Assay of Antibiotic Detection Limits in Cow's Milk Model Samples and Comparison of Sensitivity of Various Detection Systems (Disk Diffusion Method, Delvotest SP and Penzym S 100)

BERNADETTA HOZOVÁ and MARTA KRATMÜLLEROVÁ

Slovak University of Technology – Faculty of Chemical Technology, Bratislava, Slovak Republic

Abstract

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The work was aimed at estimating the detection limits of 21 antibiotics (β-lactam antibiotics, cephalosporins, aminoglycosides, macrolides and others) by means of three types of detection systems (disk diffusion method with *Bacillus stearothermophilus* var. *calidolactis* C 953, Delvotest SP and Penzym S 100) and to verify their sensitivity in the evaluation of the admissible maximum residual limits (MRL). The high sensitivity and the good correlation of results have been achieved mainly by applying the rapid methods such as Delvotest SP and Penzym S 100 versus the less sensitive disk diffusion method. In the next stage of the work, detection limits of the mutual combinations of antibiotics in milk were estimated. In the model experiment, the synergic effect between ampicillin and oxacillin, cefuroxime and trimethoprim and between cephalosporins (combination of cefazolin with cefoperhazon) was observed.

Keywords: antibiotics; disk diffusion method; Delvotest SP; Penzym S 100; synergism

Milk as one of the basic foods that are consumed by the population of all age and social groups should correspond primarily to the demanded quality criteria (microbiological, nutritional, sensory). The occurrence of milk inhibitors is considered to be undesirable and detrimental to health. Their presence causes a lot of disorders heredity and can also have some carcinogenic effects.

In the technological processing of milk the presence of inhibitors deteriorates its basic property – lactic fermentation ability. Therefore it is necessary to monitor the presence of inhibitors in raw milk regularly (antibiotics, cleaning and disinfectant agents, hydrogen peroxide, etc.) and continue milk processing in accordance with this. Several methods are used for the estimation of milk inhibitors, such as standardized conventional, or specialized and innovated miniaturized microplate methods for a rapid and reliable diagnostics. The sufficient sensitivity of applied methods is decisive for the monitoring of admissible maximum residual limits (MRL) and safety limits complying with international requirements recommended by EC 85/397 (1992).

With further reference to our previous contributions (HOZOVÁ *et al*. 1995, 1998) the present paper is aimed at:

1. Examination and the comparison of sensitivity of three methods for the detection of 21 antibiotics that are used most frequently for the treatment of mastitis in clinical veterinary practice (ß-lactam antibiotics and cephalosporins, tetracyclines, macrolides, aminoglycosides and others), namely by means of:
– classical disk diffusion method (DDM),
– DELVOTEST SP,
– PENZYM test S 100.

2. Verification of the detection limits of antibiotics published in the literature, but also of those that have not been established until now (cephalosporins), and the verification of the sufficient sensitivity of applied methods for the implementation of safety limits according to international requirements.

3. Evaluation of the interaction of some combined antibiotics and of the influence on individual detection limits with the possibility of achieving an additive or a synergic (multiplicative) effect.

MATERIAL AND METHODS

Bacterial Strain: *Bacillus stearothermophilus* var. *calidolactis* C 953 (from the collection of microorganisms at the Research Institute of Veterinary Medicine in Bratislava), propagated on GTK agar before its application and subsequently in the liquid substrate of the same composition (producer – Imuna, Šarišské Michaľany, SR). The twenty-four hour inoculum contained 10^3 cells/ml.

Antibiotics: Table 1 gives a list of the concentration ranges of 21 tested antibiotics (6 to 10 of these were used in the experiment); deionized water was used for the preparation of stock and working solutions. Model milk samples for the estimation of the detection limits of tests were prepared from the reconstituted skim milk (PROMIL-PML, Inc. Nový Bydžov, Czech Republic) and deionized water (1:9); respective antibiotics were added to the milk prepared in this way.

According to the recommendations of IDF (1991), the pH of reconstituted milk was higher than 6; natural inhibitors were subjected to thermal inactivation for 5 min at 80°C. The reconstituted dried milk without any antibiotics was used as a control.

Methods (STN 57 0531, 1995)

Disk diffusion method: The paper disk soaked with the sample examined is placed on the surface of the agar nutrient medium with *B. stearothermophilus*. The incubation (64 ± 1°C/4–5 h), at which the tested strain is growing, results in the opacity of the agar medium. If the investigated sample contains substances inhibiting the growth of the test strain, clear zones are formed around the disk. Their size depends on the concentration and type of antimicrobials and can be compared with the size of zones created by the reference solutions of penicillin of the known concentration (the inhibition zone > 1 mm was considered as positive).

Delvotest SP (GIST-BROCADES, Holland): These methods combine the principle of agar diffusion tests with a colour change of the indicator resulting from the active metabolism of the testing microorganism in the absence of the inhibitor. The sample examined is batched into microtitration plates with pits filled with the agar nutrient medium containing *B. stearothermophilus* var. *calidolactis*. The incubation (64 ± 1°C/2.5–5 h), at which the tested strain is growing, causes that the colour of the

Table 1. Concentration ranges of tested antibiotics

Antibiotics	Producer	Producer concentration ranges (mg/ml)	
Oxacillin	Léčiva, Prague (CR)	0; 0.01, 0.02, 0.03…	1.0
Ampicillin	Biotika, Sl. Ľupča (SR)	0; 0.002; 0.003; 0.005…	0.05
Cefotaxime	Roussel (France)	0; 0.1; 0.2; 0.3…	1.0
Cefuroxime	Glaxo (England)	0; 0.03; 0.04; 0.05…	0.5
Cefamandol	Lilly France SA (France)	0; 0.002; 0.003; 0.005…	0.5
Cefoperhazone	Pfizer (Germany)	0; 0.01; 0.02; 0.03…	0.8
Cefazolin	Lilly France SA (France)	0; 0.003; 0.005; 0.01…	0.2
Cefquinome	Hoecust (Germany)	0; 0.05; 0.06; 0.08…	1.5
Ceftazidime	Glaxo (England)	0; 0.5; 1.0; 2.0…	10.0
Chlortetracycline	Biotika, Sl. Ľupča (SR)	0; 0.5; 1.0; 5.0…	10.0
Streptomycin	Biotika, Sl. Ľupča (SR)	0; 1.0; 2.0; 5.0…	8.0
Neomycin	Biotika, Sl. Ľupča (SR)	0; 0.2; 0.4; 0.5…	1.0
Gentamicin	Sanofi (SR)	0; 0.2; 0.3; 0.4…	0.8
Kanamycin	Biotika, Sl. Ľupča (SR)	0; 5.0; 10.0; 15.0…	35.0
Erythromycin	Biotika, Sl. Ľupča (SR)	0; 0.2; 0.3; 0.5. …	1.2
Spiramycin	Biotika, Sl. Ľupča (SR)	0; 1.0; 2.0; 5.0…	10.0
Novobiocin	Biotika, Sl. Ľupča (SR)	0; 1.0; 3.0; 5.0.....	10.0
Chloramphenicol	Biotika, Sl. Ľupča (SR)	0; 5.0; 10.0; 15.0…	30.0
Trimethoprim	Biotika, Sl. Ľupča (SR)	0; 0.3; 0.4; 0.5…	1.2
Bacitracin	Biotika, Sl. Ľupča (SR)	0; 0.3; 0.4; 1.0…	4.0
Enrofloxacin	Pliva (Croatia)	0; 1.0; 2.0; 3.0…	12.0

indicator (bromcresol red) will change from blue-violet to yellow. If the sample examined contains substances inhibiting the growth of the test strain, the colour of the indicator will remain blue-violet.

Penzym S 100 (HERSTELLER UCB BIO-PRODUCTS, Belgium): The method is based on the reaction of DD-carboxypeptidase reacting with b-lactam antibiotics (two-step procedure: 7.5 + 15 min/47°C). If milk does not contain any antimicrobials, the enzyme will remain fully active, which will show itself in the pink colour. In the opposite case, the enzyme will be totally inactivated and no colour reaction will occur.

Sensitivity of Tests: Reconstituted dried milk samples were used for the verification of sensitivity of the detection systems: negative reference sample – without the presence of residues of the inhibition substances; positive reference sample – with Penicillin G in the concentration of 0.0025 IU/ml of milk (disk diffusion method), or 0.003 to 0.005 IU/ml of milk (Delvotest SP and Penzym S 100). In the Delvotest SP and Penzym S 100 tests, which involve subjective colour reading, the test samples were independently evaluated by 3 different assessors. To determine the sensitivity of the screening tests each concentration/combination (blind coded) was replicated at least 3–4 times.

RESULTS AND DISCUSSION

Table 2 provides a survey of authors, the applied methods and identified detection limits in milk compared with our experimental results. The table comprises also the admissible maximum residual limits (MRL) for some antibiotics recommended by EEC (1992).

Table 3 summarizes the results of the estimation of detection limits for 21 antibiotics in milk by applying three side-by-side assays, namely the disk diffusion method (DDM), Delvotest SP and Penzym S 100. The results shown are the means of three to four parallel replications.

Table 4 shows some estimated values of detection limits of antibiotic combinations: ampicillin with oxacillin, cefuroxime with trimethoprim and cefazolin with cefoperhazon, which were achieved by means of Delvotest SP in two parallel assays.

β-Lactam Antibiotics and Cephalosporins: As follows from Table 3, the detection limits of oxacillin found by us (0.5 μg/ml) are rather different from those published in the literature, owing to the applied methods. The sensitivity of Delvotest SP and Penzym S 100 is even 25-fold higher than that of DDM. The obtained results of detection limits vary within the range of MRL.

In the determination of ampicillin the application of two rapid methods allowed us to achieve equal detection limits (0.003 μg/ml) corresponding to the requirements for MRL as well as to the published data (SUHREN *et al*. 1996).

In estimating the detection limits of 7 cephalosporin antibiotics the results obtained vary considerably. In descending order, Penzym S 100 is most sensitive, followed by Delvotest and DDM. In some antibiotics (cefamandol, cefoperhazone, cefazolin) a good correlation of the results obtained by means of Penzym S 100 and Delvotest SP was observed. As for cefoperhazone, the values corresponded to the results indicated by SCHLIEPHAKE (1998). The data gained by SUHREN *et al*. (1996) during the Penzym-test for cefapirin are in agreement with some of our results (cefamandol, cefazolin). Published data concerning MRL are only available for ceftiofur (0.1 μg/ml).

Chlortetracycline and Macrolidic Antibiotics: In the assessment of chlortetracycline and other antibiotics only 2 methods were compared, DDM and Delvotest SP, inasmuch as Penzym S 100 is sensitive only to β-lactam antibiotics and cephalosporins.

The results obtained from the estimation of chlortetracycline (Table 3) suggest that DDM is less sensitive to this antibiotic because the detection limit was achieved at the concentration as high as 6 μg/ml. Delvotest SP (0.9 mg per ml) showed to be more sensitive, because the detection limit was found almost two times higher, compared to published data.

As shown in Table 3, the detection values of erythromycin differ considerably.

The detection limit identified by means of DDM (1 μg/ml) is higher than that determined by Delvotest SP (0.3 μg/ml). The obtained values are comparable with data given in the literature (CHARM & ZOMER 1995), but they exceed the MRL limit.

In the determination of spiramycin the sensitivity of Delvotest SP (2 μg/ml) was even 5 times higher than that of DDM (10 mg/ml); our results are, however, incomparable with literature data acquired by the ELISA method (ALBRECHT *et al*. 1996) and they differ from the recommended MRL values at least by an order.

Aminoglycosides: The most frequently used antibiotic in the clinical veterinary practice is streptomycin. According to our knowledge, approximately the same sensitivity was registered in both verified methods: by using DDM the detection limit represented 4 μg/ml and by using Delvotest it was achieved at the concentration of 6 μg/ml which fitted into the range of values published (CHARM & ZOMER 1995).

The detection limit of gentamicin corresponded to the concentration of 0.4 μg/ml. The comparison has shown that the above-mentioned methods are equally sensitive and the values achieved by them comply with the data of other authors (CHARM & ZOMER 1995).

In estimating neomycin the ascertained detection limits of 0.4 μg/ml and 0.5 μg/ml indicated that DDM and also Delvotest SP were sufficiently sensitive (these values were even much lower than those recorded in the literature (CHARM & ZOMER 1995).

Table 2. Detection limits (μg/ml) of the most important antibiotics and methods according to published data and MRL (μg/ml) according to EEC 85/397 (1992)

Antibiotic		MRL (EEC1992)	Detection limit	Method	Source
β-lactam	Oxacillin	0.03	0.005–0.01	beta s.t.a.r.	MICHALSKI & ROLA (1999)
			0.003	HPLC	IBACH & PETZ (1998)
			0.007	HPLC	KUBALEC *et al*. (1997)
	Ampicillin	0.004	0.005	Penzym	SUHREN *et al*. (1996)
			0.003–0,004	Penzym S	SUHREN *et al*. (1996)
			0.001	beta s.t.a.r.	MICHALSKI & ROLA (1999)
Cephalosporins	Cefoperhazone		0.05	BRT Blue	SCHLIEPHAKE (1998)
			0.065	Star	SCHLIEPHAKE (1998)
			0.025	BRT AS	SCHLIEPHAKE (1998)
			0.005–0.008	DDM beta s.t.a.r.	MICHALSKI & ROLA (1999)
	Cefazolin		0.007	Delvotest SP	SUHREN & REICHMUTH (1998)
			0.004	Penzym S	
			0.05	BRT AS	
	Cefquinome		0.25	Delvotest SP	SUHREN & REICHMUTH (1998))
			0.045	Penzym S	
			0.25	BRT AS	
	Ceftiofur	0.1	0.09	Penzym	SUHREN *et al*. (1996)
			0.07	Penzym S	
	Cefapirin		0.005–0.007	Penzym	SUHREN *et al*. (1996)
			0.003–0.005	Penzym S	
Tetracyclines	Tetracycline	0.1	0.01	HPLC	TAGUCHI *et al*. (1997)
			≤ 0.1	HPLC	ABETE *et al*. (1997)
	Chlortetracycline		0.5	Delvotest	CHARM & ZOMER (1995)
			≤ 0.1	HPLC	ABETE *et al*. (1997)
Macrolides	Erythromycin	0.04	0.4	Delvotest	CHARM & ZOMER (1995)
			0.01	ELISA	ALBRECHT *et al*. (1998)
	Spiramycin	0.2	0.008–0.0056	ELISA	ALBRECHT *et al*. (1996)
Aminoglycosides	Streptomycin		4–10.0	Delvotest	CHARM, ZOMER (1995)
			0.008	LC	SUHREN & KANAPPSTEIN (1998)
			0.01	HPLC	HASSAN ABBAS & HELLENÄS (1998)
	Neomycin		1	Delvotest	CHARM & ZOMER (1995)
	Gentamicin		0.25	Delvotest	CHARM & ZOMER (1995)
Others	Novobiocin	0	1	Delvotest	CHARM & ZOMER (1995)
	Chloramphenicol	0	0.0002	ELISA	EL-BARADEI (1997)
			5	Delvotest	CHARM & ZOMER (1995)
	Danofloxacin		0.18	DDM	CRUZ *et al*. (1998)

DDM – disk diffusion method

Table 3. Detection limits (μg/ml) of antibiotics in respective methods

Antibiotic	Method		
	1	2	3
β-lactam and cephalosporins			
Oxacillin	0.5	0.02	0.02
Ampicillin	0.02	0.003	0.003
Cefotaxime	0.4	0.03	0.08
Cefuroxime	0.4	0.2	0.04
Cefamandol	0.2	0.005	0.003
Cefoperhazone	0.7	0.03	0.02
Cefazolin	0.1	0.005	0.005
Cefquinome	1	0.09	0.06
Ceftazidime	9	0.6	1
Tetracyclines			
Chlortetracycline	6	0.9	
Macrolides			
Erythromycin	1	0.3	
Spiramycin	10	2	
Aminoglycosides			
Streptomycin	4	6	
Gentamicin	0.4	0.3	
Neomycin	0.4	0.5	
Kanamycin	30	12	
Others			
Novobiocin	*	3	
Chloramphenicol	30	10	
Trimethoprim	1	0.4	
Bacitracin	3	0.4	
Enrofloxacin	10	3	

1 = disk diffusion method (n = 4)
2 = Delvotest SP (n = 3)
3 = Penzym S 100
* undetected

The detection limits for kanamycin were 30 μg/ml in DDM and 12 μg/ml in Delvotest SP. No corresponding data were not found in the literature.

Other Antibiotics: From among other antibiotics the detection limits were estimated for novobiocin, chloramphenicol, trimethoprim, bacitracin and enrofloxacin.

In the case of novobiocin the detection limit determined by means of DDM was unobtainable, probably due to the resistance of the test strain. The detection limit using Delvotest was found to be 3 μg/ml, i. e. three times higher than the respective published data (CHARM & ZOMER 1995). The recommended MRL value is 0.

Not even in the case of chloramphenicol was DDM sufficiently sensitive (30 μg/ml), the sensitivity of Delvotest being three times higher. When applying the ELISA method, the substantially lower detection limits were reached (EL-BARADEI 1997). The recommended MRL value equals to 0.

When testing trimethoprim by means of DDM, the determined detection limit was 1 μg/ml and by Delvotest SP its sensitivity was 2.5 – fold higher (0.4 μg/ml).

In bacitracin DDM was considerably less sensitive than Delvotest SP (3 and 0.4 μg/ml).

In enrofloxacin the detection limit found by applying DDM was 10 μg/ml and by applying Delvotest SP amounted 3 μg/ml. Neither the corresponding data nor the MRL values concerning this antibiotics were found in the literature.

Table 4. Detection limits (mg/ml) of antibiotic combinations

Antibiotic	Detection limits of combinations				
Ampicillin	0	0.001	0.002	0.003	
Oxacillin	0.02	0.006	0.004	0	
Cefuroxime	0	0.01	0.05	0.09	0.2
Trimethoprim	0.4	0.2	0.07	0.05	0
Cefazolin	0.005	0.002	0.001	0	
Cefoperhazone	0	0.008	0.01	0.03	

Interactions of Antibiotics: For estimation of the detection limits of antimicrobials the method Delvotest SP (Table 4) was used.

As the previous part of the experimental work (Table 3) showed, during the estimation of ampicillin and oxacillin (as individual substances) by means of Delvotest SP the detection limits were 0.02 μg/ml (oxacillin) and 0.003 mg/ml (ampicillin), respectively. From Table 4 it follows that the detection limit of ampicillin combined with 0.004 μg/ml of oxacillin dropped to 0.002 μg/ml The detection limit of oxacillin combined with 0.001 μg/ml of ampicillin decreased to 0.006 μg/ml. These results refer to the synergic effect of the tested β-lactam antibioticts, where the detection limits of their combinations tend to decrease, compared to those of individual substances.

The detection limit of cefuroxime determined by Delvotest SP was 0.2 μg/ml and that of trimethoprim amounted 0.4 μg/ml (Table 3). In the combination of both antibiotics the detection limits could be reduced, which also points out to the synergic effect. The detection limit of cefuroxime combined with 0.05 μg/ml of trimethoprim fell down to 0.09 μg/ml and combined with 0.07 μg/ml of trimethoprim was reduced to 0.05 μg/ml. The detection

limit of trimethoprim combined with 0.01 μg/ml of cefuroxime decreased to 0.2 μg/ml.

The last combination applied was that of cephalosporins, namely of cefazolin with cefoperhazone. The detection limit of the individually applied cefazolin was 0.005 μg/ml and after its combination with 0.008 mg/ml of cefoperhazone it was reduced to 0.002 μg/ml. The sole application of cefoperhazone revealed the detection limit 0.03 μg per ml. Its combination with 0.001 μg/ml of cefazolin decreased it to 0.01 μg/ml.

CONCLUSION

From the results achieved it can be concluded that it is important to continue the study of a wider spectrum of the non-examined effects of antibiotics individually acting on the microorganisms in milk.

The mutual combinations of antibiotics used in the search for synergic effects lead to a decline of the detection limits of antibiotics. This implies that the content of individual substances is determined within the requested detection limits (the detection of substances with synergic effects can be interesting also from the viewpoint of the influence of new starter cultures).

The above-mentioned procedure allows to increase the resistance to pathogens and thus to contribute to the prevention and occurrence of infections as well as to the improved and more effective methods of treatment of the clinical forms of mastitis.

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Súhrn

HOZOVÁ B., KRATMÜLLEROVÁ M. (2001): **Stanovenie detekčných limitov antibiotík v modelových vzorkách kravského mlieka a porovnanie citlivosti rôznych detekčných systémov (disková difúzna metóda, Delvotest SP a Penzym S 100)**. Czech J. Food Sci., **19**: 125–131.

Cieľom práce bolo stanovenie detekčných limitov 21 antibiotík (β-laktámové a cefalosporíny, aminoglykozidy, makrolidy, ostatné) pomocou troch typov detekčných systémov (disková difúzna metóda s *Bacillus stearothermophilus* var. *calidolactis* C 953, Delvotest SP a Penzym S 100) a overenie ich citlivosti pre splnenie maximálne prípustných množstiev rezíduí (MRL). Vysoká citlivosť a dobrá korelácia výsledkov sa dosiahli najmä pri aplikácii rýchlometód – Delvotestu SP a Penzymu S oproti menej citlivej diskovej difúznej metóde. V ďalšej časti práce boli stanovené detekčné limity vzájomných kombinácií antibiotík v mlieku. V modelovom pokuse bol pozorovaný synergický efekt medzi ampicilínom a oxacilínom, cefuroximom a trimetoprimom a medzi cefalosporínmi (kombinácia cefazolínu s cefoperazonom).

Kľúčová slová: antibiotiká; disková difúzna metóda; Delvotest SP; Penzym S 100; synergizmus

Corresponding author:

RNDR. BERNADETTA HOZOVÁ, CSc., Slovenská technická univerzita, Chemickotechnologická fakulta, Radlinského 9, 812 37 Bratislava, Slovenská republika

tel.: + 421 2 59 32 54 78, fax: + 421 2 49 31 98, e-mail: hozova@chelin.chtf.stuba.sk