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Comparison of the sensitivity of antibiotic residue screening methods to sulphonamide standards and their presumptive identification by para-aminobenzoic acid*)

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Summary

The sensitivity of three microbiological antibiotic residue screening methods – the Four Plate Test (FPT), the Screening Test for Antibiotic Residues (STAR) and the Premi®Test – was compared for the detection of 10 different standards from the sulphonamide (SA) group. Phtalylsulphathiazole (PHT), sulphadimidine (SD), sulphaguanidine (SG), sulphachlorpyridazine (SCHP), sulphamerazine (SRZ), sulphamethoxazole (SMX), sulphanilamid (SAM), sulphanilic acid (SAC), sulphaquinoxaline (SQ) and sulphathiazole (STZ) were tested using the concentrations from 0.05 μg.ml^{-1} to 1 μg.ml^{-1}. The detection sensitivity of the methods represented by minimum inhibiting concentrations (MIC) of SA standards was evaluated. The MIC of SAs represent the detection limits of methods (LOD) for individual substances. The MIC of SA standards detected by the FPT was 0.2 μg.ml^{-1} for SMX, STZ, SQ and SRZ, 0.3 μg.ml^{-1} for SCHP, and 0.4 μg.ml^{-1} for SD. The MIC of SA standards detected by the STAR was 0.05 μg.ml^{-1} for SMX, STZ, SQ and SCHP, 0.1 μg.ml^{-1} for SRZ, and 0.3 μg.ml^{-1} for SD. No detection sensitivity of either method was observed for SAM, SAC, SG and PHT standards. The MIC of SA standards detected by Premi®Test was 0.05 μg.ml^{-1} for SMX, STZ, SQ, SCHP, SRZ and SD, 0.1 μg.ml^{-1} for PHT, 0.2 μg.ml^{-1} for SAM, and 0.3 μg.ml^{-1} for SAC and SG. The results of the tests showed that Premi®Test is the most sensitive method to detect Sas, followed by the STAR and the FPT methods. The inhibitory effect of para-aminobenzoic acid (PABA) toward the residual concentrations of SA standards was evaluated. The reference PABA concentrations for the confirmation of the presence of all SAs detected by the FPT and the STAR was 1 μg.ml^{-1} and 10 μg.ml^{-1} for the Premi®Test. In the authors' opinion, the presented concentration of PABA can be recommended for a reliable confirmation of the presence of SA residues even at concentrations that present a potential risk to human health.

Keywords: detection, microbiological methods, PABA, sulphonamide residues

The occurence of foreign substances and residues of veterinary drugs in foods of animal origin is systematically monitored by both veterinary and human medicine (11, 16). Residues of veterinary drugs in foods of animal origin represent direct and indirect risks to human health and have a negative impact on technological processes in the food industry. In terms of threat to consumer health, antibiotics used in animals are included in the food chain and may cause allergies or affect resistance (13, 15, 18).

Sulphonamides (SAs) are widely used veterinary drugs that inhibit bacteria, chlamydia, toxoplazma and other protozoan agents, especially coccidia in poultry, rabbits and other economically important animal species (17).

Due to concerns related to residues of SAs in food products of animal origin, the current legislation (6) established the MRL of 0.1 mg.kg^{-1} for SAs (all compounds of the SA group) in foods of animal origin.

The MRLs are only meaningful if backed up by effective residue control programs. The FPT (4) is widely used for the detection of the presence of SA residues in foods of animal origin in the first stage of residue screening. The FPT is a microbial inhibition test with the test organism *Bacillus subtilis* BGA, con-

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taining test agar pH 7.2 and trimethoprim (TMP) at a concentration of 0.05 µg.ml^{-1}. The Community Reference Laboratory has developed a new method, called the STAR, for the detection of antibacterial residues in milk and meat. This test is a combination of five plates with an objective to improve the detection ability of the FPT (8). For sulphonamides, the test organism *Bacillus stearothermophilus* ATCC 10149 (Test agar DST pH 7.4; TMP at a concentration of 0.005 µg.ml^{-1}) is recommended. The FPT and the STAR are based on microbial growth inhibition presented by the production of the inhibition zones (IZ).

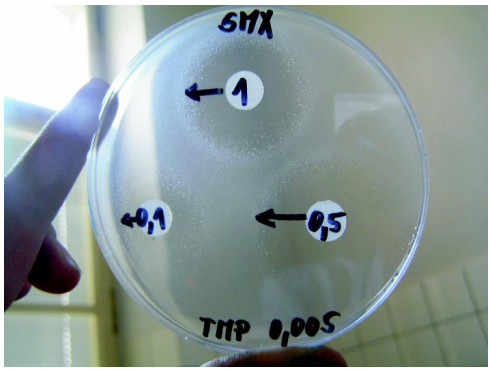

Fig. 1. STAR – IZ produced by residual concentrations of SMX standard

Fig. 2. STAR – absence of IZ after the addition of PABA

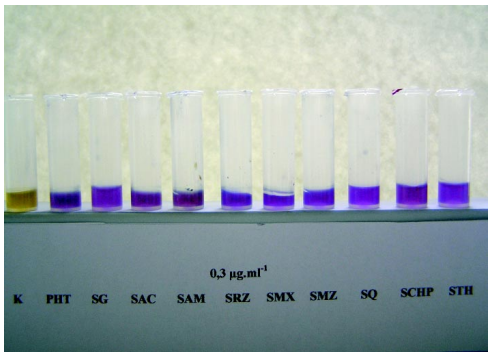

Fig. 3. Premi®Test – detection sensitivity at the residual concentration of 0.3 µg.ml^{-1}

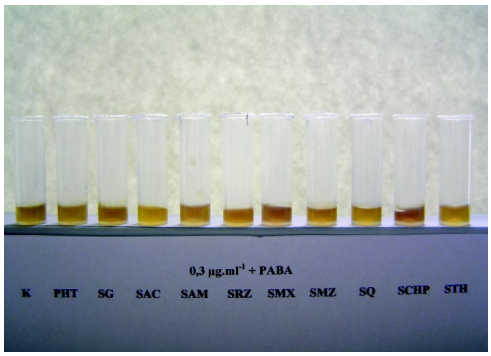

Fig. 4. Premi®Test – confirmation of SAs by PABA

The Premi®Test is based on the inhibition of the growth of *Bacillus stearothermophilus*, a thermophilic bacterium sensitive to many antibiotics and sulpha compounds. A standardized number of spores are embedded in an agar medium with selected nutrients. When heated to 64°C, the spores germinate and, in an absence of inhibitory substances, multiply and produce an acid. Using the acid-based indicator Bromocresol purple present in the agar medium, this process becomes apparent as a color change from purple to yellow. Premi®Test integrated strategy detects antimicrobial compounds at or below the EC MRL in a broad range of food products including meat, eggs, fish and honey (19).

However, the postscreening verification of the presence of SA residues in potentially positive samples must be further performed by applying an integrated test system, which utilizes more specific confirmatory techniques.

In an attempt to confirm the presence of SA residues already during the first stage of residue screening and to specify the choice of the method used for the quantitative determination of SA residues in positive samples, PABA was tested as a confirmatory solution for the SA group. As an antagonist of all SAs, an excess of PABA inhibits the bacteriostatic action of SAs. Consequently, sulphonamides become weak and lose their antibacterial activity in the presence of PABA (9, 10).

The objective of this paper was to determine which of the three aforementioned methods – the FPT, the STAR and the Premi®Test – is the most responsive and suitable for the detection of 10 standards of the SA group. To this end, LOD of the three methods for these drugs were compared and the possibility of using PABA for presumptive identification of SAs at their residue screening by applying microbiological methods was verified. The minimum confirmatory concentration (MCC) of PABA, which completely inhibited the antibacterial effects of all tested SA standards, was determined. All methods mentioned above are in Slovakia officially approved methods for screening food-producing animals and their products for residues of veterinary drugs (1-3).

Material and methods

Standard solutions. The stock solution (1000 µg.ml^{-1}) of SA standards was prepared by dissolving 10 mg of SD (Sulfamethazine sodium salt, Sigma S 5637), SG (Sulfaguanidine, Sigma S 8751), SAM (Sulfanilamide, Serva 35670), and SAC (Sulfanilic acid, Serva 35674) in 2.4 ml methanol (Merck, Germany), and PHT (Phtalylsulfathiazole, Sigma P 4258), SCHP (Sulfachlopyridazine, Sigma S 9882), SRZ (Sulfamerazine, Serva 35650), SMX (Sulfamethoxazole, Sigma S 7505), SQ (Sulfaquinoxaline sodium salt, Sigma S 7382), and STZ (Sulfathiazole, Serva 35690) in 2.4 ml ammonia (Lachema, Czech Republic), and further diluting to 10 ml with sterile deionised water. The working solutions of SAs were prepared by serial dilutions with sterile deionised water to the concentrations of 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 1 µg.ml^{-1}. Standard solutions containing a reference SD were used to verify that the operating conditions were systematically respected. The stock solution of TMP for STAR was prepared by dissolving 10 mg of TMP standard (Trimethoprim, Fluka 92131) in 1 ml 5% acetic acid (Merck, Germany) and diluting with

sterile distilled water to the concentration of 0.5 µg.ml^{-1}. The stock solution of TMP for FPT was prepared by dissolving 10 mg of TMP standard in 10 ml ethanol (Frukona, Slovakia) and diluting with sterile distilled water to the concentration of 5 µg.ml^{-1}. The stock confirmatory solution of PABA (1000 µg.ml^{-1}) was prepared by dissolving 10 mg of PABA (p-aminobenzoicacid, Sigma A 9878) powder in 10 ml distilled water. The stock and working solutions were stored in a refrigerator at +4°C.

Test organism and test agar. FPT – *Bacillus subtilis* BGA (Merck 10649), Test agar pH 7.2 (Merck 15787), STAR – *Bacillus stearothermophilus* ATCC 10149 (Merck 1.11499), Test agar DST pH 7.4 (OXOID CM 261), Premi®Test kits – *Bacillus stearothermophilus* var. *calidolactis*, solid agar medium (DSM, Netherlands).

Preparation of test agar and testing of SA standards

Study A – determination of LOD of methods. FPT – test agar was seeded with the test organism *Bacillus subtilis* BGA to obtain the final concentration of 5 × 10^4 spores.ml^{-1} in the agar medium. To obtain the final concentration of TMP in agar medium 0.05 µg.ml^{-1}, 1 ml of TMP solution (5 µg.ml^{-1}) was added to 100 ml of agar medium. Filter paper discs (Whatman No. 1, Diam 9 mm) were moistened with 100 µl of SA standards and placed in parallel on the surface of agar medium in the Petri plates. All plates were incubated at 30°C for 18-24 h. STAR – test agar was seeded with the test organism *Bacillus stearothermophilus* ATCC 10149 to give a final concentration of 5 × 10^6 spores.ml^{-1} in the agar medium. To obtain a final concentration of TMP in agar medium 0.005 µg.ml^{-1}, 1 ml of TMP solution (0.5 µg.ml^{-1}) was added to 100 ml of agar medium. Filter paper discs (Whatman No. 1, Diam 9 mm) were moistened with 100 µl of SA standards and placed parallel on the surface of agar medium in the Petri plates. All plates were incubated at 55°C for 12-15 h. Premi®Test – SA standards in a volume of 100 µl were transferred into an ampoule of Premi®Test. The ampoules were preincubated for 20 min at room temperature. After preincubation the ampoules were incubated for approximately 3 h at 64 ± 1°C.

Study B – confirmation of SAs by PABA. FPT, STAR – the plates used for the confirmatory purposes were prepared by adding 0.1, 0.3, 1, and 2 ml of stock solution of PABA to 100 ml of agar medium to obtain the tested concentrations of PABA 1, 3, 10, and 20 µg.ml^{-1}. Premi®Test – 100 µl of the PABA solution with the concentration of 100 µg.ml^{-1} was placed onto the agar in the ampoules and incubated for 20 min at the room temperature for pre-diffusion. After that, the PABA was flushed and 100 µl of SA standards were placed onto the agar in the ampoules and incubated for 20 min at room temperature for pre-diffusion. After pre-diffusion, the ampoules were incubated for approximately 3 h at 64 ± 1°C.

Interpreting the results. FPT, STAR – after incubation, the plates were evaluated and the diameters of clear IZ surrounding the filter paper discs were measured in millimeters. Both the lowest concentrations of SA standards, which inhibited the growth of the test organism by the production of the inhibition zones, and the lowest concentrations of PABA, which completely inhibited the antibacterial effect of all tested concentrations of SA standards presented by no inhibition zones, were determined. The minimum acceptable ring zone diameter for the control SD solutions set by both reference methods was 5 mm. Premi®Test – the color change was evaluated. The results were read after the evaluation of 2/3 of the height of the agar. Yellow color indicated an absence of SA residues and purple color indicated a presence of SA residues at or above the LOD of method. In the case of study B, yellow color indicated that PABA antagonized the antibacterial effect of SAs and confirmed the presence of SAs. As a control, 100 µl of sterile distilled water was used.

Tab. 1. FPT – the mean diameters of the IZ (mm) produced by SA standards (A) and no-IZ after the addition of PABA to the agar medium at the concentration of 1, 3, 10, and 20 µg.ml^{-1} (B)

SA		Mean + sd (mm) Concentration of sulphonamides (µg.ml^{-1})						
		0.05	0.1	0.2	0.3	0.4	0.5	1
SMX	A	0	0	2.04 ± 0.59	2.15 ± 0.23	2.71 ± 0.30	4.73 ± 0.07	5.83 ± 0.63
	B	0	0	0	0	0	0	0
STZ	A	0	0	1.89 ± 0.27	2.59 ± 0.50	3.45 ± 0.42	4.76 ± 0.37	6.22 ± 1.04
	B	0	0	0	0	0	0	0
SQ	A	0	0	2.96 ± 0.50	3.91 ± 0.33	4.55 ± 0.39	4.83 ± 0.46	6.99 ± 0.47
	B	0	0	0	0	0	0	0
SCHP	A	0	0	0	2.68 ± 0.20	3.40 ± 0.21	3.71 ± 0.13	5.31 ± 0.21
	B	0	0	0	0	0	0	0
SRZ	A	0	0	1.50 ± 0.27	2.38 ± 0.22	3.03 ± 0.12	3.18 ± 0.27	4.15 ± 0.63
	B	0	0	0	0	0	0	0
SD	A	0	0	0	0	2.62 ± 0.26	3.09 ± 0.17	3.67 ± 0.14
	B	0	0	0	0	0	0	0
SAM	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
SAC	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
SG	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
PHT	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0

Explanations: – the MIC of SA standards, – the lowest diameters of the IZ

Results and discussion

The sensitivity of the FPT to the residual concentrations of SA standards presented by the production of the IZ (A), and formation of no-IZ after the addition of PABA to the agar medium at all tested PABA concentrations (B) is recorded in table 1. The MIC of SA standards detected by the FPT was 0.2 μg.ml^{-1} for SMX, STZ, SQ and SRZ, 0.3 μg.ml^{-1} for SCHP, and 0.4 μg.ml^{-1} for SD. No detection sensitivity was observed for SAM, SAC, SG, and PHT standards. The MCC of PABA, which completely inhibited the antibacterial effect of all residual concentrations of SA standards, was 1 μg.ml^{-1}.

The sensitivity of the STAR to the residual concentrations of SA standards presented by the production of the IZ (A), and formation of no-IZ after the addition of PABA to the agar medium at all tested PABA concentrations is recorded in table 2. The MIC of SMX, STZ, SQ, and SCHP was 0.05 μg.ml^{-1}, the MIC of SRZ was 0.1 μg.ml^{-1}, and the MIC of SD was 0.3 μg.ml^{-1}. No detection sensitivity was observed for SAM, SAC, SG, and PHT. The MCC of PABA, which completely inhibited the antibacterial effect of all residual concentrations of SA standards, was 1 μg.ml^{-1}.

The sensitivity of the Premi®Test to the residual concentrations of SA standards (A) and confirmation their presence by PABA at the concentration of 10 μg.ml^{-1} (B) is recorded in table 3. The MIC was 0.05 μg.ml^{-1} for SMX, STZ, SQ, SCHP, SRZ, and SD, 0.1 μg.ml^{-1} for PHT, 0.2 μg.ml^{-1} for SAM, and 0.3 μg.ml^{-1} for SAC and SG. The presented MIC determined the LOD of the method to the SAs mentioned above. As table 3 shows, the presence of SA standards was confirmed by using PABA concentration of 10 μg.ml^{-1}.

Microbial inhibition tests are highly valuable in the first stage of residue screening owing to their excellent practicality and throughput. However, they provide only preliminary information about the presence of residues of certain groups of veterinary drugs in examined samples. The presence of residues presented in the potentially positive samples must be further confirmed by using a more specific physico-chemical method (7, 13, 14).

Braham et al. (5) developed a sulphonamide-sensitive rapid assay using *Bacillus stearothermophilus* inoculated PM indicator agar containing Bromocresol purple and TMP (0.04, 0.05, and 0.12 μg.ml^{-1}). The end point of the detection at this juncture was the combination of color change in the agar medium and zone of microbial growth inhibition around the sample disc. The filter paper control disc and the sample discs were applied on both non-PABA and PABA-containing agar. An IZ around the matching disc on non-PABA containing agar indicated that the tissue was suspect antimicrobial drug residue positive. The absence of the IZ around the matching disc on PABA-containing agar indicated that the antimicrobial residue was a sulphonamide. Using this method, five SAs were detected at concentrations near the MRL (from 0.08 to 0.2 μg.ml^{-1}), using 100 μg.ml^{-1} PABA for their presumptive identification.

Kožárová and Labanská (12) evaluated the detection sensitivity of Delvotest®SP, Premi®Test, the FPT and the STAR to six SA standards. They detected that the LOD of Delvotest®SP was

Tab. 2. STAR – the mean diameters of the IZ (mm) produced by SA standards (A) and no-IZ after the addition of PABA to the agar medium at the concentration of 1, 3, 10, and 20 μg.ml^{-1} (B)

SA		Mean + sd (mm) Concentration of sulphonamides (μg.ml^{-1})						
		0.05	0.1	0.2	0.3	0.4	0.5	1
SMX	A	4.67 ± 0.52	5.50 ± 0.55	7.67 ± 1.37	8.67 ± 1.37	10.00 ± 1.10	10.67 ± 0.82	12.50 ± 2.17
	B	0	0	0	0	0	0	0
STZ	A	3.83 ± 0.75	6.00 ± 2.10	8.17 ± 2.14	10.33 ± 1.75	12.00 ± 1.41	13.00 ± 1.26	15.00 ± 1.26
	B	0	0	0	0	0	0	0
SQ	A	1.67 ± 0.52	4.17 ± 1.60	7.17 ± 1.60	8.67 ± 1.51	10.17 ± 1.17	10.83 ± 1.17	12.67 ± 1.21
	B	0	0	0	0	0	0	0
SCHP	A	1.17 ± 0.75	4.17 ± 1.17	6.33 ± 1.75	9.33 ± 0.82	10.00 ± 0.63	10.50 ± 1.76	11.67 ± 1.37
	B	0	0	0	0	0	0	0
SRZ	A	0	3.00 ± 0.89	3.83 ± 1.33	5.00 ± 1.41	6.17 ± 1.47	7.00 ± 1.67	9.50 ± 1.52
	B	0	0	0	0	0	0	0
SD	A	0	0	0	2.17 ± 0.75	3.17 ± 1.17	4.00 ± 0.63	5.00 ± 0.63
	B	0	0	0	0	0	0	0
SAM	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
SAC	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
SG	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0
PHT	A	0	0	0	0	0	0	0
	B	0	0	0	0	0	0	0

Explanations: as in tab. 1.

Tab. 3. The sensitivity of the Premi®Test to SA standards (A) and the confirmation of SAs by PABA at the concentration of 10 µg.ml^{-1} (B)

SA		Concentration of sulphonamides (µg.ml^{-1})						
		0.05	0.1	0.2	0.3	0.4	0.5	1
SMX	A	+	+	+	+	+	+	+
	B	–	–	–	–	–	–	–
STZ	A	+	+	+	+	+	+	+
	B	–	–	–	–	–	–	–
SQ	A	+	+	+	+	+	+	+
	B	–	–	–	–	–	–	–
SCHP	A	+	+	+	+	+	+	+
	B	–	–	–	–	–	–	–
SRZ	A	–	+	+	+	+	+	+
	B	–	–	–	–	–	–	–
SD	A	+	+	+	+	+	+	+
	B	–	–	–	–	–	–	–
SAM	A	–	–	–	+	+	+	+
	B	–	–	–	–	–	–	–
SAC	A	–	–	–	+	+	+	+
	B	–	–	–	–	–	–	–
SG	A	–	–	–	+	+	+	+
	B	–	–	–	–	–	–	–
PHT	A	–	+	+	+	+	+	+
	B	–	–	–	–	–	–	–

Explanations: – – negative result, + – positive result, ■ – the MIC of SA standards

0.1 µg.ml^{-1} for SMX, SQ, and SCHP, 0.5 µg.ml^{-1} for SD, and 1 µg.ml^{-1} for PHT and SG. The LOD of the FPT was 0.01 µg.ml^{-1} for SCHP, SMX and SQ, 0.5 µg.ml^{-1} for SG, 1 µg.ml^{-1} for SD, and 5 µg.ml^{-1} for PHT. Premi®Test detected all SAs tested at the level of MRL 0.1 µg.ml^{-1}, and SCHP even at the concentration of 0.01 µg.ml^{-1}. The STAR detected all SAs tested at the concentration of 0.01 µg.ml^{-1}, which is below the MRL set. The results obtained by the STAR and the Premi®Test showed the higher sensitivity of these methods toward all SAs tested.

From the viewpoint of hygiene and public health, monitoring of SA residues in foods of animal origin has been critical to the protection of the food chain against the penetration of residues of these drugs. Our results showed that the Premi®Test is the method most sensitive to SAs, followed by the STAR and the FPT. Premi®Test detected six SA standards at the level of the MRL 0.1 µg.ml^{-1} set for the SA group, the STAR detected five SAs at the level of the MRL, while the FPT is not sensitive enough to detect SAs at the MRL level.

As for PABA, our observations showed that PABA antagonized the inhibitory effect of all SAs detected by the FPT and the STAR even after being added to the agar medium at the concentration of 1 µg.ml^{-1}. For the Premi®Test, the confirmatory PABA concentration was 10 µg.ml^{-1}. The utilized PABA concentration can be used for a reliable identification of SAs at their residue screening by using the FPT, the STAR, and the Premi®Test.

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