Microbial system for identification of antibiotic residues in milk

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ABSTRACT

The aim of this study was to evaluate the ResScreen® microbiological system for the identification of antibiotic residues in milk. This microbiological system consists of two methods, the "BT" (betalactams and tetracyclines) and "BS" (betalactams and sulfamides) bioassays, containing spores of *G. stearothermophilus* subsp. *calidolactis*, culture media and indicators (acid-base and redox). The detection limits of 29 antimicrobial agents were calculated using a logistic regression model.

Both methods detect residues of penicillin-G, ampicillin, amoxicillin, cloxacillin, oxacillin, cephalexin, cefoperazone and ceftiofur® at levels close to their Maximum Residue Limits (MRL). The "BT" bioassay also presents good sensitivity to tetracycline and oxytetracycline residues, whereas the "BS" bioassay detects sulfadiazine, sulfamethoxazole and sulfathiazole residues in milk.

The simultaneous use of both bioassays identifies betalactam, tetracycline and sulfamide residues in milk. Neomycin, tylosin and lincomycin residues can also be detected, but these molecules are positive with the “BT” and “BS” bioassays, e.g., betalactams, given the microorganisms’ sensitivity to these molecules.

Key words: screening test, microbiological inhibition system, betalactams, tetracyclines, sulfamides, milk.

INTRODUCTION

The presence of certain antibiotic residues in milk is a potential risk for consumers because they may be toxic and dangerous for human health, and may potentially cause antimicrobial resistance(1-2) and technological problems during dairy product manufacturing (3-5).

For this purpose, several commercially available tests have been developed for the swift, precise detection of the presence of antibiotic residues in milk (6-7). Many screening tests are based on the inhibition of microorganism growth by the presence of drug residues. Among the most widely used microorganisms, we find *Geobacillus stearothermophilus* subsp. c*alidolactis* in the following tests: Delvotest®(8), BRT® AiM(9), Eclipse®(10) and Charm® AIM-96(11).

These methods can nonspecifically detect the “presence” or “absence” of antibiotic residues in milk. To identify β-lactam or sulfonamide compounds however, “positive” and “doubtful” samples are tested using penicillinase and p-aminobenzoic acid (PABA) solutions. Thus, antibiotic residues can be classified into betalactam antibiotics or sulfamides(12).

However, the penicillinase and PABA methods do not suffice to identify other antimicrobial agents such as tetracyclines. So, when Yamaki *et al.*(13) investigated 2686 samples of ewe’s milk, 47 samples were found to be positive with the Delvotest "SP" test. When using penicillinase and PABA methods, only 29.8% of the samples were identified as containing betalactam residues, while the remaining milk samples (70.2%) remained unidentified. These authors suggested that this methodology is insufficient for a complete identification of milk antibiotic residues.

In order to identify a higher number of antibiotic groups, Althaus and Nagel[14] proposed to use a microbiological system which not only complies with the International Standardization Organization guidelines[15], but also identifies betalactam, tetracycline and sulfonamide residues.

This microbiological system consists of two methods, the "BT" (betalactams and tetracyclines) and "BS" (betalactams and sulfamides) bioassays, containing spores of *G. stearothermophilus* subsp. *calidolactis*, culture media and indicators (acid-base and redox). Moreover, this system includes synergistic components that improve the sensitivity of tetracycline [16] and sulfamide [17] residues in milk.

Thus, the objective of this research was to evaluate the ResScreen® system for the identification of antimicrobial agent residues in milk by means of studying detection limits.

MATERIALS AND METHODS

Animals and milk samples

The animals came from cattle herds of Las Colonias (Santa Fe, Argentina). For this study, milk samples corresponding to the morning machine milking session (6 am) of 16 cows were collected in the 60-90 day postpartum period. The animals received no pharmacological treatment throughout the sampling period[18].

The chemical composition and pH values of the selected samples were normal for bovine milk, with low somatic cell counts (SCC < 400000 cells ml^{-1}) and an acceptable bacterial count for cow's milk (CFU < 100000 cfu ml^{-1}).

Antimicrobial solutions and spiked samples

The drugs used for the preparation of antimicrobial solutions were stored and handled according to the manufacturers' instructions before use. All the dilutions were prepared in 100 ml volumetric flasks at the time the analyses were carried out in order to avoid the possibility of unstable solutions.

Antimicrobial solutions were prepared using antimicrobial-free milk[18], as determined by the Delvotest®. The final drug concentrations in milk ($\mu g\ l^{-1}$) were achieved after serial dilutions so that the volume of the antimicrobial agent solution did not exceed 1 % of the volume of the final solution to be analyzed[18].

ResScreen® test

The system consists of two microbial bioassays using *Geobacillus stearothermophilus* subsp. *calidolactis* C-953 spores. The microbiological method is based on growth inhibition of bacteria-test when milk containing residues of antibiotics.

The BT bioassay (Betalactams and Tetracyclines) is composed of a culture medium containing spores of thermophilic microorganism, chloramphenicol and bromocresol purple indicator[16]. If the milk sample is free of antibiotics and allows bacteria-test growth and changes in color of the acid base indicator (purple to yellow). Otherwise the test will remain the same color.

Moreover, the BS bioassay (Beta-lactams and Sulfonamides) use a medium inoculated with a microorganism spore suspension, brilliant black indicator, toluidine blue and trimethoprim[17]. So, the absence of antibiotic residues in milk causes bacteria-test growth, producing a color change of indicators from black to amber.

The ResScreen® system was carried out according to the manufacturer's instructions. Thus, 50 µl milk sample was added to individual plates of the "BT" and "BS" ResScreen® methods. Plates were incubated in a water bath at 64±1 ºC for 3 ("BT" ResScreen®) and 4 hours ("BS" ResScreen®) until the color change of the negative samples had taken place.

Visual interpretation was performed independently by 3 trained persons, and was assessed visually as "negative" and "positive"; "doubtful" qualifications were interpreted as positive[19].

Detection limits and cross specificity

Detection limits: The following substances (Sigma Chemical Co, St. Louis, MO) were used to determine the ResScreen® system detection limits:

- Ten betalactams: amoxicillin, ampicillin, cloxacillin, oxacillin, penicillin "G", cefadroxil, cephalexin, cefoperazone, cefuroxime and ceftiofur®.
- Four sulfonamides: sulfadiazine, sulfadimethoxine, sulfamethoxazole and sulfathiazole.
- Three tetracyclines: chlortetracycline, oxytetracycline and tetracycline.

The detection limits of the antimicrobial agents were established according to the Codex Alimentarius guidelines[18]. For this purpose, 12 concentrations were prepared with different levels of each drug. For each concentration, 16 replicates were prepared using antibiotic-free milk samples.

Cross specificity: The Codex Alimentarius guidelines[18] were used to calculate the detection limits of the following antibiotics (Sigma Chemical Co, St. Louis, MO):

- Four aminoglycosides: gentamycin, kanamycin, neomycin and streptomycin.
- Four macrolides: erythromycin, lincomycin, tylosin and spiramycin.
- Four quinolones: ciprofloxacin, norfloxacin, enrofloxacin and marbofloxacin.

Statistical analysis

The results were obtained by following the SAS® Logistic procedure[20]. A logistic regression model was also done to calculate the detection limits, as follows:

$$L_{ij} = \text{logit}\,[P_{ij}] = \beta_0 + \beta_1\,[A]_i + \varepsilon_{ij}$$

where: L_{ij} = lineal logistic model; $[P_{ij}] = \text{logit}\,[P_p/(1-P_p)]$: the probability of "positive" response / probability of "negative" response); β_0, β_1 = coefficients estimated for the logistic regression models; $[A]_i$ = antimicrobial concentration. ε_{ij} = residual error. The concordance coefficient[20] was applied as the rank correlation between the observed responses and the predicted probabilities.

The detection limit of the visual interpretation of the ResScreen® system was estimated as the concentrations at which 95 % of the results were assessed as "positive" or "doubtful"[19,21].

RESULTS AND DISCUSSION

Detection limits

The results of applying the logistic regression model to the positive relative frequency of the "BT" and "BS" ResScreen® system for the different antimicrobial agents assayed are shown in Table 1.

The concordance coefficients obtained by applying the logistic model were high, between 89.2 % for oxytetracycline ("BT" ResScreen®) and 99.4 % for tetracycline ("BS" ResScreen®), demonstrating the correct adjustment achieved by the logistic model.

The "β_1" coefficient represents the sensitivity of *G. stearothermophilus* to the antibiotics studied. This parameter reached higher values for penicillin antibiotics (amoxicillin, ampicillin, cloxacillin, oxacillin and penicillin "G") than for the rest of the antimicrobial agents assayed, demonstrating the sensitivity of *G. stearothermophilus* to detect the residues of these antimicrobials.

The "β_1" coefficients values of cephalosporins (cefadroxil, cephalexin, cefoperazone, ceftiofur® and cefuroxime) were similar to those calculated for tetracyclines ("BT" ResScreen®) and sulfamides ("BS" ResScreen®). In contrast, the "β_1" parameter of tetracyclines ("BS" ResScreen®) and sulfonamides ("BT" ResScreen®) were very low, showing low sensitivity for detection purposes.

Figures 1 and 2 show the effect of penicillin and cephalosporin concentrations on the visual interpretations of the ResScreen® system, as well as the curves constructed by the logistic model ("β_0" and "β_1" coefficients, Table 1). The concentrations of ampicillin, amoxicillin, oxacillin and penicillin "G" (high "β_1" coefficient values) underwent a slight increase to produce 100 % positive results, whereas the concentrations of cephalosporins (Figure 2) had to undergo greater increments to obtain positive results in both methods (lower "β_1" coefficient values).

The dose-response curves for tetracyclines (“BT” ResScreen®, Figure 3) and sulfonamides (“BS” ResScreen®, Figure 4) showed adequate sensitivity to detect the residues belonging to both antibiotic groups. Conversely, high concentrations of tetracyclines (“BS” ResScreen®, Figure 3) and sulfonamides (“BT” ResScreen®, Figure 4) were needed given the low “β_1“ coefficients values (Table 1).

The detection limits of the ResScreen® system calculated by means of logistic regression models for betalactams, tetracyclines and sulfonamides are shown in Table 2.

Amoxycillin, ampicillin, cloxacillin, oxacillin, penicillin “G”, cephalexin, cefoperazone and ceftiofur® showed similar detection limits (Table 2) for the ResScreen® system to their respective Maximum Residue Limits (MRLs).

For betalactam antibiotics, other microbiological methods such as BRT® AiM[22-23], Charm®[24], Delvotest® “SP”[22, 25] , Eclipse® 100ov[26] have similar detection limits to the ResScreen® system.

With regard to tetracyclines, Table 2 indicates how the “BT” ResScreen® method presented detection limits near at the MRLs, unlike the “BS” method which required higher concentrations of these antibiotics for them to be detected.

The detection limits calculated for the three tetracyclines with the “BT” ResScreen® method were lower than those reported by other authors with the BRT® AiM[22-23], Charm® AIM-96[24], Delvotest® “SP”[22,25] and Eclipse® 100ov[26] methods due to improved sensitivity from adding chloramphenicol to the culture medium[16].

Also, Table 2 indicates how the detection limits of sulfonamides for “BS” ResScreen® approached their MRLs, while the “BT” method was not sensitive enough to detect these drugs in milk (detection limits higher than 12000 $\mu g\ l^{-1}$).

The "BS" ResScreen® method detection limits of sulfonamides were slightly higher than those observed for BRT® AiM[27], although other authors have reported higher detection limits for BRT® AiM[22-23], Delvotest® "SP"[22,25] and Eclipse® 100[26].

Cross specificity

The detection limits calculated by the logistic model for other antimicrobial agents (aminoglycosides, macrolides and quinolones) with the ResScreen® system are provided in Table 3. Of all these antibiotics, only neomycin, lincomycin and tylosin residues were detected by the ResScreen® system at levels approaching their MRLs.

Various authors have indicated similar detection limits to those calculated in Table 3 by other methods using *G. stearothermophilus* subsp. *calidolactis*, such as BRT® AIM[22-23], Charm® AIM-96[24] and Delvotest® "SP"[22,25] indicating good sensitivity to these three antibiotics (neomycin, lincomycin, tylosin) and a low detection capacity for the rest of antimicrobials.

Identification of antibiotic residues by the ResScreen® system

Table 4 summarizes the results of Table 2 and Table 3 by collectively and simply presenting the interpretation of the results of both bioassays.

Milk samples that led to changes in color of both bioassays indicate the absence of antimicrobials (or substances that were not detected by this system). Beta-lactam antibiotics were identified by the persistence of both methods' original colors. The fact that the original color of the "BT" bioassay remained and the original color of the "BS" bioassay changed denotes the presence of tetracycline residues. Conversely, milk samples that have sulfamides brought about a change in the color of the “BT” method but maintained the color of the “BS” method.

Finally, those milk samples containing neomycin, lincomycin or tylosin residues were detected by the ResScreen® system, but were identified as beta-lactams because the "BT" and "BS" bioassays were sensitive enough to detect such substances (Table 3). The difficulty owing to the cross specificity of the ResScreen® system could be resolved by implementing subsequent tests with penicillinase and cephalosporinase enzymes.

CONCLUSIONS

To summarize, the ResScreen® system uses only two bioassays and provides a simple, economical solution to identify residues in milk. Moreover, this microbiological system identifies a larger number of antibiotic families (beta-lactams, tetracyclines and sulfamides) compared with current penicillinase and p-aminobenzoic acid methodologies (beta-lactams and sulfamides).

In the future, new bioassays can be incorporated into the ResScreen® system in order to increase its identification capacity to other antibiotic groups (macrolides, aminoglycosides or quinolones).

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Table 1. Summary of the logistic regression model parameters of antibiotics in milk for the ResScreen® system

Antibiotics	ResScreen® "BT		ResScreen® "BS"	
	Logit = $\beta_0 + \beta_1$*[A]	C	Logit = $\beta_0 + \beta_1$*[A]	C
Betalactams				
Amoxycillin	Logit = -11.3966 + 1.5185*[A]	96.7	Logit = -15.8159 + 3.7160*[A]	97.7
Ampicillin	Logit = -14.7862 + 2.3659*[A]	98.5	Logit = -21.6358 + 6.8009*[A]	99.1
Cloxacillin	Logit = -13.1755 + 0.3835*[A]	97.9	Logit = -10.9673 + 0.3371*[A]	97.5
Oxacillin	Logit = -18.4151 + 1.2483*[A]	98.6	Logit = -22.3155 + 1.5409*[A]	98.9
Penicillin "G"	Logit = -16.1514 + 6.1636*[A]	98.9	Logit = -22.5024 + 8.1827*[A]	99.4
Cefadroxil	Logit = -7.9435 + 0.0683*[A]	95.7	Logit = -16.0260 + 0.0970*[A]	97.5
Cephalexin	Logit = -10.0512 + 0.1313*[A]	97.4	Logit = -9.9664 + 0.0767*[A]	97.0
Cefoperazone	Logit = -11.0985 + 0.2277*[A]	98.3	Logit = -12.6755 + 0.1669*[A]	97.9
Ceftiofur®	Logit = -12.1451+ 0.1438*[A]	98.8	Logit = -6.7069 + 0.0841*[A]	94.3
Cefuroxime	Logit = -13.07 + 0.3282*[A]	98.9	Logit = -20.0044 + 0.1321*[A]	99.6
Tetracyclines				
Clortetracycline	Logit = -9.4066+ 0.0556*[A]	90.7	Logit = -10.1408 + 0.0036*[A]	97.7
Oxytetracycline	Logit = -10.8242 + 0.0933*[A]	89.2	Logit = -9.9616 + 0.0153*[A]	97.0
Tetracycline	Logit = -9.0156 + 0.0627*[A]	89.8	Logit = -26.5938 + 0.0309*[A]	99.4
Sulfonamides				
Sulfadiazine	Logit = -8.2241 + 0.0002*[A]	95.0	Logit = -22.089+ 0.1525*[A]	91.0
Sulfadimethoxine	Logit = -18.8281 + 0.0018*[A]	98.7	Logit = -11.9029 + 0.0577*[A]	90.0
Sulfamethoxazole	Logit = -16.7196 + 0.0015*[A]	97.9	Logit = -11.0868 + 0.1167*[A]	89.3
Sulfathiazole	Logit = -20.2747 + 0.0017*[A]	98.6	Logit = -9.0399 + 0.1246*[A]	89.3

β_0, β_1 = coefficients estimated for the logistic regression models; [A]: antimicrobial concentrations; C: concordance coefficients.

Table 2. The ResScreen® system detection limits (µg l^{-1}) for antibiotics in milk

Antibiotics	ResScreen®		BRT® AIM		Delvotest®SP		Charm® AIM	Eclipse® 100ov	MRLs[a]
	BT	BS	Charm y Ruth (1993)[(22)]	Heeschen *et al.* (1995)[(23)]	Charm y Ruth (1993)[(22)]	Althaus *et al.* (2002)[(25)*]	Linage *et al* (2007)[(24)*]	Montero *et al.* (2005)[(26)*]	
Beta-lactams									
Amoxycillin	8	5	5	---	10	5	---	7	4
Ampicillin	7	4	10	5	10	3	6	---	4
Cloxacillin	42	40	100	35	50	23	42	68	30
Oxacillin	17	16	---	---	---	---	---	28	30
Penicillin "G"	3	3	10	1.5	2.5	1.4	4	5	4
Cefadroxil	159	190	---	---	---	63	---	86	---
Cephalexin	99	160	---	---	---	68	202	115	100
Cefoperazone	62	94	---	---	---	41	82	110	50
Ceftiofur®	105	115	100	---	50	59	107	---	100
Cefuroxime	42	170	---	---	---	41	---	85	---
Tetracyclines									
Clortetracycline	275	3600	>1000	---	420	---	3989	1500	100
Oxytetracycline	150	850	1000	---	200	420	501	560	100
Tetracycline	158	720	1000	450	420	450	257	480	100
Sulfonamides									
Sulfadiazine	49000	164	1000	100-1000	>1000	260	---	---	100
Sulfadimethoxine	12000	260	100	100-1000	>1000	---	119	170	100
Sulfamethoxazole	14000	120	---	---	---	110	---	---	100
Sulfathiazole	13000	100	1000	100-1000	>1000	---	151	250	100

[a] MRLs (µg l^{-1}), EU maximum residue limits, [*]: Decision limits in ewe milk.

Table 3. The ResScreen® system detection limits (µg l^{-1}) for other antimicrobials in milk

Other antimicrobials	ResScreen®		BRT® AIM		Delvotest®SP		Charm® AIM	Eclipse® 100ov	MRLs[a]
	BT	BS	Charm y Ruth (1993)[22]	Heeschen *et al.* (1995)[23]	Charm y Ruth (1993)[22]	Althaus *et al.* (2002)[25]*	Linage *et al* (2007)[24]*	Montero *et al.* (2005)[26]*	
Aminoglycosides									
Gentamycin	320	530	>500	---	150	1200	382	3140	100
Kanamycin	5600	6200	---	---	---	---	---	18700	150
Neomycin	600	1200	>500	300	150	3300	1084	9100	1500
Streptomycin	2300	3600	>1000	---	>1000	10000	3593	10100	200
Macrolides									
Erythromycin	210	190	1000	2250	400	980	522	750	40
Lincomycin	150	220	---	---	---	---	---	---	150
Tylosin	74	50	50	---	100	120	51	230	50
Spiramycin	3400	2600	---	---	---	---	1346	18100	200
Quinolones									
Ciprofloxacin	1750	1710	---	---	---	---	---	5100	100
Enrofloxacin	2000	2300	---	---	---	---	46000	4000	100
Marbofloxacin	2700	4400	---	---	---	---	---	---	75
Norfloxacin	7100	6800	---	---	---	---	---	9500	---

[a] MRLs (µg l^{-1}), EU maximum residue limits, *: Decision limits in ewe milk.

Table 4. Interpretation of the ResScreen® system results

Antibiotics	Results	
	ResScreen® "BT"	ResScreen® "BS"
Absence (or not detect)	-	-
Betalactams	+(*)	+(*)
Tetracyclines	+	-
Sulfamides	-	+

(*) Interference due to neomycin, lincomycin and tylosin.

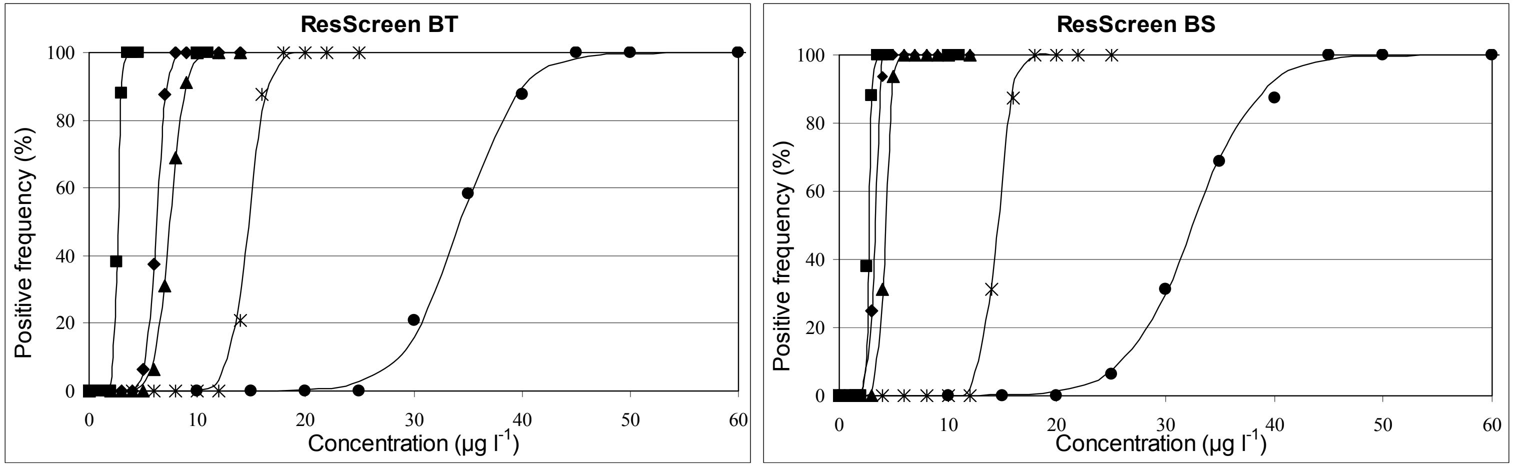

Fig. 1 Dose-response curves for different penicillin concentrations in milk analyzed by the Rescreen® system (▲: amoxycillin, ♦: ampicillin, ●: cloxacillin, **X**: oxacilina, ■: penicillin "G").

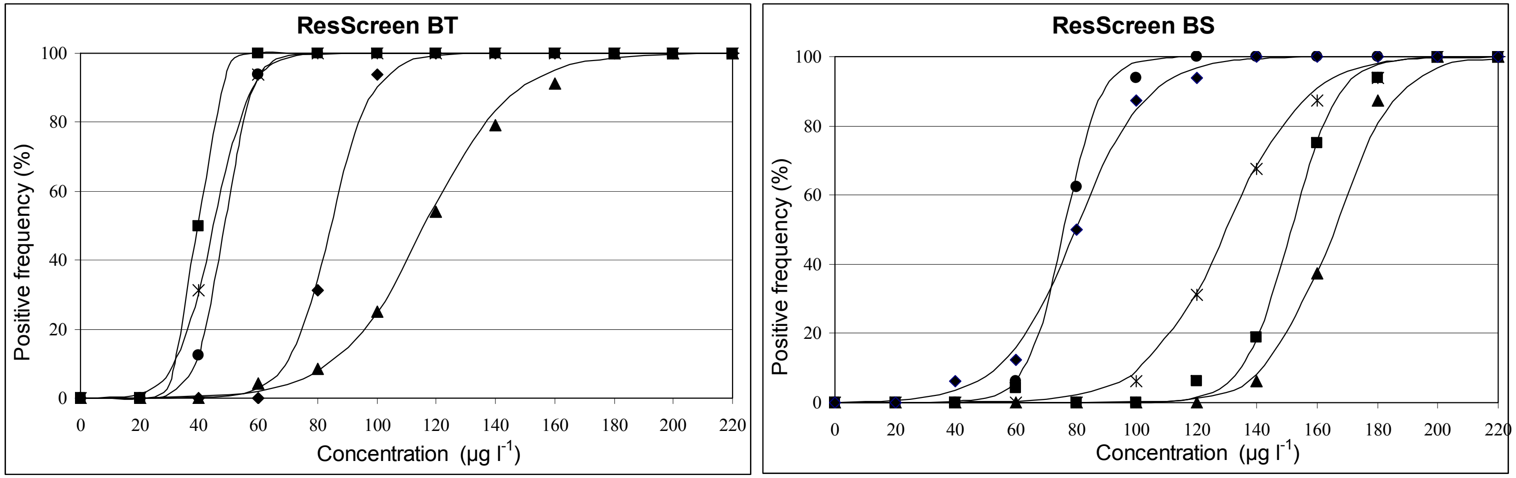

Fig. 2 Dose-response curves for different cephalosporin concentrations in milk analyzed by the Rescreen® system (▲: cefadroxil, X: cephalexin, ●: cefoperazone, ♦: ceftiofur®, ■: cefuroxime).

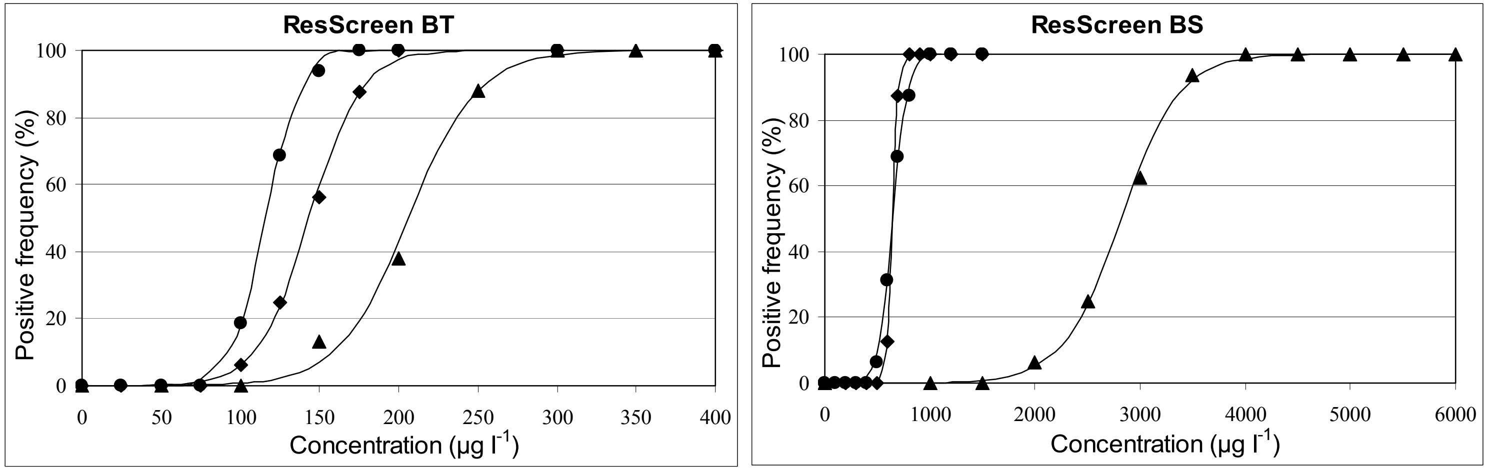

Fig. 3 Dose-response curves for different tetracycline concentrations in milk analyzed by the Rescreen® system (▲: chlortetracyline, ●: oxytetracycline, ♦: tetracycline).

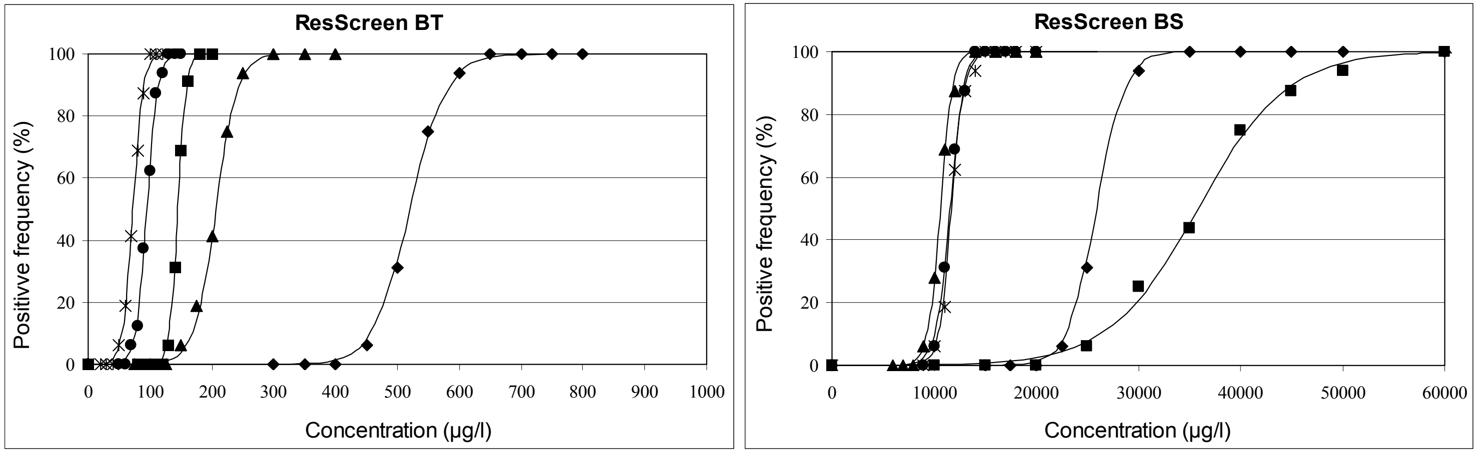

Fig. 4 Dose-response curves for different sulphamide concentrations in milk analyzed by the Rescreen® system (■: sulfadiazine, ▲: sulfadimethoxine, ♦: sulfamethazine, ●: sulfamethoxazole, X: sulfatiazole).