The utilization of disk diffusion method and the Delvotest® for determining synergistic effects of cephalosporin combinations in milk

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Summary
Antibiotics including β-lactam antibiotics are used in veterinary practice for the treatment of mastitis in milking cows. This study suggests that a suitable combination of antibiotics may result in synergistic action that might cause reduction of dosages with subsequent decrease in the maximum residue limits (MRL) as specified by the European Economic Commission of the European Union and the United States Food and Drug Administration recommendations. This study used disk diffusion assay and tested various combinations of 6 cephalosporins (cefuroxime, cefamandole, cefazoline, cefoperazone, cefotaxime and ceftazidime) for synergistic activity. Two of the antibiotic combinations were also tested by means of Delvotest® (The SP kit). In our model experiments with milk, an increase in antimicrobial activity indicating synergistic effect between tested cephalosporins was observed.

Keywords
cephalosporins; Delvostest SP; disk diffusion method; milk; synergistic effect

Milk, one of the basic foods that are consumed by the population of all ages and social groups, should correspond primarily to the demanded quality criteria (microbiological, nutritional, and sensory). Milk is an excellent source of vitamins and minerals, particularly calcium. It has long been recognised for its important role in bone health. It contains a good balance of protein, fat and carbohydrate and is a very important source of essential nutrients. Nutritionists recommend that milk and other dairy products should be consumed daily as part of a balanced diet.

Antibiotic residues in milk above tolerance levels, i.e. maximum residue limits (MRL) established by the European Union (EU) and the United States Food and Drug Administration (FDA) [1] interfere with milk product processing such as cheese manufacturing, and they present potential health risks to the consumer. Their presence also may cause allergic reactions, interference with the intestinal flora, and resistant populations of bacteria in the general population, thereby rendering antibiotic treatment ineffective [2, 3]. Antibiotic residues are of great concern to dairy farmers, milk processors, regulatory agencies, and consumers.

The antibiotic residue detection assay systems that are currently available can be classified as microbiological and special identification and detection methods (e.g. chromatographic, enzymatic, and immunological). The most common microbiological methods monitor inhibition of the growth of a test organism. Well-known tests in this category include Charm Farm (Charm Sciences, Lawrence, Massachusetts, USA), Delvotest® (DSM Food Specialties, Delft, Netherlands) and the regulatory standard *Bacillus stearothermophilus* var. *calidolactis* disk assay (BSDA) [2, 4].

The *Bacillus stearothermophilus* var. *calidolactis* has a good sensitivity to the groups of β-lactam antibiotics. It is used as the target microorganism in commercial tests throughout the world [5, 6].

The disk diffusion method is inexpensive and highly sensitive to β-lactam antibiotics. However, it is hard to perform and it takes long time to obtain results. Even so many researchers have been using disk diffusion method with *Bacillus stearothermophilus* in their work worldwide during the past few years [2, 5-7].

Some researchers have used the disk diffusion method or Delvotest® to find the interactions

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among antibiotics in model experiments [8-10]. In our previous studies [8, 9] the synergistic effect was found in model experiments with Delvotest® (The SP kit) and disk diffusion method with *Bacillus stearothermophilus* var. *calidolactis* C 953 in combination of penicillin G with β-lactams, cephalosporins, aminoglycosides, peptides, other antibiotics and sulphonamides. Combinations of cephalosporines were also tested for synergistic effect.

The present paper is aimed at the evaluation of the interactions of the cephalosporin group of antibiotics (also of those in which the detection limit has not been established until now) and of the influence on individual detection limits with the possibility of achieving a synergistic effect.

The detection limits of 6 cephalosporins and also the detection limits of their mutual combinations were estimated by means of the disk diffusion method with *Bacillus stearothermophilus* var. *calidolactis* C 953. Two of these combinations were tested for synergistic effect by means of Delvotest® (Delvotest SP kit).

MATERIAL AND METHODS

Bacterial strain

Bacillus stearothermophilus var. *calidolactis* C953 was obtained from the collection of microorganisms at the State Veterinary and Food Administration of the Slovak Republic in Bratislava, propagated on GTK agar (Glucose, Tryptone, Yeast agar; producer - Imuna Pharm, Šarišské Michaľany, Slovakia) and subsequently in the liquid substrate of the same composition prior to its application.

Antibiotics

Six tested cephalosporins and their concentration ranges used in our study are summarized in Tab. 1. Three of these cephalosporins (cefazoline, cefotaxime and cefoperazone) were also tested by Delvotest® (Delvotest SP kit) by applying lower concentration ranges in compliance with the detection limits obtained by Delvotest SP (Tab. 2). Before use all antibiotics were stored and handled according to the manufacturer's instructions. The tested antibiotics in concentrations of 100 μg.ml^{-1} were prepared by dissolving 0.0100 g of the antibiotic in sterile, deionized water in a sterile 100 ml volumetric flask. Samples were prepared freshly on the day of analysis to avoid possible inconvenience due to instability of the solutions.

The standard (stock) solution of penicillin with the concentration 60 μg.ml^{-1} (100 IU.ml^{-1}) was prepared according to STN 57 0531 [4] by dissolving the potassium salt of benzylpenicillin in sterile deionized water in a sterile volumetric flask. All working solutions of penicillin were prepared from the stock solution of penicillin according to STN 57 0531 [4] freshly on the day of analysis.

Milk samples

Model milk samples for the estimation of the detection limits of tests were prepared freshly on the day of analysis. Ten grams of the reconstituted skim milk (PROMIL-PML, Nový Bydžov, Czech Republic) was dissolved in 90 ml of sterile deionized water; respective antibiotics were added to the milk prepared in this way such that the addition of a required aliquot of the antibiotic solution to a 10 ml final volume of milk gave the targeted concentration.

According to the recommendations of the International Dairy Federation (IDF) [11], the pH of reconstituted milk was higher than 6; natural inhibitors in milk (such as lysozyme or lactoferrin) that can cause false-positive results were subjected to thermal inactivation for 5 min at 80 °C [4]. The reconstituted dried milk without any antibiotics was used as a control.

Methods

Disk Diffusion Method

The sterile paper disk (prepared according to STN 57 0531 [4] from the filter paper Whatman 1, diameter 9-13 mm, soaking ability approx. 130 mg

Tab. 1. Concentration ranges of cephalosporins and their producers.

Cephalosporin	Producer	Concentration ranges [μg.ml^{-1}]
Cefuroxime	GlaxoSmithKline (Middlesex, England)	0.0000–0.3000
Cefamandole	Lilly France SA (Suresnes, France)	0.0000–0.0800
Cefazoline	Lilly France SA (Suresnes, France)	0.0000–0.0800
Cefoperazone	Pfizer (Karlsruhe, Germany)	0.0000–0.2500
Cefotaxime	Roussel Uclaf (Paris, France)	0.0000–0.4000
Ceftazidime	GlaxoSmithKline (Middlesex, England)	0.0000–7.0000

Tab. 2. Detection limits of cephalosporins obtained by disk diffusion method, Delvotest® (Delvotest SP kit) and their comparison with the EU maximum residue limits (EEC Regulation 2377/90 [1]).

Cephalosporin	EEC Reg. 2377/90 [μg.ml^{-1}]	Detection limit [μg.ml^{-1}]	Method
Cefuroxime	Not established	0.3000	DDM
Cefamandole	Not established	0.0800	DDM
Cefazoline	0.0500	0.0800 / 0.0070	DDM / Delvotest SP
Cefoperazone	0.0500	0.2500 / 0.0300	DDM / Delvotest SP
Cefotaxime	Not established	0.4000 / 0.0100	DDM / Delvotest SP
Ceftazidime	Not established	7.0000	DDM

DDM - disk diffusion method (n = 10), Delvotest SP (n = 3).

of milk) soaked with the sample examined was placed on the surface of the agar nutrient medium (GTK agar) with *B. stearothermophilus*. After incubation (64 °C ± 1 °C for 4–5 h), during which the tested strain grows, the agar medium turns opaque. The inhibition of *Bacillus stearothermophilus* is indicated by a clear zone around the paper disc. The size of the clear zone depends on the level of antibiotic residues in milk and can be compared with the size of the zones created by the reference solutions of penicillin of known concentration (0.0040, 0.0060, 0.0300 and 0.0600 μg.ml^{-1}). An inhibition zone ≥ 2 mm is considered to be positive [4]. The disk diffusion method has the sensitivity to penicillin G of 0.0025 IU.ml^{-1} milk. The inhibition zones were measured by a digital caliper (DIOXO, Prague, Czech Republic). Technical specification: measuring range: 0–150 mm, repeatibility 0.01 mm. In our experiments the analysis of each concentration was repeated 10 times (n = 10).

Delvotest® (Delvotest SP kit)

The commercial Delvotest SP (manufactured by DSM Food Specialties, Delft, Netherlands) was carried out according to the instructions of the manufacturer.

The sample examined is batched into microtitration plates with pits filled with the agar nutrient medium containing *B. stearothermophilus* var. *calidolactis.* The incubation (64 °C ± 1 °C for 2.5–5 h), during which the tested strain grows, the colour of the indicator (bromcresol purple) changed from blue-violet to yellow. If the sample contained substances that inhibit the growth of the test strain, the colour of the indicator remained blue-violet. This is the positive result. Delvotest SP has a sensitivity to penicillin G of 0.0030–0.0050 IU.ml^{-1} of milk. The analysis of each concentration was repeated three times (n = 3).

RESULTS AND DISCUSSION

The detection limits of individual cephalosporins obtained by the disk diffusion method and Delvotest SP in our study are summarized in Tab. 2.

However, the values for cefazoline and cefoperazone obtained by disk diffusion method are higher than 0.0500 μg.ml^{-1}, which are the MRL's established by EU [1]. The MRL for cefuroxime, cafamandole, cefotaxime and ceftazidime in milk has not been established.

Tab. 3 presents the detection limits of cephalosporin combinations obtained by the disk diffusion method.

At each concentration of cephalosporins at first each cephalosporin at its detection limit and 0.0000 μg.ml^{-1} of the other cephalosporin was tested. The next choice of cephalosporin concentrations was made in accordance with the concentration ranges of individually applied cephalosporins. A wider concentration spectrum was examined to obtain the desired concentration of the other cephalosporin that could lead to a decrease of the detection limit of the first cephalosporin in the combination. The obtained values of the concentrations are presented in Tab. 3.

In our experiments made by disk diffusion method, the detection limits were 0.3000 μg.ml^{-1} for cefuroxime and 0.0800 μg.ml^{-1} for cefamandole. Tab. 3 shows that the detection limit of cefuroxime in combination with cefamandole at 0.0267 μg.ml^{-1} decreased to 0.2000 μg.ml^{-1}. The detection limit of cefamandole decreased from 0.0800 μg.ml^{-1} to 0.0587 μg.ml^{-1} after its combination with cefuroxime at 0.0800 μg.ml^{-1}.

For cefazoline the detection limit of 0.0800 μg.ml^{-1} was found; after its combination with 0.1250 μg.ml^{-1} cefoperazone the limit decreased to 0.0400 μg.ml^{-1}. The obtained value 0.0400 μg.ml^{-1} for cefazoline represents a value

Tab. 3. Detection limits of cefalosporin combinations obtained by disk diffusion method (n = 10) and their correlation coefficient.

Cephalosporin	Detection limit in combination [μg.ml^{-1}]				Correlation coefficient (*r*)
Cefuroxime Cefamandole	0.3000 0.0000	0.2000 0.0267	0.0800 0.0587	0.0000 0.0800	0.9742
Cefazoline Cefoperazone	0.0800 0.0000	0.0400 0.1250	0.0200 0.1900	0.0000 0.2500	0.9893
Cefotaxime Cefoperazone	0.4000 0.0000	0.2500 0.0938	0.1000 0.1875	0.0000 0.2500	0.9206
Cefuroxime Ceftazidime	0.3000 0.0000	0.1714 3.0000	0.0857 5.0000	0.0000 7.0000	0.9958
Cefazoline Ceftazidime	0.0800 0.0000	0.0457 3.0000	0.0229 5.0000	0.0000 7.0000	0.9953
Cefotaxime Cefuroxime	0.4000 0.0000	0.3000 0.0750	0.1000 0.2250	0.0000 0.3000	0.9689
Cefuroxime Cefoperazone	0.3000 0.0000	0.2000 0.0833	0.0900 0.1750	0.0000 0.2500	0.9790
Cefotaxime Ceftazidime	0.4000 0.0000	0.2857 2.0000	0.1143 5.0000	0.0000 7.0000	0.9990
Cefazoline Cefamandole	0.0800 0.0000	0.0600 0.0225	0.0200 0.0675	0.0000 0.0800	1.0000

Tab. 4. Detection limits of cephalosporin combinations obtained by Delvotest® (Delvotest SP kit) (n = 3) and their correlation coefficient.

Cephalosporin	Detection limit in combination [μg.ml^{-1}]				Correlation coefficient (*r*)
Cefazoline Cefoperazone	0.0070 0.0000	0.0050 0.0100	0.0020 0.0200	0.0000 0.0300	0.9908
Cefotaxime Cefoperazone	0.0100 0.0000	0.0070 0.0080	0.0065 0.0100	0.0000 0.0300	0.8798

lower than 0.0500 μg.ml^{-1} for cefazoline, which is the MRL established by EU [1]. This combination shows a synergistic effect. The rest of combinations in Tab. 3 show the decline of the detection limits which is a manifestation of a synergistic effect.

For better understanding of our data basic correlation analysis was performed. The results of correlation analysis between detection limits of both antibiotics used in combination are presented in the Tab. 3. The values of correlation coefficient are close to 1 which indicates that the antibiotics in combination are strongly related. According to the values of correlation coefficient in Tab. 3 the combination between cefazoline and cefamandole and cefotaxime and ceftazidime show the strongest relation.

However, in our laboratory experiments done by disk diffusion method, we also found the opposite of synergistic effect, the antagonistic effect. By combining 0.4000 μg.ml^{-1} cefotaxime with 0.2500 μg.ml^{-1} cefoperazone the inhibition zone was 0.1000 mm, which is much lower than 2 mm. It means that these two antibiotics at their combined concentrations did not reach the detection limit which is a clear sign of an antagonistic effect. By combining 0.3000 μg.ml^{-1} cefuroxime with 7.0000 μg.ml^{-1} ceftazidime the inhibition zone was 0.0600 mm, which also shows that this combination leads to an antagonistic effect.

In our work the combinations of cefazoline with cefoperazone and cefotaxime with cefoperazone were tested by disk diffusion method with *Bacillus stearothermophilus* var. *calidolactis* C953 and also by Delvotest SP.

Tab. 4 presents the detection limits of cephalosporin combinations obtained by Delvotest SP and the values of their correlation coefficient. In both tested combinations the value of correlation coefficient was close to 1 indicating that the antibiotics in a combination are closely related. We observed

the decline of the detection limits in the combination among cephalosporins which also points out to the synergistic effect.

However, the detection limits of individual substances obtained by Delvotest SP were lower than the detection limits obtained by disk diffusion method (Tab. 2).

By using Delvotest SP the detection limit of cefazoline (0.0070 μg.ml^{-1}) was the same as the one determined by SUHREN and REICHMUTH [12] and higher than the 0.0050 μg.ml^{-1} reported by HOZOVÁ and KRATMÜLLEROVÁ [9]. In the case of cefoperazone, the level detected in this work (0.0300 μg.ml^{-1}) was the same as the one detected by HOZOVÁ and KRATMÜLLEROVÁ [9], lower than the 0.0600 μg.ml^{-1} determined by Delvotest SP kit manufacturer and higher than the 0,020 μg.ml^{-1} detected by ALTHAUS et al. [13]. In our study, cefotaxime presented the detection limit of 0.0100 μg.ml^{-1}, lower than 0.0300 μg.ml^{-1} which is the value reported by HOZOVÁ and KRATMÜLLEROVÁ [9]. For the cephalosporins tested no other detection limits were found in the literature.

In our previous study [8] we reported that the sensitivity of Delvotest SP for cephalosporins tested was as much as 40-fold higher than that of the disk diffusion method. This fact was confirmed again by testing cefotaxime, cefazoline and cefoperazone. In determination of cefotaxime the sensitivity of Delvotest SP (0.0100 μg.ml^{-1}) was as much as 40 times higher than that of the disk diffusion method (0.4000 μg.ml^{-1}). In the determination of cefazoline the sensitivity of Delvotest SP (0.0070 μg.ml^{-1}) was as much as 11.4 times higher than that of the disk diffusion method (0.0800 μg.ml^{-1}). In the cefoperazone evaluation, the detection limit found by applying the disk diffusion method was 0.2500 μg.ml^{-1}, and that found by Delvotest SP was 0.0300 μg.ml^{-1} (8-fold increase in sensitivity over the disk diffusion method).

In summary, from the results achieved it can be concluded that in our model experiments with disk diffusion method and Delvotest® (Delvotest SP kit) the detection limits of cephalosporins, used in combinations were lower than detection limits of individual cephalosporins, testifying to the presence of a synergistic effect. However, when the MRL's for cephalosporins established by EU were considered, only the combination of cefazoline with cefoperazone presented a synergistic effect. (The detection limit of cefazoline 0.0800 μg.ml^{-1} after its combination with 0.1250 μg.ml^{-1} cefoperazone decreased to 0.0400 μg.ml^{-1}).

Furthermore, no studies on the action of combined cephalosporins on *B. stearothermophilus* var. *calidolactis* have been found in the literature. Therefore, the results of our model experiments allow us to introduce the synergy hypothesis operating in some combinations of cephalosporins

CONCLUSIONS

The disk diffusion method can be used in searching for the detection limits of individual substances or in searching for the detection limits of their mutual combinations. However, the sensitivity of this detection system is not sufficient. Considering the high values of the detection limits obtained in our experiments, more rapid and effective screening methods are better used.

Residues of antibiotics in milk are undesirable from the health point of view (allergies), and also from the technological aspect (they deteriorate or make the processing of milk to dairy products impossible). Searching for detection limit for each antimicrobial agent deserves special attention, with the aim of preventing milk with drug residuals in excess of maximum residue limits from being marketed and reaching the consumer. It is also worthwhile to study mutual antibiotic combinations at various research levels (e.g. the affect on cheesemaking process) in searching for synergistic effects. This will ensure not only the safe production of dairy products but also the innovated and more effective mode of treatment of the clinical forms of mastitis.

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