Validation studies with commercially available inhibitor tests for the detection of inhibitors and antibiotic residues in milk

By G. Suhren und K. Knappstein

Institute for Hygiene and Food Safety, Federal Research Centre for Nutrition and Food – Location Kiel, P.O. Box 6069, D-24121 Kiel

1. Introduction

The implications of the use of antimicrobials in animal husbandry is of significance for the food industry, not only because of beneficial therapeutic properties but also because of the influence of residues on the technological properties of milk, the toxicological significance for consumers and the potential for increasing antibiotic resistance in pathogenic and environmental micro-organisms. Under toxicological aspects the safety of milk and milk products is described by indices such as maximum residue limits (MRLs) or safe/tolerance levels. Regulation 2377/90 EEC specifies MRLs of veterinary drugs in foods of animal origin for the European Community (1). To date MRLs have been established for more than 40 antimicrobial substances or groups (sulfonamides) in milk from different species with concentrations between 4 µg/kg (penicillin, ampicillin, amoxicillin) and 1 500 µg/kg (neomycin); the β-lactam antibiotic group contains 16 substances with fixed MRLs in a range between 4 µg/kg and 125 µg/kg (cefacetrile). This requires appropriate testing methods which are able to detect the MRL concentrations. Further on, banned substances as e.g. chloramphenicol need to be detected by methods which fulfil minimum required performance limits (MRPL) (2), which is e.g 0.3 µg/kg in the case of chloramphenicol.

To ensure the high technological quality and safety of milk for the processor and consumer, an integrated detection system was developed by the International Dairy Federation (IDF), which is composed of two different aspects (3, 4):

1. defining the shared responsibilities of veterinarians, farmers, food processors and food inspection services (Fig. 1) and
2. application of different methods which provide different levels of “analytical depth” and complementary “detection patterns” (Fig. 2).

Under shared responsibilities the Proposed Draft Revised Guidelines for the Establishment of a Regulatory Program for the Control of Veterinary Drug Residues in Foods of the Codex Alimentarius (5) states very clearly that the control of residues of veterinary drugs including antibiotics should begin at the farm level by using veterinary drugs approved for use in food producing animals with appropriate withholding times; for lactating animals food safety measures have to provide sustainable assurance on a daily basis that milk is harvested only from those animals considered to have an acceptable status. In addition to fulfilling the aims of food regulations (e.g. within the EU one sample per 15 000 tonnes of milk produced must be analysed for compliance with the MRLs (6)) and of quality payment, also the dairy processor benefits from testing of milk for antimicrobial residues

by ensuring that the milk is suitable for the production of fermented dairy products (7-9). As there is no single method available for the detection of the antibiotics at or below MRL concentrations the IDF integrated system includes an appropriate combination of methods which complement each other in their “detection pattern” and the required “analytical depth”.

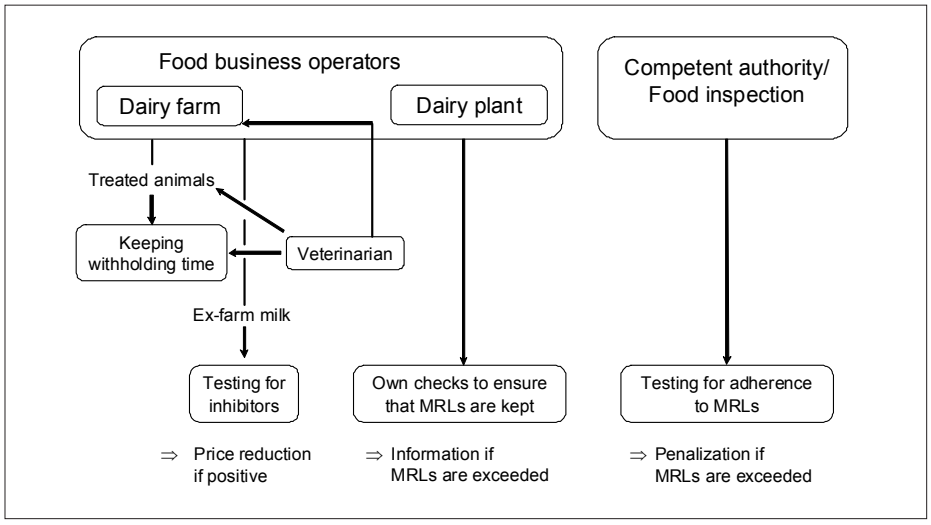

Fig. 1: Integrated detection system for antibiotic residues in milk – shared responsibilities

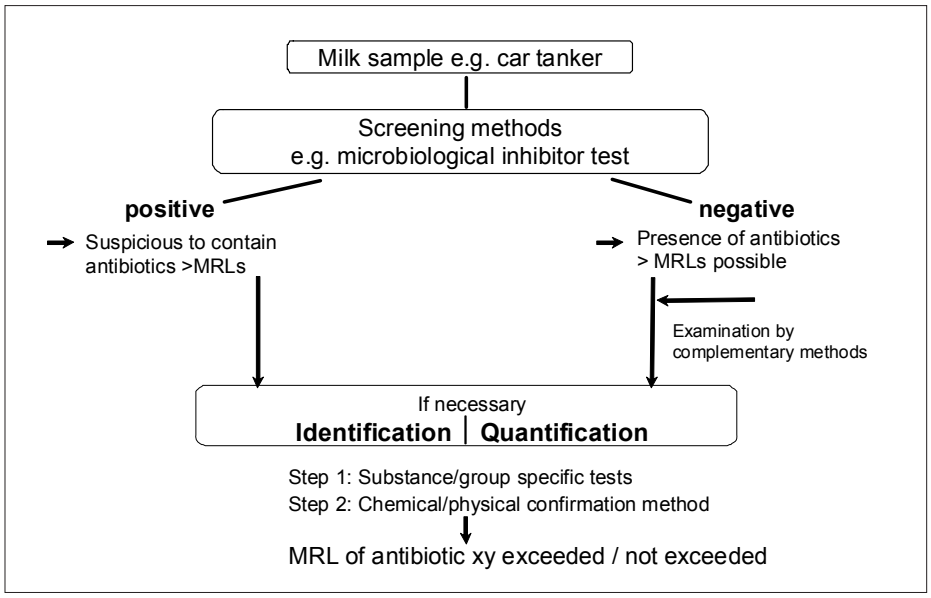

Fig. 2: Integrated detection system for antibiotic residues in milk – method combinations

Inhibitor tests with *Geobacillus stearothermophilus* – former name *Bacillus stearothermophilus* (10) – as test micro-organism in various test formats are in widespread use for the detection of inhibitors/residues of antibiotics in milk for several purposes as e.g. cow-side test within quality management systems, inhibitor test on ex-farm milk for quality payment purposes, self-control measures of food business operators, screening method for food inspection. The demands on the test and the interpretation of test results depend on the intended field of application. The tests are described either in literature (11-14), in official collections of analytical methods (e.g. Official Collection of Analytical Methods according to § 64 of the German Food and Feed Code), international standards (15) or are commercially available as proprietary techniques of several manufacturers. The test micro-organism *Geobacillus stearothermophilus* is especially sensitive for the detection of β-lactam antibiotics and sulfonamides in the case of trimethoprim addition to the agar.

The ideal screening test yields no false negative results at the levels of concern whereas a positive result indicates that follow-up action is required. In table 1 features of microbial inhibitor tests are summarized which characterize them as "ideal" as screening methods within an integrated detection system.

Tab. 1: Features of "ideal" microbial inhibitor and/or screening test

Detection	Broad variety of antimicrobials of concern Limits according to requirements, e.g. MRLs
Performance	Easy performance, e.g. no educated persons, no sophisticated equipment needed Low price Suitability for mechanized analysis of large sample numbers Robustness, e.g. testing of milk from individual cows Short test time Documented results
Susceptibility to interference factors	Low susceptibility to sample composition, e.g. lysozym content, microflora Minor influence of test procedure
Possibilities of preliminary identification of antimicrobials	Easy to perform, e.g. β-lactamase and PABA test
Standardization	Low variability within and between batches Low variability within and between users

The increasing demands regarding stability of test performance and sensitivities of detection in the last decade led to the situation that single application labs are no longer in the situation to prepare their own test systems with the required test performance criteria and that the tests used in practice are most often commercially available ones. Further on the details and knowledge behind the production of such tests can no longer be described in international or national standards in such a way that users are in the position to prepare their own tests when following the method description. Therefore about 10 years ago the responsible working group at the International Dairy Federation

(IDF) decided to work on guidelines for the evaluation of screening tests for the detection of residues of antibiotics in milk instead (16,17). In the validation studies presented in the following these guidelines were followed as closely as appropriate.

Microbial inhibitor tests with indicator are most often evaluated visually in different steps (e.g. negative, questionable, positive). Visual evaluation is subjective and the different modes of action of antimicrobials as well as variation in sample composition can lead to differing colouring of the indicator thus making the interpretation sometimes difficult. In validation studies, e.g. for the determination of detection limits, shelf life of test kits, differences between batches or during development of a new or modified test, slight differences and developments are necessary information which is hardly detectable by justifiable expenditure. Therefore the need for an evaluation method in an objective way which allows more subtle differentiation than the visual reading in few steps was discussed for several years (18-20). Today test kit manufacturers provide software which allows for objective instrumental colour measurement by means of scanner technology. The scanner is calibrated by a world-wide available colour reference (Kodak™ Professional). The software provided by the manufacturers calculates the colour of the test from three coordinates L*= lightness, a*= green-to-red chromaticity and b*= blue-to-yellow chromaticity by algorithms, for which patents are pending. The results are indicated as z-values in the case of tests from DSM (Table 2) or as colour image factor (cif)-values in the case of Copan tests (table 2, today also distributed by DSM). First results with the evaluation of test results by scanner technology are published (21-23).

The aim of the study was to validate commercially available inhibitor tests with *Geobacillus stearothermophilus* as test-microorganism and either bromcresol purple or brilliant black as indicator. In addition, the BetaStar test[1], a protein receptor test for the detection of β-lactam antibiotics was used in some cases as rapid and as preliminary confirmation test. Evaluation was by visual reading in 4 steps, by photometric reading and evaluation as relativated absorption in % (19) and/or by colour measurement with scanner technique and proprietary algorithms.

2. Experimentals

2.1 Material

2.1.1 Microbial inhibitor test formats

In table 2 commercially available inhibitor test formats with *Geobacillus stearothermophilus* as test micro-organism, which were included in the validation study, are summarized.

2.1.2 Inhibitor free milk

Composite milk of at least 10 German Holstein cows, black and white, of the experimental farm Schaedtbek of the Federal Research Centre for Nutrition and Food (BfEL), which were not treated with antibiotics within at least 3 weeks preceding the experiment was used. The somatic cell count was less than 150 000/ml. The milk was stored under cooling conditions and used on the day of collection. Before preparing test samples the milk was tested for the absence of inhibitors by microbial inhibitor tests.

---

[1] UCB Bioproducts, Chemin du Foriest, 1420 Braine-L'Alleud, Belgium

Tab. 2: Microbial inhibitor tests included in the validation study

Trade name	Manufacturer	Batch no.	Incubation time at 64°C
BRT	AIM GmbH, Kaiser-Ludwig Platz 2, 80336 München/Germany	9 batches	2 h 25 min
CMT Copan Milk Test*	COPAN, Via Perotti 10, 25125 Brescia/Italy	968 970 F2 0290 F3 0530 F3 1081 F3 1080 F4 1598	3 h 15 min
Delvo MCS	DSM Food Specialities, P.O. Box 1, 2600 MA Delft/Netherlands	Without number 04I 28A 38 04K 11A 38 05A 27A 38 05H 16B 38 05J 19C 38 06A 03A 38 06D 05A 38 06E 10A 38 06H 28C 38 06J 31A 38	2 h 45 min
Delvo SP**	DSM Food Specialities, P.O. Box 1, 2600 MA Delft/Netherlands	05D 25B 22 05G 05A 22 05G 20C 22 05H 22A 22 05I 23A 22	2 h 35 min
BR-AS special	DSM Food Specialities, P.O. Box 1, 2600 MA Delft/Netherlands	05D 11A 60 05F 30B 60 05H 24A 60 05K 14A 60 05L 20A 60 06D 24A 60 06G 05A 60 06F 27A 60 06J 17A 60 06K 21A 60	2 h 35 min
BR-AS Brilliant	DSM Food Specialities, P.O. Box 1, 2600 MA Delft/Netherlands	05D 11A 26 05I 05A 26 05J 13B 26 05K 09A 26 05L 19D 26 06C 09B 26 06G 31/26 06J 16/26 06L 18/26	2 h 15 min

* today distributed by DSM Food Specialities, P.O. Box 1, 2600 MA Delft/Netherlands

** No longer available on European market

Tab. 3: Antimicrobials used for the preparation of stock solutions (1 000 000 µg/l distilled water) for spiked milk samples

Substance	Abbreviation	Manufacturer	Order number	50 mg pure substance	Solvent*
β-lactam antibiotics					
Amoxicillin sodium salt	AMOX	Sigma	A8523	57.5 mg	H_2O
Ampicillin sodium salt	AMP	Sigma	A9518	54.5 mg	H_2O
Cefoperazone sodium salt	CPZ	Sigma	C4292	57.6 mg	H_2O
Cefquinome	CEF	Intervet		63.3 mg	0.1% acetic acid
Ceftiofur sodium salt	CFT	Pharmacia Upjohn		56.0 mg	H_2O
Cefalexin hydrate	CEX	Sigma	C4895	53.2 mg	H_2O
Cefapirin sodium salt	CEP	Sigma	C8270	52.6 mg	H_2O
Cefazolin sodium salt	CEZ	Sigma	C5020	53.0 mg	H_2O
Cloxacillin sodium salt	CLX	Sigma	C9393	55.1 mg	H_2O
Oxacillin sodium salt	OXA	Sigma	O1002	56.2 mg	H_2O
Penicillin G potassium salt	PEN	Sigma	PEN-K	56.0 mg	H_2O
Nafcillin sodium salt	NAF	Sigma	N3269	57.0 mg	H_2O
Tetracyclines					
Chlortetracycline hydrochloride	CTC	Sigma	C4881	59.7 mg	Methanole
Oxytetracyline hydrochloride	OTC	Sigma	O5875	54.0 mg	Methanole
Tetracyline	TC	Sigma	T3258	56.0 mg	Methanole
Macrolides					
Erythromycin	ERY	Sigma	E6376	52.0 mg	Methanole
Spiramycin	SPIRA	Sigma	S9132	61.5 mg	H_2O
Tylosin tartrate	TYL	Sigma	T6134	55.6 mg	H_2O
Aminoglycosides					
Dihydrostreptomycin sesquisulfate	DHS	Sigma	D7253	72.6 mg	H_2O
Gentamicin sulfate	GENT	Sigma	G3632	72.3 mg	H_2O
Neomycin sulfate	NEO	Serva	30250	116.0 mg**	H_2O
Sulfonamides					
Sulfadiazine	SDZ	Serva	35633	50.0 mg	Methanole/ 5ml 1N NaOH
Sulfadimidine	SDM	Serva	35635	50.0 mg	Methanole/ 5ml 1N NaOH
Sulfadoxine	SDX	Serva	35640	50.0 mg	Methanole/ 5ml 1N HCl
Sulfamethoxazole	SMX	Sigma	S7507	50.0 mg	Methanole/ 5ml 1N NaOH
Various					
Dapsone	DAP	Merck	821073	50.0 mg	0.1 N HCl
Lincomycin hydrochloride	LINCO	Sigma	L6004	56.5 mg	H_2O
Trimethoprim	TRIM	Sigma	T7883	50.0 mg	5% acetic acid

* 50 mg pure substance in 1 ml solvent, further dilution with H_2O

** 2 000 000 µg/kg stock solution

2.1.3 Antimicrobials

In table 3 the antimicrobials used for the preparation of spiked samples are summarized. Stock solutions with a concentration of 1 000 000 µg/kg (exception neomycin) were prepared; all further dilutions were with distilled water. Final dilution for the targeted concentrations was 1:100 with inhibitor free milk.

2.1.4 Veterinary drugs

In order to obtain incurred milk samples healthy cows as well as cows suffering from mastitis (German Holstein, black and white) from the experimental farm of the BfEL were treated with veterinary drugs. The following drugs were applied:

- Cobactan LC (mastitis treatment in lactation): Intervet International (former Hoechst Roussel Vet), Unterschleissheim/DE; substance: cefquinome
- Cobactan DC (dry cow therapy), Intervet International, see above, substance: cefquinome
- Procain Penicillin G 3 Mio udder injector (mastitis treatment in lactation): WDT, Garbsen/DE; substance: procain benzylpenicillin
- Nafpenzal MC (mastitis treatment in lactation): Intervet International, Boxmeer/NL; substances: penicillin sodium, nafcillin sodium, dihydrostreptomycin
- Omnygram (mastitis treatment in lactation): Virbac S.A., Carros/FR; substances: ampicillin trihydrate, colistin sulfate
- Peracef (mastitis treatment in lactation): Pfizer, Karlsruhe/DE, substance: cefoperazone
- Vetriclox TS (dry cow therapy): CEVA/DE; treatment during dry period; substance: cloxacillin sodium

2.1.5 Test samples

- Control milk samples: The control samples (negative control and positive control with 4 µg penicillin/kg, one batch each) were prepared following the instructions of ISO 13969/IDF 183 (16), preserved by lyophilization and stored at 6°C in the dark.
- Spiked milk samples: For the determination of the detection limits inhibitor free milk was spiked with different concentrations of antibiotics. The test samples were used within 2 days at 6°C or after deep freezing within 3 weeks. The antibiotic/concentrations tested are summarized in table 5. Test samples for control of test kit batches (2 test sample batches: July 2000 and October 2006) were preserved by lyophilization and stored at 6°C in the dark.
- Test samples (negative and incurred): Composite milk samples from treatment trials (experimental farm of the BfEL) were collected at every milking during anamnestic period, treatment period and withholding time plus at least 6 milking times after the end of the withholding time. The samples were stored at 6°C for 60 hours at maximum. For further investigations cow composite samples were stored at -20°C for 3 weeks at maximum. Samples for later re-examinations were preserved by lyophilization and stored at 6°C in the dark.
- Influence of pH-values of milk: In order to obtain milk samples with different pH-values different volumes of sodium hydroxide or lactic acid, respectively were added to inhibitor free bulk tank milk (experimental farm of the BfEL). The dilution of the milk was ≤ 1:100.

- Influence of individual cows: Composite milk samples from the experimental farm of the BfEL were collected after at least 6 days after calving. Samples from treatment trials (see incurred samples) of the anamnestic period and after the end of the withholding time were used under the conditions that the concentrations of the antibiotics applied were below the detection limits of the corresponding HPLC-methods and of microbial inhibitor tests.
- Influence of days in milk: Composite milk samples (experimental farm, BfEL) were taken at each milking time after calving for at least 7 days. If dry cow therapy was applied the samples were analysed by HPLC analyses in the case of suspicious or positive screening test results. Only the results of those samples were included in the evaluation, which were negative in preliminary confirmation tests – penicillinase, BetaStar – and/or by HPLC analysis.
- Ex-farm-milk: Samples from farm bulk tank milk were provided by the Milk Control Station Schleswig-Holstein and analysed by a method combination.
- Car tanker milk samples: Milk samples from car tankers of the northern part of Germany were provided by the Milcherzeugervereinigung Schleswig-Holstien and analysed by a method combination.
- Spiked milk samples from ADR[2]-proficiency test 2006: Milk samples were spiked with different concentrations of penicillin G potassium, dispensed and distributed deep frozen under the auspices of Milchpruefring Bayern in co-operation with the Veterinary Faculty of the University of Munich.

2.2 Methods

2.2.1 Microbial inhibitor tests

For sample inoculation (100 µl) into the wells of the microtitre plates an Eppendorf pipette was used. On each test plate four wells were inoculated with positive control (4 µg penicillin/kg) and four wells with the negative control sample. The plates were sealed by an adhesive foil. Incubation was performed in a water bath adjusted to 64°C. At the end of the indicated incubation time – see table 2 – the colour of the control samples was checked. Before reading the foil was removed, the milk discarded and the wells washed with water. All plates were read visually, by photometric measurement and in dependence on the test by colour measurement with a scanner. All test kits included in this study were inoculated from the same sample vial (with exceptions for some of the incurred samples), incubated and evaluated in parallel.

Visual reading

The test plates were evaluated visually in 4 steps:

1= negative,

2= colour slightly deviating from that of negative control sample,

3= colour deviating from that of the negative control sample and

4= colour at least as intensive as that of the positive control sample.

---

[2] Arbeitsgemeinschaft Deutscher Rinderzüchter (German Cattle Breeders Association), Adenauer Allee 174, D-53113 Bonn

For quality payment purposes in Germany only those samples which are evaluated as positive (step 4) are indicated as inhibitor positive (24). If microbial inhibitor tests are used for screening purposes samples with colours deviating from the negative control are defined as suspicious to contain antibiotics (step 2, 3 and 4); further analyses for identification and quantification are required (25). If not indicated otherwise all plates were read by one trained person.

Photometric measurement

In order to get results of instrumental reading with the same procedure for all test formats the plates were evaluated by the method of relativated absorption in % by photometric measurement with an ELISA-reader (550nm/690nm measurement/ reference wavelength) (19). In order to obtain a standardized scale, the difference between the mean absorption values of negative and positive controls was set as 100% and the absorption values of the wells of the corresponding plates calculated in 10%-steps with respect to the control values ("relativated absorption in %"). The cut-off value for all test kits was set at 55%.

Colour measurement

DSM and Copan offer software for the evaluation of microbial inhibitor tests by colour measurements by means of a scanner. The provided software calculates by an algorithmus the colour of the test from three coordinates L*= lightness, a*= green-to-red chromaticity and b*= blue-to-yellow chromaticity. The scanners are calibrated by a world-wide available colour reference (Kodak™ Professional). The algorithms are not revealed by the test kit manufacturers.

The results of DSM-tests (Delvo MCS, Delvo SP and BR-AS special) are indicated as z-values with a proposed cut-off value of $z \geq 0$. The results of Copan test are indicated as cif (colour image factor)-values and the proposed cut-off level is $cif \geq 4.5$. The latter algorithm includes fine tuning in the area $3.5<cif<5.5$.

2.2.2 HPLC methods

For the identification and quantification of the antibiotics in incurred milk samples published HPLC-methods (26-29) or *in-house* validated method (cefoperazone) were applied. The experimental parameters are summarized in table 4.

Tab. 4: Criteria of HPLC-methods applied

Criterion	CEF	PEN	NAF	CLX	CPZ	DHS	AMP	COL
Recovery (in %)*	93	64	48	75	50	95	71	106
Repeatability, s (µg/kg)*	0.9	0.4	1.1	1.0	1.3	8.5	0.4	7.2
LOD** (µg/kg)	2	1	1	0.7	1	29	1	9
LOQ*** (µg/kg)	3	1	1	1.2	10	54	1	14

* Level of MRL-concentrations; ** Limit of detection;*** Limit of quantification

2.2.3 Preliminary confirmation test – Beta Star

The commercially available Beta Star test is a receptor assay for rapid detection of β-lactam antibiotic residues in milk. The test is interpreted by visual comparison of a test band with a reference band. Results are coded in 4 levels; step 1 is interpreted as negative and steps 2, 3 and 4 are interpreted as positive.

2.2.4 Somatic cell count

Somatic cell count was measured in milk samples according to IDF Standard 148 A (30) by Fossomatic 5000.

2.2.5 Lyophilization of test samples

2.2 ml of negative control or of spiked milk samples were dispensed into polypropylene vials by a dispensette (Brand Easy Cal III, adjustable to 0.5-5.0 ml). The samples were deep frozen at -18°C for 24 hours and afterwards lyophilized within 40 hours (pressure 0.65 bar, temperature 30°C) plus additional 2 hours. The vials were capped by screw caps and stored at 6°C in the dark. The samples were resuspended by adding 2 ml distilled water and used on the day of resuspension.

2.3 Results

2.3.1 Detection limits

Spiked milk samples

For the determination of detection limits the design of a blind coded study was followed with test plates of just one batch of each test format. The concentrations included are summarized in table 5 and 6. Fig. 3 and 4 show dose-response curves of two different β-lactam antibiotics established by the different types of reading on two different test formats. In Fig. 5 and 6 the results of visual evaluation as inhibitor test or screening test of all test formats for two antimicrobials are demonstrated as examples. The detection limits evaluated by visual reading are summarized in table 5 in the case of evaluation as inhibitor test (24) and in table 6 in the case of evaluation as screening method (25). The tables include the MRLs fixed according to Regulation 2377/90 EEC (1).

Proficiency test

Within the laboratories responsible for the examination of ex-farm milk for milk quality payment purposes (German Milk Quality Payment Ordinance) once a year a proficiency test for the detection of penicillin in frozen milk samples by the microbial inhibitor tests applied in the labs is performed. The results are presented in Fig 7. In this proficiency study 17 labs participated with different numbers of tests.

Incurred milk samples

In fig. 8 and 9 the relation between antibiotic concentrations as determined by HPLC-methods and the results of Delvo MCS and Copan test in incurred milk samples for the relevant concentration ranges are demonstrated as examples; the concentrations

determined by HPLC-methods include the corresponding recovery factors. From these graphs detection limits by the different modes of reading are derived and are summarized in table 7 For comparison purposes the detection limits for spiked samples (see table 5 and 6) are additionally included. As it was not possible to examine all samples by all methods and mode of test reading the table contains some gaps.

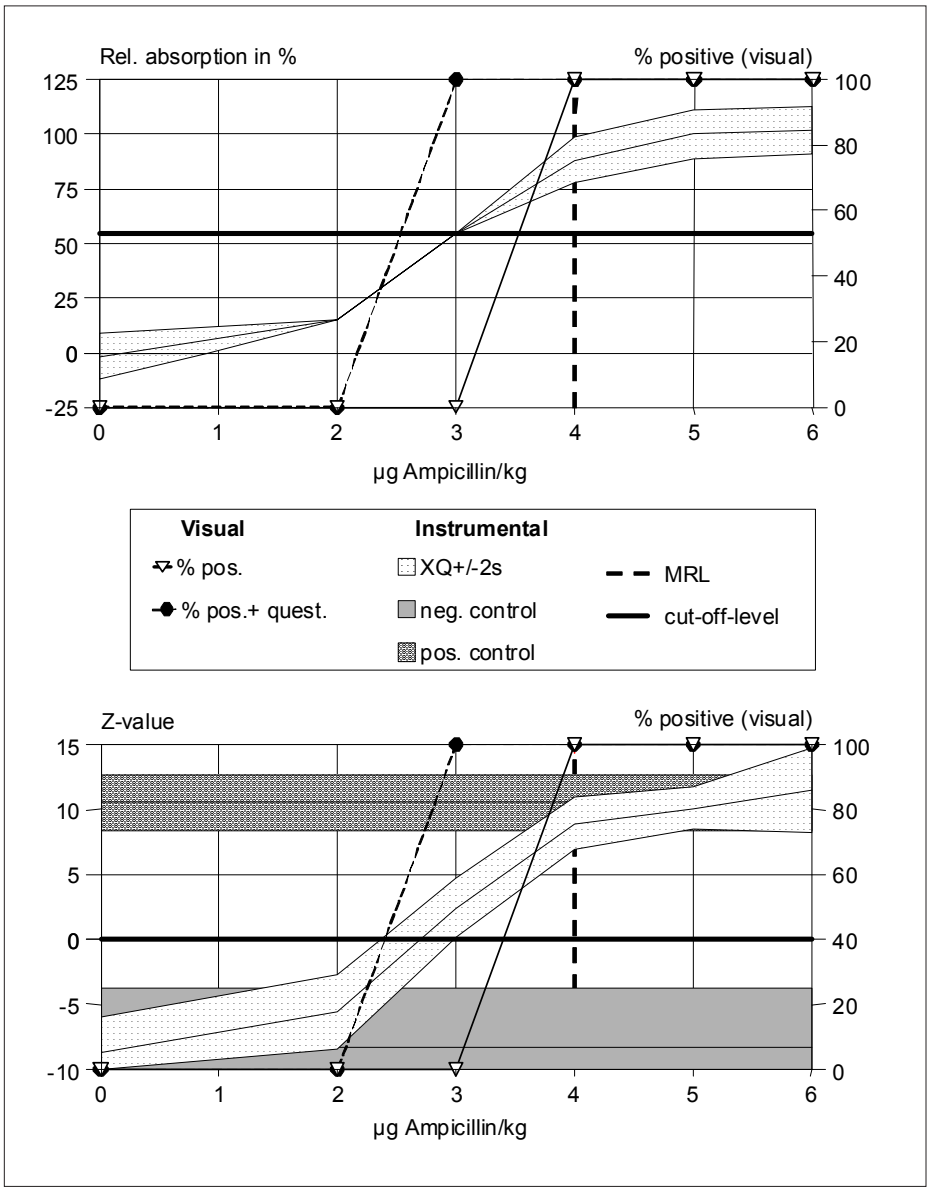

Fig. 3: Dose response curve of ampicillin on Delvo MCS (n = 6-18/concentration)

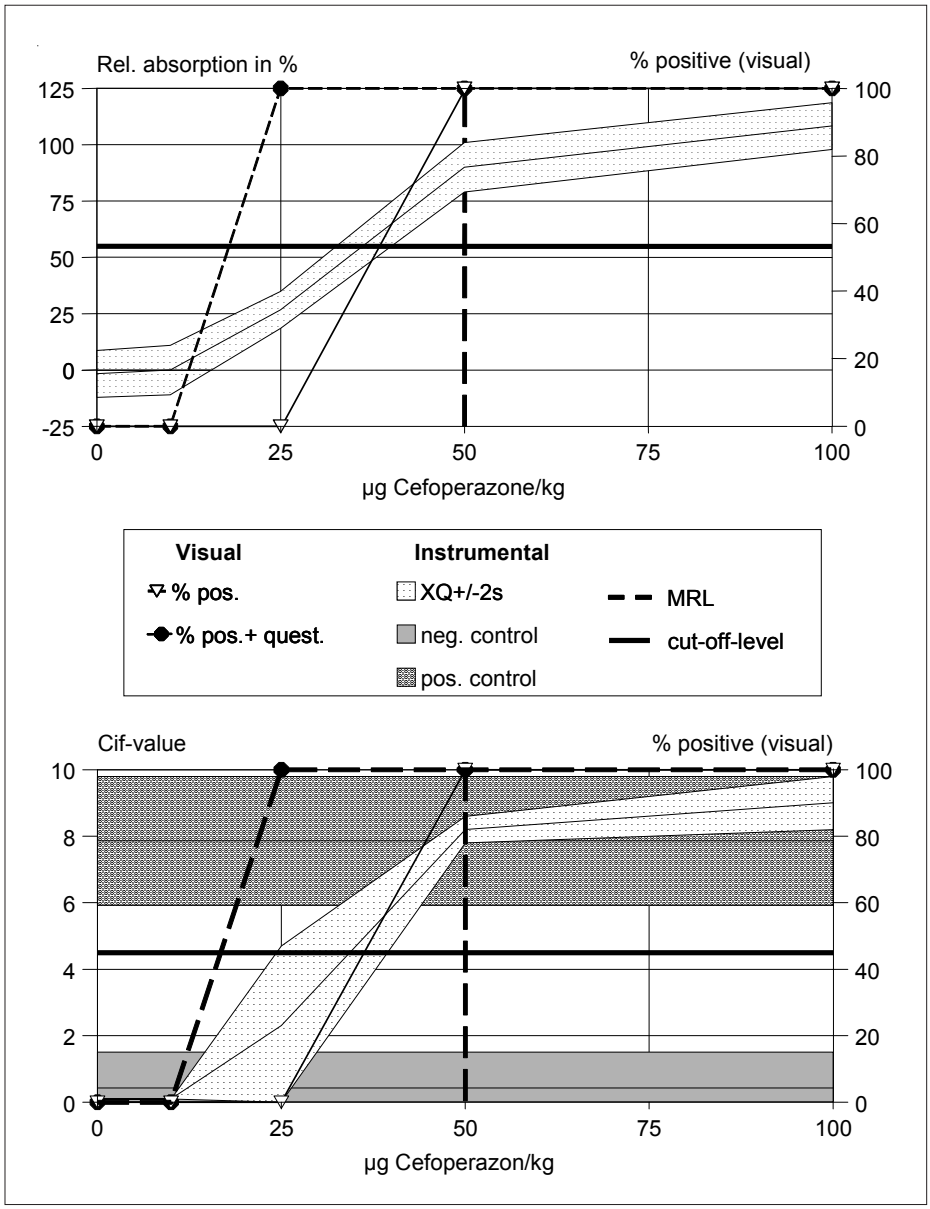

Fig. 4: Dose response curve of cefoperazone on Copan Milk Test (n = 6-18/concentration)

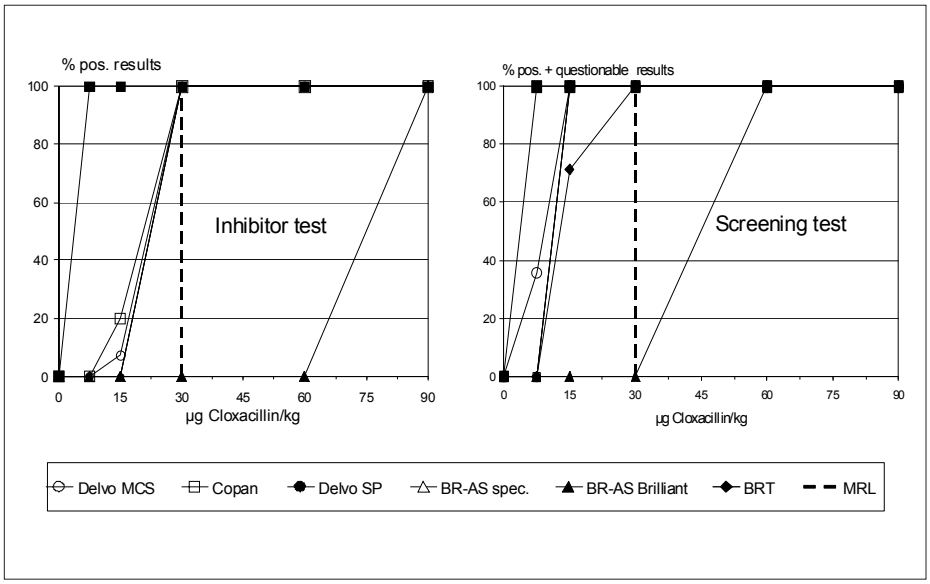

Fig. 5: Dose response curve on various microbial inhibitor tests - cloxacillin (n = 18/concentration)

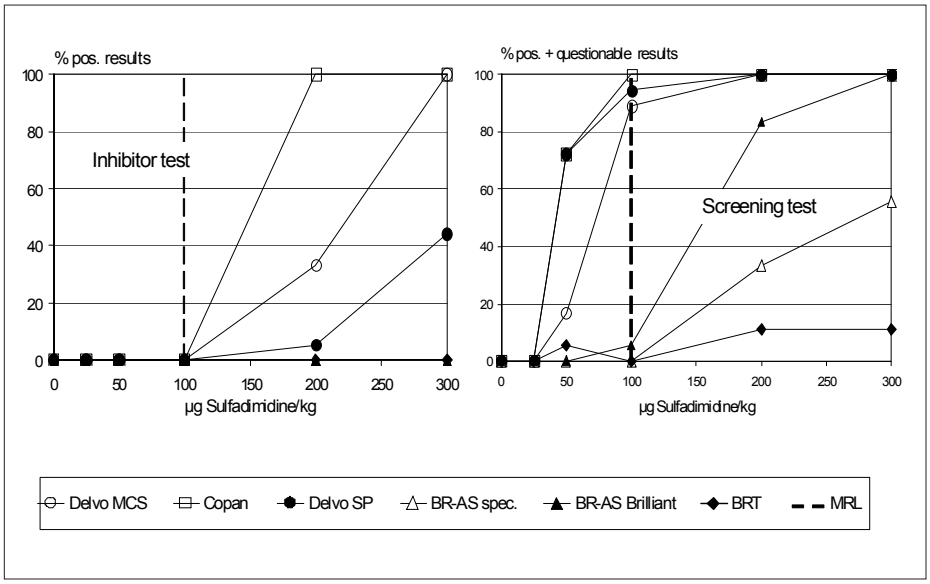

Fig. 6: Dose response curve on various microbial inhibitor tests - sulfadimidine (n = 18/ concentration)

Tab. 5: Detection limits of microbial inhibitor tests – Visual evaluation as inhibitor test*

Substance	MRL**	Concentrations	Delvo MCS	Copan	Delvo SP	BR-AS special	BR-AS Brilliant	BRT	Beta Star
Amoxicillin	4	1, 2, 4, 5, 6	4	3	3	3	4	3	4
Ampicillin	4	2, 3, 4, 5, 6	4	4	5	3	4	5	6
Cefoperazone	50	12.5, 25, 50, 100	50	50	100	50	100	50	10
Cefquinome	20	50, 100, 150, 200, 250	150	150	200	>250	150	>250	15
Cloxacillin	30	7.5, 15, 30	30	30	30	30	90	30	7.5
Penicillin	4	2, 3, 4, 5, 6, 7, 8	2	2	2	2	4	2	2
Oxytetracyline	100	100, 200, 400, 600	>600	600	600	>600	>600	>600	-
Tetracycline	100	100, 200, 400, 600	>600	>600	600	>600	>600	>600	-
Erythromycin	40	40, 80, 160, 320, 600	200	320	400	200	320	320	-
Tylosin	50	25, 50, 75, 100	75	100	50	75	100	100	-
Gentamicin	100	100, 200, 400, 600	400	500	>600	400	>600	>600	-
Neomycin	1500	750, 1500, 3000	1500	>3000	>3000	3000	3000	3000	-
Sulfadimidine	100	25, 50, 100, 200, 400	300	200	>300	>300	>300	>300	-
S-methoxazole	100	25, 50, 100, 200, 400	100	50	100	>300	200	>300	-
Colistin	50	500, 1000, 5000, 10000	>10000	>10000	>10000	>10000	>10000	>10000	-
Dapsone	0	1, 2, 4, 6	6	3	>6	>6	>6	>6	-
Lincomycin	150	75, 150, 300, 450, 600	300	600	450	450	450	>600	-
Trimethoprim	50	50, 100, 200, 400	400	200	>400	>400	>400	>400	-

* Visual evaluation step 4; ** Regulation 2377/90 EEC ; Detection limit ≤ MRL-concentration

Tab. 6: Detection limits of microbial inhibitor tests – Visual evaluation as screening test*

Substance	MRL**	Concentrations	Delvo MCS	Copan	Delvo SP	BR-AS special	BR-AS Brilliant	BRT	Beta Star
Amoxicillin	4	1, 2, 4, 5, 6	3	2	2	2	3	2	3
Ampicillin	4	2, 3, 4, 5, 6	3	3	3	2	3	4	5
Cefoperazone	50	12.5, 25, 50, 100	25	25	25	25	100	25	10
Cefquinome	20	50, 100, 150, 200, 250	100	100	100	200	100	150	10
Cloxacillin	30	7.5, 15, 30	15	15	15	15	60	30	7.5
Penicillin	4	2, 3, 4, 5, 6, 7, 8	2	2	2	2	3	2	2
Oxytetracyline	100	100, 200, 400, 600	400	400	200	400	400	>600	-
Tetracycline	100	100, 200, 400, 600	600	400	400	600	600	>600	-
Erythromycin	40	40, 80, 160, 320, 600	160	160	160	80	160	200	-
Tylosin	50	25, 50, 75, 100	50	50	50	50	50	50	-
Gentamicin	100	100, 200, 400, 600	200	200	400	200	400	400	-
Neomycin	1500	750, 1500, 3000	750	750	750	750	750	750	-
Sulfadimidine	100	25, 50, 100, 200, 400	200	100	100	300	300	>300	-
S-methoxazole	100	25, 50, 100, 200, 400	50	25	25	200	50	>300	-
Colistin	50	500, 1000, 5000, 10000	>10000	>10000	>10000	>10000	>10000	>10000	-
Dapsone	0	1, 2, 4, 6	2	2	4	>6	>6	>6	-
Lincomycin	150	75, 150, 300, 450, 600	150	300	150	300	300	300	-
Trimethoprim	50	50, 100, 200, 400	200	100	200	400	400	>400	-

* Visual evaluation steps 2, 3 and 4; ** Regulation 2377/90 EEC; Detection limit ≤ MRL-concentration

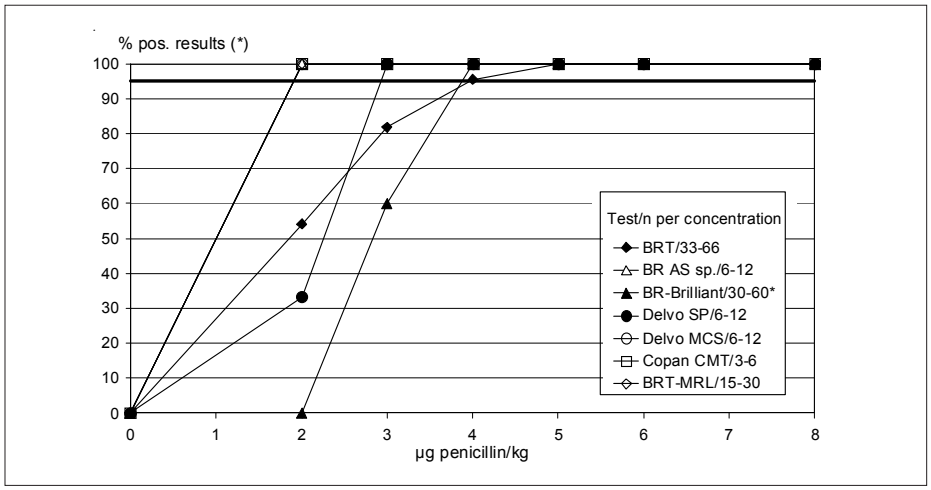

Fig. 7: Ring trial of ADR (German Cattle Breeders Association) 2006: Dose response curves of penicillin on various inhibitor tests: percentage of positive results of 1-11 test series (1 - 10 labs)

*) Evaluation as inhibitor test

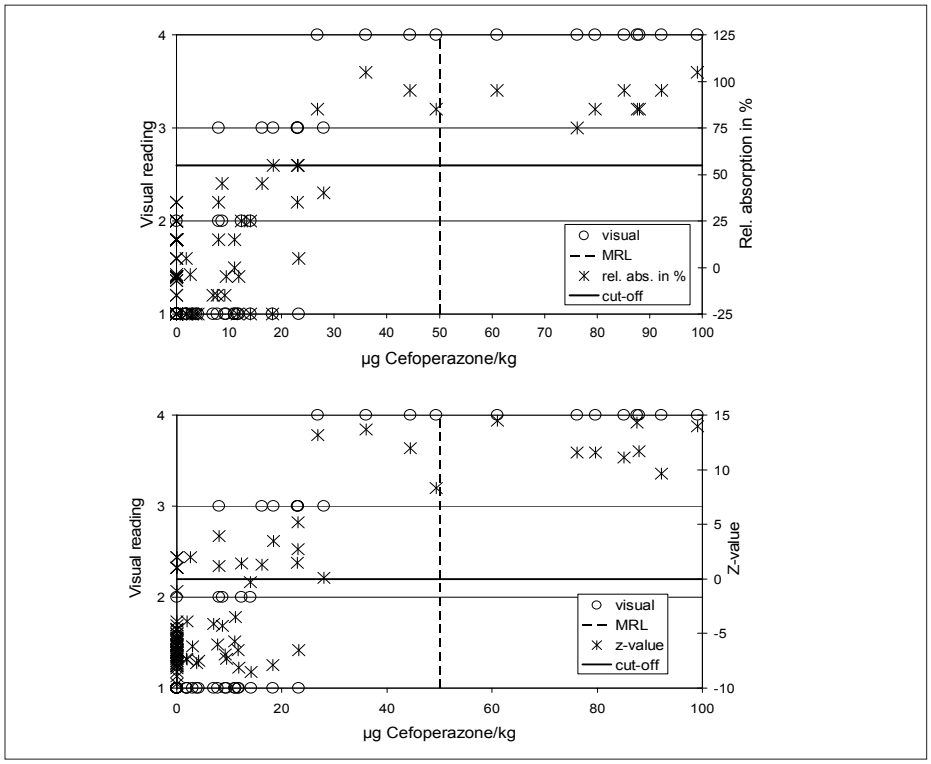

Fig. 8: Incurred milk samples: relation between cefoperazone concentration and results of Delvo MCS

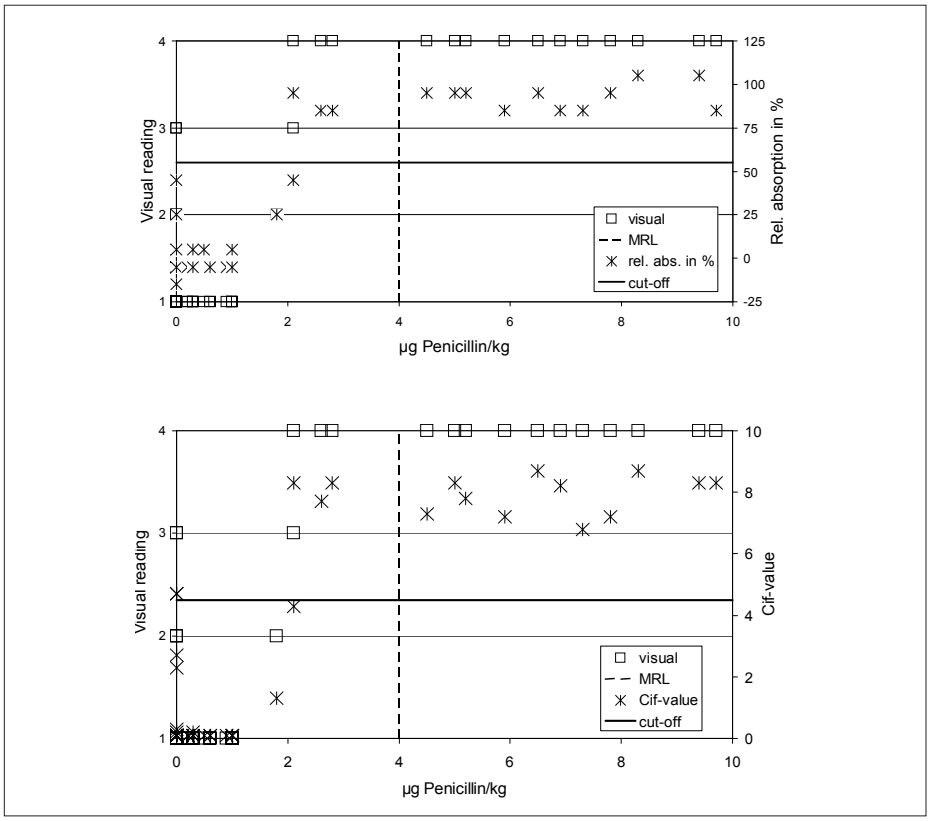

Fig. 9: Incurred milk samples: relation between penicillin concentration and results of Copan Milk Test

Tab. 7: Detection limits (≤ µg/kg) of different tests in incurred milk samples

Substance	Visual reading		Rel. Absorp. in %	Z-/cif-value	Visual reading - spiked samples	
	Inhibitor	Screening			Inhibitor	Screening
Delvo MCS*						
Ampicillin	4	3	4	4	4	3
Cefoperazone	30	25	30	30	50	25
Cefquinome	200	150	150	150	150	100
Penicillin	2	1.5	2	2	2	2
Copan**						
Ampicillin	6	4	6	6	4	3
Cefoperazone	30	20	30	30	50	25
Cefquinome	150	150	150	150	150	100
Penicillin	2.5	2	2.5	2	2	2
Delvo SP*						
Ampicillin	7	5	7	-	5	3
Cefoperazone	40	30	40	40	100	25
Cefquinome	400	300	350	-	200	100
Penicillin	3	2	3	2	2	2
BR-AS special*						
Ampicillin	6	3	5	-	3	2
Cefoperazone	40	30	40	30	50	25
Cefquinome	150	75	250	-	>250	200
Penicillin	2.5	2	3	3	2	2
BR-AS-Brilliant						
Ampicillin	-	-	-	-	4	3
Cefoperazone	60	50	40	-	100	100
Cefquinome	225	175	175	-	150	100
Penicillin	4	3	3	-	4	3
BRT						
Ampicillin	8	5	6	-	5	4
Cefoperazone	50	25	40	-	50	25
Cefquinome	350	75	250	-	>250	150
Penicillin	3	2	2.5	-	2	2
Beta Star						
Ampicillin	8	5	-	-	6	5
Cefoperazone	20	15	-	-	10	10
Cefquinome	20	20	-	-	15	10
Penicillin	2.5	2.5	-	-	2	2

* z-value; ** cif-value

2.3.2 Ruggedness testing

Incubation period of test plates

In order to test the influence of lengthening of incubation period test plates inoculated with spiked milk samples were read at the indicated incubation time and after additional incubation for 20 and 40 min respectively. The results for Delvo MCS and Copan test are presented in fig. 10.

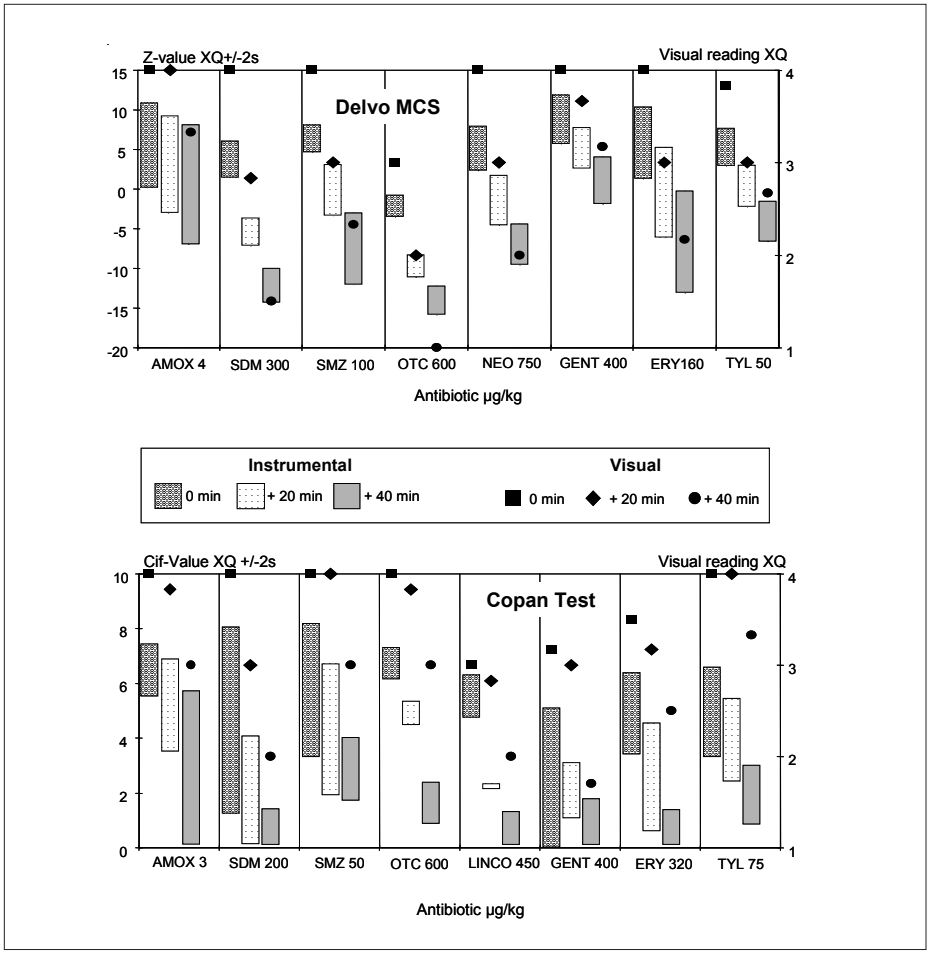

Fig. 10: Influence of deviation of recommended incubation period on instrumental and visual readings

pH-value of samples

The test samples with adjusted different pH-values were analysed on various microbial inhibitor tests in the experimental design of a blind coded study. The results are summarized in table 8.

Tab. 8: Results of microbial inhibitor tests in dependence on pH-value of the samples

pH-value n	5.2-5.6 18		5.7-6.2 18		6.3-6.8 21		6.9-7.2 12		≥7.3 18	
	XQ	% neg.	XQ	% neg.	XQ	% neg.	XQ	% neg.	XQ	% neg.
Delvo MCS										
Visual	1.00	100	1.00	95	1.05	100	1.00	100	1.11	89
Rel Abs. %	-5.0	100	-5.0	100	-3.1	100	1.7	100	6.7	100
z-value	-13.5	100	-13.8	100	-13.9	100	-13.3	100	-11.6	100
Copan										
Visual	1.00	100	1.00	100	1.00	100	1.75	25	2.83	0
Rel Abs. %	-4.4	100	-5.0	100	-0.7	100	10.0	100	27.8	100
cif-value	0.1	100	0.1	100	0.1	100	0.1	100	0.3	100
Delvo SP										
Visual	1.00	100	1.00	100	1.57	43	2.50	0	4.00	0
Rel Abs. %	-23.3	100	-9.4	100	-4.5	100	17.9	100	54.4	39
z-value	-10.6	100	-9.5	100	-8.3	100	-5.5	100	-0.7	78
BR-AS special										
Visual	3.88	0	1.83	44	1.14	95	1.00	100	1.00	100
Rel Abs.	66.8	0	22.8	100	6.9	100	1.7	100	0.0	100
z-value	2.9	6	-5.9	100	-7.1	100	-7.5	100	-8.9	100
BR-AS Brilliant										
Visual	3.72	0	1.72	28	1.05	95	1.00	100	1.00	100
Rel Abs.	44.4	61	7.2	100	4.5	100	-0.8	100	-3.9	100
BRT										
Visual	1.50	72	1.08	94	1.10	90	1.08	92	1.22	89
Rel Abs.	21.1	100	2.8	100	3.6	95	4.2	92	1.1	89

Somatic cell count

The results of readings of individual cows' milk of the treatment trials – anamnestic period and after the concentrations of antibiotics were below detection limits – and of the trial testing the influence of days in milk and lactation stage >6 days in milk – were grouped with respect to the somatic cell content (see table 9).

Tab. 9: Results of microbial inhibitor tests in individual cows' milk in dependence on somatic cell count

		Somatic cells in 1 000/ml			
		<125	**125-<500**	**500-<1 000**	**>1 000**
n		240	200	72	112
Lactation no.	XQ	2.3	2.6	3.0	2.5
	s	1.4	1.6	1.7	1.0
Days in milk	XQ	40	55	79	104
	s	76	89	123	110
pH	XQ	6.56	6.59	6.59	6.63
	s	0.08	0.10	0.09	0.11
Milk yield per	XQ	15.2	14.0	12.8	12.4
milking (kg)	s	4.2	3.6	4.2	4.2
Delvo MCS					
Visual	XQ	1.01	1.02	1.10	1.09
	% neg.	99.2	98.0	87.5	91.1
Rel. abs. %	XQ	-1.3	-1.7	-0.7	1.4
	% neg.	100	100	100	100
z-value	XQ	-10.4	-10.3	-9.4	-8.9
	% neg.	100	100	99	99
Copan					
Visual	XQ	1.00	1.03	1.06	1.06
	% neg.	100	98.0	95.8	95.5
Rel. abs. %	XQ	-2.3	-1.8	0.3	-0.9
	% neg.	100	100	100	100
Cif-value	XQ	0.11	0.22	0.19	0.15
	% neg.	100	100	100	100
Delvo SP					
Visual	XQ	1.01	1.06	1.03	1.33
	% neg.	98.8	94.5	97.2	67.9
Rel. abs. %	XQ	3.9	5.7	10.0	14.9
	% neg.	100	100	100	100
z-value	XQ	-7.9	-8.0	-7.6	-7.0
	% neg.	100	100	100	100
BR-AS special					
Visual	XQ	1.00	1.02	1.13	1.27
	% neg.	100	98.5	88.9	81.3
Rel. abs. %	XQ	-0.3	2.4	7.3	7.5
	% neg.	100	100	100	100
z-value	XQ	-5.7	-5.8	-5.4	-4.8
	% neg.	100	100	100	99
BR-AS Brilliant					
Visual	XQ	1.02	1.01	1.07	1.11
	% neg.	97.9	99.0	93.1	89.3
Rel. abs. %	XQ	3.3	0.5	1.7	3.2
	% neg.	100	100	100	100
BRT					
Visual	XQ	1.08	1.22	1.32	1.82
	% neg.	92.5	81.0	72.2	44.6
Rel. abs. %	XQ	-5.1	4.7	8.61	26.0
	% neg.	98	96	93	73

Tab. 10: Results of microbial inhibitor tests in individual cows' milk in dependence on days in milk

		Days in milk										
		≤1	≤2	≤3	≤4	≤5	≤6	≤7	≤10	≤15	≤20	≤30
n		127	226	221	223	260	231	226	312	158	68	48
pH	XQ	6.47	6.35	6.36	6.42	6.48	6.48	6.53	6.55	6.55	6.60	6.63
	s	0.16	0.13	0.11	0.08	0.08	0.38	0.09	0.07	0.48	0.06	0.06
Milk yield per	XQ	3.6	7.4	11.4	12.8	13.9	14.1	14.1	16.4	17.2	18.4	18.3
milking (kg)	s	2.6	4.2	4.0	3.8	4.2	4.4	4.5	4.7	4.1	4.3	3.6
Somatic cells[1]	XQ[2]	1643	813	479	339	251	200	186	191	162	71	89
	s[2]	3.1	3.5	3.6	3.6	4.0	4,0	3.9	4.3	4.0	2.3	4.7
Delvo MCS												
Visual	XQ	1.18	1.06	1.05	1.01	1.00	1.00	1.04	1.01	1.00	1.00	1.00
	% neg.	85.1	95.9	97.2	100	99.5	99.5	98.4	99.0	100	100	100
Rel. abs. %	XQ	3.0	-2.1	-4.3	-5.0	-4.3	-3.5	-3.3	-3.3	-3.8	-4.9	-3.5
	% neg.	97.6	99.6	99.5	100	100	100	100	100	100	100	100
z-value	XQ	-11.2	-11.4	-11.5	-11.4	-11.0	-11.0	-11.1	-10.8	-10.5	-11.6	-11.7
	% neg.	97.6	99.6	99.1	100	100	100	100	99.7	100	100	100
Copan[3]												
Visual	XQ	1.12	1.04	1.04	1.01	1.00	1.00	1.01	1.00	1.00	1.00	1.00
	% neg.	89.9	96.5	98.4	99.5	100	100	98.9	100	100	100	100
Rel. abs. %	XQ	5.8	-2.2	-6.0	-6.9	-6.9	-5.9	-5.3	-5.2	-4.8	-3.9	-4.6
	% neg.	98.4	99.6	100	100	100	100	100	100	100	100	100
Cif-value	XQ	0.20	0.14	0.17	0.11	0.13	0.14	0.12	0.13	0.10	0.11	0.15
	% neg.	99.2	100	99.5	100	100	100	100	100	100	100	100
Delvo SP[3]												
Visual	XQ	1.11	1.11	1.01	1.00	1.01	1.01	1.01	1.00	1.01	1.00	1.00
	% neg.	84.0	90.5	99.1	100	98.6	99.2	99.2	100	98.6	100	100
Rel. abs. %	XQ	-13.2	-1.1	-0,1	1.1	2.8	2.7	6.0	3.84	12.5	5.9	7.7
	% neg.	98.7	99.1	100	100	100	100	100	100	100	100	100
z-value	XQ	-9,3	-9.9	-10.7	-10.3	-9.6	-9.2	-8.8	-9.5	-9.2	-9.3	-9.5
	% neg.	98.7	99.1	100	100	100	100	100	100	100	100	100
BR-AS special												
Visual	XQ	2.16	1.50	1.13	1.04	1.05	1.04	1.05	1.02	1.01	1.00	1.00
	% neg.	29.0	84.3	90.1	95.8	94.9	96.7	97.4	97.3	99.2	100	100
Rel. abs. %	XQ	49.5	24.3	9.4	4.8	4.5	4.0	3.5	2.5	0.4	-2.2	-2.9
	% neg.	84.3	87.6	98.6	100	100	100	99.6	99.4	100	100	100
z-value	XQ	-1-2	-3.9	-4.9	-5.7	-5.9	-5.8	-5.8	-5.9	-5.8	-5.7	-5.9
	% neg.	86.6	95.1	98.7	100	100	100	100	99.7	100	100	100
BR-AS Brilliant												
Visual	XQ	1.71	1.27	1.11	1.03	1.02	1.03	1.05	1.11	1.15	1.04	1.00
	% neg.	45.2	77.9	94.5	98.5	98.2	98.2	97.1	94.2	94.6	98.2	100
Rel. abs. %	XQ	34.3	19.6	10.6	7.9	6.7	4.6	5.6	8.0	2.4	2.7	-4.2
	% neg.	75.6	91.2	98.2	99.6	100	99.6	98.7	95.5	96.2	100	100
BRT												
Visual	XQ	1.77	1.32	1.10	1.08	1.08	1.06	1.09	1.17	1.17	1.04	1.00
	% neg.	55.6	76.6	94.9	95.8	92.8	95.7	93.1	87.3	93.4	96.4	100
Rel. abs. %	XQ	44.6	19.6	7.5	6.0	6.4	4.4	6.5	9.4	8.9	14.5	7.9
	% neg.	33.1	90.3	97.3	99.1	99.2	96.3	96.9	95.5	96.2	100	100

[1] in 1 000/ml; [2] Geometric mean and coefficient of standard deviation; [3] not available during whole test period

Days in milk

In order to evaluate the influence of the stage of lactation on the "false" positive rate milk samples of individual cows at each milking time after calving were collected and analysed on different microbial inhibitor tests. Only those results were included in the calculations presented in table 10, which were from non-treated cows or from cows after the end of the withholding time of dry-cow therapy.

2.3.3 Visual versus instrumental reading

In order to test for the variability of test results numerous plates were visually read independently by three trained persons or measured repeatedly by instruments within short time. The results are summarized in table 11.

Tab. 11: Variability of test results for different reading procedures

	Inhibitor test					
	Delvo MCS	**Copan**	**Delvo SP**	**BR-AS special**	**BR-AS Brilliant**	**BRT**
Relativated absorption in % – Differences between 2 measurements						
n	1031	1032	828	995	166	166
XQ	0.14	0.04	0.62	0.30	22.77	1.51
s	2.62	3.11	3.61	2.53	33.42	5.24
Colour measurement – Differences between 2 measurements						
n	944	1032	828	996		
XQ	0.08	0.01	0.14	0.06		
s	0.21	0.11	0.38	0.13		
Visual evaluation – Percentages of deviating results of 3 independent readings						
n	471	471	471	471	471	471
1 step	13.0	17.8	36.9	23.4	20.2	21.4
2 steps	0	0.6	1.1	0	0.6	0.6
3 steps	0	0	0	0	0	0

Spiked milk samples

In tables 12 to 17 the results of instrumental readings of spiked milk samples on Delvo MCS-, Copan-, Delvo SP-, BR-AS-special-, BR-AS-Brilliant- and BR-test within visual evaluation steps in dependence on the antibiotic family, which was used for spiking of the milk samples are summarized.

Cow composite milk

In table 18 the results of instrumental readings of cow composite milk samples on various microbial inhibitor tests within visual evaluation steps are summarized.

Tab. 12: Results of instrumental readings of spiked milk samples on Delvo MCS test within visual evaluation steps in dependence on antibiotic group

Antibiotic group	Instrumental reading		Visual evaluation step 1	2	3	4
β-Lactams		n	79	40	69	205
	Rel. Abs. in %	XQ	5.7	22.3	59.7	91.5
		s	8.1	11.8	22.6	17.9
		% pos.[1]	0	5.0	71.0	99.5
	Z-value	XQ	-7.2	-2.5	3.9	10.6
		s	3.2	3.1	4.0	2.6
		% pos.[2]	2.5	22.5	91.3	100
Sulfonamides		n	31	23	23	68
	Rel. Abs. in %	XQ	8.2	22.6	43.3	76.9
		s	7.3	14.5	13.0	14.7
		% pos.[1]	0	0	30.4	95.6
	Z-value	XQ	-7.5	-2.5	1.0	8.2
		s	2.2	3.0	2.6	3.0
		% pos.[2]	0	21.7	65.2	98.5
Tetracyclines		n	46	21	19	15
	Rel. Abs. in %	XQ	10.3	32.1	55.3	81.0
		s	8.7	16.3	17.6	16.5
		% pos.[1]	0	9.5	57.9	93.3
	Z-value	XQ	-6.5	-1.6	2.1	4.9
		s	3.2	2.7	2.3	2.0
		% pos.[2]	2.2	38.1	79.0	100
Macrolides		n	33	13	20	66
	Rel. Abs. in %	XQ	10.5	21.5	62.3	82.1
		s	9.7	7.5	17.9	15.4
		% pos.[1]	0	0	80.0	98.5
	Z-value	XQ	-7.3	-3.5	3.7	8.6
		s	7.7	2.8	3.0	2.9
		% pos.[2]	0	7.7	95.0	100
Aminoglycosides		n	17	9	21	68
	Rel. Abs. in %	XQ	8.8	27.2	51.7	84.5
		s	9.8	13.3	20.6	17.5
		% pos.[1]	0	0	42.9	95.6
	Z-value	XQ	-6.8	-1.6	2.9	9.2
		s	2.4	2.7	3.2	3.2
		% pos.[2]	0	22.2	85.7	100
Various		n	30	17	23	46
	Rel. Abs. in %	XQ	3.3	19.1	43.0	65.3
		s	6.7	8.5	10.0	13.8
		% pos.[1]	0	0	17.4	87.0
	Z-value	XQ	-7.9	-4.5	2.0	7.2
		s	2.4	2.3	1.9	2.5
		% pos.[2]	0	0	82.6	100
Total		**n**	**236**	**140**	**175**	**468**
	Rel. Abs. in %	**XQ**	**7.8**	**30.0**	**53.2**	**84.2**
		s	**8.5**	**22.4**	**17.6**	**18.5**
		% pos.[1]	**0**	**11.3**	**54.5**	**98.1**
	Z-value	**XQ**	**-7.1**	**-1.6**	**2.8**	**9.3**
		s	**3.0**	**4.3**	**2.9**	**3.1**
		% pos.[2]	**0.8**	**28.6**	**84.6**	**99.8**

[1] ≥55; [2] ≥0

Tab. 13: Results of instrumental readings of spiked milk samples on Copan test within visual evaluation steps in dependence on antibiotic group

Antibiotic group	Instrumental reading		Visual evaluation step 1	2	3	4
β-Lactams		n	81	24	41	193
	Rel. Abs. in %	XQ	7.6	21.3	51.2	88.9
		s	7.3	10.8	17.4	16.9
		% pos.[1]	0	0	41.4	95.9
	Cif-value	XQ	0.6	2.3	4.0	6.8
		s	0.8	1.2	1.4	1.7
		% pos.[2]	0	0	31.1	89.6
Sulfonamides		n	26	8	21	76
	Rel. Abs. in %	XQ	9.0	25.0	43.8	88.9
		s	6.2	7.1	10.7	14.3
		% pos.[1]	0	0	16.0	98.7
	Cif-value	XQ	1.0	1.8	4.1	6.5
		s	1.0	1.3	1.4	1.7
		% pos.[2]	0	0	42.9	86.8
Tetracyclines		n	40	18	18	16
	Rel. Abs. in %	XQ	5.5	26.1	43.3	70.6
		s	7.6	10.1	10.4	12.0
		% pos.[1]	0	5.6	16.7	100
	Cif-value	XQ	1.0	2.1	3.6	5.2
		s	0.9	1.1	0.9	1.0
		% pos.[2]	0	0	11.1	68.8
Macrolides		n	51	23	19	29
	Rel. Abs. in %	XQ	6.2	22.6	35.8	70.5
		s	8.8	10.2	8.7	20.6
		% pos.[1]	0	4.4	5.3	89.7
	Cif-value	XQ	1.1	1.7	3.5	5.9
		s	7.0	1.2	1.5	1.0
		% pos.[2]	0	0	26.3	93.1
Aminoglycosides		n	29	23	25	26
	Rel. Abs. in %	XQ	5.7	21.7	36.0	65.4
		s	8.0	8.6	7.6	11.7
		% pos.[1]	0	0	0	92.3
	Cif-value	XQ	1.0	1.7	3.6	5.2
		s	0.9	1.0	1.2	1.6
		% pos.[2]	0	0	20.0	73.1
Various		n	21	17	17	54
	Rel. Abs. in %	XQ	11.9	25.3	47.4	79.8
		s	76.0	8.0	10.0	13.0
		% pos.[1]	0	0	23.5	100
	Cif-value	XQ	1.5	3.0	5.0	7.7
		s	1.2	1.4	1.20	1.28
		% pos.[2]	0	11.8	70.6	98.2
Total		**n**	**248**	**113**	**141**	**394**
	Rel. Abs. in %	**XQ**	**7.5**	**24.3**	**44.5**	**83.6**
		s	**7.3**	**9.5**	**13.4**	**17.5**
		% pos.[1]	**0**	**3.5**	**25.4**	**96.7**
	Cif-value	**XQ**	**1.0**	**2.1**	**4.0**	**6.6**
		s	**1.0**	**1.3**	**1.3**	**1.7**
		% pos.[2]	**0**	**1.8**	**32.6**	**89.5**

[1] ≥55; [2] ≥4.5

Tab. 14: Results of instrumental readings of spiked milk samples on Delvo SP test within visual evaluation steps in dependence on antibiotic group

Antibiotic group	Instrumental reading		Visual evaluation step 1	2	3	4
β-Lactams		n	87	55	51	116
	Rel. Abs. in %	XQ	1.6	26.6	53.2	95.7
		s	14.2	14.9	19.7	18.6
		% pos.[1]	0	0	70.6	99.1
	Z-value	XQ	-6.9	-2.9	0.5	6.2
		s	2.2	2.1	2.9	1.9
		% pos.[2]	0	5.5	72.6	100
Sulfonamides		n	22	24	45	37
	Rel. Abs. in %	XQ	8.6	24.4	54.4	74.2
		s	11.9	12.3	13.6	13.5
		% pos.[1]	0	0	68.9	94.6
	Z-value	XQ	-4.1	-2.1	1.6	4.6
		s	2.3	2.1	2.1	1.5
		% pos.[2]	4.6	16.7	73.3	100
Tetracyclines		n	36	7	21	28
	Rel. Abs. in %	XQ	10.8	27.1	55.0	90,4
		s	11.8	5.7	11.8	16.3
		% pos.[1]	0	0	57.1	96.4
	Z-value	XQ	-5.6	-2.5	1.4	5.4
		s	3.2	2.3	2.3	1.4
		% pos.[2]	0	14.3	85.7	100
Macrolides		n	46	25	25	26
	Rel. Abs. in %	XQ	1.1	26.8	48.2	73.8
		s	14.5	12.5	11.9	12.9
		% pos.[1]	0	0	40.0	92.3
	Z-value	XQ	-7.8	-3.2	-0.2	3.2
		s	8.0	1.7	1.7	1.1
		% pos.[2]	0	0	52.0	100
Aminoglycosides		n	39	27	10	27
	Rel. Abs. in %	XQ	-2.4	23.5	48.0	81.4
		s	9.6	14.6	14.4	24.9
		% pos.[1]	0	3.7	50.0	92.6
	Z-value	XQ	-6.1	-2.4	-0.7	5.2
		s	2.2	2.3	1.3	2.7
		% pos.[2]	0	14.8	50.0	100
Various		n	36	39	17	17
	Rel. Abs. in %	XQ	6.4	28.1	46.5	73.8
		s	12.1	13.0	14.4	12.6
		% pos.[1]	0	2.6	47.1	100
	Z-value	XQ	-6.1	-2.6	0.2	3.4
		s	2.0	1.8	1.4	1.5
		% pos.[2]	0	5.13	64.7	100
Total		**n**	**266**	**177**	**169**	**251**
	Rel. Abs. in %	**XQ**	**3.6**	**27.1**	**52.4**	**86.0**
		s	**13.3**	**13.0**	**14.9**	**67.1**
		% pos.[1]	**0**	**1.7**	**60.9**	**96.4**
	Z-value	**XQ**	**-6.4**	**-2.5**	**0.7**	**5.3**
		s	**2.5**	**2.0**	**2.4**	**2.1**
		% pos.[2]	**0.4**	**9.2**	**70.8**	**100**

[1] ≥55; [2] ≥0

Tab. 15: Results of instrumental readings of spiked milk samples on BR-AS special test within visual evaluation steps in dependence on antibiotic group

Antibiotic group	Instrumental reading		Visual evaluation step 1	2	3	4
β-Lactams		n	47	37	39	186
	Rel. Abs. in %	XQ	5.5	35.9	58.2	103.5
		s	10.4	12.9	20.9	13.6
		% pos.[1]	0	8.1	59.0	100
	Z-value	XQ	-5.7	-2.1	0.3	7.6
		s	1.5	2.3	3.0	1.4
		% pos.[2]	0	15.5	56.4	100
Sulfonamides		n	54	37	19	18
	Rel. Abs. in %	XQ	6.2	17.5	52.8	79.7
		s	12.6	16.0	22.1	18.0
		% pos.[1]	0	8.1	57.9	100
	Z-value	XQ	-5.1	-5.1	-0.5	3.1
		s	1.3	2.2	3.1	2.9
		% pos.[2]	0	13.3	52.6	88.9
Tetracyclines		n	54	24	7	1
	Rel. Abs. in %	XQ	5.4	22.9	52.1	75
		s	10.0	14.9	15.7	
		% pos.[1]	0	4.2	28.6	100
	Z-value	XQ	-6.2	-5.7	-0.7	1.4
		s	1.1	2.3	1.6	
		% pos.[2]	0	0	42.9	100
Macrolides		n	27	17	23	55
	Rel. Abs. in %	XQ	3.8	19.1	63.2	104.3
		s	10.1	15.8	12.1	11.7
		% pos.[1]	0	0	82.6	100
	Z-value	XQ	-5.2	-4.8	0.9	6.00
		s	6.2	2.0	2.1	1.5
		% pos.[2]	0	0	60.9	100
Aminoglycosides		n	11	12	23	57
	Rel. Abs. in %	XQ	12.7	30.8	58.7	106.5
		s	9.8	13.6	17.5	14.4
		% pos.[1]	0	8.3	56.5	100
	Z-value	XQ	-5.7	-3.8	-0.2	6.0
		s	0.8	2.3	2.4	1.9
		% pos.[2]	0	8.3	52.2	100
Various		n	50	34	11	14
	Rel. Abs. in %	XQ	4.2	14.1	57.7	87.1
		s	7.3	14.5	9.3	8.9
		% pos.[1]	0	0	72.7	100
	Z-value	XQ	-5.9	-5.9	0.9	5.0
		s	1.3	2.5	1.5	1.0
		% pos.[2]	0	5.9	81.8	100
Total		**n**	**243**	**161**	**122**	**331**
	Rel. Abs. in %	**XQ**	**6.3**	**24.7**	**58.3**	**102.1**
		s	**10.9**	**18.2**	**18.5**	**15.1**
		% pos.[1]	**0**	**8.1**	**62.8**	**100**
	Z-value	**XQ**	**-5.7**	**-4.4**	**0.3**	**6.7**
		s	**1.34**	**2.9**	**2.7**	**2.1**
		% pos.[2]	**0**	**7.5**	**60.2**	**100**

[1] ≥55; [2] ≥0

Tab. 16: Results of instrumental readings of spiked milk samples on BR-AS Brilliant test within visual evaluation steps in dependence on antibiotic group

Antibiotic group	Instrumental reading		Visual evaluation step 1	2	3	4
β-Lactams		n	152	30	50	165
	Rel. Abs. in %	XQ	-3.3	14.8	44.1	108.4
		s	10.2	14.8	22.0	55.1
		% pos.[1]	0	6.7	28.0	100
Sulfonamides		n	63	39	11	15
	Rel. Abs. in %	XQ	-3.4	5.6	47.2	96.6
		s	10.9	9.9	22.1	16.8
		% pos.[1]	0	0	63.6	100
Tetracyclines		n	63	18	11	0
	Rel. Abs. in %	XQ	-7.1	6.9	31.3	
		s	14.2	6.2	12.8	
		% pos.[1]	0	0	0	
Macrolides		n	54	27	15	26
	Rel. Abs. in %	XQ	-1.7	23.8	84.0	169.0
		s	10.5	12.5	47.5	56.1
		% pos.[1]	0	0	60.0	100
Aminoglycosides		n	41	23	16	23
	Rel. Abs. in %	XQ	5.3	35.6	81.8	135.8
		s	11.09	15.0	32.6	89.5
		% pos.[1]	0	17.4	81.3	100
Various		n	70	18	12	9
	Rel. Abs. in %	XQ	-3.3	6.3	35.0	112.7
		s	8.0	8.0	15.3	13.9
		% pos.[1]	0	0	25.0	100
Total		**n**	**443**	**155**	**115**	**238**
	Rel. Abs. in %	**XQ**	**-2.6**	**16.6**	**53.9**	**117.2**
		s	**10.9**	**16.3**	**33.0**	**60.1**
		% pos.[1]	**0**	**4.7**	**43.1**	**100**

[1] ≥55;

Tab. 17: Results of instrumental readings of spiked milk samples on BRT within visual evaluation steps in dependence on antibiotic group

Antibiotic group	Instrumental reading		Visual evaluation step 1	2	3	4
β-Lactams		n	103	79	56	165
	Rel. Abs. in %	XQ	4.5	24.6	47.3	98.3
		s	12.6	14.6	18.1	20.7
		% pos.[1]	0	3.8	26.8	97.8
Sulfonamides		n	102	24	2	0
	Rel. Abs. in %	XQ	-1.7	6.5	90.0	
		s	15.6	20.0	21.2	
		% pos.[1]	0.1	8.3	100	
Tetracyclines		n	75	12	1	4
	Rel. Abs. in %	XQ	0.3	20.4	55	92.5
		s	16.5	27.6	-	5.0
		% pos.[1]	0	16.7	100	100
Macrolides		n	42	25	15	40
	Rel. Abs. in %	XQ	2.8	22.8	53.0	77.6
		s	11.8	16.8	11.3	18.0
		% pos.[1]	0	0	46.7	100
Aminoglycosides		n	40	16	14	33
	Rel. Abs. in %	XQ	2.6	19.7	42.1	82.6
		s	12.6	16.1	27.5	19.6
		% pos.[1]	0	6.3	21.4	96.7
Various		n	77	28	4	0
	Rel. Abs. in %	XQ	-3.5	6.4	32.5	
		s	9.1	15.0	5.0	
		% pos.[1]	0	3.6	0	
Total		n	439	184	92	242
	Rel. Abs. in %	XQ	0.7	20.3	47.3	92.7
		s	13.6	20.0	18.7	21.7
		% pos.[1]	0	7.2	27.6	97.9

[1] ≥55

Tab. 18: Results of instrumental readings of individual cows' milk samples on microbial inhibitor tests within visual evaluation steps

Test	Instrumental reading		Visual evaluation step 1	2	3	4
Delco MCS		n	299	29	14	217
	Rel. Abs. in %	XQ	1.87	25.7	48.2	94.9
		s	13.0	11.0	10.3	14.8
		% pos.[1]	0	0	42.9	100
	z-value	XQ	-7.8	-2.5	2.3	11.9
		s	2.7	4.1	2.0	3.7
		% pos.[2]	0	27.6	85.7	100
Copan		n	286	24	9	216
	Rel. Abs. in %	XQ	-1.7	15.4	38.3	90.3
		s	6.6	8.1	5.0	11.2
		% pos.[1]	0	0	0	100
	Cif-value	XQ	0.1	0.8	3.3	7.7
		s	0.04	0.9	1.1	1.0
		% pos.[3]	0	0	22.2	99.5
Delvo SP		n	169	25	6	153
	Rel. Abs. in %	XQ	3.1	27.0	53.3	114.1
		s	13.3	13.2	24.8	17.1
		% pos.[1]	0	0	50.0	100
	z-value	XQ	-6.4	-2.3	0.8	9.17
		s	4.1	3.3	3.1	2.7
		% pos.[2]	0.6	12.0	50.0	100
BR-AS special		n	193	9	4	146
	Rel. Abs. in %	XQ	-18.5	27.2	55.0	101.6
		s	25.5	13.9	11.6	12.6
		% pos.[1]	0	0	50.0	100
	z-value	XQ	-4.8	-3.1	-0.0	7.0
		s	2.1	1.1	4.4	1.4
		% pos.[2]	0	0	75.0	100
BR-AS Brilliant		n	340	32	2	207
	Rel. Abs. in %	XQ	5.0	25.3	60.0	112.2
		s	24.7	12.0	7.1	34.6
		% pos.[1]	4.1	0.6	100	100
BRT		n	194	120	40	226
	Rel. Abs. in %	XQ	-16.0	33.8	69.0	94.9
		s	29.7	18.6	17.3	24.9
		% pos.[1]	0	15.8	85.0	98.7

[1] ≥55; [2] ≥0; [3] ≥4.5

Ex farm-milk

Ex-farm milk samples were made available by the Milk Control Station Schleswig Holstein. The samples were selected in order to obtain as many suspicious and positive results as possible by BRT. In table 19 the results of instrumental readings on various microbial inhibitor tests are summarized. Delvo SP was available only for the analysis of a limited number of samples.

Tab. 19: Results of instrumental readings of ex-farm milk on microbial inhibitor tests within visual evaluation steps

Test	Instrumental reading		Visual evaluation step 1	2	3	4
Delco MCS		n	642	1	2	18
	Rel. Abs. in %	XQ	-4.2	15	40	87.8
		s	4.2		7.1	12.7
		% pos.[1]	0	0	0	100
	z-value	XQ	-12.2	-9.4	-0.9	6.8
		s	1.4		0.9	2.1
		% pos.[2]	0	0	0	100
Copan		n	642	1	3	17
	Rel. Abs. in %	XQ	-1.6	25	45	82.1
		s	6.7		10,0	19.0
		% pos.[1]	0	0	33.3	100
	Cif-value	XQ	0.4	1.3	3.1	5.6
		s	0.3		0.6	1.2
		% pos.[3]	0	0	0	94.1
Delvo SP[4]		n	85	0	1	2
	Rel. Abs. in %	XQ	7.1		55	105.0
		s	8.9			28.3
		% pos.[1]	0		100	100
	z-value	XQ	-9.6		1.2	5.1
		s	1.4			4.0
		% pos.[2]	0		100	100
BR-AS special		n	642	1	1	19
	Rel. Abs. in %	XQ	-0.1	5	65	101.3
		s	7.7			13.0
		% pos.[1]	0	0	100	100
	z-value	XQ	-5.0	-7.9	1.6	6.6
		s	1.0			1.4
		% pos.[2]	0	0	100	100
BR-AS Brilliant		n	650	4	1	9
	Rel. Abs. in %	XQ	-6.6	8.3	55	89.4
		s	6.2	5.8		10.1
		% pos.[1]	0	0	100	100
BRT		n	642	0	1	20
	Rel. Abs. in %	XQ	-2.8		45	92.0
		s	7.9			14.2
		% pos.[1]	0		0	100

[1] ≥55; [2] ≥0; [3] ≥4.5; [4] Test available only for a limited number of samples

Car tanker milk

In table 20 the results of instrumental readings of car tanker milk samples on various microbial inhibitor tests within visual evaluation steps are summarized.

Table 21 summarizes the results of 26 car tanker samples evaluated as suspicious and positive respectively in at least one of the microbial inhibitor screening test formats applied.

Tab. 20: Results of instrumental readings of car tanker milk samples on microbial inhibitor tests within visual evaluation steps

Test	Instrumental reading		Visual evaluation step 1	2	3	4
Delvo MCS		n	3763	4	6	10
	Rel. Abs. in %	XQ	-4.9	22.5	43.5	82.0
		s	4.5	9.6	9.9	19.0
		% pos.[1]	0	0	33.3	100
	z-value	XQ	-13.5	-3.9	1.0	9.1
		s	2.5	4.0	1.4	4.2
		% pos.[2]	0	25.0	83.3	100
Copan		n	3763	8	4	7
	Rel. Abs. in %	XQ	-0.6	21.3	30.0	87.9
		s	5.8	7.4	5.8	9.9
		% pos.[1]	0	0	0	100
	Cif-value	XQ	0.1	2.3	0.7	7.7
		s	0.1	1.2	0.6	1.7
		% pos.[3]	0	0	0	100
Delvo SP		n	3763	6	3	1
	Rel. Abs. in %	XQ	-1.9	30.6	45.0	125
		s	10.8	14.9	10.0	
		% pos.[1]	0	0	33.3	100
	z-value	XQ	-10.6	-3.6	-1.7	6.8
		s	2.5	3.9	6.6	
		% pos.[2]	0	33.3	33.3	100
BR-AS special		n	3763	9	1	14
	Rel. Abs. in %	XQ	-1.1	21.1	35	88.2
		s	5.9	19.0		13.1
		% pos.[1]	0	11.1	0	100
	z-value	XQ	-6.4	-3.7	-2.3	7.0
		s	2.1	2.6		2.0
		% pos.[2]	0	11.1	0	100

[1] ≥55; [2] ≥0; [3] ≥4.5

Tab. 21: Results of car tanker milk samples suspicious or positive in at least one test

No. of sample	Delvo SP vis	Delvo SP Rel. Abs.	Delvo SP Z-value	Delvo MCS Vis	Delvo MCS Rel. Abs.	Delvo MCS Z-value	BR AS special vis	BR AS special Rel. Abs	BR AS special Z-value	Copan vis	Copan Rel. Abs.	Copan cif-value	Identification
1	1	5	-8.9	4	45	3.3	3	35	-2.3	2	15	0.1	not identified
2	1	5	-9.9	2	25	-0.3	1	5	-7.5	1	5	0.1	not identified
3	2	15	-6.8	4	65	6.8	4	65	3.7	3	25	0.7	β-lactam, not identified
4	2	15	-6.2	4	75	7.3	4	65	4.0	3	25	0.3	β-lactam, not identified
5	4	125	6.8	4	105	7.2	4	115	6.4	4	95	5.2	5.1 µg Pen G/kg
6	2	30	-5.1	2	15	-7.3	2	30	1.3	2	15	3.1	not identified
7	1	20	-2.0	2	15	-7.1	1	5	-7.3	2	15	1.5	not identified
8	2	45	0.7	2	35	-1.7	4	80	4.5	3	35	0.1	no β-lactam, not identified
9	2	50	1.9	3	40	0.9	4	85	8.4	3	35	1.5	no β-lactam, not identified
10	3	45	-5.1	4	80	6.6	4	90	7.0	4	80	7.3	2.2 µg Pen G/kg
11	3	35	-5.9	4	70	4.6	4	85	6.8	4	70	6.1	2.2 µg Pen G/kg
12	1	-15	-11.9	1	-5	-12.3	2	-5	-4.4	1	-5	0.1	not identified
13	1	15	-9.1	1	-5	-12.7	2	-5	-4.8	1	-5	0.1	not identified
14	1	0	-1.8	1	10	-3.9	2	35	-2.3	1	5	0.1	not identified
15	1	-5	-7.2	2	35	-2.5	2	15	-5.7	1	10	0.1	not identified
16	1	10	-0.9	1	25	-3.4	2	55	0.6	1	15	0.2	not identified
17	3	55	5.9	3	55	3.3	4	95	10.3	2	35	3.3	60 µg TC/kg
18	2	25	-6.3	2	35	-3.0	2	15	-7.5	1	5	0.1	20 µg TC/kg
19				4	85	14.4	4	95	9.6	4	95	8.2	7 µg AMP/kg; 33 µg CLX/kg
20				4	95	14.1	4	95	9.3	4	95	7.7	7 µg AMP/kg; 33 µg CLX/kg
21				3	55	1.8	4	95	7.4	2	25	2.7	2 µg AMP/kg; 17 µg CLX/kg
22				3	35	0.1	2	25	-3.5	2	25	3.2	1 µg AMP/kg; 4 µg CLX/kg
23				3	35	0.5	2	25	-3.0	2	25	3.2	3 µg AMP/kg; 6 µg CLX/kg
24				3	35	-0.8	4	80	5.7	2	15	1.1	not identified
25				4	95	13.5	4	95	7.2	4	95	9.2	84 µg CLX/kg
26				4	105	13.0	4	95	7.0	4	85	10.0	85 µg CLX/kg

2.3.4 Stability of lyophilized control samples and test kit batches

Lyophilized control samples

During the period of test kit validation the same batches of lyophilized positive (4 µg penicillin/kg) and negative control samples were applied on each test tablet. The results of instrumental readings for the different test formats and batches are summarized in table 22.

Tab. 22: Results of absorption and colour measurement of negative and positive control samples (lyophilised milk) of 6 inhibitor test formats

	Absorption			Z- and cif-value resp.*		
	Negative	Positive	Difference	Negative	Positive	Difference
Delvo MCS n=11 batches						
n	998	999		999	995	
XQ	0.25	0.72	0.47	-7.7	10.4	18.1
s	0.03	0.06		2.2	2.0	
Min	0.20	0.57		-13.2	4.7	
Max	0.40	1.12		-0.5	18.2	
Copan n=7 batches						
n	903	903		901	893	
XQ	0.25	0.83	0.55	0.4	7.7	7.3
s	0.04	0.11		0.5	1.0	
Min	0.20	0.47		0.1	4.7	
Max	0.45	1.35		0.3	10.0	
Delvo SP n=5 batches						
n	540	540		538	540	
XQ	0.39	0.72	0.33	-6.6	7.8	14.4
s	0.04	0.04		2.0	1.1	
Min	0.28	0.60		-12.8	4.9	
Max	0.51	0.86		-0.9	15.7	
BR-AS special n=10 batches						
n	1003	1008		1005	1008	
XQ	0.21	0.74	0.53	-4.7	7.6	12.3
s	0.03	0.49		1.7	1.6	
Min	0.09	0.51		-8.9	2.8	
Max	0.44	9.68		-0.3	12.0	
BR-AS Brilliant n=9 batches						
n	412	407				
XQ	0.14	0.52	0.38			
s	0.04	0.11				
Min	0.07	0.21				
Max	0.45	0.98				
BRT n=9 batches						
n	425	427				
XQ	0.14	0.52	0.38			
s	0.06	0.09				
Min	0	0.33				
Max	0.50	0.99				

* Z-values: Delvo MCS, Delvo SP and BR-AS special; cif-value: Copan

For the evaluation of variation of test sensitivities between and within test kit batches milk samples were spiked with different antibiotics in 3-5 concentrations, lyophilized and stored at 6°C. In fig. 11 the results of photometric readings of 6 different test samples as examples on BR-AS special and Delvo SP during a storage period of the test samples up to about 6 years at 6°C are demonstrated.

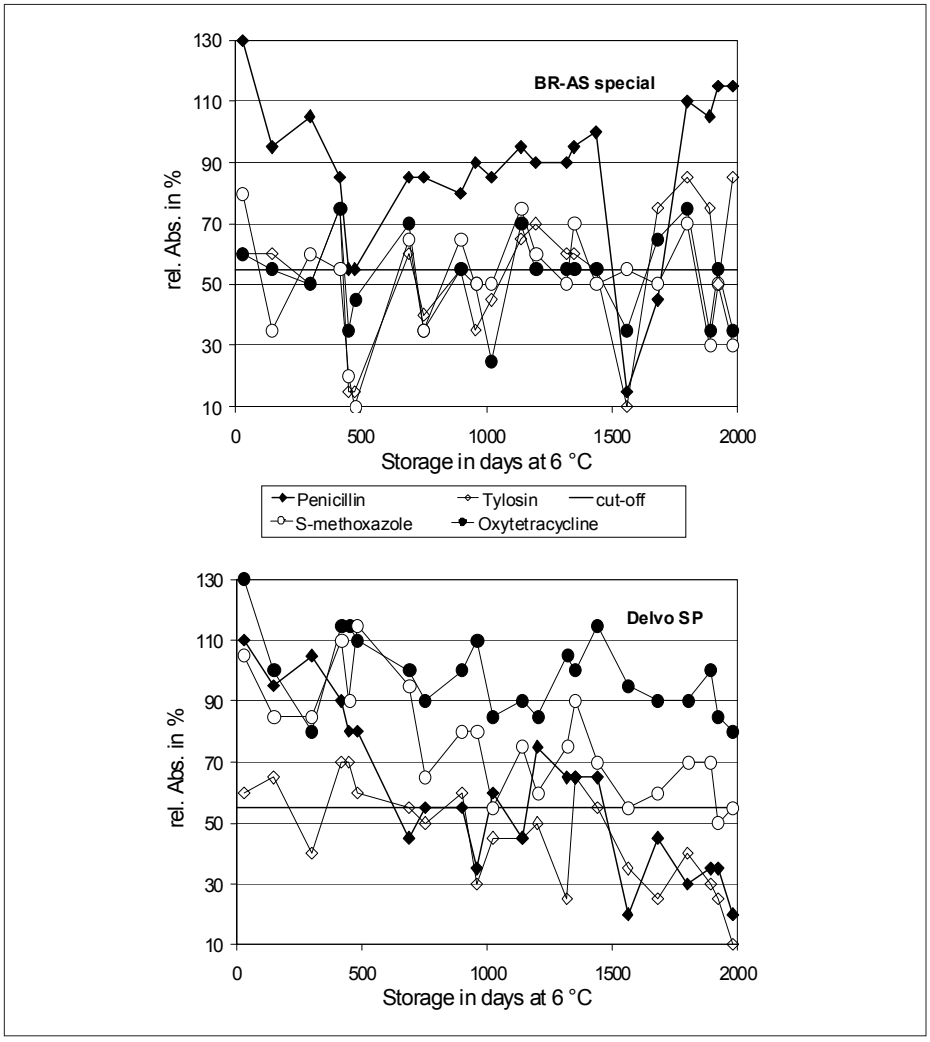

Fig. 11: Influence of storage period of lyophilized milk standards on instrumental reading results on microbial inhibitor tests

During the validation experiments new test samples had to be prepared. The lyophilized test samples stored for about 6 years at 6 °C and the new batch of test samples were analysed on test plates of the same test kit batches on the same examination day. In fig. 12 the average values of relativated absorption in % of comparable test samples of four different antibiotic groups are demonstrated.

It becomes obvious that in most cases the average values of stored and new test sample batches are similar; in some cases the average value of stored test samples is less than of the new batch – e.g. Delvo SP and β-lactams and aminoglycosides – and in some cases the average value of the new batch are lower than of the stored – e.g. Delvo MCS and tetracyclines and sulfonamides. From these data it can be derived that the response of lyophilized test samples did not change systematically during the storage period.

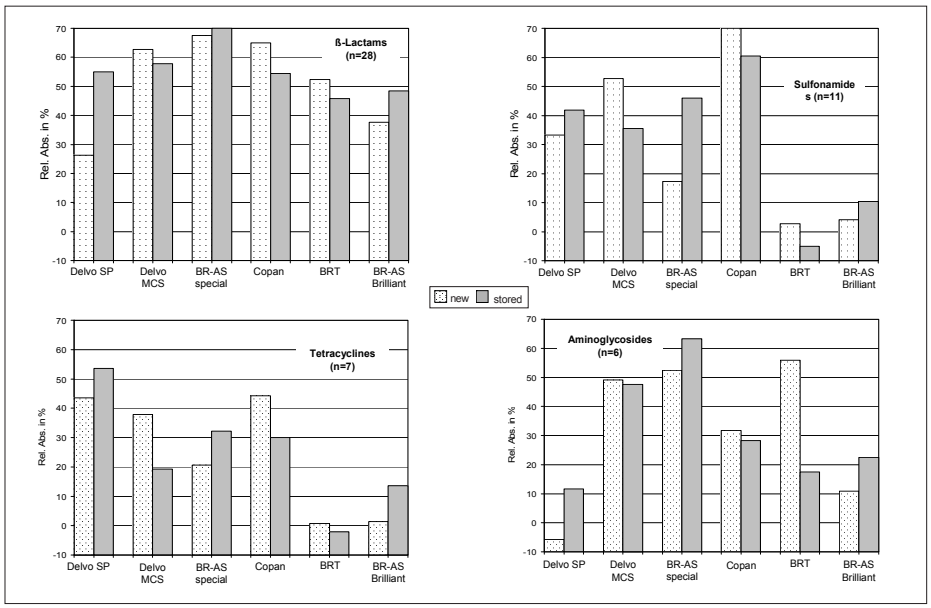

Fig. 12: Results (XQ) of instrumental readings on various microbial inhibitor tests with stored (6 years/6°C) and new lyophilized test samples

Variation of test sensitivities between test kit batches

Each test kit batch applied in experimental studies is tested with milk samples, which were spiked with various antibiotic/concentration combinations and lyophilized. During validation experiments two different batches of lyophilized test samples were used (July 2000 and Oct 2005). As the antibiotic/concentration combinations of the two batches of test samples partly differed in table 23 and table 24 only the results with the test sample batch Oct 2005 are summarized. The antibiotic/concentrations chosen for the latter batch of test samples were optimized with respect to the test sensitivities of Delvo MCS, Copan and BR-AS special. In table 23 the average values of visual and instrumental readings of the test samples belonging to the same antibiotic group are given.

Variation of test sensitivities during storage of test plates

Test plates of Delvo MCS and Copan test, which were stored at 6° C up to one year, were tested with spiked, lyophilized test samples several times during storage period in order to evaluate the influence of storage time on test sensitivities. In table 24 the average values of visual and instrumental readings within antibiotic group at the different examination dates are summarized.

Tab. 23: Variation of test sensitivities between batches in dependence on antibiotic group (XQ)

Batch	β-lactams n=40			Tetracylines n=9			Sulfonamides n=12			Macrolides n= 11			Aminoglycosides n=9		
	Vis.	Rel.Abs. value*	Z-/cif	Vis.	Rel.Abs. value*	Z-/cif	Vis.	Rel.Abs. value*	Z-/cif	Vis.	Rel.Abs. value*	Z-/cif	Vis.	Rel.Abs. value*	Z-/cif
Delvo MCS															
05H	3.0	68	4.3	1.6	17	-6.5	2.7	46	-0.1	2.8	57	2.1	3.1	71	5.2
05J	3.2	59	6.4	1.5	17	-4.4	2.6	39	1.7	3.0	50	4.3	3.2	54	4.7
06A	3.1	60	4.8	1.3	13	-6.8	2.2	38	-1.5	2.6	49	0.5	3.1	65	3.8
06D	3.1	66	5.8	1.6	26	-2.5	2.8	57	3.5	2.6	54	2.9	3.1	61	4.9
06E	3.1	64	4.8	1.8	28	-2.5	2.9	53	2.2	2.9	53	2.8	3.2	55	4.4
06H	3.1	65	4.7	2.1	32	-1.1	3.0	55	2.9	2.8	55	3.0	3.0	59	4.3
Copan															
F3 05	3.2	61	4.0	1.7	14	0.4	2.4	37	2.3	1.6	7	0.3	2.1	20	0.6
F3 08	2.7	53	4.3	1.4	22	1.8	2.4	48	3.4	1.3	6	0.8	1.9	26	1.6
F3 10	2.9	63	5.4	1.6	22	1.5	2.5	46	3.9	1.4	11	0.8	2.2	33	2.0
F3 12	3.0	64	6.4	2.0	27	2.7	3.0	58	5.5	1.6	15	1.7	2.3	30	2.6
BR AS special															
05K	3.3	89	4.8	1.4	27	-4.6	2.1	51	-2.1	2.6	69	0.5	2.9	72	1.9
05L	3.1	71	3.8	1.7	20	-4.9	2.2	37	-2.8	2.5	51	0.4	3.1	76	3.9
06D	2.8	51	1.8	1.2	11	-6.2	1.7	25	-4.8	2.2	39	-1.4	3.0	60	1.7
06E	3.2	71	4.2	1.6	19	-4,4	2.3	45	-0.3	2.6	55	1.6	3.0	71	4.2
06F	2.7	59	1.6	1.3	9	-6.0	1.8	25	-4.6	2.3	40	-1.1	3.4	75	3.2
06G	3.0	65	2.9	1.7	22	-4.5	2.3	41	-1.9	2.7	54	0.6	3.7	87	5.9
06J	2.6	59	1.1	1.5	20	-0.2	1.8	29	-5.2	2.3	44	-1.8	3.1	69	1.8
06K	2.8	55	1.1	1.3	12	-7.1	2.1	20	-5.9	2.4	38	-2.7	3.0	59	0.9
BR AS Brilliant															
05K	2.7	45		1.6	11		1.9	11		2.4	39		2.1	35	
05L	2.7	41		1.5	7		2.7	39		2.6	42		2.6	41	
06C	2.5	41		1.3	11		2.2	33		2.2	38		2.1	36	
06G	2.2	48		1.2	2		1.8	29		1:9	38		1.9	44	
06J	2.2	56		1.2	13		1.8	32		1.9	40		1.9	44	
06L	2.5	50		1.4	11		2.5	43		2.0	34		1.8	26	
BRT															
18-11	3.0	47		1.4	-3		1.0	-5		2.1	13		1.9	21	
28-12	2.9	65		1.3	2		1.2	3		1.8	30		2.3	39	
07-03	3.0	60		1.3	0		1.3	-3		2.6	50		2.4	42	
27-10	2.3	40		1.0	1		1.0	-1		1.7	23		2.1	31	
29-12	2.6	52		1.3	9		1.0	4		2.4	40		2.6	48	

* z-Values: Delvo MCS, BR-AS special; cif-value: Copan

Tab. 24: Variation of test sensitivities within batches of Delvo MCS and Copan in dependence on storage period at 6°C in days and antibiotic group (XQ)

Storage in days	β-lactams n=40			Tetracylines n=9			Sulfonamides n=12			Macrolides n= 11			Aminoglycosides n=9		
	Vis.	Rel.Abs.	Z-/cif value*	Vis.	Rel.Abs.	Z-/cif value*	Vis.	Rel.Abs.	Z-/cif value*	Vis.	Rel.Abs.	Z-/cif value*	Vis.	Rel.Abs.	Z-/cif value*
Delvo MCS Batch 05H															
120	3.0	66	4.3	1.6	17	-6.5	2.7	56	-0.1	2.8	59	2.1	3.1	71	5.2
240	3.1	66	5.9	1.7	25	-1.9	3.0	58	4.2	3.0	70	5.8	3.8	79	8.9
360	3.0	61	4.9	1.8	32	-1.0	3.1	58	4.7	3.1	66	6.1	3.8	77	8.9
Delvo MCS Batch 06A															
30	3.0	59	2.8	1.3	13	-6.8	2.2	38	-1.5	2.6	50	0.5	3.1	65	3.8
240	3.1	60	4.8	1.8	23	-2.2	3.1	58	3.9	3.1	60	4.1	3.8	83	7.9
270	2.9	52	0.8	1.2	8	-9.5	2.0	26	-6.3	2.4	39	-3,4	3.0	58	0.4
Copan Batch F2 02															
120	3.5	55	7.0	2.1	26	4.2	3.5	62	7.1	2.0	21	4.0	2,6	31	4.6
180	3.2	67	5.7	2.1	27	2.3	3.1	63	5.2	1.8	17	1.7	2.3	32	2.7
270	2.8	56	4.8	1.7	25	1.9	2.8	55	5.2	1.6	13	1.0	2.1	27	1.9
Copan Batch F3 05															
60	3.2	61	4.0	1.7	14	0.4	2.4	37	2.3	1.6	7	0.3	2.1	20	0.6
150	2.8	54	4.0	1.4	16	0.4	2.3	37	2.6	1.2	3	0.2	1.3	11	0.4
270	2.8	56	3.6	1.4	21	0.6	2.2	41	2.5	1.2	5	0.2	1.3	14	0.4
Copan Batch F3 08															
60	2.7	53	4.3	1.4	22	1.8	2.4	48	3.4	1.3	6	0.8	1.9	56	1.6
180	2.7	52	5.4	1.7	24	2.3	2.6	52	4.7	1.4	10	1.1	2.1	29	2.6
200	2.7	56	4.2	1.8	28	1.9	2.4	46	3.2	1.5	10	0.8	2.2	28	1.7

* z-Values: Delvo MCS; cif-value: Copan.

3. Discussion and conclusions

Due to their easy performance, low price and the the broad range of detection of residues of antimicrobials in milk microbial inhibitor tests with *Geobacillus stearothermophilus* as test micro-organism are in wide-spread application either for the detection of inhibitors in milk in quality payment schemes or as screening methods for the detection of residues of antibiotics with respect to food hygiene legislation. The tests underlie continuous modification with the aim to adjust the detection pattern to requirements as e.g. the MRLs as fixed in the EU (1, 14), to increase the stability of test performance, e.g. expiry date or to facilitate objective instrumental reading with the possibilities of documentation as demanded by some accreditation bodies instead of subjective visual evaluation (18-21). *Geobacillus stearothermophilus* tests most often used are proprietary techniques. For validation of microbial inhibitor tests an internationally accepted guidance was established (16).

During June 2005 and March 2007 validation experiments with several commercially available inhibitor tests with *Geobacillus stearothermophilus* as test microorganisms – BR-AS-Brilliant, BR-AS special, BRT, Copan Milk Test, Delvo MCS, Delvo SP – of three test kit manufacturers – AIM/Munich (DE), Copan/Brescia (IT), DSM Food specialities/ Delft (NL) – and two different indicators – pH-indicator bromcresol purple, redox-indicator brilliantblack - were performed following the experimental design of ISO 13969/IDF 183: Milk and milk products – Guidance for the standardized description of microbial inhibitor tests (16) as closely as possible and appropriate. In order to obtain a more differentiated scale visual evaluation was performed in 4 steps (1 = negative, 2 = slightly suspicious, 3 = suspicious, 4 = positive). In Germany the interpretation of microbial inhibitor tests depends on the purpose of analysis:

- For quality payment purposes results are evaluated as positive only when the colour is as intense as that of the positive control sample with 4 µg penicillin/kg. This corresponds to visual evaluation step 4 in this study. Further analysis for identification and/or quantification of the inhibitor is not demanded (24).
- For screening purposes, e.g. analysis of samples within the national control plan according to Council Directive 96/23 EEC (6), samples with colours deviating from that of the negative control are interpreted as suspicious to contain antibiotics; further analyses for identification and quantification of the antimicrobial are required (25). This corresponds to visual evaluation steps 2, 3 and 4 in this study.

Instrumental reading for all tests was by photometric measurement by an ELISA-reader, expression of results in relative absorption in % and a cut-off level of 55 (19) and by scanner technology and the algorithms provided by the manufacturers for the corresponding test kits. The results of the test kits from the manufacturer DSM Food Specialities – BR-AS special, Delvo MCS and Delvo SP – are indicated as z-value with a cut-off level of ≥ 0 and those of the Copan test as cif-values and a proposed cut off level of ≥ 4.5.

Test procedure

There were no problems observed with the test procedure in any test kit. The indicated incubation times were always sufficient; in no case it was necessary to lengthen the incubation period due to the colour of the control samples. Visual reading was easy due to good contrasts of the colours of negative and positive control sample with the exception of one batch of BR-AS Brilliant. The evaluation by scanner technology and the software provided by the manufacturers was comfortable and worked without any problem during the validation period.

Detection limits with spiked milk samples

The detection limits of 18 antimicrobials were determined by establishing dose-response curves with spiked milk samples and visual evaluation. The results are summarized in tables 4 and 5. In fig. 13 and 14 the results of 17 substances and of 4 test formats included in this study are presented as "detection pattern" with a MRL-standardized scale (Regulation 2377/90 EEC (1)). The ideal test should detect 1 x MRL-concentration of all antimicrobials of concern. This corresponds to the bold middle circle in the graphs. The more sensitive an antimicrobial can be detected with respect to MRL-requirement the further is the distance from the centre on the standardized scale and *vice versa*. The results of dapsone as a banned substance are not presented in the graphs. Fig. 13 demonstrates the detection pattern when the test results were interpreted as inhibitor test (corresponding to visual evaluation step 4) and fig. 14 when interpreted as screening method (corresponding to visual evaluation steps e"2). Out of the 18 antibiotics tested the following numbers were detected at ≤ MRL concentration when evaluated as inhibitor/ screening method:

BR-AS Brilliant	3 / 4
BR-AS special	5 / 7
BRT	4 / 7
Copan	6 / 9
Delvo MCS	7 / 9
Delvo SP	5 / 10.

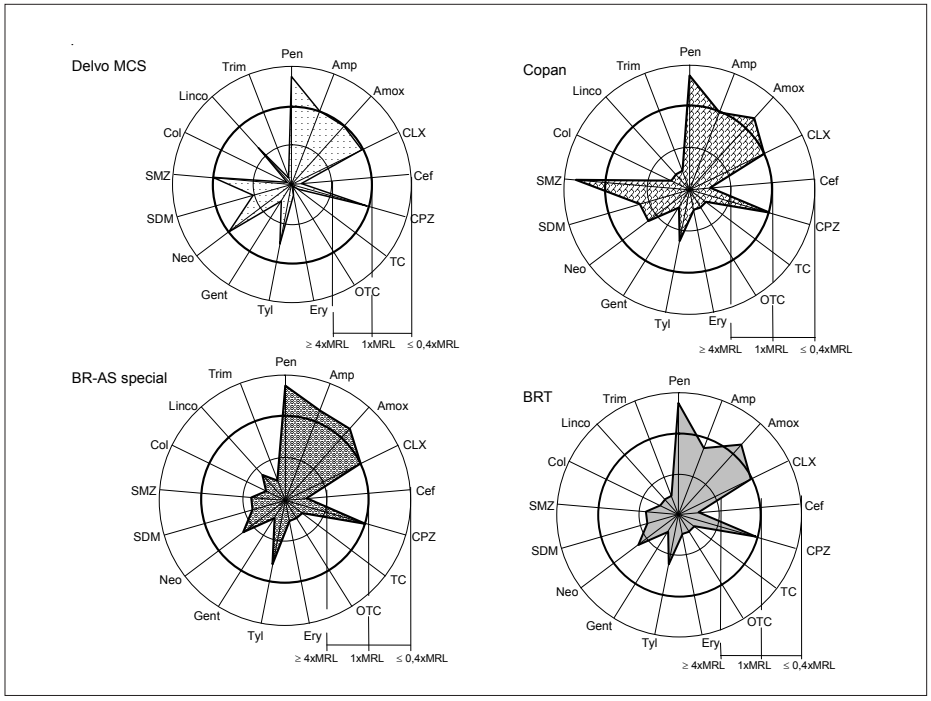

Fig 13: "Detection pattern" of inhibitor tests for various antibiotics – detection limits expressed as n-fold MRL-concentration

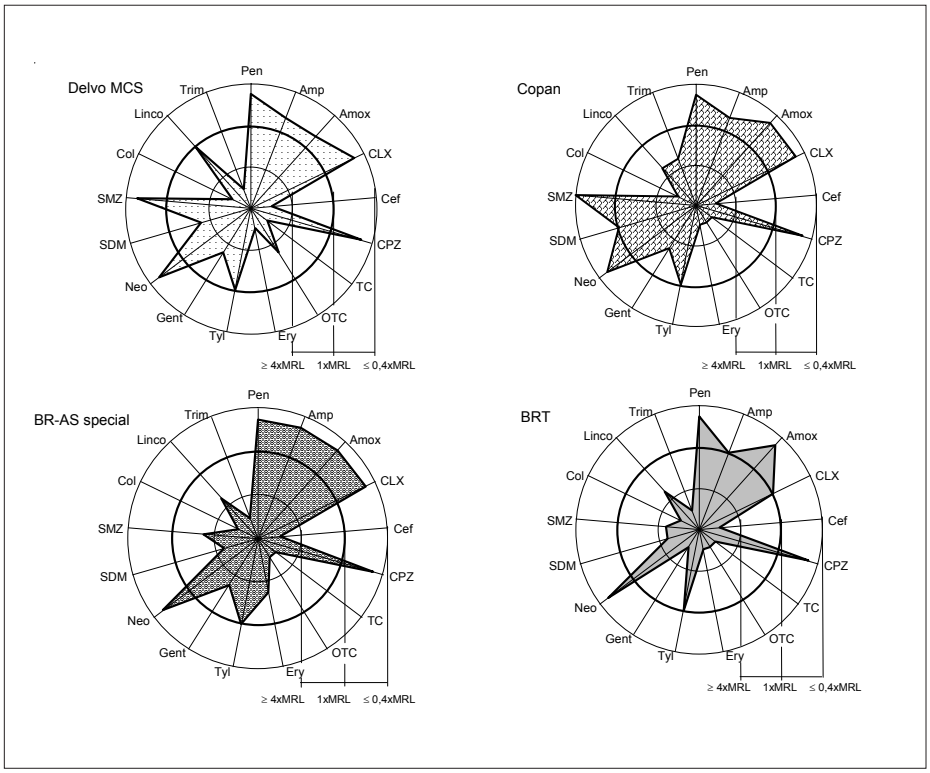

Fig. 14: "Detection pattern“ of screening methods for various antibiotics – detection limits expressed as n-fold MRL-concentration

In a proficiency test the laboratories responsible for the examination of ex-farm milk for quality payment purposes were involved. All laboratories applied their routinely used test with frozen milk samples spiked with different concentrations of penicillin. With the exception of BRT-test and one lab samples spiked with 4 µg penicillin/kg were evaluated as positive in every case and fulfilled by this the demands of L01.01-5 of the Official Collection of Methods according to the German Food and Feed Code (24).

Detection limits with incurred samples

In the validation study with incurred milk samples the substances ampicillin, cefoperazone, cefquinome and penicillin were included. The detection limits in incurred and spiked samples determined by the different ways of test reading are most often in the same order of magnitude (see table 7). There is the tendency that ampicillin is detected a little bit less sensitive and cefoperazone a little bit more sensitive in incurred samples than in spiked samples. Interpreting the results it has to be kept in mind that the determination of concentrations by HPLC-methods and the determination of detection limits include measurement uncertainties. In addition, not in every test kit format/antibiotic combination a sufficient number of results in the area of the individual detection limits was available.

Ruggedness testing

With milk samples spiked with 8 different substances representing different groups of antibiotics the influence of lengthening the incubation time on test sensitivities was tested (see fig. 10). It became obvious that for all substances tested and for all three types of test evaluation the incubation period is of influence on test sensitivities: Detection limits increase with increasing incubation time. The influence of incubation period on test sensitivities is dependent on the kind of antibiotic. It is e.g. less pronounced in the case of amoxicillin, a bactericidal antibiotic, and distinctly pronounced in the case of sulfadimidine, a bacteriostatically acting antimicrobial. That means that lengthening of incubation period which is sometime recommended to avoid “false” positive results e.g. when testing milk of other species than the cow (31, 32) coincides with increasing detection limits. The influence of shorter incubation periods was not included in this study.

In order to evaluate the influence of pH-value of samples on test results lactic acid or sodium hydroxide were added to bulk tank milk samples. From table 8 and fig. 15 it becomes obvious that the results of the test formats with bromcresol purple as indicator have the tendency to be influenced by alkaline pH-value of the samples whereas the test formats with brilliantblack as indicator are influenced by acid pH-values. Within the group of bromcresol purple tests Delvo MCS and within the group of brilliantblack reduction tests BRT were the ones most rugged with respect to pH-value of the samples. "False suspicious or false positive“ results could be avoided by lengthening the incubation period of test plates by 20 minutes in about 50% of the cases, but this also decreases detection sensitivities. The risk to read a test result as "false suspicious or false positive“ is higher with visual reading than with instrumental reading.

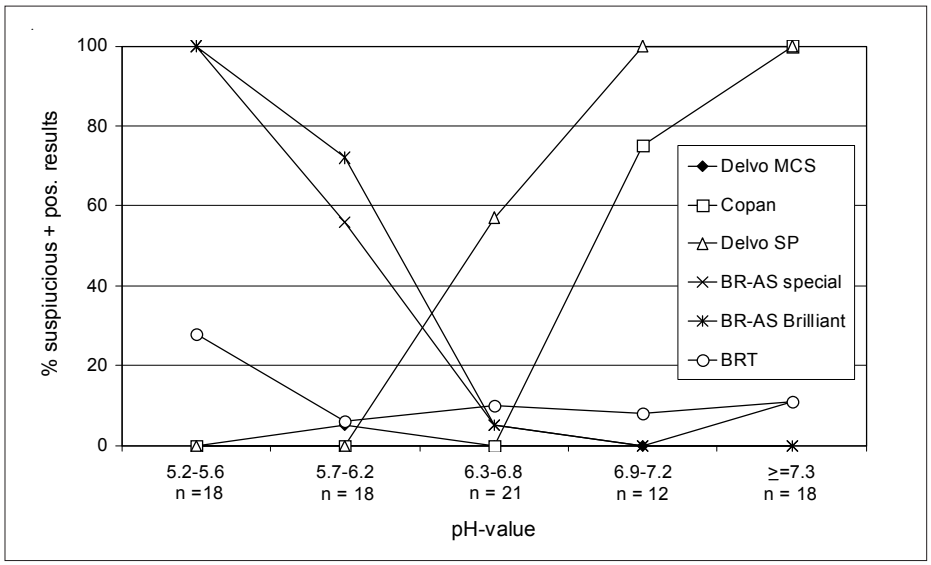

Fig. 15: Results of microbial inhibitor tests in cow composite milk in dependence on pH-value of the samples (pH adjusted by addition of lactic acid/sodium hydroxide)

With milk of individual cows the influence of somatic cell count on test results was studied. From table 9 it can be derived that for all test formats included the percentage of samples evaluated by visual reading as "false suspicious and false positive“ increases

with increasing somatic cell count; this effect is less pronounced with Copan test and most obvious with BRT. The „false suspicious or positive“ rate is dependent on the mode of reading and - with the exception of BRT - most pronounced for visual test interpretation.

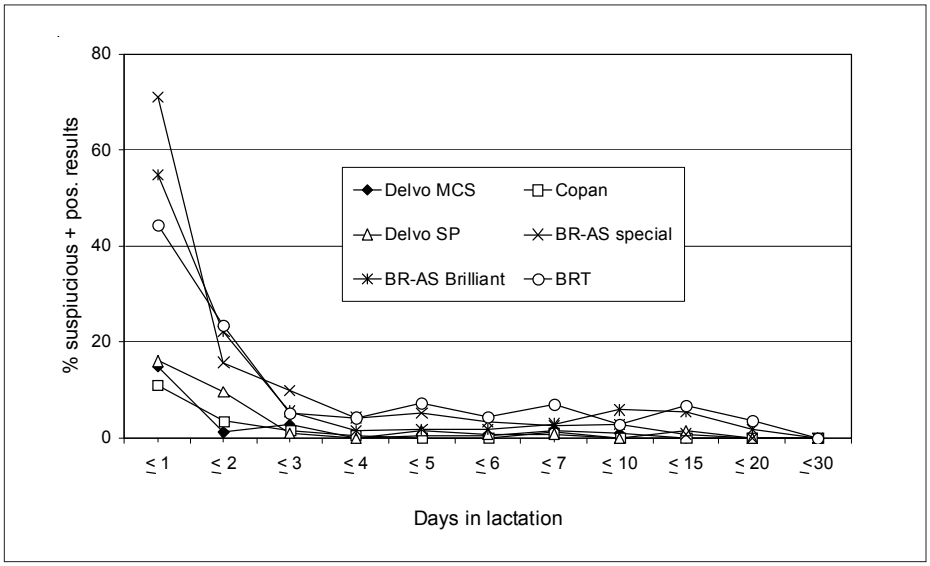

Fig. 16: Results of microbial inhibitor tests in cow composite milk in dependence on days in milk

In some regional and/or national farm quality payment systems it is recommended to test milk of individual cows by microbial inhibitor tests after antibiotic treatment. This means the necessity of suitable methods for screening of antibiotic residues. Due to recent EU-food hygiene legislation (33) colostrum and colostrum-based products are included as food with the requirement that food business operators ensure that MRLs are not exceeded. In order to evaluate the rate of "false positives“ in milk samples of cow composite milk samples at each milking time after calving were collected, analysed and evaluated with respect to the influencing factor days in milk (see table 10). Samples evaluated as suspicious and positive respectively were not always identical on the test formats applied. With all tests the percentage of samples evaluated as "false suspicious and false positive“ by visual reading decreased with increasing days in milk as demonstrated in fig. 16. The percentage of „false positive/suspicious“ samples was lower when instrumental reading of test results was applied. The test formats with brilliantblack as indicator are much more influenced by the stage of lactation than the tests with bromcresol purple.

Visual versus instrumental reading

Repeated instrumental reading of plates within short time led only to minor differences in values (see table 11). Nevertheless in the range of cut-off-values this may lead to deviating test interpretation. Therefore it is recommended to repeat the measurement of those results which are in the range of +/- 5% in the case of relativated absorption in % (cut-off value 55%) and +/- 0.2 in the case of cif- and z-value respectively. With independent visual reading of n=471 samples by 3 trained persons within short time in no

case a difference of 3 visual evaluation steps was observed between persons; a deviation of 2 steps was rather seldom (0% - 1.1%); a deviation of 1 step was most pronounced with Delvo SP (36.9%) and most seldom with Delvo MCS (13.0%). The variability of test results has to be kept in mind, when in the following the relation between visual and instrumental reading is described.

Table 25 summarizes the percentages of positive results of instrumental readings within visual evaluation steps (1=negative, 2=slightly suspicious, 3=highly suspicious, 4=positive) in dependence on the antibiotic family, which was used for spiking.

When the samples evaluated as negative by visual evaluation were positive with instrumental reading and vice versa the measuring values were always close to the cut-off value. Only with the Copan test greater deviations were observed.

The discrepancy between visual evaluation step "positive“ and instrumental readings was most pronounced for Copan-test and scanner measurement for the antibiotic families tetracyclines and aminoglycosides. This can be interpreted as a lower sensitivity of colour measurement for this test format compared to visual evaluation. In the visual evaluation steps "suspicious“ (step 2 and 3) overlapping is expected. With Delvo MCS and colour measurement the highest percentage of positive results within visual evaluation step "highly suspicious“ (step 3) was observed. This can be interpreted as slightly higher sensitivities of Delvo MCS when read by colour measurement instead of visual reading.

The relation between visual and instrumental reading was studied in cow composite milk, ex-farm milk by all test kits, and for car tanker milk only by BR-AS special, Copan, Delvo MCS and Delvo SP. Tab. 26 summarizes the percentages of positive findings within visual evaluation steps.

With the exception of cow composite milk and Delvo SP (colour measurement) and BR-AS Brilliant there is very good agreement between the results of instrumental readings within visual evaluation step “negative”. The results within visual evaluation steps "suspicious“ (step 2 and 3) have to be interpreted with caution due to the low sample numbers within these groups. Within visual evaluation step "positive“ with the exception of rare cases with Copan test (colour measurement), in which results were close to cut-off level, all samples were evaluated as positive by instrumental readings.

Car tanker samples evaluated as suspicious or positive in at least one of the applied tests were further examined (table 21). In most cases there was good agreement between visual and instrumental reading results. Exceptions are two samples in Delvo SP (no. 8 and 9), which were visually evaluated as slightly suspicious, but positive by scanner reading. In addition, one sample on BR-AS special (no. 16) was visually evaluated as slightly suspicious, but interpreted as positive by both kinds of instrumental reading. In no case of visual evaluation as negative or positive, respectively, a deviating result was obtained by instrumental measurements with the cut-off levels applied.

In all samples, which were read as slightly suspicious by visual reading the inhibitor could not be identified by the method combination applied (28). In 10 samples, which were evaluated as positive in at least one of the tests or mode of reading applied, ß-lactam antibiotics were identified. In two samples (no. 17 and 18) tetracyclines were identified, but the concentrations determined by HPLC-method do not allow to explain the suspicious and positive test results when compared to the test sensitivities (tables 5 and 6).

Tab. 25: Percentage of positive test results of instrumental readings* in spiked milk samples within visual evaluation steps

	Photometric measurement Visual evaluation step				Colour measurement Visual evaluation step			
	1	2	3	4	1	2	3	4
Delvo MCS								
β-lactams	0	5.0	71.0	99.5	2.5	22.5	91.3	100
Sulfonamides	0	0	30.4	95.6	0	21.7	65.2	98.5
Tetracyclines	0	9.5	57.9	93.3	2.2	38.1	79.0	100
Macrolides	0	0	80.0	98.5	0	7.7	95.0	100
Aminoglycosides	0	0	42.9	95.8	0	22.2	85.7	100
Various	0	0	17.4	87.0	0	0	82.6	100
Total	**0**	**11.3**	**54.5**	**98.1**	**0.8**	**28.6**	**84.6**	**99.8**
Copan								
β-lactams	0	0	41.4	95.9	0	0	31.1	89.6
Sulfonamides	0	0	16.0	98.7	0	0	42.9	86.8
Tetracyclines	0	5.6	16.7	100	0	0	11.1	68.8
Macrolides	0	4.4	5.3	89.7	0	0	26.3	93.1
Aminoglycosides	0	0	0	92.3	0	0	20.0	73.1
Various	0	0	23.5	100	0	11.8	70.6	98.2
Total	**0**	**3.5**	**25.4**	**96.7**	**0**	**1.8**	**32.6**	**89.5**
Delvo SP								
β-lactams	0	0	70.6	99.1	0	5.5	72.6	100
Sulfonamides	0	0	68.9	94.6	4.6	16.7	73.3	100
Tetracyclines	0	0	57.1	96.4	0	14.3	85.7	100
Macrolides	0	0	40.0	92.3	0	0	52.0	100
Aminoglycosides	0	3.7	50.0	92.6	0	14.8	50.0	100
Various	0	2.6	47.1	100	0	5.1	64.7	100
Total	**0**	**1.7**	**60.9**	**96.4**	**0.4**	**9.2**	**70.8**	**100**
BR-AS special								
β-lactams	0	8.1	59.0	100	0	15.5	56.4	100
Sulfonamides	0	8.1	57.9	100	0	13.3	52.6	88.9
Tetracyclines	0	4.2	28.6	100	0	0	42.9	100
Macrolides	0	0	82.6	100	0	0	60.9	100
Aminoglycosides	0	8.3	56.5	100	0	8.3	52.2	100
Various	0	0	72.7	100	0	5.9	81.8	100
Total	**0**	**8.1**	**82.8**	**100**	**0**	**7.5**	**60.2**	**100**
BR-AS Brilliant								
β-lactams	0	6.7	28.0	100				
Sulfonamides	0	0	63.6	100				
Tetracyclines	0	0	0					
Macrolides	0	0	60.0	100				
Aminoglycosides	0	17.4	81.3	100				
Various	0	0	25.0	100				
Total	**0**	**4.7**	**43.1**	**100**				
BRT								
β-lactams	0	3.8	26.8	97.8				
Sulfonamides	0.1	8.3	100					
Tetracyclines	0	16.7	100	100				
Macrolides	0	0	46.7	100				
Aminoglycosides	0	6.3	21.4	96.7				
Various	0	3.6	0					
Total	**0**	**7.2**	**27.6**	**97.9**				

* cut-off-values: photometric measurement: ≥55% rel. absorp. in %; colour measurement: z-value ≥0 (Delvo MCS, Delvo SP and BR-AS special) and cif-value ≥4.5 (Copan)

Tab. 26: Percentage of positive results of instrumental readings* in cow composite, ex-farm and car tanker milk within visual evaluation steps

Samples	Photometric measurement Visual evaluation step				Colour measurement Visual evaluation step			
	1	2	3	4	1	2	3	4
Delvo MCS								
Cow composite	0	0	42.9	100	0	27.6	85.6	100
Ex-farm	0	0	0	100	0	0	0	100
Car tanker	0	0	33.3	100	0	25.0	83.3	100
Copan								
Cow composite	0	0	0	100	0	0	22.2	99.5
Ex-farm	0	0	33.3	100	0	0	0	94.1
Car tanker	0	0	0	100	0	0	0	100
Delvo SP								
Cow composite	0	0	50.0	100	0.6	12.0	50.0	100
Ex-farm	0		100	100	0		100	100
Car tanker	0	0	33.3	100	0	33.3	33.3	100
BR-AS special								
Cow composite	0	0	50.0	100	0	0	75.0	100
Ex-farm	0	0	100	100	0	0	100	100
Car tanker	0	11.1	0	100	0	11.1	0	100
BR-AS Brilliant								
Cow composite	4.1	0.6	100	100				
Ex-farm	0	0	100	100				
BRT								
Cow composite	0	15.8	85.0	98.7				
Ex-farm	0		0	100				

* cut-off-values: photometric measurement: ≥55% rel. absorp. in %; colour measurement: z-value ≥0 (Delvo MCS, Delvo SP and BR-AS special) and cif-value ≥4.5 (Copan)

Stability of lyophilized control samples and test kit batches

On each test tablet lyophilized negative and positive control samples (4 µg penicillin/kg) of one batch each were analysed (see table 22). The values of negative and positive control samples were distinctly different from the proposed cut-off values. The low variation of instrumental readings between examination days/plates/batches indicate consistent test kits. Only for two batches of BR-AS Brilliant the differences in absorption of positive and negative control samples was <0.3. The results of these batches were difficult to evaluate.

To check for variation of test sensitivities within and between test kit batches milk samples were spiked with different antibiotic/concentration combinations, lyophilized and stored at 6°C up to 6 years. By the results of these samples with various batches of BR-AS special and Delvo SP during this storage period with none of the tests and none of the substances a systematic trend during storage was obvious (see fig. 11). After a storage period of the test samples of about 500 and 1600 days results of BR-AS special were lower than expected. This was not the case with the results of Delvo SP and therefore probably

lower test sensitivities of BR-AS batches tested at that time and not a systematic change in response of the test samples were responsible. These results make obvious that lyophilization of milk samples is a good tool for the preparation of stable test samples for the control of microbial inhibitor tests.

In order to check for the variability of test sensitivities within and between test kit batches, each test kit batch was analysed once or several times during storage of test plates at 6°C by use of spiked, lyophilized test milk samples. In table 23 and fig. 17 the results of various test kit batches of all test formats included in this study are presented. Table 24 shows results of the same batch (of Copan and Delvo MCS) during storage.

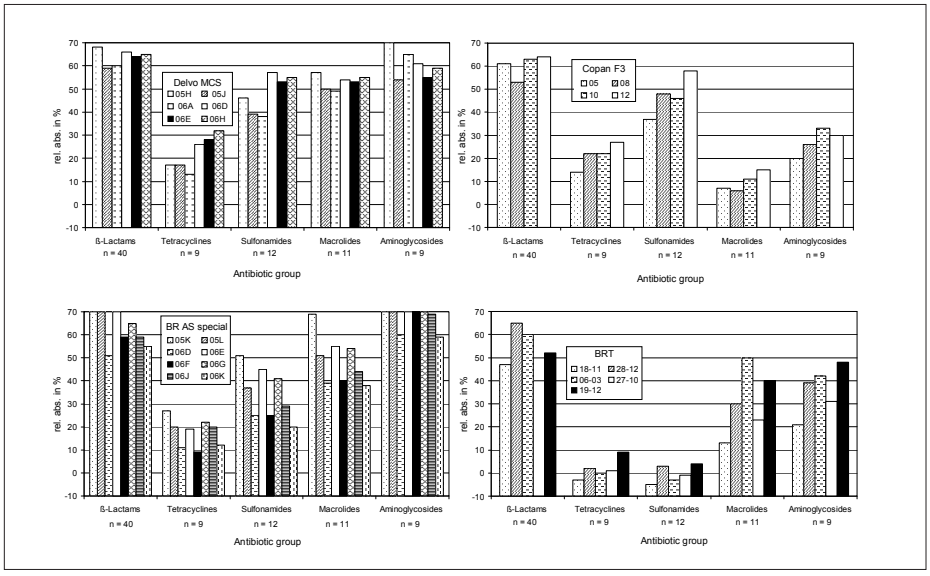

Fig. 17: Variation of test sensitivities between batches of inhibitor tests in dependence on antibiotic group (XQ)

In most cases the variation between the results of various batches of the same test kit format is low. This means that the test sensitivities do not distinctly vary between batches. The results of the test formats with bromcresol purple are more consistent than those of the test with brilliantblack as indicator. With BR-AS special and β-lactam antibiotics the tendency of decreasing sensitivities becomes obvious.

From the results obtained during the storage period of test plates of Copan and Delvo MCS – see table 24 – it can be derived that there is no strong influence of storage time up to one year on the test sensitivities. After a storage period of 240 days of Delvo MCS batch 06A within all antibiotic groups higher sensitivities are observed than in the examination before and after that storage time; in this case it cannot be explained whether this is due to storage influence of test plates or to the test plate examined at that time; an influence of test samples can be excluded as the results obtained from test plates of other test formats at the same time and with the same samples are within the expected range. With one batch of Copan test (F2 02) slightly decreasing test sensitivities were observed for all antibiotic groups whereas this was not the case with the two other batches. The results indicate a shelf life of at least 9 months when stored at 6°C.

Position of microbial inhibitor tests within the integrated detection system

As mentioned before no single method is available for the broad variety of antibiotics and broad spectrum of concentrations which need to be detected due to the MRL-concept. The IDF-integrated system (3,4) includes an appropriate combination of methods that complement each other in their "detection pattern" and the required "analytical depth". Due to their easy test performance and low price microbial inhibitor tests play a prominent role as screening tests within such an integrated system: Test kits with *Geobacillus stearothermophilus* as test micro-organism are in widespread use and are commercially available. Validation data of six different test formats presented here indicate that these tests

- are suitable for the detection of residues of antibiotics in spiked and incurred milk samples with different detection patterns
- are more or less robust with respect to incubation period of test plates, pH-value and somatic cell count of milk samples and lactation stage of cows
- show low variation in test sensitivities between batches and during storage of test plates (shelf life)
- offer the possibility of objective instrumental reading of test results instead of subjective visual evaluation.

The choice of a test depends on the field of application. E.g. if cow composite milk at the beginning of the lactation period has to be examined test formats with bromcresol purple will be preferred due to the lower „false" positive rate compared to the test formats with brilliantblack as indicator. If as many antibiotics as possible shall be detected below MRL concentrations the tests with the broadest spectrum of sufficient detection limits will be chosen.

The test micro-organism *Geobacillus stearothermophilus* is especially sensitive for the detection of β-lactam antibiotics. If other antibiotics need to be detected microbial inhibitor tests with different test micro-organisms as e.g. *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli* can be used to complement the detection pattern (34-36). One disadvantage of the latter test micro-organisms is the long incubation period of test plates needed (up to 24 hours).

The risk of antibiotic residue concentrations exceeding MRLs in milk depends beside other factors on

- concentration of the applied veterinary drug,
- carry-over rate into milk and
- MRL-concentration.

In mastitis therapy β-lactam antibiotics still play an important role. They are often applied as single substance or in combination with other antibiotics. Due to own experiments the β-lactams within combination drugs are the substances with the longest excretion time (37). The MRLs of some β-lactams are extremely low (e.g. penicillin 4 µg/kg) and therefore the carry over of small volumes of contaminated milk can cause violations of the MRLs. A rough calculation on the basis of own experiments (37, 38) shows the risk for exceeding the MRL in bulk tank milk if carry over of milk of treated cows occurs (see table 27). The highest risk is for penicillin due to high concentrations in milk after udder treatment and the low MRL.

Experiences with the application of a method combination for the detection of a broad variety of antimicrobials, for which MRLs are fixed according to Regulation 2377/90 EEC (1) on car tanker samples from the Northern part of Germany underline the predominance

of β-lactams as residues (28). Therefore microbial inhibitor tests with *Geobacillus stearothermophilus* as test micro-organism are suitable screening methods which need to be supplied by other tests which complement the "detection pattern".

Tab. 27: Risk of contamination of milk at MRL concentration by carry over of milk from treated quarters in dependence of antibiotic substance

Antibiotic	MRL in milk (µg/kg)	Concentration in milk of treated quarter* (µg/kg)	Volume (ml) needed to contaminate 1 000 l milk >MRL
Penicillin G	4	400 000	10
Ampicillin	4	100 000	40
Nafcillin	30	20 000	1 500
Cefquinome	20	20 000	1 000
DHstreptomycin	200	30 000	6 700
Colistin	50	30 000	1 700

* Highest concentration in cow composite milk after treatment of 4 quarters

4. References

(1) Council Regulation (EEC) No. 2377/90 of 26 June 1990 laying down a community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. Official Journal of the European Communities No. L 224/1, 18.08.1990

(2) Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal of the European Communities No. L221/8; 17.08.2002

(3) Heeschen, W., Suhren, G.: IDF integrated detection system for antimicrobials: introductory statement and practical experiences in Germany. International Dairy Federation: Proceedings of the Symposium on residues of antimicrobial drugs and other inhibitors in milk, Kiel/DE, August 28-31, 1995, 310-318

(4) Heeschen, W., Suhren, G.: Principles of and practical experiences with an integrated system for the detection of antimicrobials in milk. Milchwissenschaft **51** 154-159 (1996)

(5) Codex Committee on Residues of Veterinary Drugs in Foods: Proposed draft revised guidelines for the establishment of a regulatory program for the control of veterinary drug residues in foods. CX/RVDF 06/16/8, December 2005

(6) Council Directive 96/23 of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664. Official Journal of the European Communities No. L125, 23.05.1996

(7) Mäyrä-Mäkinen, A.: Residues and quality impairment of fermented milk products and cheeses. IDF workshop on residues of antibiotics and other antimicrobials inhibitors in raw and heat-treated milk, Copenhagen/DK, December 8-10, 1993, 136-143

(8) Suhren, G.: Untersuchungen zum Einfluss von Rückständen von antimikrobiell wirksamen Substanzen in Milch auf kommerziell eingesetzte Starterkulturen in Modellversuchen. Kieler milchwirtschaftliche Forschungsberichte **48** (2) 131-149 (1996)

(9) Suhren, G., Knappstein, K.: What do MRL levels of antibiotics mean with respect to technological safety? Poster D1-05, Congrilait - 26th IDF World Dairy Congress, Paris/F, September 24-27, 2002

(10) Nazina, T., Tourova, T., Polaraus, A., Novikova, E., Gregoryan, A., Ivanaova, A., Belyaev, S., Ivanaov, M.: Taxonomic study of aerobic thermophilic bacilli: Descriptions of *Geobacillus uzensis sp. Nov*. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus kaustophilus*, *Bacillus thermodenitrificans* to Geobacillus as the new combinations of *G. stearothermophilus*. International Journal of Systematic and Evolutionary Microbiology **51** (2) 433-446 (2001)
(11) Galesloot, T.E., Hassing,F.: A rapid and sensitive paper disc method for the detection of penicillin in milk. Netherlands Milk and Dairy Journal **16** 89-95 (1962)
(12) Kraak, J., Tolle, A.: Brilliantschwarz-Reduktionstest mit Bac. stearothermophilus var. calidolactis zum Nachweis von Hemmstoffen in der Milch. Milchwissenschaft **22** 669-673 (1967)
(13) Gudding, R.: The suitability of some media and peptones for sulfonamide testing. Acta Veterinaria Scandinavica **15** 366-380 (1974)
(14) Nouws, J., van Egmond, H., Smulders, I., Loeffen, G., Stegeman, H.: Microbiological assay system for assessment of raw milk exceeding residue limits. International Dairy Journal **9** 85-90 (1999)
(15) International Standard Organisation: Milk and Milk products – determination of antimicrobial residues – Tube diffusion test. ISO/TS 26844/IDF/RM 215:2006
(16) International Dairy Federation: Milk and milk products – Guidance for the standardized description of microbial inhibitor tests. ISO/13969/IDF 183:2003
(17) International Dairy Federation: Milk and milk products – Guidelines for the standardized description of immunoassays or receptor assays for the detection of antimicrobial residues. ISO 18330:2003
(18) Schiffmann, A.P.: Methodische und rechtliche Probleme beim Nachweis von Hemmstoffen in Milch. Diss. med. vet., Tierärztliche Hochschule Hannover, 1992
(19) Suhren, G., Luitz, M.: Evaluation of microbial inhibitor tests with indicator in microtitre plates by photometric measurements. Milchwissenschaft **50** 467-470 (1995)
(20) Adriany, A.: Entwicklung eines modifizierten Brilliantschwarz-Reduktionstests (BRT) zum verbesserten Nachweis von Tetracyclinen und Sulfonamiden in Milch. Diss. med. vet., Ludwig-Maximilians-Universität München, 1996
(21) Stead, S., Richmond, S., Sharman, M., Stark, J., Gejp, E.: A new approach for detection of antimicrobial drugs in food by Premi® Test coupled to scanner technology. Analytica Chimica Acta **529** 83-88 (2005)
(22) Suhren, G., Walte, H.-G.: Instrumentelle Auswertung von mikrobiologischen Hemmstofftests mit Indikator in Mikrotiterplatten. Kieler Milchwirtschaftliche Forschungsberichte **57** (2) 73-79 (2005)
(23) Le Breton, M.-H., Savoy-Perroud, M.-C., Diserens J.-M. : Validation and comparison of the Copan Milk Test and Delvotest SP-NT for the detection of antimicrobials in milk. Analytica Chimica Acta **586** 280-283 (2007)
(24) Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG (§ 64 LFGB): Nachweis von Hemmstoffen in Milch – Agar-Diffusionsverfahren (Brillantschwarz-Reduktionstest); L01.01-5, Februar 1996
(25) Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG (§ 64 LFGB): Such-verfahren auf das Vorhandensein von Antiinfektiva in Milch – Agar-Diffusionsverfahren mit Bacillus stearothermophilus (Brillantschwarz-Reduktionstest), L01.00-11, Februar 1996
(26) Suhren, G., Knappstein, K.: Detection of incurred dihydrostreptomycin residues in milk by liquid chromatography and preliminary confirmation methods. Analyst **23** 2797-2801 (1998)
(27) Suhren, G., Knappstein, K.: Detection of cefquinome in milk by liquid chromatograph and screening methods. Analytica Chimica Acta **483** 363-372 (2003)
(28) Suhren, G., Walte, H.-G.: Experiences with the application of method combinations for the detection of residues of antimicrobial drugs in milk from collecting tankers. Milchwissenschaft **58** 536-540 (2003)
(29) Suhren, G., Knappstein, K.: Detection of colistin in spiked and incurred milk samples by LC- and ELISA-technique. Analytica Chimica Acta **529** 97-101 (2005)
(30) International Dairy Federation: Milk – Enumeration of somatic cells – Part 2: Guidance on the operation of fluoro-opto-electronic counters. ISO 13366-2/IDF 148-2 (2006)

(31) Barbosa, M., Suhren, G., Beukers, R.: Suitability and application of available test kits for the detection of residues of antimicrobials in milk from other species than the cow – a review. Bulletin of the IDF 390/2004, 29-40
(32) Zaadhof, K.J., Märtlbauer, E., Strasser, A., Schulze, S.: Nachweis von Hemmstoffen in Milch verschiedener Tierarten. Deutsche Molkereizeitung **19** 27-29 (2005)
(33) Commission Regulation (EC) No 1662/2006 of 6 November 2006 amending Regulation (EC) No 853/2004 of the European Parliament and of the Council laying down specific rules for food of animal origin. Official Journal of the European Union L 320/1; 18.11.2006
(34) Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG (64 LFGB): Suchverfahren auf das Vorhandensein von Antiinfektiva in Milch – Agar-Diffusionsverfahren mit Bacillus cereus (TTC-Reduktionstest). L01.00-62 (Sept. 1997)
(35) Suhren, G., Hammer, P., Heeschen, W.: Detection of residues of quinolones in milk. In: Haagsma, N., Ruiter, A. (eds.): Residues of veterinary drugs in food – Proceedings of the EuroResidue III conference, Veldhoven (NL), May 6-8, 1996, ISBN 90-6159-023-x, 917-921
(36) Gaudin,V., Maris, P., Fuselier, R., Ribouchon, J.L., Cadieu, N., Rault, A.: Validation of a microbial method: the STAR protocol, a five-plate test for the screening of antibiotic residues in milk. Food Additives and Contaminants **21** 422-433 (2004)
(37) Knappstein, K., Suhren, G., Walte, H.-G.: Influence of milking frequencies in automatic milking systems on excretion characteristics of different antibiotics in milk. Kieler milchwirtschaftliche Forschungsberichte **57** (4) 215-261 (2005)
(38) Knappstein, K., Suhren, G., Walte, H.-G., Slaghuis, B.A., Ferwerda-van Zonneveld: Prevention of antibiotic residues: Appropriate management of antibiotic treatment of cows in automatic milking systems. Deliverable D12, http://www.automaticmilking.nl

Acknowledgement

The authors are grateful to the companies Copan Italia, Brescia/Italy, DSM Food Specialities, Düsseldorf/Germany and Chr. Hansen, Nienburg/Germany for providing test kits of microbial inhibitor tests. The Milcherzeugervereinigung Schleswig-Holstein and the Landeskontrollverband Schleswig-Holstein are acknowledged for providing milk samples from car tankers and ex-farm milk. We thank the milking personnel of the experimental farm Schaedtbek and the laboratory technicians Heinz Braun and Sabine Holler-Niehus for excellent technical assistance in sampling and laboratory analysis.

5. Summary

Suhren, G., Knappstein, K.: **Validation studies with commercially available inhibitor tests for the detection of inhibitors and antibiotic residues in milk**. Kieler Milchwirtschaftliche Forschungsberichte **59** (4) 227-283 (2007)

06 Veterinary medicine and hygiene (Inhibitor test, antibiotic residues, raw milk)

Microbial inhibitor tests with *Geobacillus stearothermophilus* as test micro-organism are in wide-spread application for the detection of inhibitors in milk in quality payment schemes or as screening methods for the detection of residues of antibiotics with respect to food hygiene legislation. The tests underlie continuous modifications in order to improve the detection pattern or to facilitate the evaluation. Often the commercially available tests are proprietary techniques. Validation is necessary according to internationally accepted protocols.

In this study validation experiments were performed with 6 different commercially available microbial inhibitor tests: BRT (AIM; Munich/Germany), CMT Copan Milk Test (Copan, Brescia/Italy), Delvo MCS, Delvo SP, BR-AS special and BR AS Brilliant (all from DSM Food Specialities, Delft/Netherlands). Instrumental evaluation of the tests by ELISA reader or scanner was used in addition to visual evaluation.

The results are summarized as follows:

– No problems ere observed with the test procedure recommended by the manufacturer w. Visual evaluation was easy, but the scanner technology applied by two companies facilitated the evaluation of test results.

Detection limits

– Detection limits depended on test kit and type of evaluation: from 18 antibiotics 4 to 10 substances were detected at ≤ MRL concentrations when tests were evaluated as screening tests, but only 3 to 7 when tests were evaluated as inhibitor tests.
– Detection limits for ampicillin, cefoperazone, cefquinome and penicillin were similar in spiked and incurred samples.
– The detection limits may increase by prolonging the incubation time; this effect was dependent on the antibiotic substance.

Influences on results

– Influences by pH of samples were dependent on type of indicator used in the test kit: Tests with bromcresol purple as indicator were sensitive to alkaline pH, whereas tests with brilliantblack as indicator were influenced by acidic pH.
– In cow composite milk samples an influence of somatic cell count on test results was detected with higher numbers of "false suspicious or positive“ results with increasing somatic cell count. This was only the case with visual test interpretation.
– "False suspicious or positive“ results were also observed with milk samples collected after calving. The number of "false suspicious or positive“ samples decreased with days in milk and was in general lower with instrumental evaluation. A more pronounced influence was observed in test formats with brilliantblack as indicator.

Consistency of test results

– Only slight deviations were observed when tests were evaluated visually by trained persons or read by instruments repeatedly within short time. Differing results between visual and instrumental readings were only detected in cases when the test results were close to the cut-off value of instrumental readings. Only the Copan test showed greater deviations, especially for residues of tetracyclines and aminoglycosides. In general there was good agreement between visual and instrumental readings.
– The lyophilized control samples showed consistent results over the examination period which makes them a suitable tool for the control of microbial inhibitor tests.
– Low variation of instrumental readings was observed in repeated evaluation at different examination days and with different plates or batches. This indicates consistent test kits with the following exceptions: two batches of BR AS Brilliant were difficult to interprete because of small differences between positive and negative control samples; one batch of Delvo MCS and Copan test each showed inconsistent sensitivities which might be due to the fact that the validation studies started from the very beginning of test kit manufacture by the companies.

Role of inhibitor tests in an integrated detection system

Based on the results of this validation study it can be summarized that commercially available microbial inhibitor tests are suitable for detection of antibiotic residues in raw milk. They show similar sensitivity with spiked and incurred samples. Differences in detection patterns as well as – in dependence on the indicator used – different sensitivities to influences by incubation period, pH and somatic cell count of milk samples have to be considered. Comparable results are produced with different batches and during storage of test kits. Some manufacturers offer the possibility for objective evaluation by instrumental reading. In addition microbial inhibitor tests are easy to use and cheap when compared to other detection methods.

As no single method is available to detect all antibiotics used in veterinary therapy at MRL concentrations an integrated system is needed consisting of a combination of methods which complement each other. Due to their above mentioned properties microbial inhibitor tests play an important role for the detection of antibiotic residues in raw milk within an integrated system. The appropriate choice of test is dependent on the field of application.

Zusammenfassung

Suhren, G., Knappstein,K.: **Untersuchungen zur Validierung von kommerziell erhältlichen Hemmstofftests für den Nachweis von Hemmstoffen und Antibiotika-Rückständen in Milch**. Kieler Milchwirtschaftliche Forschungsberichte **59** (4) 227-283 (2007)

06 Veterinärmedizin und Hygiene (Hemmstofftest, Antibiotika-Rückstände, Rohmilch)

Mikrobielle Hemmstofftests mit *Geobacillus stearothermophilus* als Testkeim werden weitverbreitet für den Nachweis von Hemmstoffen in Milch verwendet, sowohl im Rahmen von Qualitätsbezahlungssystemen als auch als Screening-Verfahren für den Nachweis von Antibiotika-Rückständen in Bezug auf rechtliche Vorgaben der Lebensmittelhygiene.

Die Tests unterliegen fortdauernden Modifikationen, um die Nachweismuster zu verbessern oder die Auswertung zu vereinfachen. Oftmals handelt es sich bei den kommerziell verfügbaren Tests um firmengebundene Methoden. Eine Validierung entsprechend den Vorgaben international akzeptierter Protokolle ist daher notwendig.

In der vorliegenden Studie wurden Untersuchungen zur Validierung von 6 verschiedenen, kommerziell erhältlichen mikrobiellen Hemmstofftests durchgeführt: (AIM, München/Deutschland), CMT Copan Milk Test (Copan, Brescia/Italien), Delvo MCS, Delvo SP, BR-AS Spezial and BR AS Brilliant (alle DSM Food Specialities, Delft/Niederlande). Zusätzlich zur visuellen Auswertung wurden instrumentelle Auswertungen mittels ELISA Reader oder Scanner durchgeführt.

Die Ergebnisse lassen sich wie folgt zusammenfassen:

– Bei Befolgung der von den Herstellern empfohlenen Vorgehensweisen wurden keine Probleme in der Testdurchführung beobachtet. Die visuelle Ablesung war einfach, wurde aber durch die Scanner-Technik, die von zwei Firmen zur Auswertung angeboten wird, erleichtert.

Nachweisgrenzen:

- Die Nachweisgrenzen waren abhängig vom jeweiligen Test und von der Art der Auswertung: Von 18 antibiotisch wirksamen Substanzen wurden 4 bis 10 Substanzen in Konzentrationen ≤ MRL nachgewiesen, wenn die Tests als Suchtest ausgewertet wurden, aber nur 3 bis 7 Substanzen, wenn die Tests als Hemmstofftest ausgewertet wurden.
- Die Nachweisgrenzen für Ampicillin, Cefoperazon, Cefquinom und Penicillin waren in künstlich kontaminierten Proben (spiked) und in Milchproben von Behandlungsversuchen (incurred) ähnlich.
- Durch eine Verlängerung der Inkubationszeit können sich die Nachweisgrenzen erhöhen; dieser Effekt ist abhängig von der antibiotisch wirksamen Substanz.

Einflüsse auf die Ergebnisse

- Der Einfluss des pH-Wertes von Proben war abhängig vom verwendeten Indikator: Tests mit Bromkresolpurpur als Indikator reagierten empfindlich auf alkalische pH-Werte während Tests mit Brilliantschwarz als Indikator durch saure pH-Werte beeinflusst wurden.
- In Kuh-Gesamtgemelksproben wurde ein Einfluss des Gehaltes an somatischen Zellen festgestellt: der Anteil „falsch verdächtiger oder positiver" Ergebnisse nahm mit dem Zellgehalt zu. Allerdings war dies nur bei visueller Ablesung der Fall.
- Ebenso wurden bei Proben im Zeitraum nach der Kalbung „falsch verdächtige oder positive" Ergebnisse beobachtet. Die Anzahl „falsch verdächtiger oder positiver" Proben nahm mit der Anzahl der Laktationstage ab und war bei instrumenteller Auswertung geringer als bei visueller Ablesung. Der Einfluss des Laktationsstadiums war bei Tests mit Brilliantschwarz als Indikator ausgeprägter als bei solchen mit Bromkresolpurpur.

Konsistenz der Ergebnisse

- Wurden die Tests visuell durch geschulte Personen oder instrumentell innerhalb kurzer Zeit wiederholt abgelesen, traten bei den Ergebnissen nur geringfügige Abweichungen auf. Unterschiede zwischen visueller und instrumenteller Auswertung traten nur auf, wenn die Ergebnisse nahe der Grenzwerte der instrumentellen Auswertung lagen. Nur beim Copan-Test wurden größere Abweichungen beobachtet, insbesondere bei Rückständen von Tetracyclinen und Aminoglykosiden. Allgemein stimmten visuelle und instrumentelle Auswertung gut überein.
- Die lyophilisierten Kontrollmilchproben zeigten über den Untersuchungszeitraum konsistente Ergebnisse. Sie sind somit ein geeignetes Werkzeug für die Kontrolle mikrobieller Hemmstofftests.
- Bei der wiederholten Auswertung an verschiedenen Untersuchungstagen and mit verschiedenen Testplatten oder Chargen wurde nur eine geringfügige Variation der instrumentell ermittelten Ergebnisse festgestellt. Dies zeigt beständige Testkits mit folgenden Ausnahmen an: Zwei Chargen von BR AS Brilliant waren schwer zu interpretieren, da zwischen Positiv- und Negativkontrolle nur geringe Unterschiede bestanden; je eine Charge von Delvo MCS und Copan Test zeigten abweichende Testempfindlichkeiten, was darauf zurückzuführen sein könnte, dass die Validierungsstudien bereits ab Beginn der Testkit-Produktion durch die Hersteller durchgeführt wurden.

Bedeutung von Hemmstofftests in einem integrierten Nachweissystem

Basierend auf den Ergebnissen der vorliegenden Validierungsstudie kann festgehalten werden, dass kommerziell verfügbare mikrobielle Hemmstofftests für den Nachweis von Antibiotika-Rückständen in Rohmilch gut geeignet sind. Sie zeigen ähnliche Empfindlichkeiten bei künstlich und natürlich kontaminierten Proben. Unterschiede im Nachweismuster ebenso wie – in Abhängigkeit vom verwendeten Indikator – unterschiedliche Empfindlichkeit gegenüber Inkubationszeit, pH-Wert und Zellgehalt der Proben sind zu berücksichtigen. Mit unterschiedlichen Testkit-Chargen wie auch während deren Lagerung werden vergleichbare Ergebnisse erzielt. Einige Hersteller bieten zudem instrumentelle Verfahren zur objektiven Auswertung an. Darüber hinaus sind die Hemmstofftests im Vergleich zu anderen Nachweisverfahren leicht zu handhaben und kostengünstig.

Da keine Methode verfügbar ist, mit der alle in der veterinärmedizinischen Therapie verwendeten Antibiotika im Bereich der MRL-Konzentrationen nachgewiesen werden können, ist ein integriertes System bestehend aus einer Kombination von Methoden, die sich gegenseitig ergänzen, erforderlich. Auf Grund der vorgenannten Eigenschaften spielen die mikrobiellen Hemmstofftests in einem integrierten System eine wichtige Rolle für den Nachweis von Antibiotika-Rückständen in Rohmilch. Die Auswahl eines geeigneten Testkits ist abhängig vom Anwendungsbereich.

Résumé

Suhren, G., Knappstein,K.: **Etudes de validation avec des tests inhibiteurs commercialisés pour la détection d'inhibiteurs et de résidus antibiotiques dans le lait**. Kieler Milchwirtschaftliche Forschungsberichte **59** (4) 227-283 (2007)

06 Médecine vétérinaire et hygiène (test inhibiteur, résidus antibiotiques, lait cru)

Pour la détection d'inhibiteurs dans le lait, on utilise généralement des tests inhibiteurs microbiens avec *Geobacillus stearothermophilus* comme germe de référence. Ceci se fait dans le cadre des systèmes de paiement de qualité et de méthodes de screening pour la détection de résidus antibiotiques conformément à la législation pour l'hygiène alimentaire.

Pour améliorer les échantillons justificatifs ou simplifier l'évaluation, les tests sont soumis à des modifications permanentes. Dans le cas des tests commercialisés, il s'agit souvent de méthodes liées à des sociétés. Une validation conforme aux normes de protocoles admis au niveau international est donc nécessaire.

Dans la présente étude, des examens portant sur la validation de 6 tests inhibiteurs microbiens différents et commercialisés ont été réalisés. (AIM, Munich/Allemagne), CMT Copan Milk Test (Copan, Brescia/Italie) Delvo MCS, Delvo SP, BR-AS Spezial et BR AS Brilliant (tous les DSM Food Specialities, Delft/Pays-Bas). En plus de l'évaluation visuelle, on a fait des évaluations instrumentales avec ELISA Reader ou par scanner.

Les résultats sont résumés comme suit:

– En suivant les procédures recommandées par les fabricants, il n'y avait pas de problèmes dans la mise en œuvre des tests. Bienque la lecture visuelle fût simple, elle était en plus facilitée par la technique de scanner offerte pour l'évaluation par deux sociétés.

Seuils de détection:

- Les seuils de détection dépendaient du test respectif et du type d'évaluation: De 18 substances à effet antibiotique, 4 à 10 substances étaient détectées dans des concentrations ≤ LMR (Limites Maximales de Résidus) en évaluant les tests comme screening et seulement 3 à 7 substances en les évaluant comme test inhibiteur.
- Les seuils de détection pour ampicilline, céphopérazone, cefquinome et pénicilline étaient similaires dans les échantillons contaminés artificiellement (spiked) et dans les échantillons prélevés d'animaux contaminés par un traitement antibiotique.
- Par une prolongation de la période d'incubation, il est possible d'augmenter les seuils de détection; cet effet dépend de la substance à effet antibiotique.

Influences sur les résultats

- L'influence du pH physiologique des échantillons dépendait de l'indicateur utilisé: Des tests avec du pourpre de bromocrésol comme indicateur ont réagi sensiblement à des pH physiologiques alcalins tandis que des tests avec du noir brilliant comme indicateur ont été influencés par des pH physiologiques acides.
- Dans les échantillons de traite complète de lait de vache, une influence de la comptage de cellules somatiques a été constatée: les résultats „faux suspects ou positifs" ont augmenté avec la teneur en cellules. Toutefois, cela n'était le cas que lors d'une lecture visuelle.
- Aussi des résultats „faux suspects ou positifs" ont été observés dans des échantillons pendant la période après le vêlage. Le nombre d'échantillons „faux suspects ou positifs" diminuait avec le nombre des jours de lactation, et était plus faible lors de l'évaluation instrumentale que lors de la lecture visuelle. L'influence de la phase de lactation était plus prononcée dans des tests avec du noir brilliant comme indicateur que dans des tests avec du pourpre de bromocrésol.

Consistance des résultats

- Quand les tests étaient lus visuellement à plusieurs reprises par des personnes qualifiées ou avec des instruments dans un court délai de temps, on n'a observé que de légères déviations dans les résultats. Des différences entre une évaluation visuelle et instrumentale ne sont apparues que si les résultats étaient dans les alentours des valeurs limites de l'évaluation instrumentale. Ce n'est qu'avec le Copan Milk Test que des déviations plus importantes, en particulier dans des résidus de tétracyclines et d'aminoglycosides, étaient observées. En général l'évaluation visuelle et instrumentale concordaient bien.
- Les échantillons de lait de contrôle lyophilisés fournissaient des résultats cohérents sur la période d'étude. Ils sont ainsi un outil approprié pour le contrôle de tests inhibiteurs microbiens.
- Lors de l'évaluation répétée à différents jours d'étude et avec différentes plaques d'essai ou lots, on n'a constaté qu'une légère variation des résultats déterminés instrumentellement. Cela indique des kits d'essai constants avec les exceptions suivantes: Deux lots de BR AS Brilliant étaient difficiles à interpréter, puisqu'il n'y avait que de faibles différences entre le contrôle négatif et positif; un lot de Delvo MCS et du Copan Milk Test ont montré des sensibilités divergentes par rapport au test, ce qui pourrait être dû au fait que les études de validation ont déjà été réalisées par les fabricants dès le début de la production de kits d'essai.

Importance des tests inhibiteurs dans un système justificatif intégré

En se basant sur les résultats de la présente étude de validation, on peut retenir que des tests inhibiteurs microbiens commercialisés sont bien appropriés pour la détection des résidus d'antibiotique dans du lait cru. Ils font preuve d'une sensibilité semblable dans des échantillons artificiellement et naturellement contaminés. Des différences dans l'échantillon justificatif et – en fonction de l'indicateur utilisé – une sensibilité différente par rapport à la période d'incubation, à la valeur pH et à la comptage de cellules des échantillons doivent être prises en considération. Des résultats comparables sont obtenus avec des lots de kits d'essai différents et aussi pendant leur stockage. Certains fabricants offrent en outre des méthodes instrumentales pour une évaluation objective. En plus, en comparaison avec d'autres techniques de détection, les tests inhibiteurs sont faciles à manipuler et sont bon marché.

Puisqu'aucune méthode disponible n'est capable de détecter, dans les limites maximales de résidus (LMR), tous les antibiotiques utilisés dans la thérapie vétérinaire, un système intégré, composé d'une combinaison de méthodes se complétant mutuellement, est nécessaire. A cause des caractéristiques susmentionnées, les tests inhibiteurs microbiens jouent un rôle important dans un système intégré pour la détection de résidus antibiotiques dans du lait cru. Le choix d'un kit d'essai appropriée dépend du champ d'application.