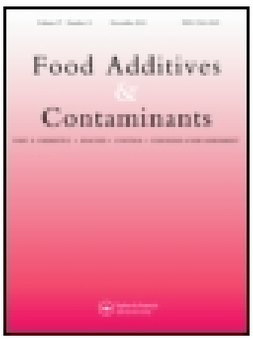

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Analysis of 27 antibiotic residues in raw cow's milk and milk-based products – validation of Delvotest® T

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ABSTRACT

Delvotest® T was evaluated for its capability at detecting residues of 27 antibiotics in raw cow's milk and in some dairy ingredients (skimmed and full-cream milk powders). The kit was used as a screening tool for the qualitative determination of antibiotics from different families in a single test. Results delivered by such a method are expressed as 'positive' or 'negative', referring to the claimed screening target concentration (STC). Validation was conducted according to the European Community Reference Laboratories' (CRLs) residues guidelines of 20 January 2010 and performed by two laboratories, one located in Europe and the other in Asia. Five criteria were evaluated including detection capability at STC, false-positive (FP) rate, false-negative (FN) rate, robustness and cross-reactivity using visual reading and Delvoscan®. STCs were set at or below the corresponding maximum residue limit (MRL), as fixed by European Regulation EC No. 37/2010. Four antibiotics (nafcillin, oxytetracycline, tetracycline and rifaximin) out of 27 had a false-negative rate ranging from 1.7% to 4.9%; however, it was still compliant with the CRLs' requirements. Globally, Delvotest T can be recommended for the analysis of the surveyed antibiotics in raw cow's milk, skimmed and full-cream milk powders. Additional compounds were tested such as sulfamethazine, spiramycin and erythromycin; however, detection at the corresponding MRL was not achievable and these compounds were removed from the validation. Other drugs from the sulfonamide, aminoglycoside or macrolide families not detected by the test at the MRL were not evaluated in this study. Regarding the reliability of this rapid test to milk-based preparations, additional experiments should be performed on a larger range of compounds and samples to validate the Delvotest T in such matrices.

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Validation; rapid test kit; antibiotic residues; raw cow's milk; milk-based products

Introduction

Veterinary drugs are widely used for therapeutic and preventive treatments of infections of food-producing cows (e.g. mastitis). These chemicals also have been used in animal feed to improve feed efficiency. According to the Codex Alimentarius CAC/MISC 5-1993, veterinary drugs are defined as 'any substance applied or administered to any food-producing animal, such as meat or milk producing animals, poultry, fish or bees, whether used for therapeutic, prophylactic, or diagnostic purposes, or for modification of physiological functions or behaviour'. Functionally, veterinary drugs can be divided into six broad classes: antimicrobials (antibiotics), anti-parasites, anti-inflammatory drugs, tranquillisers, drugs with growth promotional effect and others. Antibiotics are inhibitory substances that kill or slow down growth of both bacteria and microbial parasites and represent the largest group of veterinary drugs.

Drug residues can be found in raw cow's milk when inappropriate withdrawal times or when prohibited drugs are used by negligence or fraud. Excessive residues in resulting finished products may thus represent a risk for the health of consumers, including allergic reactions and/or antibiotic resistance.

In Europe, antibiotics (β-lactams, tetracyclines, sulfonamides, aminoglycosides, fluoroquinolones, macrolides etc.) in raw milk are regulated by Commission Regulation (EU) No. 37/2010, typically with MRLs ranging from 4 to 1500 μg kg^{-1}.

Numerous analytical methods have been proposed to detect antibiotic residues in milk and/or milk-based derivatives. The most efficient ones make use of LC-MS/MS and allow the detection of 150–255 compounds at very low LODs (Ortelli et al. 2009; Zhan et al. 2012). However, such an approach still requires expensive instrumentation and specialised analysts. Additionally, a sample workup step is mandatory to extract and

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concentrate the analytes being surveyed. To prevent antibiotics entering the food chain, their detection in food ingredients should ideally be performed as soon as possible. For raw cow's milk, this means at milk collection points such as farms and milk collection centres. For semi-processed dairy ingredients (e.g. skimmed milk), it means at the factory entrance. Consequently, rapid tests with minimal sample preparation and able to screen for a large range of antibiotics are highly desirable. Many test kits are already commercially available. Their different detection principles include either microbiological (Le Breton et al. 2007) or biochemical interactions (Reybroeck et al. 2010). Results can be delivered within less than 10 min with dipstick tests and by up to 3 h with inhibitory tests.

The Delvotest® T from DSM-Food specialties (Delft, the Netherlands) is a broad-spectrum microbiological inhibitor test. It is devoted mainly to β-lactams, tetracycline detection and is also claimed to detect some sulfonamides, aminoglycosides, macrolides and rifamicins in a single test. Other drugs from the sulfonamide, aminoglycoside or macrolide families not detected by the test at the MRL were not evaluated in this study. This test is applicable to fresh raw cow's milk but also to milk ingredients such as skimmed and full-cream milk powders. The Delvotest T kit is a new version of the Delvotest SP-NT with an improved detection capability for tetracyclines. The present paper describes the full validation of the Delvotest T for the qualitative analysis of 27 antibiotics in fresh raw cow's milk, skimmed and full-cream milk powders. Validation according to the Community Reference Laboratory Laboratories Residue (CRLs)' guidelines was conducted by two partners, an ISO 17025-accredited laboratory located in Europe and a dairy factory laboratory in Asia. Five criteria were evaluated including detection capability at the screening target concentration, false-positive rate, false-negative rate, robustness and cross-reactivity.

Materials and methods

Chemicals and reagents

Amoxicillin, ampicillin, cloxacillin, dicloxacillin, nafcillin, oxacillin, penicillin-G, cefazolin, cefoperazone, ceftiofur, cefalexin, cefalonium, cefapirin, doxycycline, oxytetracycline, tetracycline, sulfadiazine, sulfadimethoxine, sulfathiazole, sulfaquinoxaline, sulfamethoxazole, gentamycin, neomycin, rifaximin, sulfadoxine, tylosin, ivermectin and enrofloxacin were obtained from Sigma Aldrich (Fluka, Buchs, Switzerland). Cefacetril was from Chemos GmbH (Regenstauf, Germany). β-Lactam-positive control (4 μg kg^{-1} penicillin-G), sulfonamide-positive control (1000 μg kg^{-1} sulfadiazine) and a negative control were from DSM-Food specialties. Methanol, acetonitrile, water and 0.1 M sodium hydroxide (NaOH) solution were supplied by Merck (Darmstadt, Germany).

Standard solutions

Individual stock standard solutions at 100 or 1000 μg ml^{-1} concentrations were prepared by dissolving each analyte (at least 10 mg) either in water (penicillin and aminoglycoside families), in water–acetonitrile (1:1) (cephalosporin family), in methanol (tetracycline, sulfonamide and macrolide families), in water–methanol (1:1) (rifamycin family), in acetonitrile (avermectin family) or in 1% NaOH (0.1 M) in methanol (fluoroquinolone family). Stock standard solutions were kept at −20°C protected from light for up to 12 months, except for the β-lactam family which was stored for only 1 month. Dilutions at 1 and 0.5 μg ml^{-1} were prepared on a monthly basis in water, except for the β-lactam family which was prepared on a weekly basis. All solutions were allowed warming at RT before use.

Delvotest® T

The Delvotest T is supplied with individual tubes and/or with multi-well microplates filled with an agar medium. In this study, individual tubes were considered along the validation. The agar is pre-seeded with spores of *Geobacillus stearothermophilus* var. *calidolactis* and contains fermentable sugar (glucose) and a pH indicator (bromocresol purple). A total of 100 μl of milk is added onto the surface of the agar and the test is incubated at 64 ± 2°C for 3 hours ± 15 minutes. Incubation time depends on the control time, i.e. the time at which the blank sample turns from purple to yellow. If there is no inhibitory substance in the milk sample or at a concentration lower than the LOD, bacillus spores germinate, grow and acid produced from the fermentation changes the purple colour of the indicator bromocresol in the medium to yellow. Alternatively, if inhibitory substances are present in the sample under test, germination and growth of the bacillus spores are inhibited. No fermentation occurs, leading to no acid production and no change of the bromocresol purple indicator. Colour formation can be read either visually or electronically with a high-resolution scanner controlled by the Delvoscan® software.

Samples

Normal fresh raw cow's milk samples were collected from local farms in Europe and Asia. Composition range was from 0.255 to 0.498 g l^{-1} of fat and from 0.304 to 0.408 g l^{-1} of total nitrogen content. Total germ count (TGC ml^{-1}) was in the 2000–228 000 range, whilst the somatic cell count (SCC ml^{-1}) was from 43 000 to 658 000. Powdered dairy ingredients such as skimmed milk, demineralised whey (DWP), modified sweet whey (MSWP), whey protein concentrate (WPC) and lactose were obtained from different dairy suppliers worldwide. Full-cream milk powders were provided by the Chinese dairy factory. The absence of antibiotics residues in all samples was checked by using the Delvotest T.

Milk sample preparation

Fresh raw cow's milk samples were analysed without any preparation step. Milk powder ingredients were first reconstituted in water before analysis. For skimmed and full-cream milk powders, 3.0 g were weighed in a 50-ml polypropylene tube and then 20.0 ml of water were added. For whey powders, 1.5 g were weighed in a 50-ml Erlenmeyer then 8.5 ml of water were added and subsequently warmed at 40°C. For lactose, 1.0 g was weighed in a 50-ml Erlenmeyer flask then 9.0 ml of water were added and the solution warmed at 40°C. For all powdered dairy ingredients, the slurry was vigorously shaken using a magnetic stirrer for 10 min until lumps disappearance. Reconstituted milk powders and fresh raw cow's milk samples were cooled at 4°C until testing. Spiking fresh raw cow's milk and reconstituted milk powders at screening target concentrations (STCs) was done on the day of analysis.

Validation of the Delvotest T

Validation according to CRLs' guidelines was conducted by two partners: an external ISO 17025-accredited laboratory located in Europe (lab 1) and a dairy factory laboratory located in Asia (lab 2). A β-lactam-positive control (4 µg kg^{-1} penicillin-G), a sulfonamide-positive control (1000 µg kg^{-1} sulfadiazine) and a negative control were systematically included during experiments each working day to ensure the reliability of the kit. Incubation of each individual tube was done using a water bath and a colour reading was performed using a visual and/or a Delvoscan reading. Five criteria were evaluated and included detection capability at the STCs, false-positive rate, false-negative rate, robustness and cross-reactivity.

Table 1. MRLs fixed by European Commission Regulation No. 37/2010 and corresponding screening target concentrations (STCs) set for the validation of the Delvotest® T.

			Lab 1 (Europe)	Lab 2 (Asia)
Family	Compounds	MRL (µg kg^{-1})	STC level (µg kg^{-1})	
β-Lactam (penicillin)	Amoxicillin	4	4	4
	Ampicillin	4	4	–
	Cloxacillin	30	15	15
	Dicloxacillin	30	10	–
	Nafcillin	30	10	–
	Oxacillin	30	30	30
	Penicillin-G	4	2	3
β-Lactam (cephalosporin)	Cefacetril	125	50	–
	Cefazolin	50	10	–
	Cefoperazone	50	40	40
	Ceftiofur	100	30	20
	Cefalexin	100	40	30
	Cefalonium	20	10	–
	Cefapirin	60	10	–
Tetracycline	Doxycycline	Banned[a]	50	–
	Oxytetracycline	100	100	100
	Tetracycline	100	80	80
Sulfonamide	Sulfadiazine	100	50	40
	Sulfadimethoxine	100	50	40
	Sulfathiazole	100	50	40
	Sulfaquinoxaline	100	30	–
	Sulfamethoxazole	100	30	–
	Sulfadoxine	100	–	90
Aminoglycoside	Gentamycin	100	90	90
	Neomycin	1500	200	100
Rifamycin	Rifaximin	60	60	
Macrolide	Tylosin	50	–	50

Note: [a]Banned because it is not to be uses in animals from which milk is produced for human consumption.

Screening target concentration (STC)

According to the CRLs' guidelines, the STC is the concentration at which a screening test categorises the sample as 'screen positive' (potentially non-compliant) and triggers a confirmatory test. For authorised drugs, the STC has to be set at or below the related MRL. At the STC, a spiked/contaminated sample should yield a positive result at least 95% of the time (< 5% of false-negative results). Lab 2 applied the lowest STC level for validation (when possible), which explains some differences in STC level between labs 1 and 2. Table 1 summarises the STCs considered for validation.

False-positive and -negative rates

Milk samples were analysed both unspiked and spiked at the STC level to assess false-positive and -negative rates. According to the CRLs' guidelines, if the STC is set at 50% of the MRL or lower, the number of screen-positive sample must be 20. If the STC is set between 50% and

90% of the MRL, the number of screen-positive sample must be at least 40. If the STC is set between 90% and 100% of the MRL, the number of screen-positive sample must be at least 60. Calculations were based on the following formulae:

$$\text{False positive rate: } \frac{\text{False positives}}{\text{All trully negative samples}} \qquad \text{False negative rate: } \frac{\text{False negatives}}{\text{All trully positive samples}}$$

Related to the CRLs' guidelines, the false-negative rate must have a target value less than 5%. For a false-positive rate, the target value was established internally and should be no higher than 10% for economic reasons (raw product losses). Truly negative samples are blank samples free of antibiotic residues and checked using the Delvotest T. Truly positive samples are samples spiked at the STC level.

Validation scheme

- Lab 1 (Europe): 25 target analytes were analysed by two analysts operating over several days, using three different batches of the Delvotest T. Half the samples were fresh raw cow's milk samples and half were skimmed milk powders. Both visual and Delvoscan reading were considered.
- Lab 2 (Asia): 16 target analytes were analysed by five different analysts operating over several days using one batch of the Delvotest T. One-third of samples were fresh raw cow's milk samples, the second one-third were skimmed milk powders and the last one-third of samples were full-cream milk powders. Visual reading at the control time according to the instructions from the supplier was used.

Robustness

Robustness was performed by lab 1. Three criteria were evaluated including incubation temperature, delay of reading and impact of milk type. All milk samples were analysed as such and spiked at the STC level with four compounds: penicillin-G, cloxacillin, oxytetracycline and sulfadiazine. Interpretation of results was done by both visual and Delvoscan reading at the control time.

Incubation temperature

One fresh raw cow's milk was analysed in five replicates at incubation temperatures (62 and 66°C) above and below that recommended by the supplier (i.e. 64°C).

Delay of reading

Reading time recommended by the supplier at the control time was extended by 15, 30 and 45 min, respectively. For this, individual tubes were removed from the water bath, cooled using a cold bath or water with ice to stop the colour change, and left standing at RT for the time delays given above. Fresh raw cow's milk was considered for these experiments and analysed five times at each time delay.

Impact of milk type

The Delvotest T was tested on 20 different milk-based preparations. Selected powdered samples were four DWPs, three MSWPs, 10 WPCs and three lactose samples.

Cross-reactivity

Cross-reactivity is the extent to which other closely related substances interfere with the test results. A check of this parameter was performed by lab 1 (Europe) for two families not covered by the Delvotest T, typically 'avermectins' and 'fluoroquinolones'. Five fresh raw cow's milk samples were analysed as such and spiked at a high concentration level with one avermectin (ivermectin at 100 µg kg^{-1}) and one fluoroquinolone (enrofloxacin at 1000 µg kg^{-1}). Interpretation of results was done by both visual and Delvoscan reading at the control time.

Results and discussion

Screening target concentration (STC)

All collected STCs for the 27 compounds were set at or below the corresponding MRL (Table 1) by both laboratories. However, the Delvotest T was not able to detect additional compounds such as sulfamethazine, spiramycin and erythromycin with an STC below or equal to the respective MRL. Detection was only possible for sulfamethazine at 150 µg kg^{-1} (MRL = 100 µg kg^{-1}), spiramycin at 685 µg kg^{-1} (MRL = 200 µg kg^{-1}) and erythromycin at 160 µg kg^{-1} (MRL = 40 µg kg^{-1}). These three compounds were not considered for method validation anymore. All other sulfonamides, macrolides and aminoglycosides not tested in this validation were known to be undetectable with the Delvotest T at the MRL, so they were not evaluated in this study.

False-positive and -negative rates

Twenty-three antibiotics out of 27 gave false-positive and -negative rates of 0% when tested at the STC. Four antibiotics (nafcillin, oxytetracycline, tetracycline and rifaximin) had a false-negative rate ranging from 1.7% to 4.9% (Table 2). However, such rates are

Table 2. Results of false-positive (FP) and false-negative (FN) rates for the analysis of 27 antibiotics in cow's milk, skimmed and full-cream milk powders.

Analyte	Performance characteristic (FP or FN rate)	Lab 1 (Europe)			Lab 2 (Asia)	
		Number of samples	Visual reading (%)	Scanner reading (%)	Number of samples	Visual reading (%)
Amoxicillin	FP	60	0	0	30	0
	FN		0	0		0
Ampicillin	FP	60	0	0		–
	FN		0	0		–
Cloxacillin	FP	20	0	0	30	0
	FN		0	0		0
Dicloxacillin	FP	41	0	0		–
	FN		0	0		–
Nafcillin	FP	23	0	0		–
	FN		4.3	4.3		–
Oxacillin	FP	60	0	0	30	0
	FN		0	0		0
Penicillin-G	FP	32	0	0	30	0
	FN		0	0		0
Cefacetril	FP	20	0	0		–
	FN		0	0		–
Cefazolin	FP	23	0	0		–
	FN		0	0		–
Cefoperazone	FP	42	0	0	30	0
	FN		0	0		0
Ceftiofur	FP	23	0	0	30	0
	FN		0	0		0
Cefalexin	FP	27	0	0	30	0
	FN		0	0		0
Cefalonium	FP	23	0	0		–
	FN		0	0		–
Cefapirin	FP	24	0	0		–
	FN		0	0		–
Doxycycline	FP	28	0	0		–
	FN		0	0		–
Oxytetracycline	FP	60	0	0	30	0
	FN		1.7	3.3		0
Tetracycline	FP	41	0	0	30	0
	FN		4.9	0		0
Sulfadiazine	FP	22	0	0	30	0
	FN		0	0		0
Sulfadimethoxine	FP	25	0	0	30	0
	FN		0	0		0
Sulfathiazole	FP	24	0	0	30	0
	FN		0	0		0
Sulfaquinoxaline	FP	24	0	0		–
	FN		0	0		–
Sulfamethoxazole	FP	24	0	0		–
	FN		0	0		–
Gentamycin	FP	60	0	0	60	0
	FN		0	0		0
Neomycin	FP	20	0	0	30	0
	FN		0	0		0
Rifaximin	FP	60	0	0		–
	FN		1.7	1.7		–
Sulfadoxine	FP		–	–	60	0
	FN		–	–		0
Tylosin	FP		–	–	60	0
	FN		–	–		0

still compliant with the CRLs' guidelines. Nafcillin analysis of fresh raw cow's milk gave a false-negative result in both visual (4.3%) and Delvoscan reading (4.3%). For oxytetracycline in raw cow's milk, a false-negative result was obtained in both visual (1.7%) and a Delvoscan reading, whilst when analysing a second raw cow's milk a false-negative result was delivered only when using a Delvoscan reading (3.3%). For tetracycline, two skimmed milk powders gave false-negative results, but only when using a visual reading (4.9%). On the other hand, Delvoscan reading gave no false-negative results. For rifaximin in cow's milk, a false-negative result was obtained using both visual (1.7%) and a Delvoscan reading (1.7%). No correlation of false-negative results with milk composition (TGCs and SCCs, fat and total nitrogen contents) or batch of the Delvotest T could be evidenced.

Robustness

Incubation temperature and delay of reading

A lower (62°C) or a higher (66°C) incubation temperature compared with that recommended (64°C) had no influence on the performance of the Delvotest T since no false-positive or -negative results were observed. Also, reading after delays of 15–45 min did not impact the reliability of the results.

Impact of sample type

Analysis of penicillin-G and cloxacillin in 20 different milk-based preparations did not generate false-negative results and were compliant. False-negative results were only observed for oxytetracycline and sulfadiazine. For oxytetracycline, false-negative results were found at 33% for MSWPs, 60% for WPCs and 25% for DWPs. Regarding sulfadiazine, false-negative results were evaluated at 30% for WPCs and 33% for MSWPs. To validate the reliability of the Delvotest T in such matrices, a deeper investigation should be performed on a larger range of compounds coming from different families and on a larger number of samples.

Cross-reactivity

Analyses of compounds out of the Delvotest T scope did not generate interferences during the analysis. All generated data gave no false-positive and no false-negative results.

Conclusions

To provide customers with safe dairy products, compliance of raw cow's milk and/or milk ingredients can be done using the Delvotest® T. All during the validation it was demonstrated that this multi-residue test was easy to use (no specialised analyst required), cost-effective (no sample preparation), fast in terms of result delivery (3 h) and robust since incubation temperature change and delay of reading did not impact the final result. The Delvotest T was shown to detect 27 antibiotics mainly from the β-lactam and tetracycline families at their European Union MRL, and some sulfonamides, aminoglycosides, macrolides and rifamicins also at their European Union MRL. The test was not able to detect sulfamethazine (sulfonamide family), spiramycin and erythromycin (macrolide family) compounds with an STC below or equal to the respective MRL. All other sulfonamides, macrolides and aminoglycosides not tested in this validation were known to be undetectable with the Delvotest T at the MRL. The applicability of the Delvotest T to milk-derivate-based preparations (DWP, MSWP, WPC, lactose powder) was found to be reliable for the β-lactam family. However, false-negative results were observed for oxytetracycline and sulfadiazine compounds. To validate the reliability of the Delvotest T in such matrices, a deeper investigation should be done including more antibiotics and a higher number of samples.

Disclosure statement

No potential conflict of interest was reported by the authors.

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