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EXPERIMENTAL DESIGN FOR THE MICROBIOLOGICAL FOUR-PLATE TEST FOR THE DETECTION OF SULPHADIMIDINE RESIDUES AT THE LEVELS OF CONCERN

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Abstract

The aim of the study was to evaluate the sensitivity of the reference four-plate test (FPT) for the detection of sulphadimidine residues. The test agar, trimethoprim (TMP) and *Bacillus subtilis* BGA as test organism were used in the study. After determining the minimum inhibiting concentration (MIC) for sulphadimidine, the potential effects of selected parameters (pH value of agar medium, TMP concentration, addition of dextrose to the agar medium and use of distilled or deionised water as solvents) on the increasing sensitivity of the method or on lowering of the MIC for sulphadimidine were studied. It was demonstrated that the sensitivity of FPT was 1 $\mu g.ml^{-1}$ only. The addition of TMP to the agar medium at the concentration of 0.10 or 0.15 $\mu g.ml^{-1}$ significantly increased ($P<0.05$) the sensitivity of the method from the viewpoint of detecting the presence of sulphadimidine residues at the level of the MRL (0.01 $\mu g.ml^{-1}$). The using of deionised water significantly increased the diameters of inhibition zones ($P<0.05$). The different pH values of agar media had only a minor effect on the inhibition zone diameter. At pH 7.0 and 7.2, the MICs were equal.

Key words: sulphadimidine, residues, four-plate test.

Sulphonamides are one of the oldest groups of pharmacologically active substances used in veterinary medicine to date. Their discovery, in 1935, signified the beginning of a new era in the treatment of a wide range of bacterial diseases and a number of protozoal infections. In food-producing animals, sulphonamides are used not only for therapeutic but also for prophylactic purposes. Used in subtherapeutic doses, they exhibit a growth-promoting action and so are added to feedstuffs to increase productivity (11).

Sulphonamide residues present a potential risk to human health. Direct toxic or allergic reactions after administration of therapeutic doses of sulphonamides to humans have been described (4, 16). Besides, these negative effects of sulphonamides or their metabolites on the human body have been reported, particularly after a long-term consumption of animal products containing their trace amounts. The accumulation of these trace amounts in edible tissues has resulted in a build-up of resistance and the development of hypersensitivity to sulphonamides (1, 16). Sulphonamides are known also for their negative effects on the thyroid gland in relation to the development of thyroid gland tumours (2, 17).

Owing to the concern about the residues of sulphonamides in foods of animal origin, the current legislation (5, 18) established the MRL of 0.1 $mg.kg^{-1}$ for sulphonamides (all compounds of the sulphonamide group) in the foods.

Microbiological techniques are the basis of screening methods for monitoring the presence of veterinary drug residues, which possess antibiotic or antibacterial activity in foods of animal origin. The reference microbiological method for the screening of sulphonamide residues in the foods is the four-plate test (FPT) (3). The agar medium inoculated with *Bacillus subtilis* BGA spores at pH 7.2 with addition of trimethoprim at the concentration of 0.05 $\mu g.ml^{-1}$ provides the highest sensitivity of the test in the detection of sulphonamide residues. The presence of sulphonamide residues in the sample is expressed by the growth inhibition of the test organism *B. subtilis* BGA with the subsequent production of inhibition zones. The size of the inhibition zones is directly proportional to the concentration of sulphonamide within a concentration range specific for each compound of the sulphonamide group. However, from the published data we can deduce that this method is not equally sensitive to the whole sulphonamide groups (6, 9, 13, 15).

Inasmuch as the sulphadimidine is the most widely used compound of the sulphonamide group in veterinary medicine, the aim of our study was to evaluate the detection sensitivity of the test organism *B. subtilis* BGA to the residual concentrations of sulphadimidine. We decided to use the reference FPT, and the experimental design of the FPT from the viewpoint of detecting the sulphadimidine residues at or below the level of the MRL.

Material and Methods

Standards. Sulphadimidine (Sulphamethazine sodium salt, Sigma S 5637), Trimethoprim (Sigma T 7883).

Reagents and solvents. Dextrose (Difco, USA), 30% NaOH (Lachema, Czech Republic), ethanol 96% (Frukona, Slovak Republic), methanol (Merck, Germany), sterile distilled water and sterile deionized water.

Standard solutions. The first stock solution of sulphadimidine (SD-1) was prepared by dissolving 10 mg of sulphadimidine standard in 2.4 ml of methanol and diluting to 10 ml with sterile distilled water. The second stock solution of sulphadimidine (SD-2) was prepared by the same way but as a solvent a sterile deionized water was used. The working solutions of SD-1 and SD-2 were prepared by serial dilutions with the appropriate solvents to get the concentration of 50, 20, 10, 5, 0.5, 0.1, 0.01 $\mu g.ml^{-1}$. The working solutions with the concentration of 20 $\mu g.ml^{-1}$ were used to check the quality of the prepared agar medium. The stock solution of trimethoprim (TMP) was prepared by dissolving 10 mg of TMP in 10 ml of ethanol and diluting with sterile distilled water to the concentration of 5 $\mu g.ml^{-1}$. The stock and working solutions were stored in the refrigerator at 4°C.

Test organism and test agar. *Bacillus subtilis* BGA (Merck 10649) and test agar medium (Merck 15787).

Preparation of the test agar. The agar medium was adjusted to pH 7.0 and 7.2 with 30% NaOH. To obtain the final concentrations of TMP in agar medium (0.05, 0.1, 0.15, and 0.2 µg of $TMP.ml^{-1}$), 1, 2, 3, and 4 ml of TMP stock solution were added to 100 ml of the medium. The medium was further divided into two groups: with 0.4% addition of dextrose and without dextrose.

Testing of sulphadimidine standard solutions. Filter paper discs (S&S Antibiotic-Assay Discs, Diam ½ In. Aldrich Z 134104) were moistened with 0.1 ml of sulphadimidine standard solutions (SD-1, SD-2, control sulphadimidine solution at the concentration of 20 $\mu g.ml^{-1}$) and placed in parallel on the surface of agar medium in Petri plates. The plates were incubated at 30˚C for 18–24 h. After incubation, the plates were evaluated and the diameters of clear inhibition zones surrounding the filter paper discs were measured in millimetres. The lowest concentration of sulphadimidine standard solutions which completely inhibited the growth of the test organism was recorded as the minimum inhibiting concentration (MIC). The minimum acceptable ring zone diameter for the control sulphadimidine solution was 6 mm.

Statistical analysis. The data were analysed statistically by ANOVA-RBD with Statlets software.

Results

The mean diameters of the inhibition zones produced by the standard solutions of sulphadimidine in dependence of the pH value of agar medium and concentration of TMP in the medium without the addition of dextrose are summarized in Tables 1 and 2. The 0.4% addition of dextrose to the medium did not increase the sensitivity of the test organism evaluated according to the diameters of inhibition zones formed around the filter paper discs moistened with the tested concentrations of sulphadimidine. The differences in diameters of the inhibition zones were not observed.

Table 1

The mean diameters of inhibition zones (mm) of the standard solutions of sulphadimidine at pH 7.0 with the addition of TMP at the tested concentration and without addition of dextrose

c_{TMP} ($\mu g.ml^{-1}$)	SD	c_{SD} ($\mu g.ml^{-1}$)							
		50	20	10	**5**	**1**	**0.5**	**0.1**	**0.01**
0.05	SD-1	16	4	3	**2**	0	0	0	0
	SD-2*	19	7	5	3	**1**	0	0	0
0.1 *	SD-1	19	8	6	4	**2**	0	0	0
	SD-2*	23	11	8	6	3	**1**	0	0
0.15 *	SD-1	24	15	12	10	6	4	**2**	0
	SD-2*	28	17	14	12	10	8	5	**2**
0.2	SD-1	The growth of *B. subtilis* BGA was inhibited							
	SD-2	The growth of *B. subtilis* BGA was inhibited							

SD – sulphadimidine; c_{TMP} – concentration of trimethoprim; c_{SD} – concentration of sulphadimidine; bold numerals represent the lowest diameter of inhibition zones produced by the certain concentration of standard solutions of sulphadimidine (MIC); *difference statistically significant (P<0.05).

Table 2
The mean diameters of inhibition zones (mm) of the standard solutions of sulphadimidine at pH 7.2 with the addition of TMP at the tested concentration and without addition of dextrose

c_{TMP} (µg.ml^{-1})	SD	c_{SD} (µg.ml^{-1})							
		50	20	10	**5**	**1**	**0.5**	**0.1**	**0.01**
0.05	SD-1	17	5	3	**1**	0	0	0	0
	SD-2*	19	6	4	2	**1**	0	0	0
0.1 *	SD-1	20	9	6	4	**2**	0	0	0
	SD-2*	24	12	9	6	3	**1**	0	0
0.15 *	SD-1	24	15	13	10	6	4	**2**	0
	SD-2*	28	17	14	12	9	7	5	**2**
0.2	SD-1	The growth of *B. subtilis* BGA was inhibited							
	SD-2	The growth of *B. subtilis* BGA was inhibited							

SD – sulphadimidine; c_{TMP} – concentration of trimethoprim; c_{SD} – concentration of sulphadimidine; bold numerals represent the lowest diameter of inhibition zones produced by the certain concentration of standard solutions of sulphadimidine (MIC); *difference statistically significant ($P<0.05$).

Our results indicate that the concentration of TMP in the agar medium and the sterile deionized water used as the solvent for sulphadimidine standard had the significant effect on increasing of the diameters of inhibition zones produced by the standard solutions of sulphadimidine. The different pH values of agar media had only a minor effect on the diameters of inhibition zones. At both pH values, the MICs of sulphadimidine were equal.

At the addition of TMP to the agar medium at the concentration of 0.05, 0.1 and 0.15 µg.ml^{-1}, the MIC for SD-1 was 5, 1 and 0.1 µg.ml^{-1}, respectively, and for SD-2 1, 0.5 and 0.01 µg.ml^{-1}, respectively. At the addition of TMP at the concentration of 0.2 µg.ml^{-1}, the growth of *B. subtilis* BGA was inhibited.

The presented MICs determined the detection limits of the method or sensitivity of the test organism *B. subtilis* BGA to sulphadimidine. When the reference FPT was used, the detection limit of the method was only 1 µg.ml^{-1}. However, when the FPT with the experimental design based on the addition of TMP to the agar medium was used, the detection limit of the method at TMP concentration of 0.1 µg.ml^{-1} was 0.5 µg.ml^{-1}, and at the concentration of 0.15 µg.ml^{-1} was 0.01 µg.ml^{-1}.

The comparison of the diameters of inhibition zones of the standard SD-2 solution of sulphadimidine at pH 7.0 and 7.2 is presented in Fig. 1. As Fig. 1 shows, the pH value of agar media had no significant effect on increasing the sensitivity of the test organism, and on lowering the detection limit of the FPT for sulphadimidine.

The filter paper discs moistened with the control sulphadimidine solution at the concentration of 20 µg.ml^{-1} gave values which were equal to the minimum annular zone diameter stated as acceptable for the method.

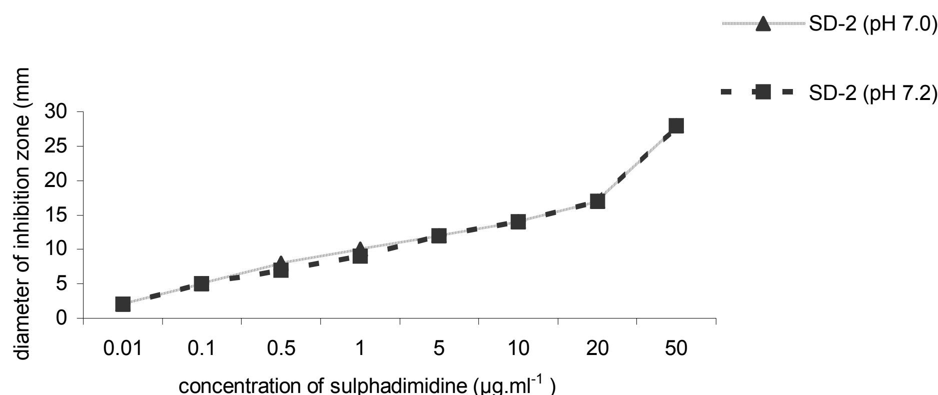

Fig. 1. Comparison of the diameters of inhibition zones of the standard SD-2 solution of sulphadimidine at pH 7.0 and 7.2.

Discussion

In the present two-tier testing system for the monitoring of sulphonamide residues in foods of animal origin, the microbiological techniques as the screening methods are highly valuable in practical routine work. However, the detection of the presence or absence of sulphonamide residues at or below the level of concern depends fully on the sensitivity of the microbiological method used (11, 12, 14).

The detection sensitivity of the FPT for sulphadimidine has been evaluated by many authors. Hrdlička (9) compared three microbiological methods of agar diffusion for the detection of sulphadimidine residues. He found that sulphadimidine residues can be detected only by the FPT, however, the detection limit of the FPT is not sufficiently sensitive to detect the sulphadimidine residues (MIC 1 $\mu g.ml^{-1}$) at the level of the MRL. Currie *et al*. (6) evaluated the FPT for all compounds of sulphonamide group. They found that seven of the 11 compounds tested had MICs less than the specified MRL, however, a notable exception from this group was sulphadimidine (MIC 0.25 $\mu g.ml^{-1}$). Other authors who evaluated the FPT as a tool for screening the sulphonamide residues were Okerman and Van Hoof (15). They found that the FPT is not sensitive enough to detect sulphonamides at the MRL. Similar results were recorded by Kožárová and Máté (13) who evaluated the sensitivity of the test organisms to the residual concentrations of anticoccidial drugs, including the sulphadimidine.

Koenen-Dierick and De Beer (10) examined the techniques for optimizing the test sensitivity by alterning the dextrose and TMP concentration in the medium, thickness of the agar medium, and preincubation time. They found out that 0.6% dextrose and 0.2% TMP in the agar medium, 1.5 mm thick, plus a 2h prediffusion time yielded the best sensitivity to sulphadimidine. For improving the detection level for sulphonamides, Ferrini *et al.* (7) raised the TMP concentration to 0.12 $\mu g.ml^{-1}$. In their experiments, it was the optimal concentration of TMP for an increase in drug sensitivity, without affecting the test organism growth.

In our study, the experimental design was applied for the FPT to detect the presence of sulphadimidine residues at the levels of concern. According to our result, obtained by the screening of the standard solutions of sulphadimidine, we confirmed that the FPT does not indicate the presence of sulphadimidine residues at the levels of concern. In spite of the MRL of 0.1 $mg.kg^{-1}$ established for sulphonamides, the detection limit of the method or sensitivity of *B. subtilis* BGA to sulphadimidine was 1 $\mu g.ml^{-1}$ only. According to the results obtained with the experimental design of the FPT we can deduce that from the selected parameters tested, the addition of TMP to the agar medium at the concentration of 0.10 and 0.15 $\mu g.ml^{-1}$ had the significant effect ($P<0.05$) on increasing the sensitivity of the test organism, and on lowering the detection limit of the method for sulphadimidine. The addition of TMP to the agar medium at the concentration of 0.10 $\mu g.ml^{-1}$ allowed to detect the presence of sulphadimidine residues near the level of the MRL (0.5 $\mu g.ml^{-1}$), and at the concentration of 0.15 $\mu g.ml^{-1}$ below the level of the MRL (0.01 $\mu g.ml^{-1}$). The use of the sterile deionized water as a solvent had resulted in increasing the solubility of sulphadimidine sodium salt. The increase in the diameters of inhibition zones was, therefore, significant ($P<0.05$). The different pH values of agar media had only a minor effect on the diameters of inhibition zones. At both pH values, the MICs for sulphadimidine were equal.

The addition of TMP to the agar medium enhances the sensitivity to sulphonamides. The synergistic effect of TMP was, for the first time, demonstrated by Gudding (8), and it has been still re-evaluated up to this day. As seen from the results obtained in our study, the optimal concentration of TMP in agar medium for the detection of the presence of sulphadimidine at the MRL levels was 0.15 $\mu g.ml^{-1}$.

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