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Comparative Examination of the Analysis of β-Lactam Antibiotic Residues in Milk by Enzyme, Receptor–Enzyme, and Inhibition Procedures

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Abstract Antibiotic residues in milk are of great concern to dairy farmers, milk processors, regulatory agencies, and consumers. The aim of this study was to compare the screening tests for residue detection in milk with the purpose of choosing the most sensitive test that could prove residue quantities at maximum residue limits (MRL). The Penzyme S, Delvo-X-press β-lactam II, Delvo SP test, and diffusion test were examined. Milk samples (218) were collected from different segments of milk production chain: farms, milk collection points, dairy, and market. The limit of detection (LOD) of all methods for penicillin G, ampicillin, and amoxicillin were within the MRL, except the LOD for cloxacillin (diffusion test and Delvo-X-press), which were above the MRL value of 30 ng/g. Agreement between test results evaluated by kappa statistic for all tests was substantial (κ=0.61–0.80). We suggest the samples to be examined by Delvo SP test in the dairy. Positive milk samples should be retested by Penzyme S or Delvo-X-press test because of the high probability that residues are β-lactams, and these tests can prove it. By recurrent examining procedure, starting with the milk samples from pickup trucks, bulk milk tanks in collection points, or bulk milk tanks at farm and from individual producers, the specific milk that was contaminated by antibiotics can be traced.

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Introduction

β-Lactam antibiotics, i.e., penicillins and cephalosporins, are essential for the control of mastitis and other infectious diseases in lactating dairy cows. Therefore, these antibiotics are the most frequent residues that may be detected in milk (Heeschen and Suhren 1996a). Although the adherence of withdrawal time is emphasized, antimicrobial drug residues occasionally occur in milk. It may be explained by various reasons, e.g., failure to observe withdrawal time, overdose, or drug misuse.

Antibiotic residues in milk are of great concern to dairy farmers, milk processors, regulatory agencies, and consumers. β-Lactam residues in milk, besides inhibiting the starter cultures in the production of milk products, can provoke allergic reactions in some hypersensitive individuals (Dewdney et al. 1991; Dayan 1993; Le Breton et al. 2007). To minimize exposure of humans to β-lactam antibiotics, maximum residue limit (MRL) values were established in the European Union Regulation 2377/90 (Council Regulation 1990) and subsequent modifications and amendments. For milk, these values ranged from 4 to 125 ng/ml, depending on the specific nature of the β-lactam antibiotics.

Systematic control of antibiotic residues includes analysis and selection of a large number of samples and requires a wide range of screening methods. Samples that are positive or suspect as to the presence of residues are further analyzed using more sophisticated methods, which allow the identification and quantification of residues (HPLC, GC, and GC/MS).

Screening tests must satisfy the following requirements: they must detect antibiotics of interest such as β-lactams in

the dairy products industry, detection limits must comply with the requirements (MRLs), they must be easy to perform and cost effective, the test results are to be obtained rapidly, and the tests must be standardized (low variability within and between batches/laboratories) (Suhren and Heeschen 1996).

Various screening tests have been developed to detect antibiotic residues at tolerance levels in milk. Microbiological agar diffusion tests are widely used as a standard for screening purposes. One of the limitations of agar diffusion test is that the results are usually obtained only after few hours of incubation. Because of the test principle, i.e., measurement of the inhibition of multiplication (diffusion test) and/or metabolism of the test microorganism (Delvo SP), the microbial inhibitor tests cannot serve as a rapid test when results are required within a few minutes. Alternatively, more rapid screening methods that retain sufficient sensitivity and reliability are called for. Among the most widely used commercially available tests are Delvo-X-press β-lactam II test (enzyme-linked receptor binding assay) and Penzyme S test (enzymatic method based on the inhibition of the DD-carboxypeptidase by β-lactams) that enable obtaining of the results within 7 and 15 min, respectively (Žvirdauskienė and Šalomskienė 2007).

The aim of this study was to compare the performance of screening tests (Delvo SP test, Delvo-X-press β-lactam II test, diffusion method with *Geobacillus stearothermophilus*, and Penzyme S test) in aspects of capability to detect β-lactams at MRL levels permitted in EU (Council Regulation 1990) and agreement between test results in the examination of milk samples collected from the different segments of the milk production chain. The possibility of identifying violative producers whose raw milk with antibiotic residues contaminated all the milk in a bulk was examined by the diffusion method.

Material and Methods

Chemicals and Reagents

Delvo SP test and Delvo-X-press β-lactam II test (Delvo-X-press) were supplied by Gist-Brocades N.V. Delft, The Netherlands. Penzyme S test was supplied by UCB-Bioproducts S.A., Belgium.

Drug standards: ampicillin trihydrate, cloxacillin benzatine, benzilpenicillin procaine (FATRO, Bologna, Italy), and amoxicillin trihydrate (Flamma, Bergamo, Italy).

Stock solutions of referent drugs were prepared in distilled water. The stock solutions were kept at 2–5 °C and used within 1 week. Working solutions were also prepared in distilled water and used on the same day.

Samples

Blank milk for fortified antibiotics residue study was collected and commingled from two milking cows that had not been treated with any drugs for last 3 months. Blank milk was kept frozen in the laboratory and used within 1 month. Fortified milk samples with known concentrations (in ng/g) of the different β-lactams were prepared by adding the antimicrobial working solution to the defrosted blank milk. Fortified milk samples were prepared and tested on the same day.

Milk samples were collected from the following sources (number of samples): farms with >10 milking cows—farm bulk milk tanks (20); farms with <10 milking cows—cans at collecting points (51), bulk milk tanks at collecting points (10), different segments of pickup tanker (68); and pickup tanker—bulk samples (29), bulk milk tank in a dairy (20), pasteurized and sterilized milk from the market (20) (Fig. 1).

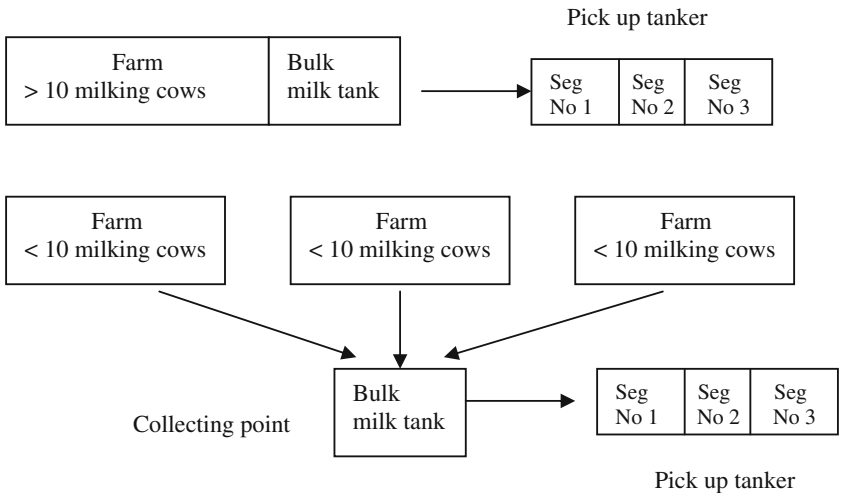

Fig. 1 Sampling scheme

Test Procedure

Delvo SP, Delvo-X-press and Penzyme S tests were used according to the instructions of the manufacturer.

Diffusion test with holes in agar and *G. stearothermophilus* var. *calidolactis* as a test microorganism was performed according to instructions of the Faculty of Veterinary Medicine Belgrade (Katić and Miljković 1996) Test agar pH 7.0 (yeast extract 2.5 g, tryptone 5 g, glucose 1 g, agar 15 g, distilled water aa 1,000 ml) was seeded with *G. stearothermophilus* (25 ml of 18-h-old culture of *G. stearothermophilus* was inoculated in 250 ml of test agar, pH 7.0). Aliquots of 10 ml of the medium were poured into the Petri dishes and left to harden on an even surface. After the medium solidified, six holes (1 cm in diameter) were made in each dish. Raw milk samples were inactivated in water bath (80 °C) for 10 min, and the pH of the milk was adjusted with 0.1 M NaOH to 7 before analysis. Each hole in the medium was filled with 0.1 ml of milk. Each sample was examined in 12 replicates. The plates were kept in a refrigerator for 2 h, and then incubated at 63 °C for 4 h. After incubation, the plates were examined for inhibition zones around the holes, and inhibition zones for all 12 replicates were recorded (2 mm width was considered positive result).

Determination of LOD

The limit of detection (LOD) of the diffusion test was determined by the method recommended by Reichmuth et al. (1997). Series of seven concentrations of each antibiotic were analyzed in 12 replicates. Milk without antibiotics and milk fortified with two to three times higher concentration of antibiotics than the expected LOD were used as negative and positive controls, respectively. The expected LOD was determined in preliminary examinations. Three different concentrations between the negative control sample and expected positive sample were analyzed. The following concentrations were examined (ng/g): penicillin G and ampicillin—0.0, 0.5, 1.0, 1.5, 2.0, 4.0, 5.0; cloxacillin—0.0, 10.0, 20.0, 30.0, 35.0, 40.0, 70.0; amoxicillin—0.0, 0.5, 1.0, 2.0, 2.5, 4.0, 5.0. The results are shown in the form of dose–response curves. For this examination, the LOD is defined as that concentration where 95% of the results were evaluated positive. The place where the line from 95% positive responses cuts the dose–response curve presents the LOD.

Statistical Analysis

Statistical analysis was performed using the Microsoft Office Excel 2000 and statistical program SPSS for Windows 8.0.0. The kappa statistic (κ) was used for the assessment of intertest agreement. Categorization of the strength of agreement for the κ values was as follows: <0.00 poor, 0.00–0.20 slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial, and 0.81–1.00 almost perfect. The interpretation of the results was performed according to the method of Landis and Kock (1977).

Results

Figure 2 demonstrates the results of the examination of the diffusion test sensitivity toward penicillin G in the form of dose–response curves. Concentrations 0.0, 0.5, 1.0, and 1.5 ng/g penicillin G did not have any positive response, whereas the concentrations 2.0, 4.0, and 5.0 ng/g penicillin G had 100% positive responses. For this examination, LODs are defined as those concentrations where 95% of the results were evaluated as positive. The LOD of penicillin G in ng/g can be derived from Fig. 2 as 2 ng/g.

The LODs for other examined antibiotics were determined in the same manner as for penicillin G (Table 1). The examination of blank milk (negative control) did not evaluate a single false-positive result. In the examination of 0.5 ng/g penicillin G, ampicillin, and amoxicillin, no zones of inhibition in all 12 replicates did not appear, as well as in examination of 10 ng/g cloxacillin. The concentrations of 1 ng/g penicillin G, ampicillin, amoxicillin, and 20 ng/g cloxacillin were also evaluated at 100% as negative responses. Positive controls, concentrations two times higher than the LOD were positive in all examined β-lactams in all 12 replicates.

Repeatability of diffusion test results was uniform (CV <30%, and SD less then one third of the average value inhibition zone width) for all examined β-lactams concentrations, except for the concentration of 30 ng/g cloxacillin. High CV was the result of the examination of concentration

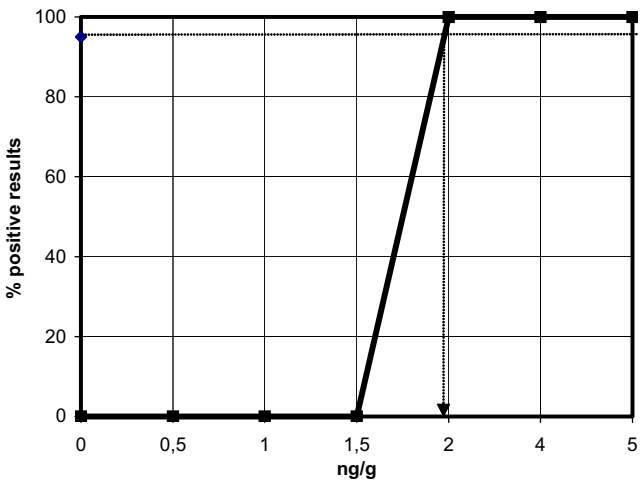

Fig. 2 LOD of diffusion test for penicillin G

Table 1 Response of diffusion test on milk samples fortified with different drugs

β-Lactam	ng/g	Mean±SD	CV
Penicillin G	1.5	1	0
	2	2	0
	4	4.0±0.389	10.44
	5	6	0
Cloxacillin	30	1.5±0.479	36.05
	35	2	0
	40	2.0±0.374	17.95
	70	6	0
Ampicillin	1.5	1.5	0
	2	2	0
	4	4	0
	5	5.0±0.360	6.98
Amoxicillin	2	1	0
	2.5	2	0
	4	4.0±0.374	8.98
	5	6	0

Mean–average value of 12 measurements inhibition zone width in mm.

that was close to LOD (35 ng/g); of 12 examined replicates 4 had positive results (2 mm zones) and 8 replicates had negative results (<2 mm).

Delvo SP, Penzyme S, and Delvo-X-press are screening tests validated and standardized by the manufacturer. Their LODs were proven by analyzing the series of five different concentrations of each β-lactam. Negative and positive control probes were performed in the same manner as for determining the LOD in a diffusion test. Each concentration was examined in three replicates. The lowest concentration of the examined antibiotic that revealed positive results in all three probes was considered LODs. The results are displayed in Tables 2, 3, and 4.

The MRLs established in the European Union Regulation 2377/90 (Council Regulation 1990), LODs determined with fortified milk samples, and LODs established by the manufacturer are shown in Table 5.

Table 2 Response of Delvo SP test on milk samples fortified with different drugs

β-Lactam	Concentration (ng/g)/response				
Penicillin G	0	0.5	2	2.5	8
	− − −	− − −	− − −	+ + +	+ + +
Cloxacillin	0	5	25	30	50
	− − −	− − −	− + +	+ + +	+ + +
Ampicillin	0	0.5	1	2	8
	− − −	− − −	− − +	+ + +	+ + +
Amoxicillin	0	0.5	1	2	8
	− − −	− − −	− − +	+ + +	+ + +

−: negative test result, +: positive test result

Table 3 Response of Penzyme S test on milk samples fortified with different drugs

β-Lactam	Concentration (ng/g)/response				
Penicillin G	0	0.5	3	4	8
	− − −	− − −	− − +	+ + +	+ + +
Cloxacillin	0	5	20	30	140
	− − −	− − −	− − −	+ + +	+ + +
Ampicillin	0	0.5	1	2	8
	− − −	− − −	− + +	+ + +	+ + +
Amoxicillin	0	0.5	2	3	8
	− − −	− − −	− + +	+ + +	+ + +

−: negative test result, +: positive test result

From Table 5, it may be observed that the residues of penicillin G, ampicillin, and amoxicillin can be detected at EU MRLs levels by diffusion, Delvo SP, Penzyme S, and Delvo-X-press tests, whereas cloxacillin must be present at higher amounts to be detected by diffusion and Delvo-X-press tests (LOD above MRL value of 5 ng/g).

Table 5 also implies that all evaluated tests are—with the exception of the Delvo SP test, Delvo-X-press, and cloxacillin—within the range claimed by the manufacturers. It was established that the LODs are 5 ng/g higher than the upper limit stipulated by the manufacturer. The LOD value of 30 ng/g cloxacillin for the Delvo SP test determined in our study corresponds with the detection limit of 22.5–30 ng/g that was established in IDF intralaboratory study (29 laboratories included) (Suhren and Beukers 1998).

Results of the comparative analysis of 70 milk samples collected from different segments of the milk production chain using Delvo SP test, Penzyme S test, and diffusion test are presented in Table 6. Intertest agreement between test results evaluated by kappa statistic for all tests was substantial (κ=0.61–0.80; Fig. 3).

To examine the traceability of positive milk sample, which could contaminate whole bulk milk tank in a dairy, we applied the diffusion test. For examinations carried out in a laboratory, the rapidity of obtaining the results is not as

Table 4 Response of Delvo-X-press test on milk samples fortified with different drugs

β-Lactam	Concentration (ng/g)/response				
Penicillin G	0	0.5	1.5	2	4
	− − −	− − −	− − +	+ + +	+ + +
Cloxacillin	0	5	30	35	50
	− − −	− − −	− + +	+ + +	+ + +
Ampicillin	0	0.5	2	3	8
	− − −	− − −	− − −	+ + +	+ + +
Amoxicillin	0	0.5	2	4	8
	− − −	− − −	− − −	+ + +	+ + +

−: negative test result, +: positive test result

Table 5 LODs of diffusion, Delvo SP, Penzyme S, and Delvo-X-press tests

Test	LOD (ng/g)			
	Penicillin G	Cloxacillin	Ampicillin	Amoxicillin
Diffusion	2	35	2	2.5
Delvo SP	2.5 (2.5[a])	30 (15–25[a])	2 (3–4[a])	2 (3–4[a])
Delvo-X-press	2 (2[a])	35 (30[a])	3 (4[a])	4 (4[a])
Penzyme S	4 (2–4[a])	30 (30–70[a])	2 (3–4[a])	3 (3–4[a])
MRL	4	30	4	4

[a] a-LOD established by the manufacturer

important as in a dairy. Far more important is the reliability and sensitivity of a method. Diffusion test with *G. stearothermophilus*, although having more variations in different countries (disc or wholes in agar, medium in Petri dish or in tube with or without pH indicator) is usually used as a standard method (Wen 1999). A total of 29 samples from the pickup tankers was examined, 4 of which were positive. Pickup tankers collects milk from bulk milk tanks on big farms (>10 milking cows) or from bulk tanks on collecting points where producers which have <10 milking cows brings milk in cans on their own. Depending on the quantity of milk collected, one to three segments of the pickup tanker can be loaded. In four positive bulk samples obtained from the pickup tanker, samples from each particular segment of the pickup tanker (a total of nine samples) were examined. The results of testing four positive pickup tanker bulk samples are displayed in Table 7.

Pickup tankers A, B, and D collect milk from three different big dairy farms (>50 milking cows). Pickup tankers C, E, and F collect milk from farms with <10 milking cows. Samples from these farms were subjected to further examination. A total of 51 samples were examined, and antibiotics were detected in 3 samples originating from 3 different farms. The first and the second farm supplied milk to pickup tankers E and F where no antibiotic residues were detected. Moreover, the analysis of the milk from each segment of pickup tanker did not reveal the presence of antibiotic residues. Inhibition zones width ranged between 1 and 2 mm in both samples. As the diffusion test is a semiquantitative method, it may be concluded that these two milk samples have contained some insubstantial quantities of antibiotics that were diluted at bulk tank at collection point by mixing with milk without antibiotics. Pickup tanker C collects milk from the third farm. This pickup tanker incorporates two segments. Antibiotic residues were detected in milk sample obtained from tanker C and in a segment of tanker no. 1, whereas they could not be detected in the segment no. 2. On examining milk supplied from all farms, the presence of antibiotic residues in milk was found only in milk supplied by this particular farm from pickup tanker C.

Discussion

The two key features of a screening test for residues are sensitivity and rapidity. The LOD of a microbiological test depends of the innate sensitivity of the test bacterium, pH, and thickness of the growth medium (Petrović 2006). The sensitivity toward all examined β-lactam antibiotics ranged between closely similar values for all four tests.

Results that correspond in this paper with respect to the sensitivity of the diffusion test toward penicillin G 1–4 ng/g (De Santis and Mazzette 1991), cloxacillin 31 ng/g (Ginn et al. 1982; Bishop and White 1984), ampicillin 1–2.5 ng/g (Vilim et al. 1979; Moretain and Boisseau 1989), and amoxicillin 6 ng/g (Oliver et al. 1990) were obtained by several other authors. Our results on sensitivity of the Delvo SP test are in accordance with the results of other researchers. Rogelj and Miklič-Andrejič (2000) established the LOD for penicillin G as being 1.5 ng/g, and Sischo (1996) determined the detection limit of 2 ng/g for amoxicillin and ampicillin. Authors that examined sensitivity of the Penzyme S test obtained similar values for penicillin G 3 ng/g, ampicillin 2–3 ng/g, amoxicillin 3 ng/g, and cloxacillin 55 ng/g (Suhren et al. 1996; Sischo 1996).

Table 6 Results of comparative analysis of milk samples using Delvo SP test, Penzyme S test, and Diffusion test

Agreement/disagreement of test results	Bulk tanks on farm	Bulk tanks on collecting points	Bulk tanks in dairy	Milk from the market
All tests positive	5	0	3	3
All tests negative	12	9	16	15
Total congruent, *n* (%)	17 (85)	9 (90)	19 (95)	18 (90)
Total noncongruent, *n* (%)	3 (15)	1 (10)	1 (5)	2 (10)
Total no. of samples	20	10	20	20

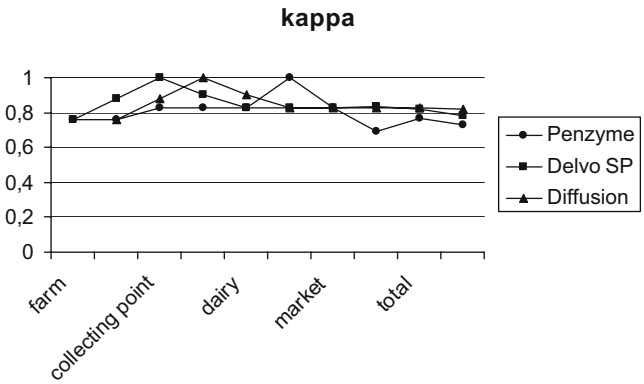

Fig. 3 Kappa statistic

Diffusion and Delvo SP tests are sensitive not only to β-lactam, but also to other antibiotics (Nouws et al. 1999). Penzyme S and Delvo-X-press tests are simple and rapid methods; however, sensitive only to β-lactams. This class of antibiotics is the most commonly applied therapy in dairy cattle. About 90–95% inhibitor positive samples were caused by penicillinase-labile β-lactams (Kress et al. 2007). The Penzyme S test is the better choice because it is faster to run, simpler to use according to the testing procedures, and easier to read. The use of the Delvo-X-press test is comparable to the Penzyme S test, but for evaluating the results the Delvo-X-press Reader device is necessary.

Agreement of diffusion test and Delvo SP test results was almost perfect (κ=0.81–1.00). There is no difference between the microbial tests, but little difference was found between microbial tests and enzymatic tests. Agreement of Penzyme S test with Delvo SP test and diffusion test results was substantial (κ=0.61–0.80). A possible explanation is that milk samples could contain other inhibitors/drugs, not only β-lactams, hence the enzymatic tests fail to detect such substances. A similar conclusion resulted from the research of Žvirdauskienė and Šalomskienė (2007) who compared the microbiological and enzyme tests.

Different results obtained in screening tests mostly result from differences in sensitivity to diverse antibiotics of different LOD values. Results can differ when milk samples are analyzed by different methods even if tests have very similar designs because the LOD could differ in 1, 2, or more ng/g (Suhren and Heeschen 1996). The test may also yield false-positive results. Delvo-X-Press false-positive results were obtained with bovine lactoferrin (≥1 mg/ml), bovine plasma (20% and 40%), and somatic cell count (>10^6/ml) (Angelidis et al. 1999). For a screening test, the absence of false-negative results is more important than the absence of false-positive results because samples positive on screening are retested.

Analysis of milk samples obtained at various sites of the milk production chain revealed a high rate of positive results: only 60% of farm bulk milk tanks were negative (30% were positive in all three tests and 10% differ in results), 10% of bulk milk tanks on collecting points (non-β-lactams drugs, diffusion and Delvo SP test positive, Penzyme S test negative); 15–20% of dairy bulk milk tank samples were positive. Results recorded in Poland correspond to the results obtained in Serbia, ranging from 13.1% to 22.4% (Rubinska et al. 1995); whereas the rate of positive results in Slovenia was significantly lower (0.42%) (Torkar and Teger 1995). Miletić and Popović (1993) in Serbia established the rate of milk samples containing antibiotic residues as being 26.2%. Analysis of pasteurized and sterilized milk revealed the presence of residues in 15–20% samples. Similar data are reported in Poland 10.5–19.5% (Rubinska et al. 1995). The research of Pešić (1990) conducted in 1987–1989 in Serbia established the presence of residues in 5.6–20.4% of samples.

High percentages of milk samples that contain antibiotic residues impose a need for introducing regular milk examination by applying appropriate screening methods. Because a large number of structurally nonrelated compounds with widely varying MRLs could theoretically be present in violative milk samples, the primary requirement of a suitable analytical strategy is the ability to efficiently detect the causative substances. We suggest that the dairy samples be examined by the Delvo SP test because this test is sensitive to many antimicrobial drugs. If the presence of antibiotics residues is proven, it is necessary to retest positive milk samples using the Penzyme S test or Delvo-X-press test because of the high probability that residues of β-lactams are present, and these two tests can prove it. In further examinations, one of these two tests may be used because it is necessary to test great number of the samples and both tests are faster than the Delvo SP test.

By recurrent examining procedure, starting with the milk samples from pickup trucks, bulk milk tanks in collection points, or bulk milk tanks at farm and from cans of small producers, the specific milk that was contaminated by antibiotics can be traced. By this recurrent procedure, it can be traced who is the producer of the milk that could

Table 7 Test results of four positive bulk milk pickup tanker samples

Samples from	Average inhibition zone width (mm)			
	Pickup tanker A	Pickup tanker C	Pickup tanker C	Pickup tanker D
Segment no. 1	3	4	4	0
Segment no. 2	1	3	0	0
Segment no. 3	/	/	/	3
bulk sample pickup tanker	3	3	3	3

0–1 mm: negative result, *>2 mm*: positive result, /: no segments

contaminate the whole bulk milk tank. Further confirmatory testing (HPLC) is not necessary in some countries. According to the German milk quality regulation in routine milk testing, a positive result obtained for bulk tank milk by microbiological inhibitor tests needs no further confirmation, but results in reduced milk payment of 0.05 Euros/kg for 1 month (Nouws et al. 1999).

Detecting the residues of veterinary drugs in raw milk could reduce public health hazard and harmful consequences that appear in milk processing. Applying penalty measures could contribute to developing responsibility of milk procedures when they observe withdrawal period and selling milk and this would reduce the presence of veterinary drug residues in milk.

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