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Chemiluminometric β-Galactosidase Detection as a Basis for a Tetracycline Screening Test in Milk**

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The observation that tetracyclines inhibit the biosynthesis of β-galactosidase in *Escherichia coli* to a greater extent than other antibacterials was exploited for the development of a chemiluminometric method to detect residues of this class of antibiotics in milk. The procedure involves the incubation of a milk sample with 10^7 CFU/ml of an *E. coli* strain in the presence of IPTG, an inducer of β-galactosidase, and of EGTA, a chelator of calcium ions, followed by a 1000-fold dilution and measurement of the residual enzymatic activity using the chemiluminogenic substrate Galacton. Chemiluminometry proved an essential tool in this procedure because the extensive dilution of the sample, necessary to avoid light quenching by turbidity, results in an insufficient level of β-galactosidase activity to be measurable by colorimetry. This tetracycline galactosidase (TG) test has been validated and compared in the field to existing commercial screening assays for antibiotics. Its detection limit for tetracyclines ranges between 40 and 65 μg/kg, which is below the European maximum residue limit (MRL = 100 μg/kg) in milk. No other antibacterials, at concentrations commonly expected in milk, were found to interfere with the TG test. Strategies to avoid false positive reactions possibly arising from very high somatic cell counts will be reported elsewhere.

Keywords: chemiluminescence; β-galactosidase; tetracyclines

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INTRODUCTION

The bioluminescent assay of ATP is becoming an increasingly important tool for the quality control of milk. Examples of its application include the determination of the bioburden, sterility testing and, occasionally, the detection of antibiotic residues (1–3). The latter are undesirable because they promote the development of bacterial resistance, may cause hypersensitivity reactions and interfere with technological processes (4, 5). In Europe, residual concentrations of antibiotics in milk for human consumption should be below the maximum residue limits (MRLs) specified by the European Council (6, 7). In spite of the availability of various assays, some antimicrobials are frequently overlooked at the screening stage (4, 8–11). Several ATP-based bioluminescent tests for the detection of antibiotics in milk and other biological fluids have

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been described (12–17). Most of them focus on penicillin detection but, as ATP measurement is non-selective, other antimicrobial agents will also react, whereas some others will remain undetected at or below MRL level. Other bioluminescent procedures for antibiotic detection suffer from a similar lack of selectivity and/or sensitivity. In the commercially available Valio T102-test (Valio Ltd., Helsinki, Finland) the presence of antibiotics in a milk sample causes a decrease in light output from a bioluminescent test bacterium. However, this method fails to detect certain compounds, e.g. tetracyclines, at MRL level (2, 9, 18).

A method using a bioluminescent bacterium specifically developed for the detection of protein synthesis inhibitors in serum does not distinguish between all classes of antibiotics (19). As early as 1962 it was demonstrated that chloramphenicol and other protein synthesis inhibitors inhibit the *de novo* biosynthesis of inducible enzymes to a greater extent than they inhibit enzyme activity (20). The observation that enzyme biosynthesis, as opposed to enzyme activity, is more affected by toxicants has been exploited for environmental toxicity screening (21–23). The ToxiChromotest (EBPI, Brampton, Ontario, Canada), based on the inhibition of the β-galactosidase biosynthesis in a selected *E. coli* strain, uses a colorimetric measurement of the residual enzyme activity (24). However, this test is inapplicable to milk because the turbidity of the milk matrix would disturb absorbance measurements and dilution would cause the concentration of the test bacterium to fall below the detection limit of the enzyme assay. This obstacle would be likely to be overcome in a chemiluminometric assay of β-galactosidase which is reportedly capable of demonstrating considerably lower activities of β-galactosidase in *E. coli* (25).

This paper reports such a chemiluminometric method specifically for the detection of tetracycline residues in milk. To the best of our knowledge this is the first example of the application of chemiluminescence for this purpose.

MATERIALS AND METHODS

Chemical reagents, test bacterium and milk samples

Chemiluminescent substrates and enhancers were obtained from Tropix Inc. (Bedford, MA, USA). Unless otherwise specified, all chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA). The test bacterium, *E. coli* ATCC 10536, came from the American Type Culture Collection (Rockville, MD, USA). Milk samples were obtained from Belgian farms and from the Faculty of Veterinary Medicine (University of Ghent, Belgium) and were stored at −20°C until analysis. Samples used as controls or for supplementation with antibiotics were pooled milks from a small herd of cows not treated with veterinary drugs for 6 weeks preceding the sampling. Selection was further based on their good microbiological quality and their normal chemical composition and somatic cell number.

Assay procedure

The TG (tetracycline galactosidase) test procedure was optimized with water and milk samples supplemented with antibiotics. To this end, the hydrochloride salts of the three tetracyclines were dissolved in phosphate buffer (0.1 mol/L, pH 4.5). Different dilutions were made in the same buffer so that constant volumes of the standard solutions (100 μL) could be added to a given volume (9.9 mL) of milk to obtain the following final concentrations: 30, 50, 80, 90, 100, 200, 300, 500, 800, 900, 1000 μg/mL. Likewise, other antibacterials were dissolved in water or a suitable solvent or buffer and dilutions were added to the milk as indicated above. The concentrations tested for each compound were in the range 50–1000 μg/kg (Table 1). Critical parameters of the TG test included the nature of the chelator, the concentration of the inducer and the bacterial concentration. The total assay time is about 3 h.

1. Induction step. Equal amounts of the sample, a suspension of *E. coli* (10^7 CFU/ml) and a chelator-inducer mixture were combined and allowed to react in a water bath (37°C) for 90 min, during which the *de novo* β-galactosidase biosynthesis takes place. The inducer-chelator mixture contained isopropyl-β-D-thiogalactopyranoside (IPTG; 0.014%) and ethylene glycol-bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA; 12 mmol/L) in a 50 mmol/L sodium phosphate buffer (pH 6.5). As a chelator to prevent inactivation of tetracyclines by calcium in milk, EGTA was chosen instead of EDTA because the latter also chelates magnesium, an essential co-factor in the enzymatic reaction. The test bacterium was added to milk and buffer in a 'minimal induction

Table 1. Inhibition percentages obtained with the TG test for antibacterials other than tetracyclines, present in milk

	Inhibition (%)										
Concentration (μg/kg)	ERY	SPIRA	TYLO	CAP	GENTA	NEO	STREP	AMPI	SPEC	SULFA	TRIM
1000	14	16	0	56	0	0	0	6	18	0	18
500	12	7	0	18	nt	0	nt	0	7	0	11
100	0	0	0	0	0	0	0	0	0	0	14
50	0	0	0	6	0	0	0	0	0	0	11
0	0	0	0	0	0	0	0	0	0	0	0

Abbreviations: ERY, erythromycin; SPIRA, spiramycin; TYLO, tylosin tartrate; CAP, chloramphenicol; GENTA, gentamicin sulphate; NEO, neomycin sulphate; STREP, dihydrostreptomycin sulphate; AMPI, ampicillin trihydrate; SPEC, spectinomycin dihydropentahydrate; SULFA, sulphadimidine sodium; TRIM, trimethoprim; nt, not tested.

medium' (MIM, pH 7.3), containing the following ingredients (quantities in g/L): K_2HPO_4, 1; NaCl, 5; $MgSO_4.7H_2O$, 0.05; sodium dodecyl sulphate (E. Merck AG, Darmstadt, Germany), 0.05; tryptone (Oxoid, Basingstoke, UK), 1; and $(NH_4)\ H_2PO_4$, 2. This medium, adapted from the low-nutrient medium described previously (25), was specifically designed to combine optimal chemiluminescence characteristics, i.e. low background and minimal light quenching, with the capability to support maximal biosynthesis of *β*-galactosidase.

2. Dilution. After induction, the mixture was diluted 1000-fold with water to prevent light quenching due to milk matrix turbidity.

3. Enzyme assay. To a 400 μL aliquot of the diluted mixture were added 50 μL aliquots of solutions of polymyxin B sulphate (100 μg/mL) and the chemiluminogenic enzyme substrate, Galacton (3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)-tricyclo-[3.3.1.1^{3,7}]decan}-4-yl)phenyl-*β*-D-galactopyranoside; 50 μg/mL). Both solutions were prepared in 250 mmol/L phosphate buffer (pH 6.5), supplemented with $MgCl_2{\cdot}6H_2O$ (10 mmol/L). The resulting mixture was incubated for 45 min in a water bath (37°C). In the course of this incubation the enzyme substrate is cleaved, as described previously for similar chemiluminogenic compounds (26, 27). The membrane permeabilizer polymyxin B promotes the bacterial uptake of the substrate (25).

4. Triggering of light emission and measurements. Light output was measured in an AutoLumat LB 953 (EG & G Berthold, Bad Wildbad, Germany) after addition of 100 μL of enhancer by the pump of the luminometer (1.6 mg Emerald per mL of 0.5 mol/L aqueous piperidine). Light emission was measured over 10 s with a delay time of 2 s and expressed as relative light units (RLUs).

5. Calculations. The enzyme activity of an unknown sample relative to that of a control was expressed by the following calculation: percentage inhibition = (1 − RLUs of the sample/RLUs of the control) × 100. The control contained control milk as well as all reagents but no antibiotic and was processed as the unknown samples. All samples were analysed in triplicate.

RESULTS AND DISCUSSION

Our observation that tetracyclines inhibit the biosynthesis of an inducible enzyme such as *β*-galactosidase essentially confirmed the report by Sypherd (20). However, the finding that other inhibitors of protein synthesis had to occur in at least tenfold higher concentrations to produce a comparable effect was unexpected. For example, oxytetracycline and chloramphenicol yielded a comparable degree of enzyme inhibition in concentrations of 45 and 500 μg/kg milk, respectively. Macrolides, aminoglycosides as well as non-inhibitors of protein synthesis, e.g. *β*-lactams and sulphonamides, had no significant effect in concentrations below 1000 μg/kg (Table 1). Hence, the TG test exhibits selectivity for tetracyclines, at least as far as 'realistic' concentrations of antibiotic residues, encountered in milk, are concerned. Inhibition of *β*-galactosidase was dose-

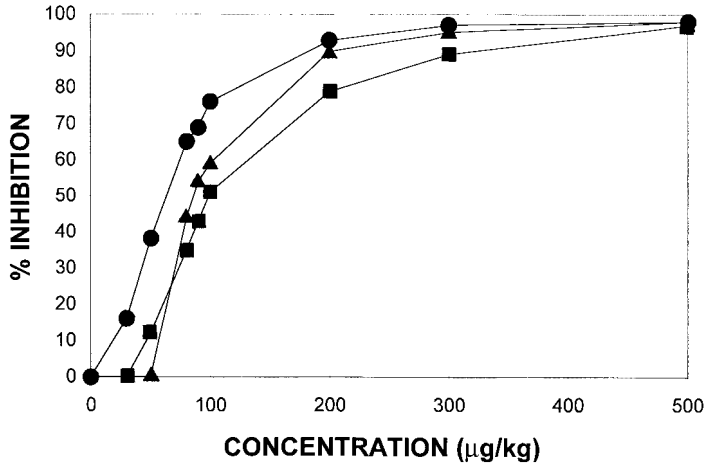

Figure 1. Dose–response curves of oxytetracycline hydrochloride (●), chlortetracycline hydrochloride (▲) and tetracycline hydrochloride (■), spiked in control milk

dependent, as shown in Fig. 1. From the dose–response curves, the detection limits, i.e. the concentrations corresponding to the threshold for positivity (26% inhibition), can be derived. The latter was calculated as twice the relative standard deviation (RSD) on the background light emission from control milk, devoid of antibiotics (RSD = 13%, $n = 40$) (28). Based on this cut-off value, the detection limits were 40 μg/kg (oxytetracycline) and 65 μg/kg (chlortetracycline and tetracycline) respectively. Method validation included the evaluation of the repeatability (RSD = 5.7% for oxytetracycline at 65 μg/kg, $n = 30$) and the between-run precision (3.5%) (28). The assay sensitivity was evaluated using milk samples from cows treated with tetracyclines. The TG test performed equally or sometimes slightly better than commercial screening methods for antibiotics. Elevated somatic cell counts may occasionally result in false positive responses, but these can mostly be eliminated by pre-heating the samples (29).

This method represents a situation where the use of a chemiluminometric rather than a more common colorimetric assay of β-galactosidase is not only justified but even indispensable. Colorimetry would in principle also be capable of measuring the inhibition of β-galactosidase biosynthesis, provided a higher number of test bacteria were used. However, to avoid quenching of light by the turbid milk, extensive dilution before the measurement was required. This would result in a level of β-galactosidase activity that would be too low to be measurable by colorimetry. Chemiluminogenic substrates for β-galactosidase, such as AMPGD (3-(4-methoxyspiro {1,2-dioxetane-3,2′-tricyclo-[3.3.1.1^{3,7}]decan}-4-yl)phenyl-β-D-galactopyranoside) and its derivatives reportedly yield a 1000-fold lower detection limit than their chromogenic counterparts and, hence, can still accommodate the β-galactosidase assay in a low (approximately 10^4/mL) number of cells. The use of the AutoLumat, with its capability of automated enhancer addition and unattended measurement of a series of samples, facilitates the practical application of the test. However, the sample chain was never filled up beyond 50 cuvettes because there was a tendency towards an increase in light output with time. This phenomenon was probably caused by an ongoing enzymatic activity at the relatively elevated temperature of the sample compartment, which could not be cooled. Attempts to reduce residual enzyme activity by pre-cooling the cuvettes in the refrigerator, or by adding an inhibitor of β-galactosidase or bactericidal chemicals, led to an increase in luminescence background. A run with 40–50 samples represented a good compromise between sample throughput and accuracy.

As the TG test can detect tetracyclines below the European MRL, it may be complementary to the existing screening methods, some of which fail to meet this goal. However, no claims regarding its application as a routine test in the dairy industry are made at present. This method is only suitable for screening purposes and is not intended to identify or quantitate tetracyclines. Samples that are found positive for tetracyclines in the Department of

Animal Product Quality and Transformation Technology (Agricultural Research Centre CLO-Ghent) are now regularly retested with this method.

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