Original scientific article

Potential applications of rapid microbiological methods for detection of antibiotic residues in wastewater, surface and well water

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ABSTRACT

The goal of the present study was to determine whether the commercial microbiological and tracer assays for the detection of antibiotics in the food were also useful and sensitive enough for testing water samples. Diffusion tests Delvotest® SP-NT and BRT-AiM showed the similar sensitivity to tested antibiotics in spiked water samples. Both tests showed the similar sensitivity to examined antibiotics in water as it was published in milk, while tracer assay BetaStar showed slightly higher minimum detection levels for penicillin and ampicillin but not for cloxacillin. The previous concentration of the samples by lyophilization took place to detect concentrations of antibiotics 100-fold lower than there were the minimum detection limits of the assays. The presence of inhibitory substances in surface and well samples was detected in 16 (16.3 %) cases out of 98 with both ampoule diffusion methods. The positive results were obtained at 15.0 % of surface water samples, while in well water the residues were found also in 16.9 % and 13.6 % samples, using Delvotest SP-NT and BRT-AiM, respectively. The β-lactams were detected with BetaStar in 7.5 % of surface water samples. The 12 wastewater samples from hospitals were contaminated with inhibitory substances in 45.5 % (Delvotest SP-NT) or in 36.4 % (BRT-AiM).

Key words: Antibiotics, microbiological methods, water, screening, contamination

Received: 28. 10. 2013
Accepted: 10. 11. 2013

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INTRODUCTION

Antibiotics are pharmaceuticals which are used widely and in large amounts in human and veterinary medicine.

In veterinary practice, they are utilized at therapeutic levels primarily to treat diseases [1]. Cabello [2] reported about the widespread use of prophylactic antibiotics in aquaculture to forestall bacterial infections.

Residues of human and veterinary pharmaceuticals, including a lot of non-biodegradable antibiotics, are introduced into the environment via a number of pathways, primarily from discharges of wastewater treatment plants from hospitals and pharmaceutical industry or land application of sewage sludge and animal manure. They have been consequently widely detected in various environmental matrices including surface water, groundwater, soils, and sediments [3]. Although wastewater treatment plants remove some pharmaceuticals during the cleaning process [2, 4] the removal efficiencies vary from plant to plant. In certain circumstances they even inhibit the working microorganisms in biological wastewater treatement plants [5, 6]. Some antibiotics seem to persist in the environment long time and cause adverse health effects in both humans and wild life [5, 7, 8, 9]. They may lead to the development of antibiotic-resistant strains of microorganisms [9, 10].

Some antibiotics seem to persist in the environment long time and cause adverse health effects in both humans and wild life. They may lead to the development of antibiotic-resistant strains of microorganisms.

Residues may enter the food chain and are found at different concentration levels not only in drinking water, but also in products of animal origin, such as milk, eggs and meat and can cause human health problems (e.g. the induction of allergic reactions in hypersensitive individuals). The prevention of antibiotic residues in milk and meat is crucial in order to avoid losses in fermentation processes using microorganisms as starter cultures [1].

Antibiotic residues in edible animal products are of great concern to regulatory agencies and consumers, so reliable screening methods for rapid, selective and sensitive detection of these residues were developed to ensure food safety [11]. In general, analytical methods for monitoring antibiotic residues in food can be classified in three groups:

Biological methods based on bacterial growth inhibition. They are not selective and can cover several chemical classes of active analytes but do not allow the identification of individual analytes.

The toxic or genotoxic effect of different substances, including antibiotics can be detected by bioassays, using bacteria *Vibrio fisheri*, *Microcystis aeruginosa* (cyanobacteria), *Brachionus calyciflorus* (rotifer), *Thamnocephalus platyurus* (crustacean anostraca), *Daphnia magna* (crustacean cladocera), *Danio rerio* (teleostei), *Pseudokirchneriella subcapitata* (green algae), and some others [12, 13].

The presence and concentrations of specific antibiotics in water samples are determined by more sensitive physicochemical methods, like solid phase extraction (SPE) and liquid chromatography-tandem mass spectrometry (LC/MS/MS) with electrospray ionization (ESI) [14, 15, 16].

Physicochemical methods (e.g. TLC, GC, LC, HPLC, capilary electrophoresis, LC/MS) distinguish the chemical structure and molecular characteristics of analytes by separation of molecules and the detection of signals related to molecular characteristics. They detect the concentration and type of antibiotics in tested sample. They are time – consuming, expensive and require complex laboratory equipement and trained personnel [11, 17].

Biochemical or tracer methods**,** like ELISA, RIA, etc., detect molecular interactions between analytes and antibodies or receptor proteins. They are either selective for a family of analytes having related molecular structures or are sometimes analyte specific [11].

The goal of the present study was to determine whether the methods for the detection of antibiotics in food were also useful and enough sensitive for testing water samples. We focused on analytical methods on commercial kit tests that allow fast, sensitive detection of antibiotic residues with minimum sample treatment. Once these procedures were optimized, they were applied to the analysis of water samples collected from some major Slovenian streams, groundwater from wells and wastewaters.

The goal of the present study was to determine whether the methods for the detection of antibiotics in food were also useful and enough sensitive for testing water samples.

MATERIAL AND METHODS

Environmental water samples

A total of 110 water samples, collected in the period from two seasons: December 2009 to March 2010 and June to September 2010 were tested for the presence of inhibitory substances. Fifty-nine out of 110 samples were groundwater samples from individual wells, 40 were surface water samples (streams, rivers) and 11 samples were wastewater samples from hospitals, clinical departments and one farmaceutical factory (**Table 3**). The sampling sites were selected randomly in rural and urban areas, distributed throughout the country. The temperatures of winter and summer samples were between 4 °C to 13 °C and 10 °C to 21 °C, respectively, the rainfall quantity was measured as well.

The sampling sites were selected randomly in rural and urban areas, distributed throughout the country.

From each of the testing sites 1–2 samples were collected, not all of them were tested in each of the sampling period.

Preparation of environmental samples

One litre of water sample was collected in duplicates into appropriate sterile glass bottle, approximately 20 cm below the surface of the water in two different sides of each stream and transferred to the laboratory at temperatures from 4 to 10 °C in maximal two hours. All samples were filtered through 0.45 μm filters (11306-50-N, SartoriusStedim, Germany) and stored at -20 °C until they were analysed. The pH values in well and surface water samples ranged between 6.5 and 7.3 while in wastewater between 6.8 and 8.5.

Spiked and standard samples

The spiked samples were prepared with defined concentrations of antibiotics. Standard solutions of antibiotics benzyl penicillin, ampicillin, cloxacillin, erythromycin, kanamycin, nalidixic acid and chloramphenicol, were prepared in concentrations, which are minimal detection limits for used methods for milk. To study the matrix effect, we prepared samples of water and milk with the same concentrations of dissolved antibiotics. Each antibiotic was dissolved in sterile distilled water in different concentrations. For the preparation of milk samples, the antibiotics were first dissolved in distilled water as stock solutions and then in reconstituted skim milk (Skim milk powder, 1.15363, Merck, Germany) as well. The proportion of the added aqueous standard solution in the final milk dilution step was less than 1 %. The selection of concentrations for the determination of the senstivity for each test was based on the detection limits mentioned by producers, together with at least one concentration step higher and two concentration steps lower [18] (**Table 1**). The spiked samples were divided into three subsamples and frozen at -20 °C ± 2 °C. The test kits with different batches were used for detecting the antibiotics in each subsample.

The selection of concentrations for the determination of the senstivity for each test was based on the detection limits mentioned by producers, together with at least one concentration step higher and two concentration steps lower.

We 100-folded the concentration of water samples and thereby detected lower concentrations of antibiotics with the same methods. For this purpose 300 ml of the sample was lyophilizated using Freeze Dryer Alpha 2-4 LSC, Christ (Germany). For each concentration of antibiotic there were prepared two parallel samples. After lyophilization one of them was dissolved in 3 ml of sterile nutrient broth [19], and the other in sterile distilled water, with intention to investigate the potential impact of the diluent on the sensitivity of the methods. All working solutions were prepared freshly at the same day of analysis. According to the recommendations of the International Dairy Federation the pH of the sample should be higher than 6 [20]. The pH values of resuspended concentrates prepared after lyophilization were between 6.8 and 8.2.

Standard samples Penicillin G Standard, full cream milk lyophilizate 4 ng/mL (9143, AiM GmbH, Germany), and Inhibitor Free Milk, full cream milk lyophilizate, (9150, AiM GmbH, Germany) were used as positive and negative control. The nutrient broth (Merck, Germany) and distilled water were used as negative control too. The antibiotic discs with gentamycin, GM 10 μg, penicillin G, P 10 IU and streptomycin, S 10 μg (Becton Dickinson, Great Britain) were used as standards for detection of the sensitivity at disc diffusion methods.

METHODS

For detection of inhibitors and medical residues in water, there were established microbiological ampoule diffusion methods BRT-AiM (tubes for single sample, 3040, Neogen Corporation, USA) and Delvotest® SP-NT (DSM Food Specialities, The Netherlands), disc diffusion methods with test bacteria *Geobacillus stearothermophilus* var.

Table 1:
The sensitivity of the methods used in the experiment and concentrations of tested antibiotics in spiked milk and water samples.

Antibiotics	Concentration of antibiotic in the sample µg/L	Concentration of antibiotic in the water after lyophilisation µg/L[a]	Delvotest SP-NT		BRT		Discs *G. s.*[b] inh. zone (mm)[c]		Discs *B. s.*[d] inh. zone (mm)[c]		Beta Star	
			Milk	Water	Milk	Water	Milk	Water	Milk	Water	Milk	Water
β-lactams												
Benzyl-Penicillin G potassium salt (13750, Sigma Aldrich, USA)	1.0		-	-	-	-	6±0	6±0	6±0	6±0	-	-
	1.5		-	-	-	-	6±0	6±0	6±0	6±0	-	-
	2.5[f]		+[f]	+	+	+	6±0	6±0	6±0	6±0	-	-
	4.0		+	+	+	+	**8±0.1**	6±0.2	6±0	6±0	+	+
	25		+	+	+	+		**37.3±0.4**	**19.2±0.2**	**18.2±0.2**	+	+
	250		+	+	+	+		40.3±0.2	NM[e]	NM[e]	+	+
	0.001	1.0		-		-		6±0		6±0		-
	0.015	1.5		-		-		6±0		6±0		-
	0.025	**2.5**		+		+		6±0		6±0		-
	0.04	4.0		+		+		6±0.7		6±0		-
	0.06	**6.0**		+		+		**65.8±1.2**		**19±0**		+
	0.24	24		+		+		48.7±2.4		18.8±0.9		+
	0.25	25		+		+		44.2±0.2		18.8±0.8		+
	2.5	250		+		+		15.8±0.5		NM[e]		+
Ampicillin sodium salt (A9518, Sigma Aldrich, USA)	1.0		-	-	-	-		6±0			-	-
	1.5		-	-	-	-		6±0			-	-
	2.5		+	+	+	+		6±0			+	-
	4.0		+	+	+	+		6±0			+	+
	16		+	+	+	+		**6.8±0.2**			+	+
	25		+	+	+	+		34±0			+	+
	250		+	+	+	+		16.5±0.5			+	+
	0.025	**2.50**		+		+		6±0				-
	0.04	4.00		+		+		6±0				-
	0.16	**16**		+		+		**6.2±0.2**				+
	0.25	25		+		+		36.0±0.9				+
	2.5	250		+		+		16.0±0.6				+
Cloxacillin sodium salt monohydrate (C9393, Sigma Aldrich, USA)	**1**		-	-	+	+	6±0	6±0	6±0	6±0	-	-
	4		-	-	+	+	6±0	6±0	6±0	6±0	+	+
	10		-	-	+	+	6±0	6±0	6±0	6±0	+	+
	20		-	-	+	+	6±0	6±0	6±0	6±0	+	+
	40		-	-	+	+	6±0	6±0	6±0	6±0	+	+
	100		+	+	+	+	6±0	6±0	6±0	6±0	+	+
	0.01	**1**		-		+		6±0		6±0		-
	0.04	**4**		-		+		6±0		6±0		+
	0.10	10		-		+		6±0		6±0		+
	0.20	20		-		+		6±0		6±0		+
	0.40	40		-		+		6±0		6±0		+
	1	100		+		+		6±0		6±0		+
	2	200		+		+		6±0		6±0		+

Antibiotics	Concentration of antibiotic in the sample µg/L	Concentration of antibiotic in the water after lyophilisation µg/L[a]	Delvotest SP-NT		BRT		Discs *G. s.*[b] inh. zone (mm)[c]		Discs *B. s.*[d] inh. zone (mm)[c]		Beta Star	
			Milk	Water	Milk	Water	Milk	Water	Milk	Water	Milk	Water
Macrolydes												
Erythromycin (E5389, Sigma Aldrich, USA)	40		-	-	-	-	6±0	6±0	6±0	6±0	-	-
	75		+	-	+	+	6±0	6±0	6±0	6±0	-	-
	150		+	+	+	+	**16±0.2**	**16±0.0**	6±0	6±0	-	-
	300		+	+	+	+	16.6±0.1	16.4±0.7	6±0	6±0	-	-
	0.001	0.1		-		-		6±0		6±0		-
	0.1	10		-		-		6±0		6±0		-
	0.4	**40**		-		+		6±0		6±0		-
	1.5	**150**		+		+		**18±0.6**		6±0		-
Amynoglicosides												
Kanamycin sulfate (K4379, Sigma Aldrich, USA)	37		-	-	-		6±0	6±0	6±0	6±0	-	-
	75		-	-	-		6±0	6±0	6±0	6±0	-	-
	378		+	+	+	+	6±0	6±0	6±0	6±0	-	-
	3785		+	+	+	+	6±0	6±0	6±0	6±0	-	-
	7570		+	+	+	+	6±0	6±0	6±0	6±0	-	-
	37850		+	+	+	+	6±0	6±0	6±0	6±0	-	-
	0.378	37.8		-		-		6±0		6±0		-
	0.757	75.7		-		-		6±0		6±0		-
	3.785	**378.5**		+		-		6±0		6±0		-
	37.85	**3785**		+		+		6±0		6±0		-
	75.70	7570		+		+		6±0		6±0		-
	378.50	37850		+		+		6±0		6±0		-
	3785	378500		+		+		6±0		6±0		-
	37850	3785000		+		+		6±0		6±0		-
Quinolones												
Nalidixic acid sodium salt (N3143, Sigma Aldrich, USA	0.05		-	-	-	-	6±0	6±0	6±0	6±0	-	-
	1		-	-	-	-	6±0	6±0	6±0	6±0	-	-
	5		+	+	+	+	**8±0.1**	6±0	6±0	6±0	-	-
	25		+	+	+	+	9±0	6±0	6±0	6±0	-	-
	0.01	1		-		-		6±0		6±0		-
	0.050	**5**		+		+		**7.7±0.5**		6±0		-
	0.25	25		+		+		10±0.2		6±0		
	25	2500		+		+		6±0		6±0		-
	255	25520		+		+		6±0		6±0		-
Others												
Chloramphenicol (CO378, Sigma Aldrich, USA)	25		-	-	-	-	6±0	6±0	6±0	6±0	-	-
	50		-	-	-	-	6±0	6±0	6±0	6±0	-	-
	250		-	-	-	-	6±0	6±0	6±0	6±0	-	-
	2500		+	+	+	+	6±0	6±0	6±0	6±0	-	-
	5000		+	+	+	+		6±0		6±0		-
	0.025	2.5		-		-		6±0		6±0		-
	0.25	25		-		-		6±0		6±0		-
	2.5	250		-		-		6±0		6±0		-
	25	**2500**		+		+		6±0		6±0		-
	50	5000		+		+		6±0		6±0		-

[a] – after 100-fold concentration with lyophilization followed by resuspension with distilled water or nutrient broth; [b] – *G. s.*: disc diffusion method with *Geobacillus stearothermophilus* var. *calidolactis*; [c] – diameter of inhibition zones in mm (mean values of 3 measures and the average deviations of the mean); [d] – *B. s.*: disc diffusion method with *Bacillus subtilis*; [e] – NM: not measured; [f] – The detection limits of the methods, representing 95 % positive results for each antibiotic in the experiment, were highlighted in the **bolt** script.

calidolactis C953 (ATCC7953, 1.11499, Merck, Germany) which is added to the melted sterile agar medium according to Kundrat (1.10662, Merck, Germany), and *Bacillus subtilis* strain BGA (DSM618, 1.10649, Merck, Germay) in Test Agar pH 7.2 for the inhibitor test (1.15787, Merck, Germany). The tracer method BetaStar (Neogen Corporation, USA) is a receptor binding assay, which detects penicillins and cephalosporins.

The procedures were carried out following manufacturer's instructions and recommendations of previous publications [1, 20, 21, 22, 23, 24, 25, 26, 27, 28].

The spiked samples were tested in triplicates using different assay batches and environmental samples in duplicates as well.

The statistical analyses were calculated by using IBM SPSS Statistics 20 programme. The statistical analysis included analysis of Pearson Chi-Square between samples. Two-sided asymptomatic significance was set at α=0.05.

RESULTS AND DISCUSSION

The surface waters and especially underground water are sources for drinking water supplies, so its physiochemical and microbiological quality is very important.

The spiked samples were tested in triplicates using different assay batches and environmental samples in duplicates as well.

Most classical bioassays for detecting genotoxic substances generally in water samples have not proven very sensitive to antibiotics or are not fast enough screening tools [12, 13], their minimal detection concentrations for antibiotics are higher than those that have proven at routine methods for the detection of antibiotics in food.

We assessed the suitability of some commercial microbiological and tracer methods routinely used in food control for detection of antibiotics in water. Their minimal detection levels for single antibiotic residues are mostly in the concentrations prescribed as MRL in food samples [29] (**Table 2**).

The concentrations of antibiotics residues are in water sources according published reports lower than MRLs for food. The concentrations of antibiotics in streams were up to 0.694 μg/L [30]; up to 1.435 μg/L [16] up to 2.3 μg/L [31, 32], or even up to 6.72 μg/L [33], depending on the type of detected antibiotic, the sample, the area and the season of sampling.

The highest concentrations of quinolones in surface water were from 0.3 to 1.3 μg/L, while the mean values of β-lactams were found around 0.25 μg/Land amynoglycosides 0.04 μg/L [9, 31]. Feitosa-Felizzola and Chiron [33] reported about the concentrations of clarithromycin and oxitetracycline in streams 0.02 and 0.08 μg/L, respectively (**Table 1**).

The maximal concentrations of antibiotics in wastewater samples from hospitals were in the range from 0.01 to 15 μg/L [31], from 11 to

69.570 ng/L [32] or from 0.0039 μg/L to approximately 27 μg/L [34]. Brown [35] and Kümmerer [36] detected β-lactams in hospital wastewater in ranges even from 0.85-80 μg/L.

These values are in most cases, particularly in waste waters, approximately 100-fold lower than the MRLs and minimal detection concentrations obtained by routine methods used in food industry. In order to use these routine microbiological methods for detection of antibiotics on the levels found in water, samples should be concentrated in this way, that we could still observe a wide range of different groups of antibiotics. Many antibiotics are sensitive to some solvents or high temperatures, so the chosen procedures of samples preparation should not change their concentration or activity. In our experiment we used the lyophilization of the samples, which is recommended for preparing of test samples for validation of microbial inhibitor tests for ISO 13969/IDF 183 [18]. This procedure would not affect the sensitivity of the method, the activity of the test bacteria, larger changing in pH, persistence of wider range of antibiotics which can be present, and composition of water samples. Hirsch [4] used this technique for preconcentration the water samples before quantification the antibiotics using HPLC-electrospray-tandem-mass spectrometry. Some other ways of concentration, like evaporation and thermization could lead the degradation of antibiotics [37].

The sensitivity of the assays for detecting antibiotics in spiked water samples

The chosen methods and concentrations of tested antibiotics as well as minimum detection limits using the standard solutions of antibiotics are represented in **Table 1**. With Delvotest SP-NT we detected penicillin and ampicillin in concentrations 2.5 μg/L of water sample. After 100-fold concentration of the samples using lyophilization this minimal detection sensitivity was 0.025 μg/L. The minimal concentrations of cloxacillin, erythromycin, kanamycin, nalidixic acid and chloramphenicol, where we obtained the positive reaction of Delvotest, were at least 100 μg/L, 150 μg/L, 378500 μg/L, 5 μg/L and 2500 μg/L of sample, respectively. These values were after concentration decresed 100-fold for each antibiotic (**Table 1**).

The detection levels of β-lactams penicillin and ampicillin were in spiked water samples the same as Mitchell [38] obtained for milk. Delvotest was slightly less sensitive to cloxacillin and chloramphenicol, and more sensitive to erythromycin as it was reported for milk samples [24, 38].

The sensitivities of BRT-AiM towards penicillin, ampicillin, cloxacillin, erythromycin, kanamycin, nalidixic acid and cloramphenicol were in concentrations of at least 2.5 μg/L, 2.5 μg/L, 1 μg/L, 75 μg/L, 378500 μg/L, 5 μg/L and 2500 μg/L of sample, and after lyophilisation 0.025 μg/L, 0.025 μg/L, 0.01 μg/L, 0.75 μg/L, 3785 μg/L, 0.05 μg/L and 25 μg/L for each antibiotic, respectively. Our results showed the lower detection limit for cloxacillin, than it is reported for BRT-AiM test for milk [39, 40] (**Table 2**).

Table 2:
Limits of detection of tested methods towards antibiotics (μg/L) used in the experiment and MRLs for cattle milk.

Drugs	Delvotest[a]	BRT[b]	Disc *G. s.*[c]	Disc *B. s.*[c]	BetaStar[d]	MRL[e]
Penicillines						
Benzylpenicillin	1-2	2-3	6	18	2-4.8	4
Ampicillin	4	2-3	5	-[g]	4-7	4
Cloxacillin	20	20-30	35	-	6-9	30
Macrolides						-
Erythromycin	40-80	40-60	225-600	100		40[f]
Others						-
Chloramphenicol	-	-	-	10000	-	-
Aminoglycosides						
Kanamycin	-	-	28000	-		150[f]
Quinolones						
Nalidictic acid	-	-	-	-	-	-

[a][24, 41]; [b][39]; [c][20];[d][28]; [e][44, 48]; [f][49]; [g] not mentioned

BRT-AiM test and Delvotest showed very similar sensitivity to spiked antibiotic concentrations in water samples, except BRT-AiM test was according our results slightly more sensitive to cloxacillin and erythromycin. *G. stearothermophilus* var. *calidolactis* is the test organism used in both assays which have consequently simmilar sensitivity. They differ among themselves only in the fact, that the color indicator at Delvotest SP-NT reacts to changes in pH values, while at the BRT-AiM test is sensitive to changes in redox potential. The minimal detection limits could be in some cases even lower and more precise if we have used a larger number of spiked samples with minnor differences in the concentrations of the antibiotics.

The satisfactory sensitivity of these two diffusion methods towards amynoglicoside kanamycin and even nalidixic acid as representative of quinolons is delightful, particularly we did not find any limits for these two antibiotics in milk.

Both assays are sensitive not only to a wide range of β-lactams but also to representatives of macrolides, amynoglycosides, lincosamides, sulphonamides etc. as well [24, 39, 40]. It is important, that they can be applicable for screening of samples with a wide range of pH values higher than 5.5 [41].

Some adaptations of the Delvotest and BRT-AiM protocols were required to produce results from environmental samples. Smith [19] recommended that the water samples should be transferred into a nutrient media to stimulate the bacterial spores to germination and then the vegetative cells to rapid growth and respiration.

We obtained some differences in results between samples, dissolved after lyophilization in water and in broth. The samples with 37.8 μg/L of kanamycin and 255 μg/L of nalidixic acid, dissolved in nutrient broth showed with BRT-AiM assay positive reaction. On the contrary, the

negative reaction at the broth sample with 0.0504 μg/L of nalidixic acid using Delvotest SP-NT was observed as well. In other spiked samples there were no differences in results between samples resuspended in nutrient broth and water.

The standard control samples with deffined concentrations of penicillin were used to check the correct procedure of Delvotest SP-NT and BRT-AiM, while the end points of incubation were determined as the time at which the blanks (distilled water, broth) turned yellow. We must point out that we had to extend the incubation for 30 minutes and it took at both assays from 3 hours 30 minutes, regardless of weather it was used nutrient broth or water for resuspendion of lyophilized samples.

BetaStar is sensitive to β-lactam antibiotics penicillin, ampicillin and cloxacillin in milk in concentrations between 2 to 9 μg/L [27, 28]. Our examination of spiked water samples using BetaStar showed slightly higher minimum detection levels for penicillin. The reaction was negative in the test samples with all β-lactams in concentrations of 2.5 μg/L and positive at 6 μg/L, 10 μg/L and 16 μg/L of penicillin, cloxacillin and ampicillin, respectively. In concentrated samples the minimal sensitivity values were 100-fold lower. We also agree with previous reports, that there was observed the equal sensitivity to cloxacillin in the comparasion to reports for milk samples [38, 42] (**Table 1, table 2**). The repeatability of the test was very good and the results were not significantly influenced by small changes (e.g. pH values) in the protocol [28].

Matrix effect was minimal and did not significantly affect on the results.

Calculation of the Chi-Square statistical tests indicate that there were statistically significant relationships between the results obtained by Delvotest SP-NT, BRT-AiM test and BetaStar ($p<0.05$). A comparisson of all three methods shows high correlation ($p<0.05$) and therefore relevance of tested methods. We also found statistically significant relationships between the results of the determination of the antibiotics in milk and water samples and in samples before and after concentration as well ($p<0.01$). Matrix effect was minimal and did not significantly affect on the results (**Table 3**).

More than 6.0 μg of penicillin per litre of water or broth was detected also with both disc diffusion methods. The inhibition zone around disc with 25 μg/L of ampicillin and 150 μg/L of erythromycin on the medium seeded with *G. stearothermophilus* var. *calidolactis* was obvious in all three repetitions, while bacteria *B. subtilis* was not inhibited. The inhibition zone was measured also arround the disc with nalidixic acid in concentration 5 μg/L, but not in higher concentrations used (**Table 1**).

The disc diffusion methods were in our experiment less sensitive than ampoule diffusion methods Delvotest and BRT-AiM. The inhibition zones were at both disc diffusion assays against expectations at higher concentrations of antibiotics in spiked samples smaller than at lower concentrations.

Disc diffusion method with *B. subtilis* was sensitive only to penicillin (**Table 1**) in spite of Okerman [43] reported about positive reaction to

cephalosporines, some quinolones, lincosamides, macrolides, aminoglycosides, and sulphonamides as well. Its sensitivity depends on the pH of the medium and the constitution of the sample matrix. The pH values of the agar medium were targeted to 7.2, because this assay is considered to be according producer's instructions under these conditions slightly less sensitive to penicillin, gentamycin and streptomycin, but extra sensitive to sulfonamides [21, 43]. All used methods were especially sensitive to β-lactam antibiotics [44]. These antibiotics still comprise roughly half of the antibiotic market worldwide. Mostly combined with clavulanic acid or other β-lactamase inhibitors are still the most frequently administered drugs in parental and intra-mamary mastitis therapy in veterinary medicine. They have been reported to dominate in human medicine and the overall antibiotic concentration in some sewage influents as well [26, 28].

In spite of these antibiotics tend to be significantly reduced in concentrations during biological process in wastewater treatment plants [31, 34], some of them showed certain anaerobic biodegradation only after 60 days [5]. Furthermore, they were sporadically reported in effluent, which may indicate that although their pseudopersistance may be occuring due to their continual discharge [31]. Huang [34] identify that antibiotics of sulphonamides and fluoroquinolones are the most likely water contaminants, followed by macrolides. These groups were still detected in wastewater treatment plants effluents, because the average removal rate of greater than 80 % for all of them [31] The representatives of these two groups of antibiotics were well detected with the methods chosen in our experiment.

Huang identify that antibiotics of sulphonamides and fluoroquinolones are the most likely water contaminants, followed by macrolides.

Antibiotic residues in well water, streams and wastewater samples

The data about the presence of antibiotics in Slovenian ground water, drinking water surface water and wastewater have not been published yet. The presence of inhibitory substances was detected by Delvotest SP-NT in 16 (16.3 %) and BRT-AiM assay in 14 (14.3 %) out of 99 surface and well samples. The positive results were obtained at 15.0 % of surface water samples, while in well water the residues were found also in 16.9 % and 13.6 % samples, using Delvotest SP-NT and BRT-AiM, respectively. The antibiotics from β-lactam group were detected with BetaStar in 7.6 % of surface water samples. As it was expected, the wastewater samples were contaminated with inhibitory substances in even 45.5 % (Delvotest SP-NT) or in 36.4 % (BRT-AiM). The β-lactams were determined in 18.1 % of them (**Table 4**). Using discs diffusion methods we did not get positive results, except at one wastewater sample. Generally there were no obvious differences in sensitivity between BRT-AiM test and Delvotest SP-NT. In three cases (2.7 %) out of 110 samples gave Delvotest SP-NT positive and BRT-AiM negative result.

The presence of antibiotics in larger number of water samples from individual wells is a major concern. In rural areas, water from domestic

Table 3:
Statistically significant relationships between Delvotest SP-NT, BRT-AiM test and BetaStar and between types of samples (Pearson Chi-Square with one degree of freedom).

Methods/samples	Chi-Square Value	2-sided asymptomatic significance (p)	R^2
Analysis of methods comparison			
Delvotest SP-NT : BRT	56.821 (min 15.52)[d]	<0.001	0.494
Delvotest SP-NT : BetaStar	7.453 (min 9.05)[d]	0.006	0.128
BRT : BetaStar	21,290 (min 3.62)[d]	<0.001	0.367
Analysis of matrix effect			
Delvotest SP-NT (M)[a] : Delvotest SP-NT (V)[b]	33.197 (min 2.21)[d]	<0.001	0.897
BRT (M) : BRT (V)	37.000 (min 3.89)[d]	<0.001	1.000
BetaStar (M) : BetaStar (V)	15.033 (min 2.21)[d]	<0.001	0.790
Analysis of concentration effect			
Delvotest (V) : Delvotest (Conc)[c]	30.00 (min 5.63)[d]	<0.001	1.000
BRT (V) : BRT (Conc)	21.232 (min 2.70)[d]	<0.001	0.707
BetaStar (V) : BetaStar (Conc)	7.350 (min 1.67)[d]	0.007	0.490

[a](M): milk sample; [b](V): water sample; [c](Conc): sample after concentration using lyophilisation; [d] The minimum expected count.

Table 4:
The presence of inhibitory substances in environmental water samples detected with methods used in the experiment.

Samples	Total	Number (%) of positive samples				
		Delvotest[a]	BRT[b]	Disc *G. s.*[c]	Disc *B. s.*[c]	Beta Star[d]
Surface water	40 (36.4)	6 (15.0)	6 (15.0)	0 (0)	0 (0)	3 (7.5)
Well water	59 (53.6)	10 (16.9)	8 (13.6)	0 (0)	0 (0)	0 (0)
Wastewater	11 (10.0)	5 (45.5)	4 (36.4)	1 (9.0)	0 (0)	2 (18.2)
Total	110 (100)	21 (19.1)	18 (16.4)	1 (9.0)	0 (0)	5 (4.5)

[a] Delvotest SP-NT ampoule format, control time: time of negative control colouring yellow [24, 41];
[b] BRT-AiM test [39];
[c] Disc diffusion method with *Geobacillus stearothermophilus* and *Bacillus subtilis* [20, 42];
[d] Tracer assay (Neogen Corporation, USA) [28];

wells, supplied mostly by groundwater, is often used by people for drinking, watering livestock and irrigation of vegetables. Groundwater is a major contributor to flow in many streams and rivers and thus, has a strong influence on river and wetland habitats for plants and animals [45].

In some countries there are no regulations requiring that livestock farms must have a wastewater treatment plants, so that their waste water with undergraded antibiotic residues passed directly through the groundwater and surface water.

Barnes [45] found the veterinary and human antibiotic sulfamethoxazole in 23 % out of 47 groundwater samples, while Arikan [30] detected the same anthibiotic in 19 % of samples in river stations. Chlortetracycline (19 % detection) and oxytetracycline (15 % detection) were the most frequently detected of the TCs group of antibiotics of the river stations in his study.

Watkinson [31] detected the antibiotics at quantifiable concentrations in more than 50 % out of the 81 surface water samples in South-East Queensland, Australia, which was three times more than in our study. Wang [46] (2010) found four fluoroquinolone antibiotics in 77.5 % of tap water samples from Guangzhou and 100 % of samples from Macao water area.

Hirsch [4] reported about presence of sulfonamide residues in four out of 59 ground samples in agricultural areas in Germany.

The larger differences in the presence of inhibitory substances between winter and summer samples were not estimated. We detected them in 15.2 % of winter samples and 18.5 % of summer samples from individual wells. The specimens from surface waters were positive in 7.7 % of cases in winter and in 29.4 % of cases in summer season. Only twice out of 99 samples the antibiotics were detected in both seasons at the same sampling place. On the contrary, Arikan [30] obtained more samples with positive detections for antibiotics from the group tetracyclines and sulfadrugs in agricultural watershed reivers in USA in the December (winter) collections, followed by collections in June and September. Higher levels of clarithromycin in winter season determined also Feitosa-Felizzola and Chiron [33] in river water in Southern France.

The larger differences in the presence of inhibitory substances between winter and summer samples were not estimated.

Tong [47] reported about average concentrations of eight tested antibiotic residues in groundwater and lake water, respectively, 1.6-8.6 and 5.7-11.6 ng/L in summer; respectively, 2.0-7.3 and 6.7-11.7 ng/L in winter.

It is difficult to compare our results with the publications of other authors, because they mainly reported about the presence of individual antibiotics in waters. Their results were observed by using the precision physico-chemical methods. In comparison with the physicochemical methods the microbiological methods used in our experiment are faster, require unexpensive apparatus and smaller amount of samples. Furthemore, they are more sensitive to antibiotics than standard bioassays for detection the toxic or genotoxic substances in water. The residues of antibiotics according to the published data are obviously very common in the waters, sometimes even in drinking water, which is a great concern.

It is difficult to compare our results with the publications of other authors, because they mainly reported about the presence of individual antibiotics in waters.

Maximum concentrations of antibiotics in the water in the international legislation have not been specified yet. So it would be necessary to define the statutory MRLs in waters too. MRLs for most antibiotics in milk are defined. The MRLs in the water should be probably similar or slightly lower, as in the milk. In this circumstances might be some commercial microbiological assays for determing the inhibitory substances including β-lactams and some other most often prescribed antibiotics in veterinary and human medicine, useful and sensitive enough for routine monitoring of water samples. These positive samples can be than confirmed by immunological or/and chemical assays.

CONCLUSIONS

We can assume that particularly Delvotest SP-NT and BRT-AiM test could be at the appropriate preparation of the samples, useful for routine screening detection of β-lactams and some other antibiotic groups in water, especially in waste waters.

Their minimum detection concentrations in water were comparable to those in milk.

The lyophilization of the samples was used to increase the sensitivity of methods.

Inhibitory substances were obtained in 15.0 % of the Slovenian surface water samples.

In well water the residues were found in 16.9 % of the samples.

ACKNOWLEDGEMENTS

This study was supported through the CRP project (V4-0512) by the Slovenian Research Agency, the Ministry of Agriculture, Forestry and Food and the Government Office for Local Self-Government and Regional Policy.

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