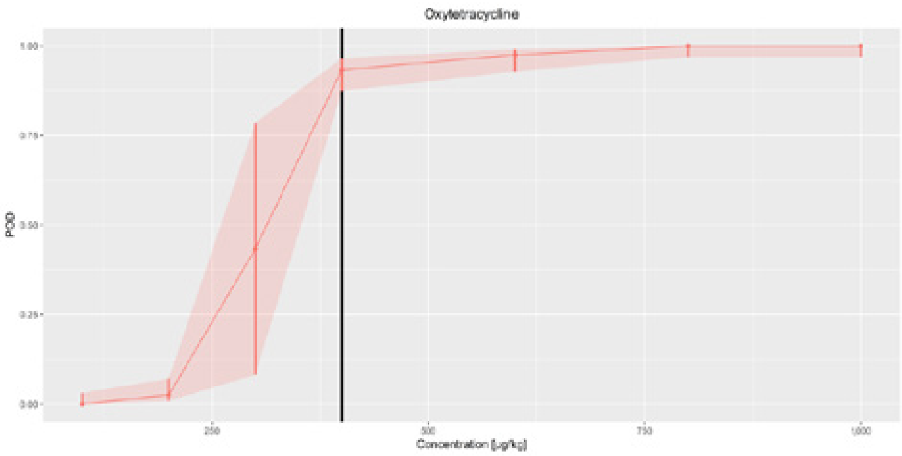

Sulfamethoxypyridazine
POD
1.00
0.75
0.50
0.25
0.00
250
500
750
1,000
Concentration [µg/kg]

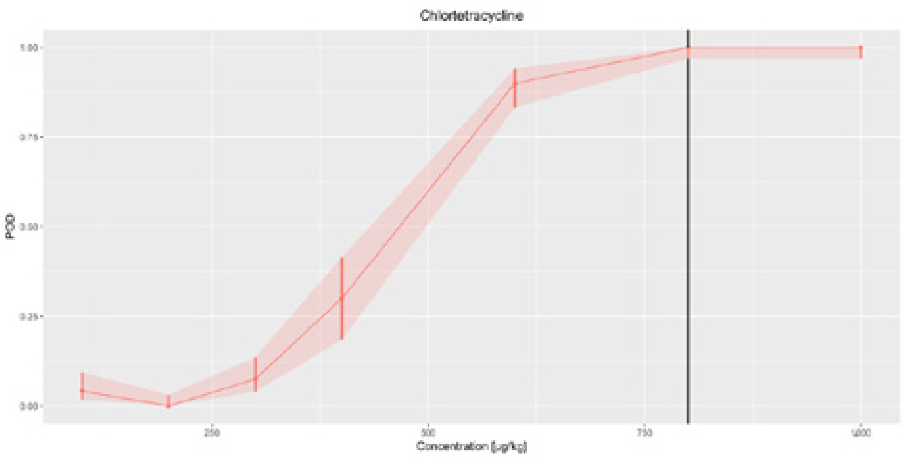

Sulfadiazine
POD
1.00
0.75
0.50
0.25
0.00
200
400
600
800
Concentration [µg/kg]

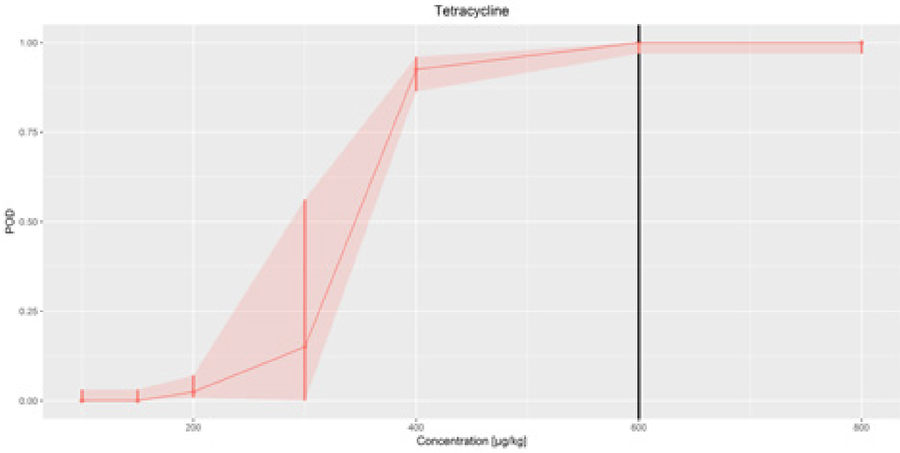

Sulfadimethoxin
POD
1.00
0.75
0.50
0.25
0.00
200
400
600
800
Concentration [µg/kg]

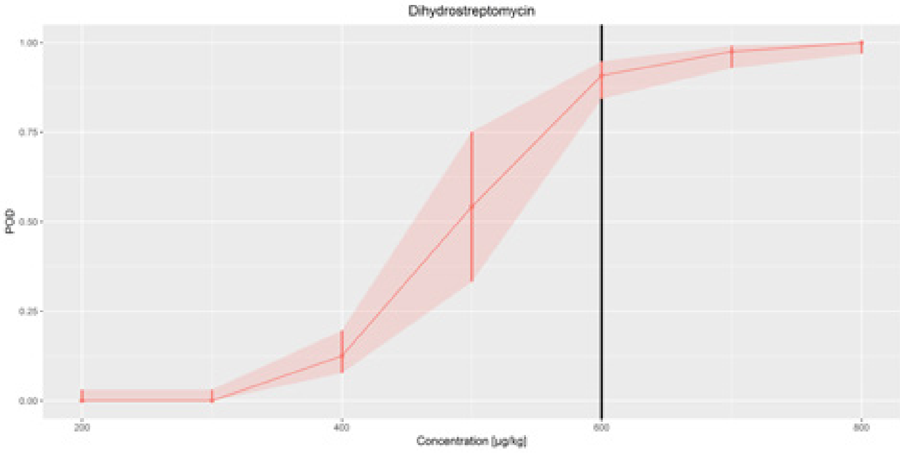

Sulfamethazine
1.00
0.75
0.50
0.25
0.00
POD
250
500
750
1,000
Concentration [µg/kg]

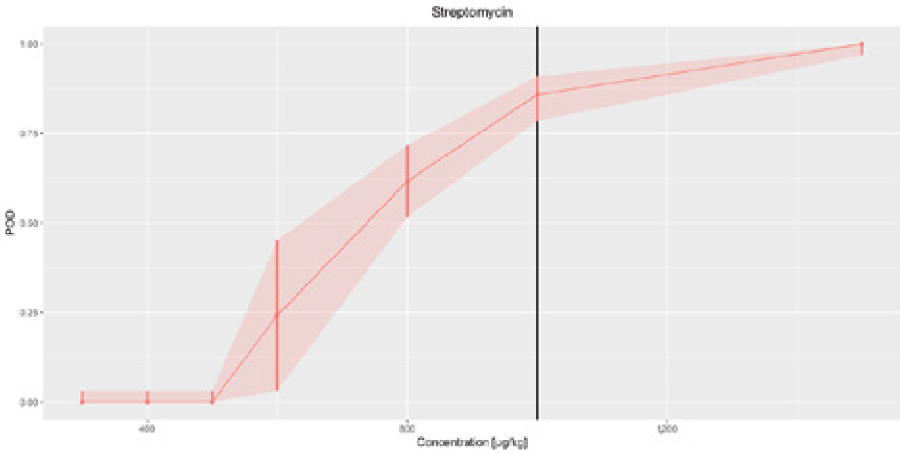

Sulfathiazol
1.00
0.75
0.50
0.25
0.00
POD
200
400
600
Concentration [µg/kg]

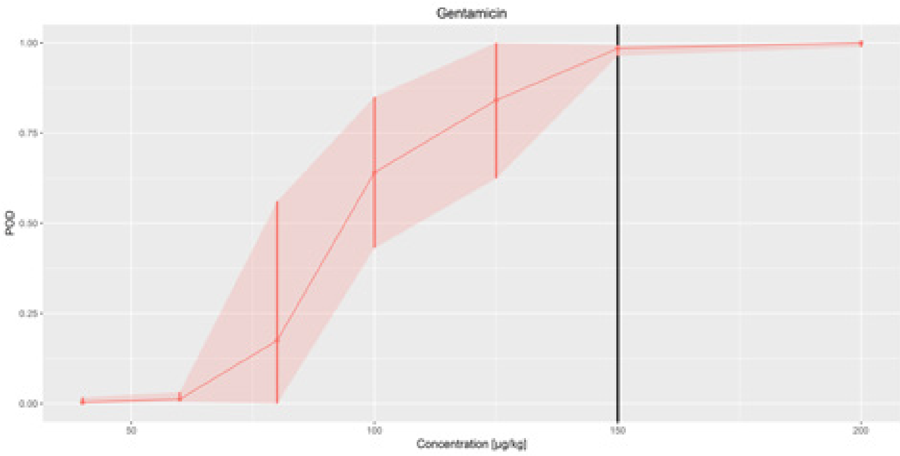

Oxytetracycline
1.00
0.75
0.50
0.25
0.00
POD
250
500
750
1,000
Concentration [µg/kg]

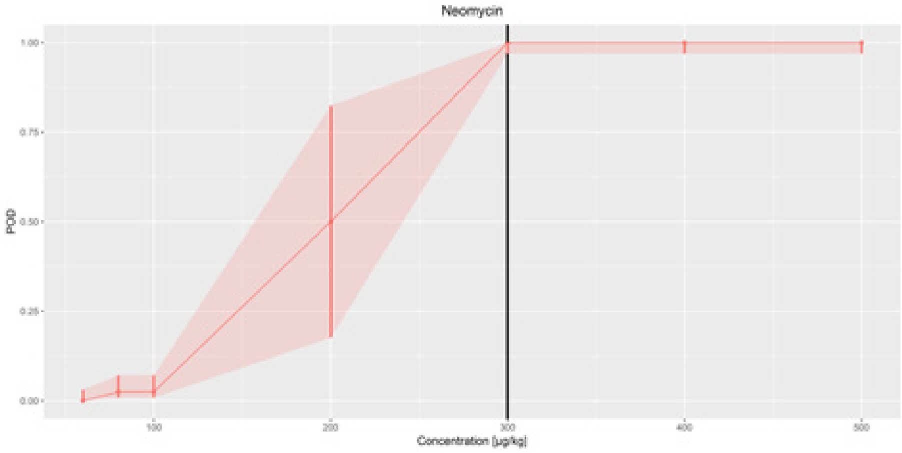

Chlortetracycline
1.00
0.75
0.50
0.25
0.00
POD
250
500
750
1,000
Concentration [µg/kg]

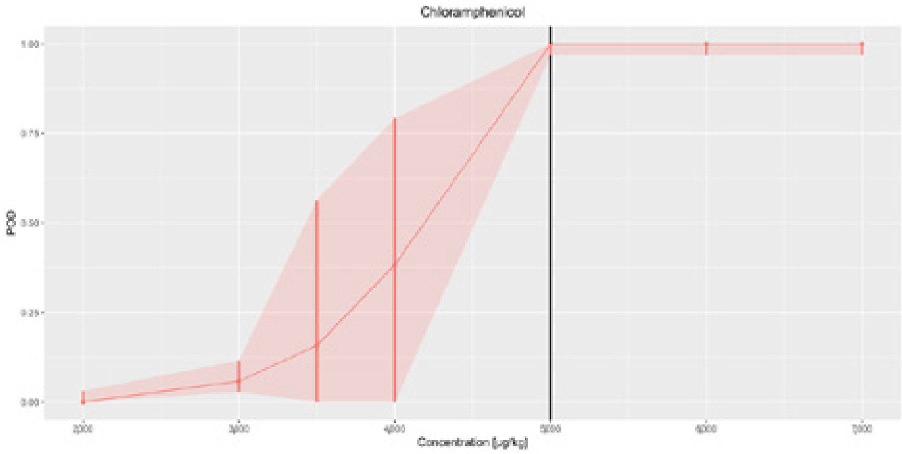

Tetracycline
1.00
0.75
0.50
0.25
0.00
POD
200
400
600
800
Concentration [µg/kg]


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


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


Gentamicin
1.00
0.75
0.50
0.25
0.00
POD
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
	Photometric + Visual	4	120	0	0.00	0.00	0.03	13	-	107
		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
	Photometric + Visual	20	200	16	0.08	0.05	0.13	173	-	11
		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
	Photometric + Visual	6	120	58	0.48	0.00	0.97	62	-	0
		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
	Photometric + Visual	100	120	8	0.07	0.03	0.13	90	-	22
		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)
Ceftiofur	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Cefalonium	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Erythromycin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Tylosin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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)
Sulfadiazine	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Sulfadi-methoxin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Sulfametha-zine	Photometric	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
	Photometric + 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
Sulfathiazol	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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)
Sulfadoxin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Sulfamethoxy-pyridazine	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Chlortetra-cycline	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Oxytetra-cycline	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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)
Tetracycline	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Dihydro-streptomycin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Streptomycin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Gentamicin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
		1	B	16	0	0	
		1	C	8	0	0	
		2	A	16	0	0	1
		2	B	16	0	0	
		2	C	8	0	0	
Ampicillin	2.5	1	A	16	0	0	1
		1	B	8	0	0	
		1	C	16	0	0	
		2	A	16	0	0	1
		2	B	8	0	0	
		2	C	16	0	0	
Amoxicillin	3	1	A	16	0	0	1
		1	B	8	0	0	
		1	C	16	0	0	
		2	A	16	0	0	1
		2	B	8	0	0	
		2	C	16	0	0	
Cloxacillin	25	1	D	16	0	0	1
		1	E	16	0	0	
		1	F	8	0	0	
		2	D	16	0	0	1
		2	E	16	0	0	
		2	F	8	0	0	
Dicloxacillin	12.5	1	A	8	0	0	1
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	
Nafcillin	10	1	A	8	0	0	1
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	
Oxacillin	8	1	A	8	0	0	1
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	
Cefalexin	300	1	D	8	0	0	1
		1	E	8	0	0	
		1	F	8	0	0	
		2	D	8	0	0	1
		2	E	8	0	0	
		2	F	8	0	0	
Cefapirin	5	1	A	8	0	0	1
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	
Cefoperazone	30	1	A	16	0	0	1
		1	B	8	0	0	
		1	C	16	0	0	
		2	A	16	0	0	1
		2	B	8	0	0	
		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
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	
Sulfamethoxy-pyridazine	500	1	A	16	0	0	1
		1	B	24	0	0	
		1	C	24	0	0	
		2	A	16	0	0	1
		2	B	24	0	0	
		2	C	24	0	0	
Chlortetra-cycline	800	1	A	8	0	0	1
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	
Oxytetracycline	400	1	A	8	0	0	1
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	
Tetracycline	600	1	A	8	0	0	1
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	
Dihydro-streptomycin	600	1	D	8	0	0	1
		1	E	8	0	0	
		1	F	7	1	0	
		2	D	8	0	0	1
		2	E	8	0	0	
		2	F	7	1	0	
Streptomycin	1,000	1	D	7	1	0	1
		1	E	8	0	0	
		1	F	8	0	0	
		2	D	8	0	0	1
		2	E	8	0	0	
		2	F	8	0	0	
Gentamicin	150	1	A	24	0	0	0.11
		1	B	21	3	0	
		1	C	16	0	0	
		2	A	24	0	0	0.33
		2	B	22	2	0	
		2	C	16	0	0	
Neomycin	300	1	A	8	0	0	1
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	
Chlor-amphenicol	5,000	1	A	8	0	0	1
		1	B	8	0	0	
		1	C	8	0	0	
		2	A	8	0	0	1
		2	B	8	0	0	
		2	C	8	0	0	

Validierungsbericht
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).

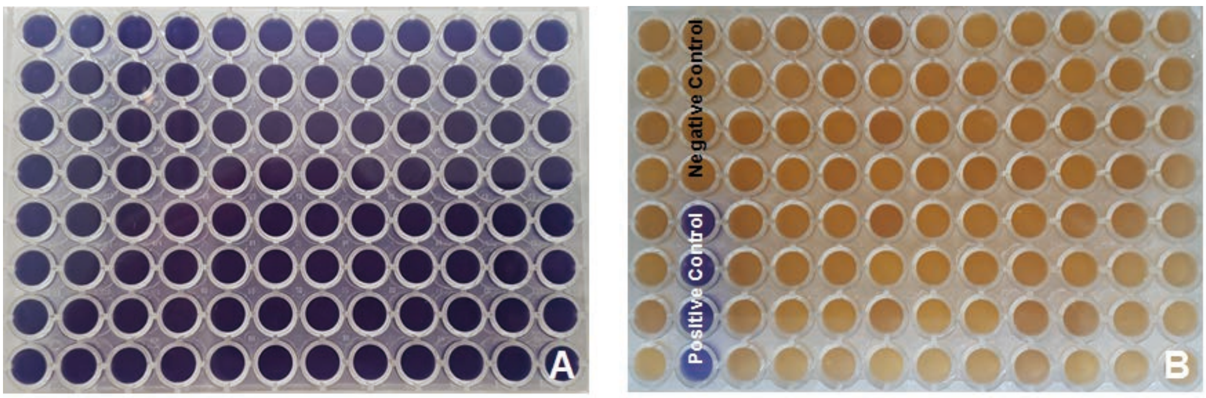


Figure 1. BRT hi-sense test plates before (A) and after (B) incubation

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 °C 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:

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

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 correct 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).

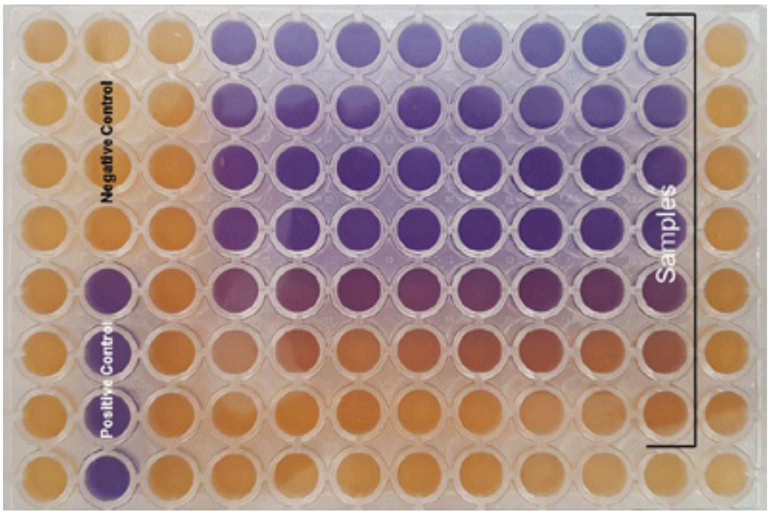


Figure 2. Incubated BRT hi-sense plate inoculated with four positive and four negative controls as well as raw milk samples spiked with 7 different concentrations of an antibiotic substance

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.

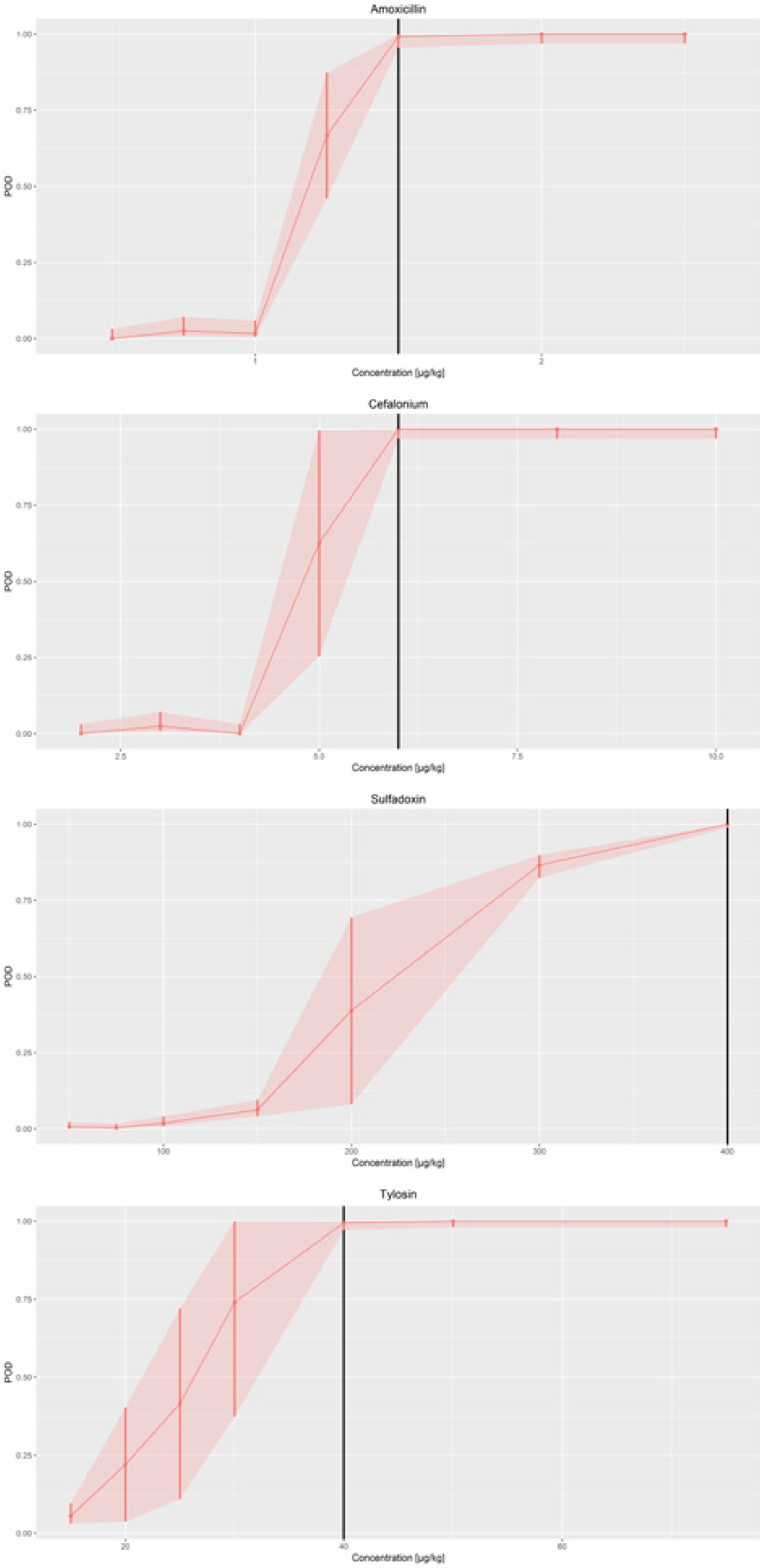


Figure 3. Dose-response curves of the bactericidal antibiotics Amoxicillin and Cefalonium and of the bacteriostatic substances Sulfadoxin and Tylosin.
Red line = dose-response curve; red shade = CI; Black line = CCß A (photometric reading);

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

Use	Drug Class	Substance	MRL [µg/kg]	Concentration [µg/kg]	False positive Results	
					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

<u>Materials and Methods</u>

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.

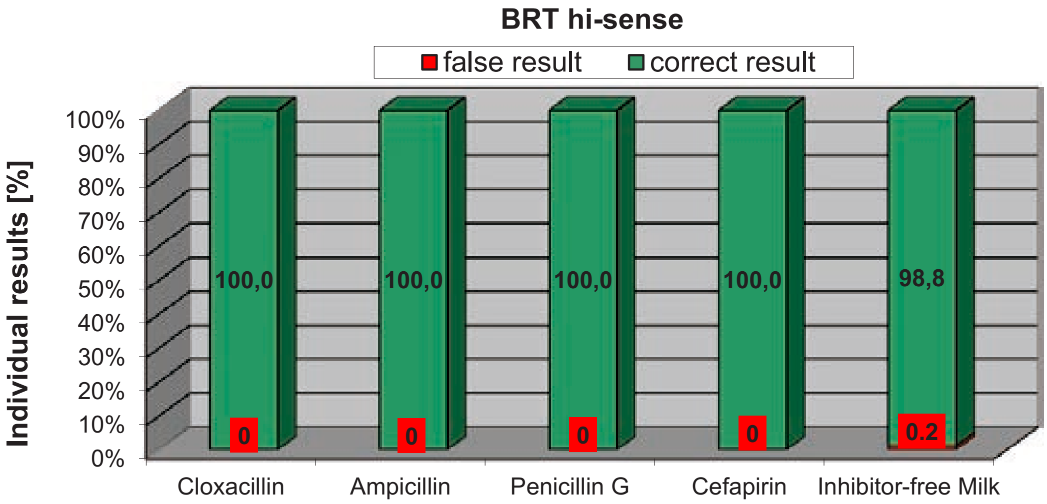


Figure 4. Rate of correct and false results (%) obtained by the interlaboratory study within the framework of the validation of the BRT hi-sense

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.

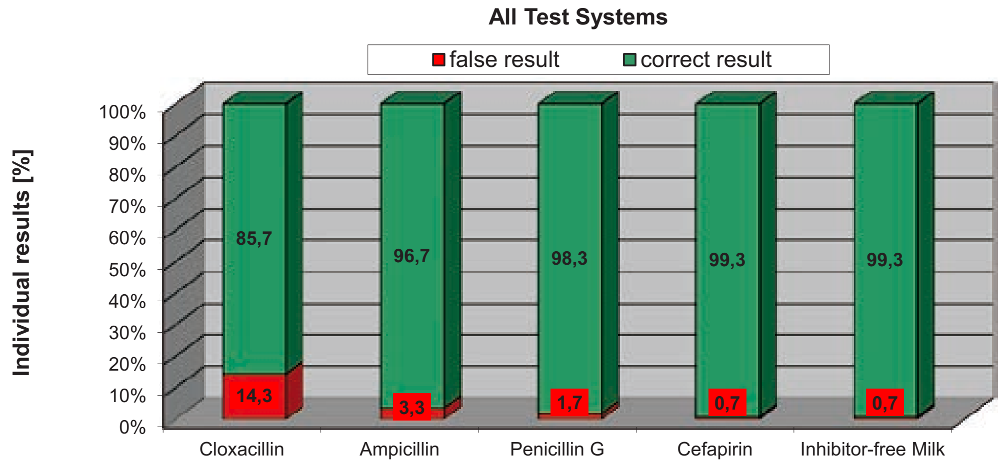


Figure 5. Rates of correct and false results (%) of all test systems (microbiological and receptor tests, 10th proficiency test)

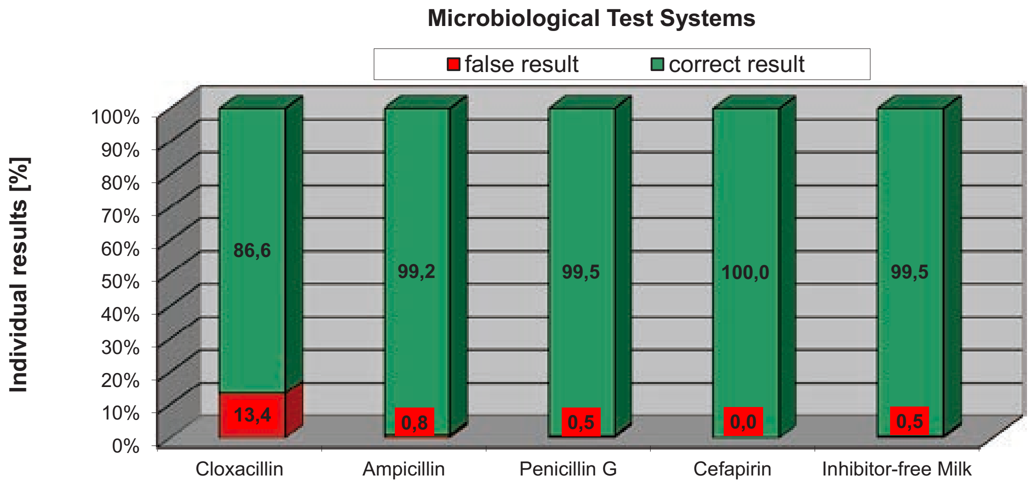


Figure 6. Rates of correct and false results (%) of microbiological test systems (10th proficiency test)

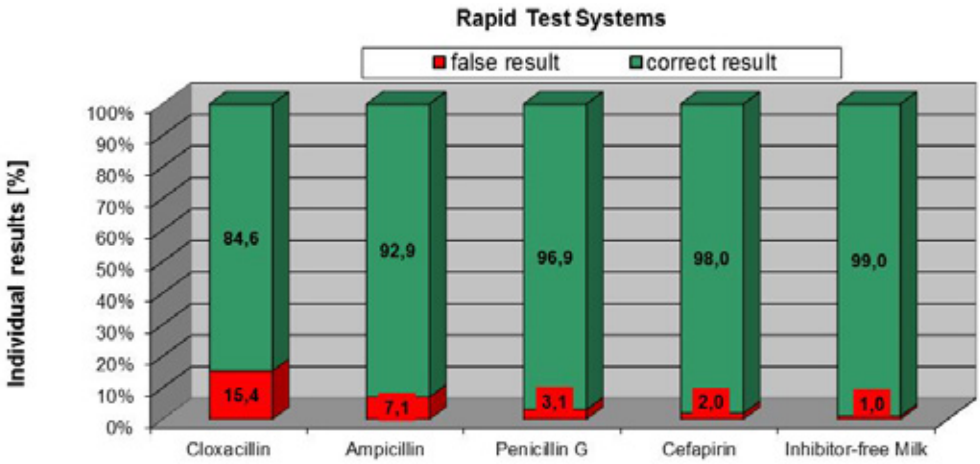


Figure 7. Rates of correct and false results (%) of receptor test systems (10th proficiency test)

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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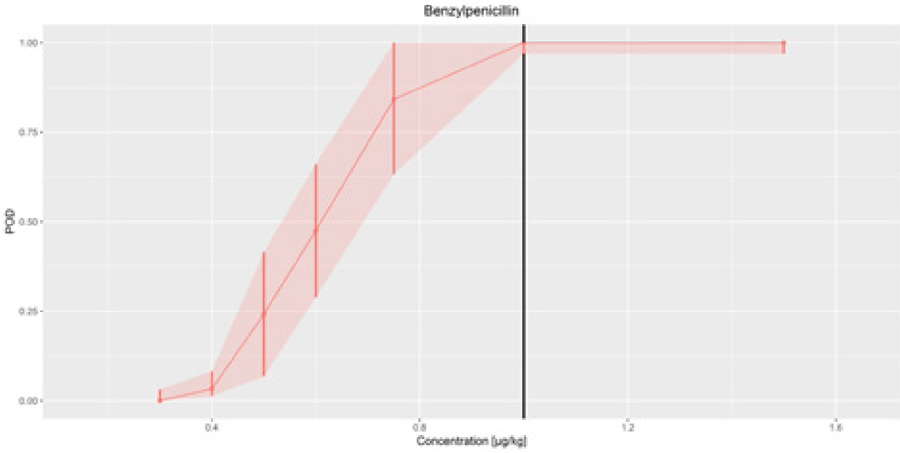
Date of publication: 09.11.2018

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	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
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0.25
0.00
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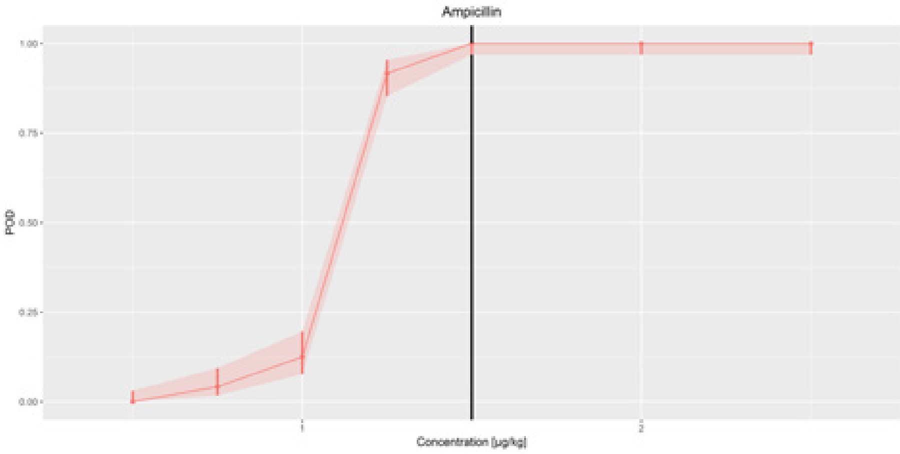
1.2
1.6
Concentration [µg/kg]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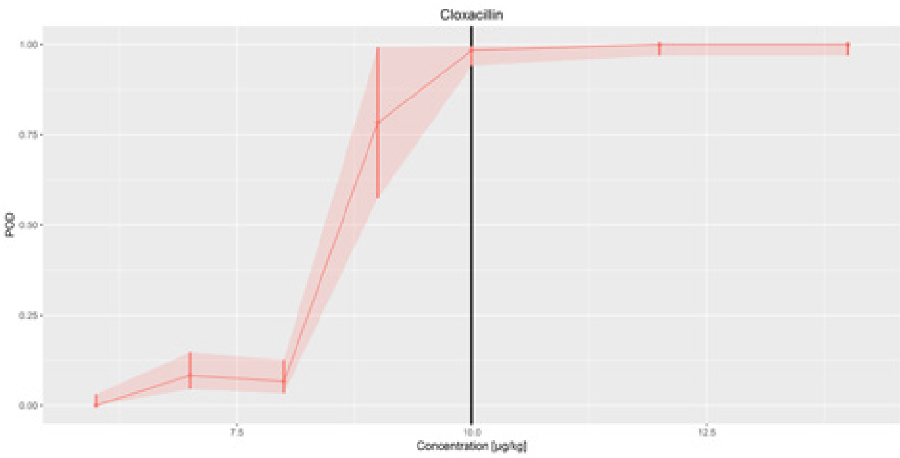
0.25
0.00
1
2
Concentration [µg/kg]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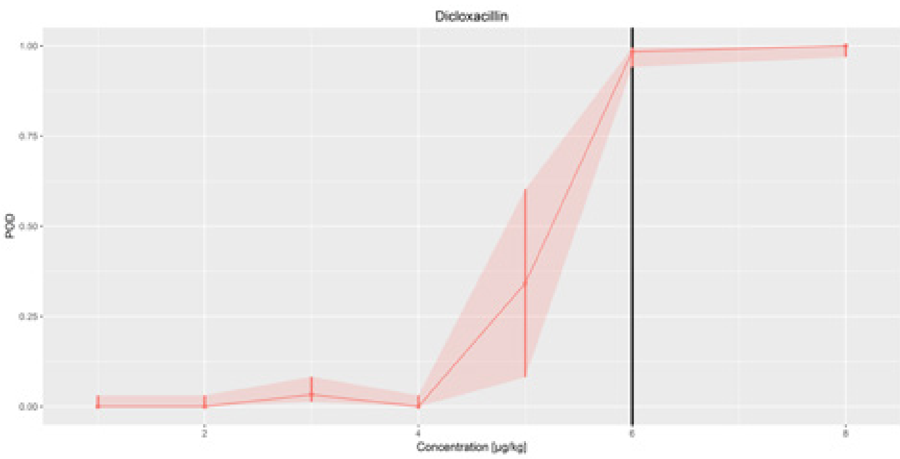
0.00
7.5
10.0
12.5
Concentration [µg/kg]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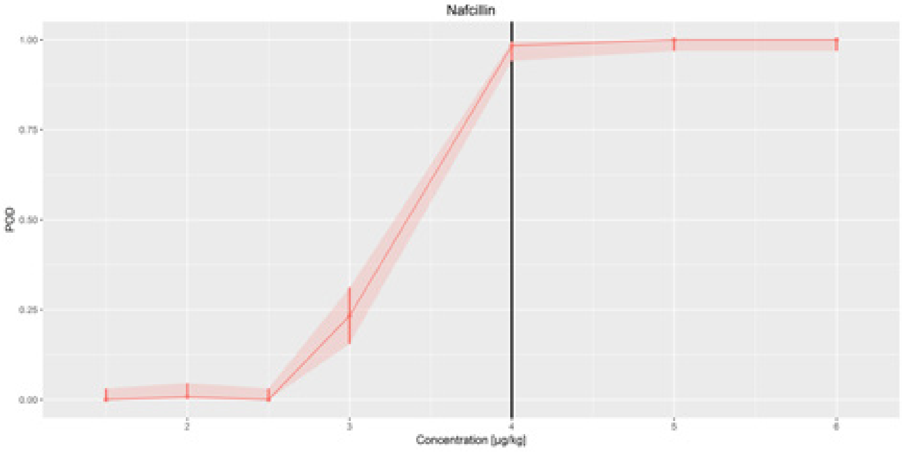
0.00
2
4
6
8
Concentration [µg/kg]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POD
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0.00
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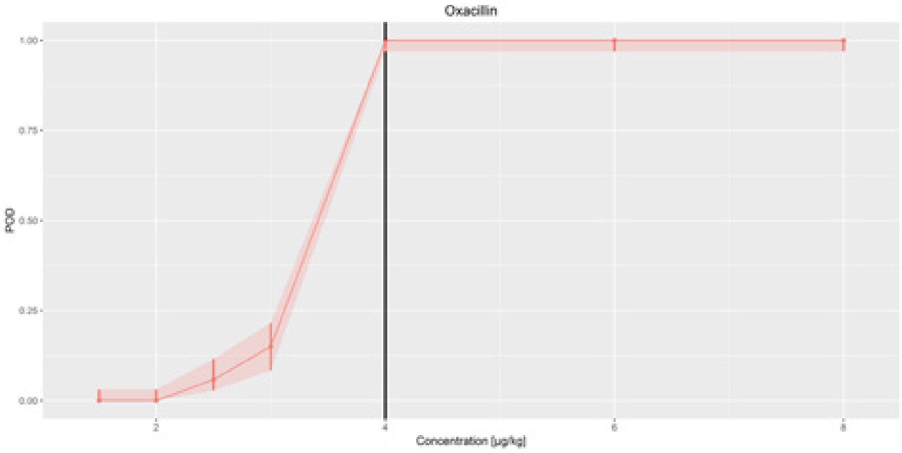
3
4
5
6
Concentration [µg/kg]


Oxacillin
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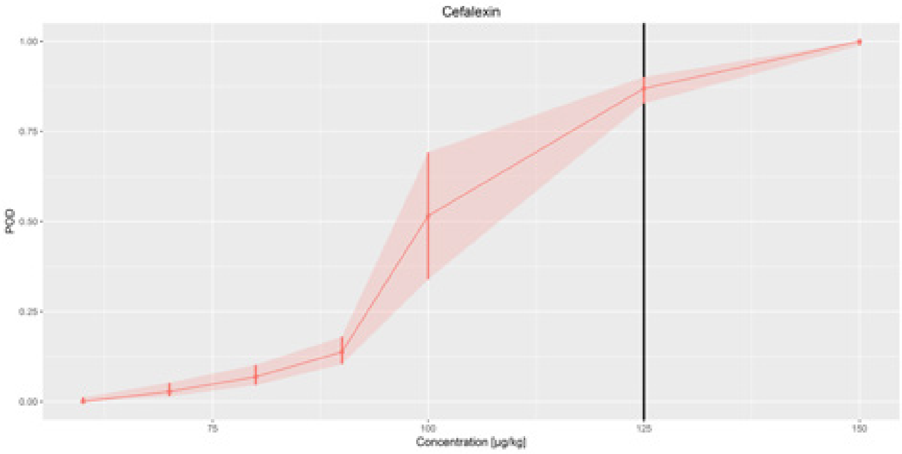
4
6
8
Concentration [µg/kg]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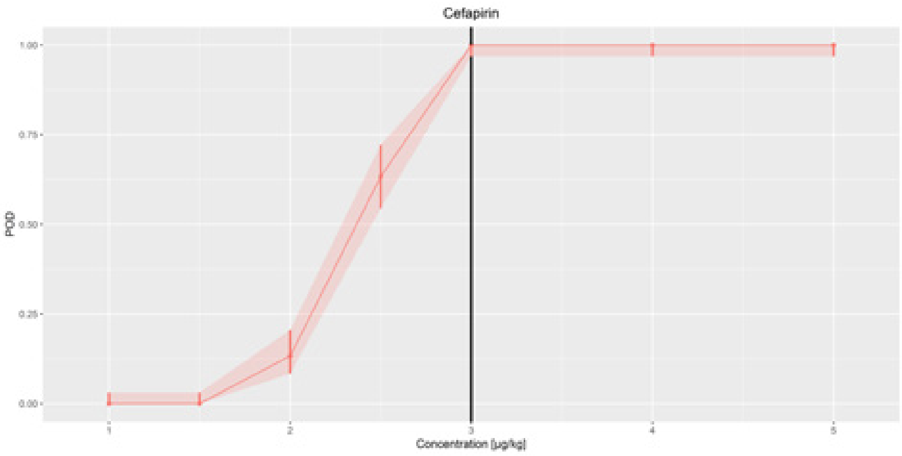
0.25
0.00
75
100
125
150
Concentration [µg/kg]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POD
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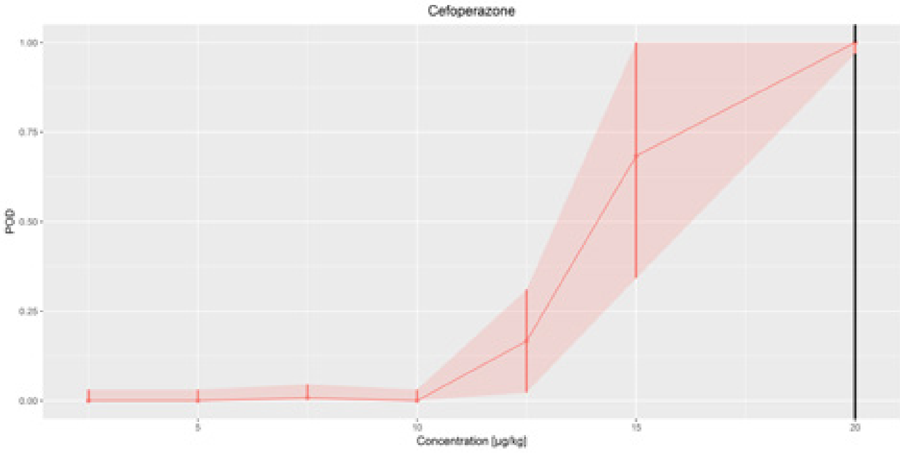
2
3
4
5
Concentration [µg/kg]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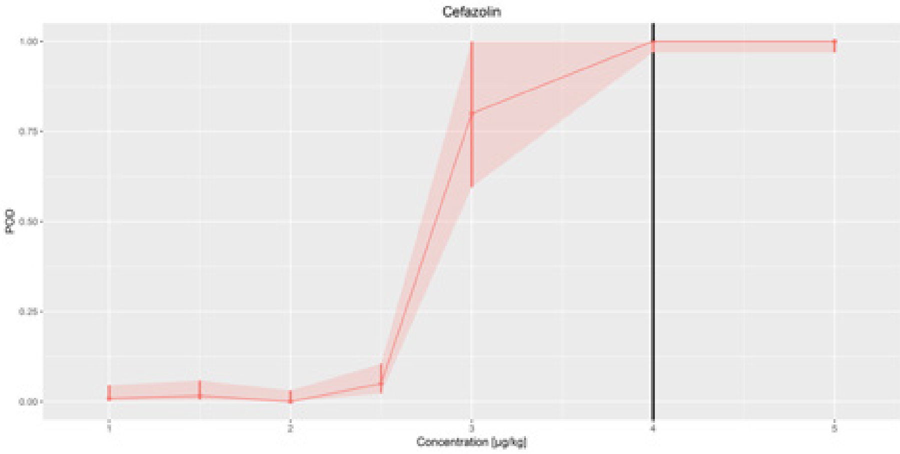
POD
5
10
15
20
Concentration [µg/kg]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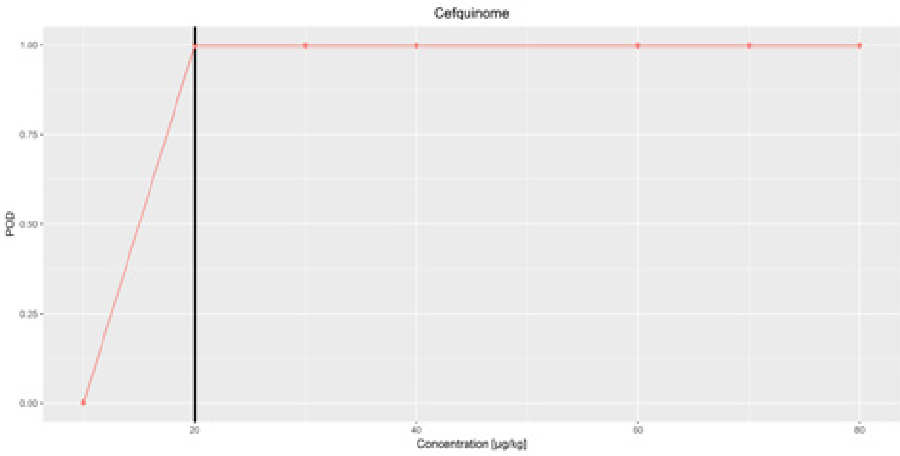
Concentration [µg/kg]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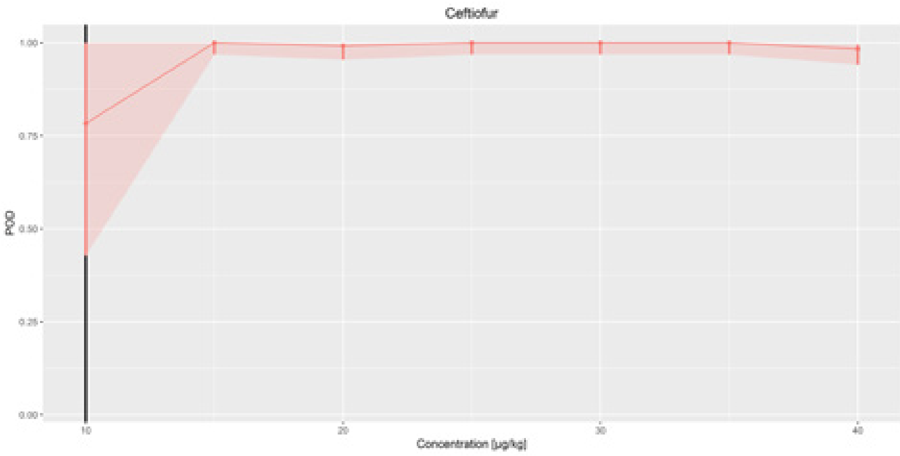
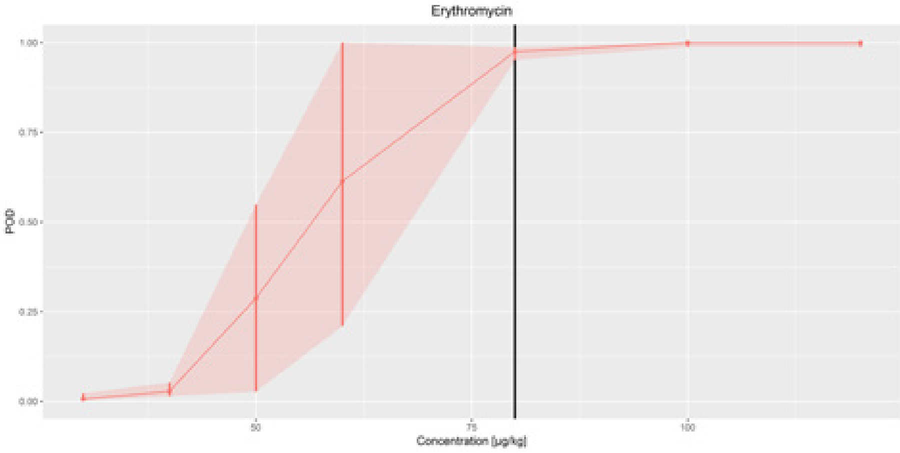
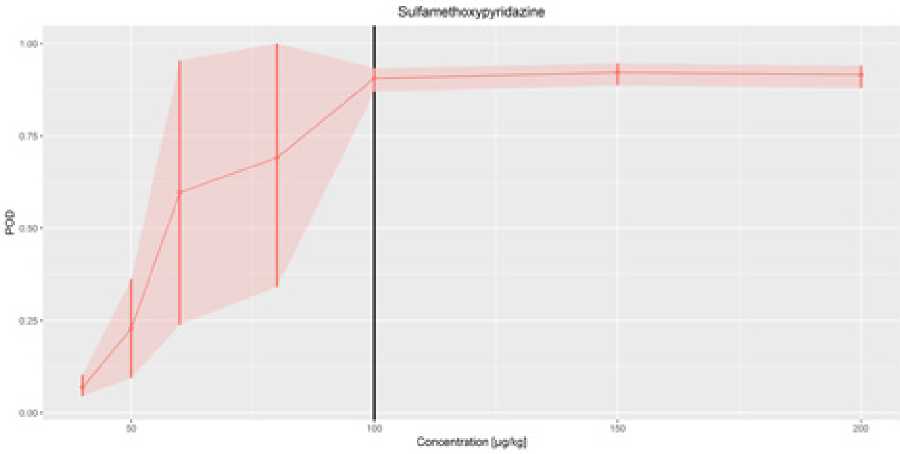
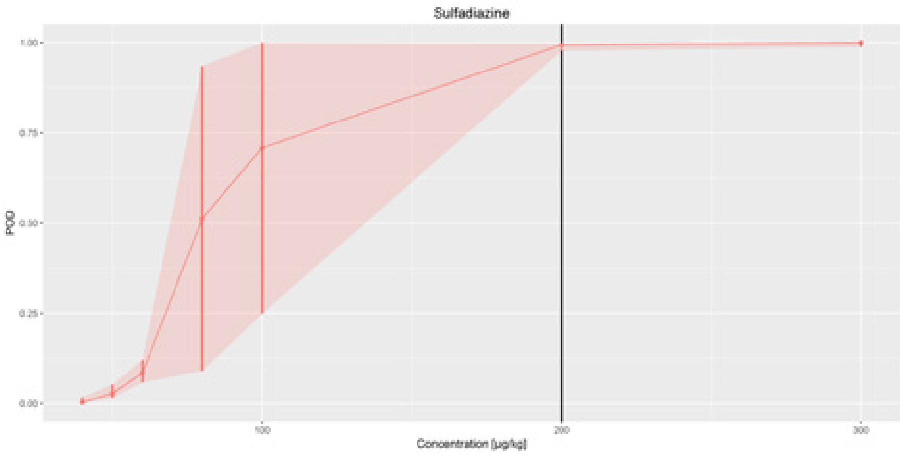
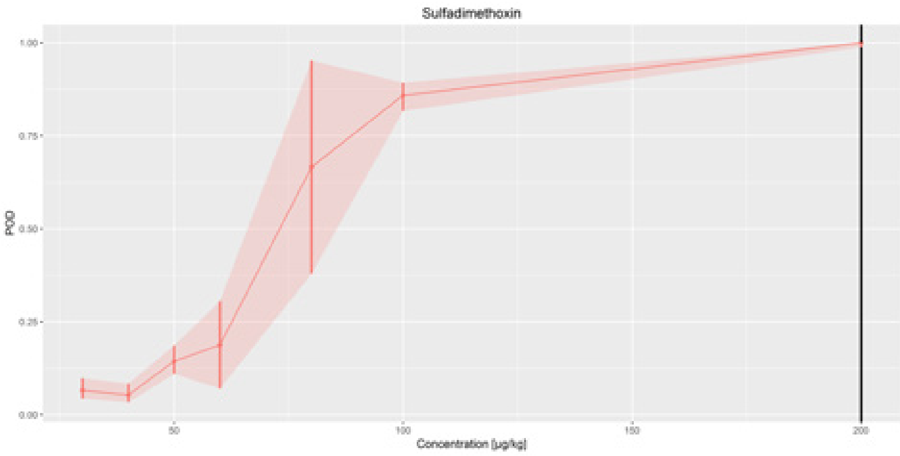
0.75
0.50
0.25
0.00
POD
20
40
60
80
Concentration [µg/kg]

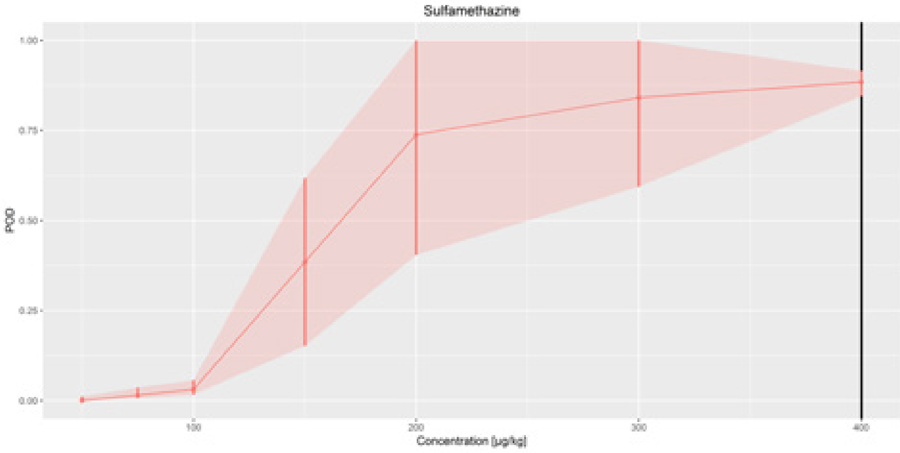

Ceftiofur
1.00
0.75
0.50
0.25
0.00
POD
10
20
30
40
Concentration [µg/kg]

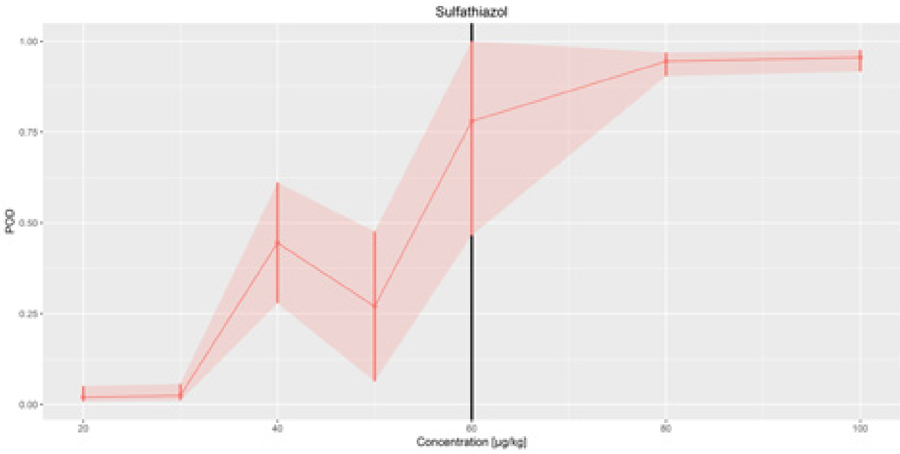

Erythromycin
1.00
0.75
0.50
0.25
0.00
POD
50
75
100
Concentration [µg/kg]

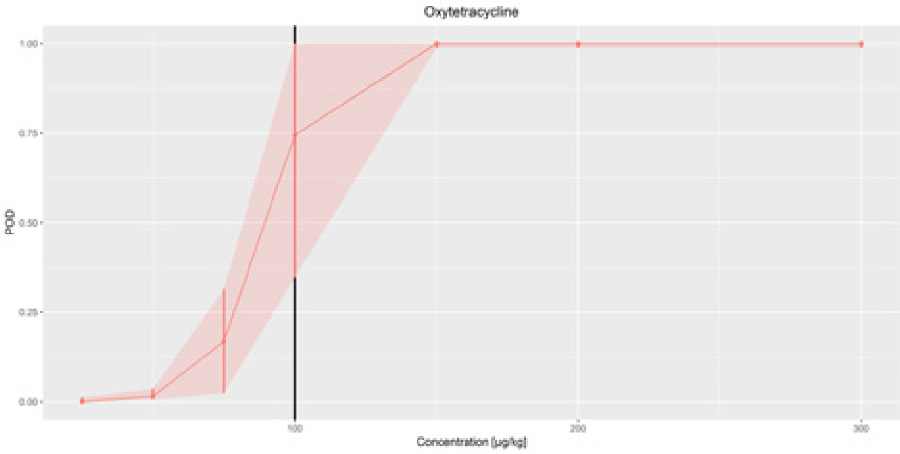

Sulfamethoxypyridazine
1.00
0.75
0.50
0.25
0.00
POD
50
100
150
200
Concentration [µg/kg]

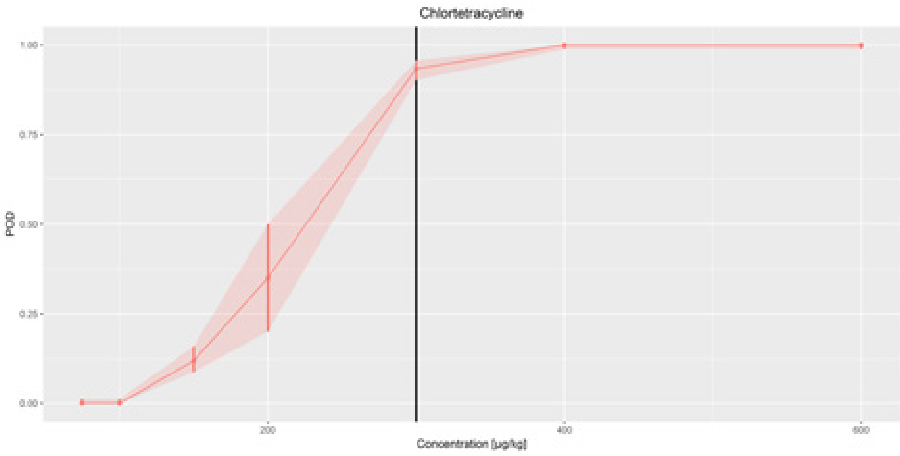

Sulfadiazine
1.00
0.75
0.50
0.25
0.00
POD
100
200
300
Concentration [µg/kg]

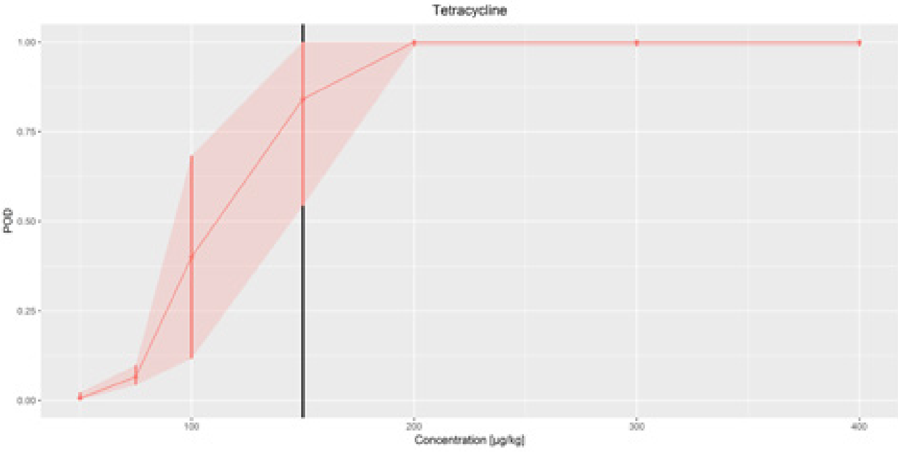

Sulfadimethoxin
1.00
0.75
0.50
0.25
0.00
POD
50
100
150
200
Concentration [µg/kg]

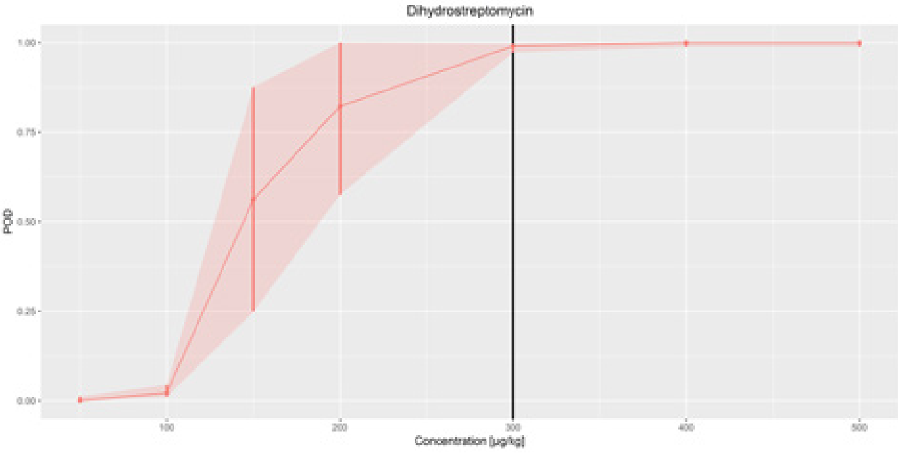

Sulfamethazine
POD
Concentration [µg/kg]

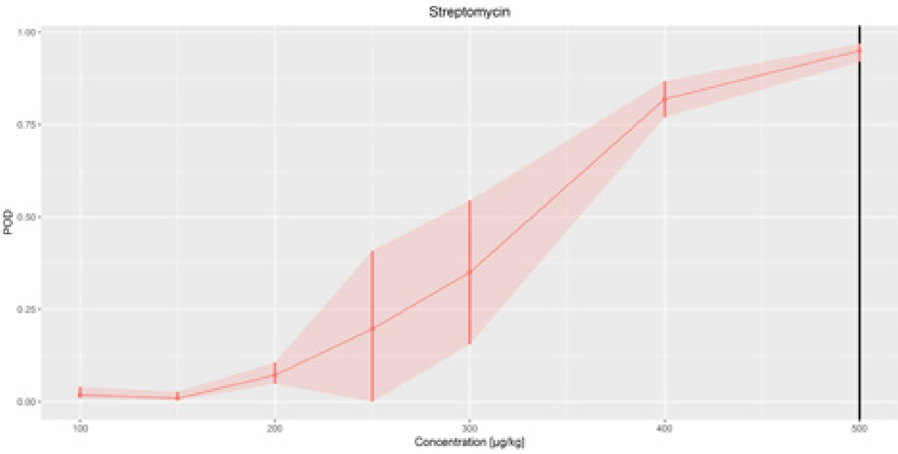

Sulfathiazol
POD
Concentration [µg/kg]

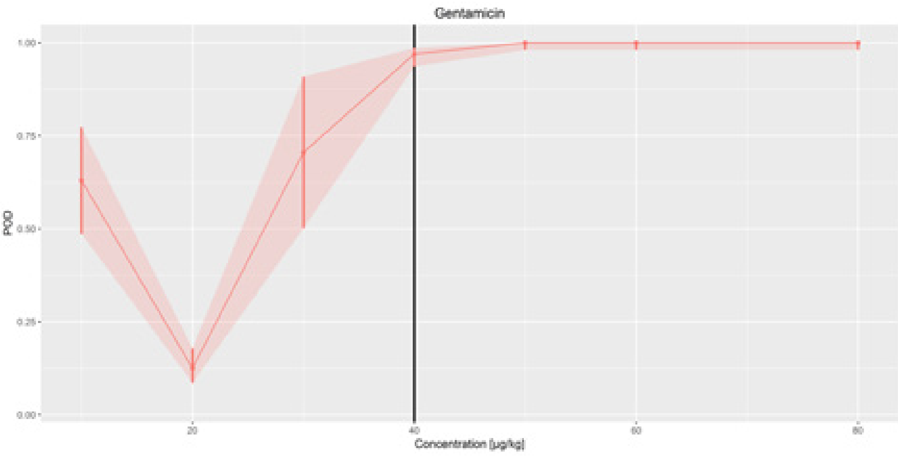

Oxytetracycline
POD
Concentration [µg/kg]

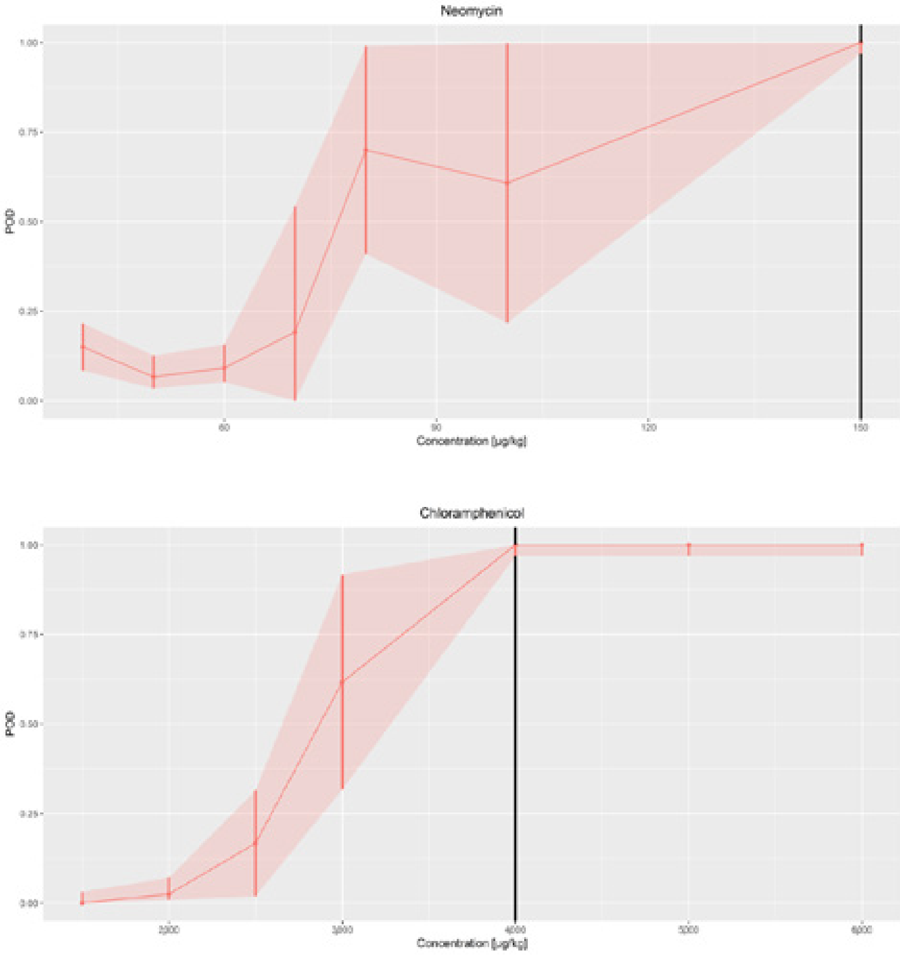

Chlortetracycline
POD
Concentration [µg/kg]


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


Dihydrostreptomycin
POD
1.00
0.75
0.50
0.25
0.00
100
200
300
400
500
Concentration [µg/kg]


Streptomycin
POD
1.00
0.75
0.50
0.25
0.00
100
200
300
400
500
Concentration [µg/kg]


Gentamicin
POD
1.00
0.75
0.50
0.25
0.00
20
40
60
80
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)
Cefapirin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Cefoperazone	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Cefazolin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Cefquinome	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
	Photometric + Visual	20	120	119	0.99	0.95	1.00	1	-	0
		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
	Photometric + Visual	4	120	0	0.00	0.00	0.03	47	-	73
		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
	Photometric + Visual	50	320	92	0.29	0.03	0.55	228	-	0
		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)
Sulfadiazine	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Sulfadi-methoxin	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Sulfametha-zine	Photometric	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
		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
	Visual	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
		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
	Photometric + Visual	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
		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
Sulfathiazol	Photometric	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
	Photometric + 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

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
		150	320	180	0.56	0.25	0.88	136	-	4
	Photometric + Visual	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	

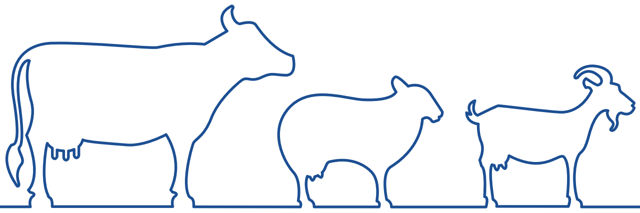
Continuation Annex Table 3

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	

Nachweisempfindlichkeiten
Detection sensitivities



Detection sensitivities of AiM BRT test systems
towards selected anti-infectives in cow milk, µg/kg (ng/ml; ppb)

Substance	BRT Inhibitor Test		BRT MRL-Screening Test		BRT hi-sense		MRL (470/2009 EEG resp. 37/2010 EEG)
	CCβ A	CCβ B	CCβ A	CCβ B	CCβ A	CCβ B	
Penicillins							
Benzylpenicillin	2.5	2	2	1.5	1	0.6	4
Oxacillin	10	8	8	8	4	3	30
Cloxacillin	25	20	25	18	10	9	30
Amoxicillin	3	2.5	3	2.5	1.5	1.3	4
Ampicillin	3.5	3	2,5	2	1.5	1.3	4
Dicloxacillin	15	12.5	12.5	10	6	5	30
Nafcillin	15	10	10	8	4	4	30
Cephalosporins							
Cefalexin	400	300	300	250	125	100	100
Cefalonium	14	12	12	10	6	5	20
Cefapirin	6	5	5	5	3	2.5	60
Cefazolin	9	7	7	6	4	3	50
Cefoperazone	35	25	30	20	20	15	50
Ceftiofur	200	150	150	100	10	10	100
Cefquinome	500	300	300	200	20	20	20
Aminoglycosides							
Streptomycin	1,500	600	1,000	500	500	250	200
DH-Streptomycin	600	400	600	400	300	150	200
Gentamicin	200	100	150	80	40	10	100
Neomycin	400	200	300	200	150	60	1,500
Macrolides							
Erythromycin	100	50	80	50	80	40	40
Tylosin	75	40	75	30	40	20	50
Sulfonamides							
Sulfadimidine	1,000	300	> 1,000	200	400	100	100
Sulfadoxine	> 1,500	400	1,500	300	400	150	100
Sulfamethoxypyridazine	500	100	500	100	100	40	100
Sulfadiazine	> 800	100	400	100	200	60	100
Sulfadimethoxine	> 800	200	600	100	200	50	100
Sulfathiazole	400	60	200	60	60	40	100
Tetracyclines							
Tetracycline	1,000	600	600	300	150	75	100
Chlortetracycline	> 1,000	800	800	400	300	150	100
Oxytetracycline	800	400	400	200	100	75	100
Others							
Lincomycin	200	150	200	100	125	75	150
Chloramphenicol	7,000	4,000	5,000	3,500	4,000	2,000	illegal

NKML – NordVal International Certificates no. 051, 052, 053 issued for BRT, valid until 01 March 2023

Data BRT Validation 2018: Reading with ELISA Reader: Measuring wavelength 450 nm, reference wavelength 620 nm
CCß: Lowest concentration obtaining a minimum of 95% positive results
CCβ A: Evaluation with regard to the colour of the positive control
CCβ B: Evaluation with regard to the colour of the negative control

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