A Group-Specific Microbiological Test for the Detection of Tetracycline Residues in Raw Milk

Jussi Kurittu,* Stefan Lönnberg, Marko Virta, and Matti Karp

Department of Biotechnology, University of Turku, Tykistökatu 6A, FIN-20520 Turku, Finland

The potentiality of using a luminescent *Escherichia coli* strain for the specific detection of tetracycline residues in raw bovine milk was investigated. The sensor cells contain a reporter plasmid carrying the bacterial luciferase operon of *Photorhabdus luminescens* under the control of the tetracycline responsive control region from transposon *Tn10*. Incubation of the cells with the sample containing tetracyclines increases the light emission of the sensor cells. The most sensitive tetracycline detection was achieved in 120 min and by using CDTA as a chelating agent in the assay. Heat-treatment of milk before the assay decreased the variations in background luminescence signals and in tetracycline-induced luminescence between different milk samples. The detection limits for tetracycline, oxytetracycline, chlortetracycline, doxycycline, methacycline, demeclocycline, and minocycline were between 2 and 35 ng/mL. Nontetracycline antibiotics did not significantly interfere with the detection of tetracyclines.

Keywords: *Tetracycline, luminescence, luxCDABE, luciferase, antibiotic residue*

INTRODUCTION

Tetracycline antibiotics are widely used in animal husbandry for the treatment of bacterial infections and in some countries at subtherapeutic levels as feed additives to enhance growth of food-producing animals. Use of tetracyclines has been intensive mainly due to their activity against a wide range of pathogens, low price, and relatively low toxicity (Chopra et al., 1992; Standiford, 1995; Roberts, 1996).

However, use of tetracyclines and other antibacterials in the farming industry has certain drawbacks: antibiotic residues in food products, such as in milk, may provoke allergic symptoms in humans, or may lead to monetary losses in the dairy industry, e.g., by inhibiting starter cultures in food technological processes (Heeschen, 1993; Mäyrä-Mäkinen, 1995). Furthermore, residues may promote the development and distribution of bacterial resistance to antibiotics (Roberts, 1996; McManus, 1997). Because of such disadvantages, food products need to be tested for residues before use.

Current methods for the detection of tetracycline residues include microbiological inhibition tests, immunoassays and chemical−physical methods. All the methods available have their characteristic disadvantages: microbiological tests are relatively slow and nonspecific for tetracyclines, whereas immunoassays are usually quite expensive. Drawbacks of chemical−physical methods based on high-priced instrumentation, such as HPLC and MS, include complexity, low amount of samples analyzed per time unit, and requirement of trained personnel. In view of these disadvantages and difficulties, there is a need for new simple, fast, inexpensive, and sensitive assay methods for the detection tetracycline residues.

An emerging concept to detect different organic and inorganic compounds is to use genetically modified bacterial cells, which respond specifically and rapidly to a certain analyte or group of analytes (Lewis et al., 1998). These sensor cells contain a reporter plasmid, in which a compound-specific regulatory unit is coupled to a reporter gene, such as luciferase or β-galactosidase. In this kind of sensing system, the expression of the reporter gene is activated when the cells are exposed to the specific analyte. Gene expression can be sensitively detected, e.g., with electrochemical or luminescent methods depending on the reporter system utilized. Specific sensor strains have been constructed e.g. for heavy metals, organic pollutants and antibiotics (Chopra et al., 1990; King et al., 1990; Virta et al., 1995; Ramanathan et al., 1997; Tauriainen et al., 1998). We have previously constructed an *Escherichia coli* sensor strain for the detection of tetracyclines (Korpela et al., 1998). The sensor plasmid of the strain contains bacterial luciferase operon from *P. luminescens* under the control of the regulatory unit of tetracycline resistance determinant from transposon *Tn10* (Hillen and Berens, 1994). The luciferase operon consists of five genes, *luxC, D, A, B,* and *E*, which are necessary and sufficient for in vivo light production of the *E. coli* cells (Meighen, 1991). In the absense of tetracyclines, the expression of bacterial luciferase genes in the plasmid is repressed, whereas the presence of tetracyclines in the sample induces luciferase gene expression, which can be seen as an increase in the light emission of the sensor cells.

Although several specific sensor bacteria for a wide range of analytes have been developed, no extensive application of these sensors for real environmental or food samples has been described. The principal objective of this study was to investigate whether the previously constructed tetracycline sensor strain, *E. coli* K12-(pTetLux1), could be used to detect tetracycline residues in raw bovine milk, which is commonly known to be a rather problematic sample matrix: it contains large amounts of organic (e.g., proteins and carbohydrates) and inorganic compounds (e.g., calcium), as well as

* To whom correspondence should be addressed. Phone: +358-2-333 8059. Fax: +358-2-333 8050. E-mail: juskur@utu.fi.

10.1021/jf9911794 CCC: $19.00
Published on Web 07/11/2000

somatic cells and bacteria, which are known to disturb analytical assay methods. Therefore, different sample pretreatments have been applied prior to, e.g., microbial residue assays. For example, heat-treatment has been used to denaturate milk proteins (Nouws et al., 1998), and assay sensitivity for tetracyclines has been improved by reducing the availability of divalent cations (Suhren and Heeschen, 1990). Another intention of this research was to clarify to what extent other antibiotics possibly present in the sample interfere with the detection of tetracyclines.

MATERIALS AND METHODS

Chemicals. Tryptone and yeast extract were obtained from Difco (Detroit, MI). CDTA (*trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid), EDTA (ethylenediaminetetraacetic acid), and MES (2-[*N*-morpholino]ethanesulfonic acid) were from Sigma (St. Louis, MO). EGTA (ethylene glycol-*O*,*O*′-bis-(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid) was from Fluka (Buchs, Switzerland). All tetracyclines, gentamicin sulfate, neomycin sulfate, spiramycin, norfloxacin, streptomycin sulfate, penicillin G, cefoperazone, sulfadiazine, and trimethoprim were obtained from Sigma (St. Louis, MO). Chloramphenicol and erythromycin were from Serva (Heidelberg, Germany). All chemicals were of analytical grade.

Milk Samples. All raw bovine milk samples were obtained from different farms in Denmark or from Maito-Aura dairy (Turku, Finland). Maito-Aura dairy milk was from an 80 000 L milk silo. It was used as sample matrix in the development and optimization of the assay concept, because it represented "average raw milk". Raw milk samples from different farms in Denmark were used to study between-sample variation of the assay. Antibiotic-spiked milk samples were prepared as follows: Tetracycline stock solutions (5 mg/mL) were prepared into 0.1 M or 0.01 M HCl or water. Stock solutions (5 mg/mL) of chloramphenicol, spiramycin, erythromycin, and trimethoprim were prepared into 99% EtOH. All other antibiotics were dissolved into water. Further dilutions of all antibiotics were prepared into water if necessary, after which raw milk samples were spiked with proper concentrations of antibiotics. Heat-treatment of milk samples was performed by keeping 1 mL of the samples in 82 °C water bath for 10 min before use in the assay. Heat-treatment was performed after the milk samples were spiked with antibiotics. Oxytetracycline was used as a sample analyte in the development of the assay, because preliminary experiments had revealed that it is the least effective inducer of luminescence of the sensor strain used.

Lyophilization of Sensor Bacteria. Sensor bacteria *E. coli* K12(pTetLux1) were cultivated in Luria-Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter, pH 7) supplemented with 100 μg/mL of ampicillin in a shaker at 30 °C overnight. An inoculum (1:100) from the overnight culture was cultivated to $OD_{600\ nm}$ 1.5 at 37 °C (Korpela et al., 1998). After this, the bacterial suspension was diluted 1:1 with 20% lactose and lyophilized (Janda and Opekarova, 1989; Sidyakina and Golimbet, 1991) in 1 mL aliquots by using a 96 h freeze-drying procedure. Freeze-dried sensor cells were stored at −20 °C before use.

Assay Conditions and Procedure. Lyophilized sensor cells were rehydrated with buffered (100 mM MES; pH 6.0) LB-medium [20 mL LB-medium per cell ampule; 120 min recovery period at room temperature (22°C)]. First, 25 μL of chelating agent solution (CDTA, EDTA, or EGTA) was pipetted into the wells of white microtitration plates (Labsystems, Helsinki, Finland), after which 100 μL of milk sample was added. Finally, 100 μL of recovered sensor cells was added on the milk samples. The microtitration plates were incubated at 37 °C without shaking for 120 min after which the luminescence was measured with Victor 1420 multilabel counter (EG & G Wallac, Turku, Finland). Optimal pH and assay temperature had been determined previously (Korpela et al., 1998). In the optimization of the assay time, luminescence was measured in 30 min intervals (after each luminescence measurement, incubation was continued).

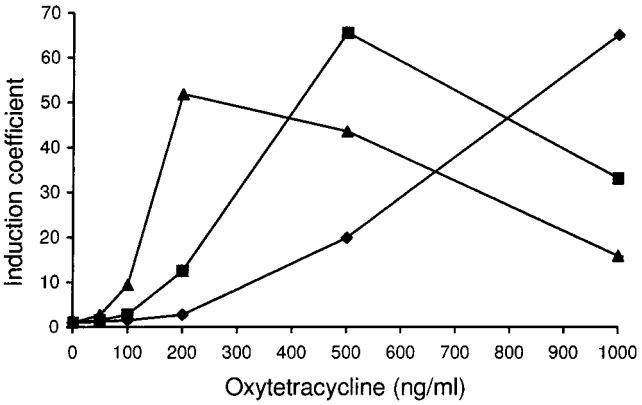

Figure 1. Effect of CDTA on the detection of oxytetracycline in raw milk with *E. coli* K12(pTetLux1) sensor strain. Sensor cells in buffered LB-medium (100 mM MES; pH 6.0) were incubated with milk samples in the presence of different CDTA concentrations for 120 min, after which luminescence was measured. Induction coefficients were calculated as described in the Materials and Methods. Symbols used: ◆ = CDTA 0 mM, ■ = CDTA 10 mM, ▲ = CDTA 25 mM. Standard deviations of two replicas were smaller than the symbols used.

Induction coefficients (IC) were calculated as in vivo luminescence ratio between tetracycline-spiked and blank milk samples, i.e., samples without tetracycline addition: ($IC = I_{spiked}/I_{blank}$).

RESULTS

Development of the Assay Concept in Raw Milk. A very important factor affecting the sensitivity of tetracycline detection was the use of chelator in the reaction mixture. Chelation of divalent cations from milk by chelating agents (CDTA, EDTA, or EGTA) increased the sensitivity of oxytetracycline detection significantly. When CDTA was present in the assay, the induction coefficients obtained with lower concentrations of oxytetracycline (50−200 ng/mL) were higher than in the absence of CDTA (Figure 1). Furthermore, the maximal induction of luminescence was achieved in a lower concentration of oxytetracycline when CDTA was used in the assay (Figure 1). Of the concentrations tested, the optimal CDTA concentration in the assay mixture was 25 mM. Almost identical sensitivity of oxytetracycline detection was achieved with EDTA (compared to CDTA), whereas EGTA had a less pronounced effect on the sensitivity of the assay (data not shown).

An essential factor affecting the performance of the sensor cells was the heat-treatment (82 °C 10 min in water bath) of the milk samples. Heating of the samples before use in the assay decreased the variation of background signal levels of different milk samples considerably (Figure 2). In addition, it was found to efficiently reduce variations in oxytetracycline-induced luminescence between different milks.

Incubation time also had a significant effect on the sensitivity of the assay (Figure 3). The highest induction coefficients and thus the most sensitive oxytetracycline detection was achieved in 120 min, after which no increase in induction coefficients was observed. Results with the other tetracyclines were identical (data not shown).

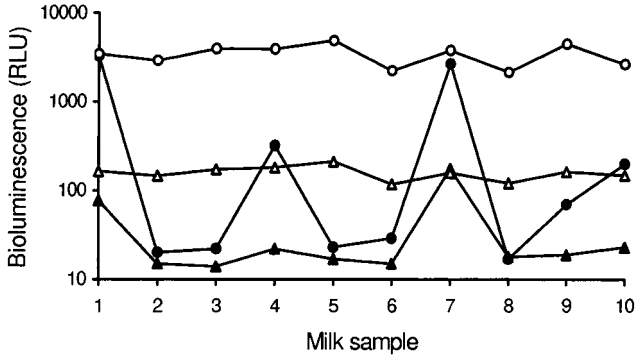

Figure 2. Performance of *E. coli* K12(pTetLux1) sensor strain with heated and nonheated milk samples. Sensor cells in buffered LB-medium (100 mM MES; pH 6.0) were incubated with heated (82 °C 10 min) and nonheated milk samples in the presence of 25 mM CDTA for 120 min, after which luminescence was measured. Symbols used: Triangles = control, nonspiked milk samples. Circles = milk samples spiked with 100 ng/mL oxytetracycline. Open symbols = heated milk samples. Closed symbols = nonheated milk samples.

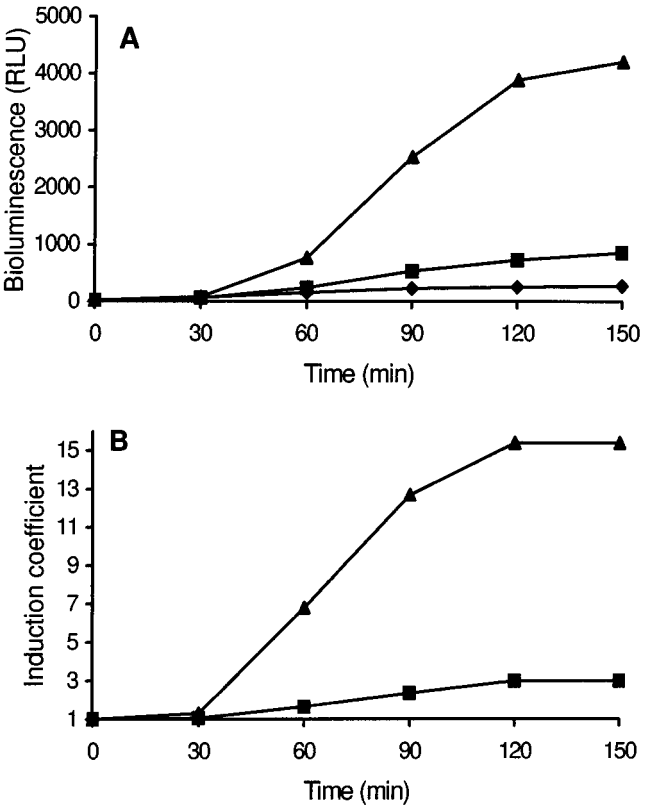

Figure 3. Effect of the incubation time on the detection of oxytetracycline in raw milk with *E. coli* K12(pTetLux1) sensor strain. Sensor cells in buffered LB-medium (100 mM MES; pH 6.0) were incubated with milk samples in the presence of 25 mM CDTA. Luminescence was measured in 30 min intervals. Results are presented as luminescence signals in relative light units (RLU) (A) and as induction coefficients (B). Induction coefficients were calculated as described in the Materials and Methods. Symbols used: (A) ◆ = oxytetracycline 0 ng/mL; ■ = oxytetracycline 50 ng/mL; ▲ = oxytetracycline 100 ng/mL; (B) ■ = oxytetracycline 50 ng/mL; ▲ = oxytetracycline 100 ng/mL. Standard deviations of two replicas were smaller than the symbols used.

Other factors tested (amount of sensor cells in the assay, different buffers) had only minor effects on the sensitivity and performance of the assay (data not shown).

Table 1. Detection Limits of *E. coli* K12(pTetLux1) Sensor Strain for Seven Tetracyclines and European Union Maximum Residue Limits and U.S. FDA Tolerance Limit for Tetracycline Residues in Milk[a]

antibiotic	detection limits (ng/mL) farm milks	detection limits (ng/mL) silo milk	MRL (EU) (ng/mL)[b]	tolerance limit (FDA)[c] (ng/mL)
tetracycline	3−8	6	100	the sum of all
oxytetracycline	12−35	25	100	residues
chlortetracycline	2−4	4	100	<300
doxycycline	2−4	3	0	−
methacycline	7−12	10	−	−
demeclocycline	3−9	5	−	−
minocycline	2−4	3	−	−

[a] Detection limit is defined as luminescence signal average + 3 times standard deviation of milk samples without tetracycline addition (blank milk samples). MRL value 0 for doxycycline means that it is not allowed to be used in animals producing milk for human consumption. The dash (−) means that no MRL or tolerance limit for the antibiotic has been established. [b] MRL values for tetracyclines (parent residue and its 4-epimer) in milk accordance with European Union regulations (EEC) No 2377/90 and (EEC) 281/96. [c] Reference: *J. Am. Vet. Med. Assoc.* **1998**, *213*, 946

On the basis of the results presented above, 25 mM CDTA was used as a chelator and the incubation time was 120 min in all further experiments. Furthermore, milk samples were always heated (10 min 82 °C) before use in the assay. Otherwise, the assays were performed as described in Materials and Methods.

Detection Limits of the Assay for Seven Tetracyclines. The detection limits of the assay for the seven tetracyclines were determined in silo milk from Maito-Aura dairy, which represented average raw milk. Furthermore, detection limits were determined in 10 raw milks collected from different farms in Denmark. The definition of the detection limit was the average luminescence signal of nonspiked blank milk samples (N = 10) plus three times their standard deviation (99% confidence). The results are presented in Table 1 together with European Union Maximum Residue Limits (MRL) and U.S. Food and Drug Administrations Tolerance Limit (sum of all tetracycline residues) for tetracyclines in milk.

In addition, the dose−response curves of the assay for oxytetracycline in 10 different farm milks are shown in Figure 4.

Effect of Other Antibiotics on the Performance of the Assay. The effect of other antibiotics on the detection of tetracyclines was studied by spiking raw milk with oxytetracycline (0, 50 and 100 ng/mL) and with other antibiotics (Table 2). Only protein synthesis inhibitors chloramphenicol and neomycin had a slight effect on the detection of oxytetracycline. However, the concentrations having an inhibitory effect were 1−2 μg/mL, which are rather high to be encountered as residues in milk. None of the nontetracycline antibiotics alone in the sample caused induction of luminescence (data not shown).

DISCUSSION

Milk is a very complex mixture of different components: it contains large amounts of lipids, proteins, vitamins, and minerals, as well as somatic cells and bacteria. These components of milk may have a significant influence on the performance of the test organism. Therefore, the complexity of milk needs to be considered when developing a microbiological assay in milk matrix.

Table 2. Effect of Other Antibiotics on the Detection of Oxytetracycline in Raw Milk with *E. coli* K12(pTetLux1) Tetracycline Sensor Strain[a]

concn (ng/mL)	relative signal (%)										
	G-PEN	CEFO	SULFA	SPIRA	ERY	STR	NEO	GENTA	CM	NOR	TRIM
0	100	100	100	100	100	100	100	100	100	100	100
100	88	96	102	99	109	97	95	94	108	106	106
500	95	93	94	102	95	98	98	98	120	131	103
1000	87	102	95	99	97	105	63	86	72	133	88
2000	79	90	82	95	82	95	54	87	47	145	89

[a] The percentage values show the remaining luminescence signal, when the milk sample contains 100 ng/mL oxytetracycline and a certain concentration of another antibiotic. The first row describes the situation in which there is only 100 ng/mL of oxytetracycline in the sample (this luminescence signal has been given the value 100%). Results obtained with 50 ng/mL of oxytetracycline were identical. Abbreviations: G-Pen, penicillin-G; CEF, cefoperazone; SULFA, sulfadiazine; SPIRA, spiramycin; ERY, erythromycin; STR, streptomycin; NEO, neomycin; GENTA, gentamycin; CM, chloramphenicol; NOR, norfloxacin; TRIM, trimethoprim; concn, concentration.

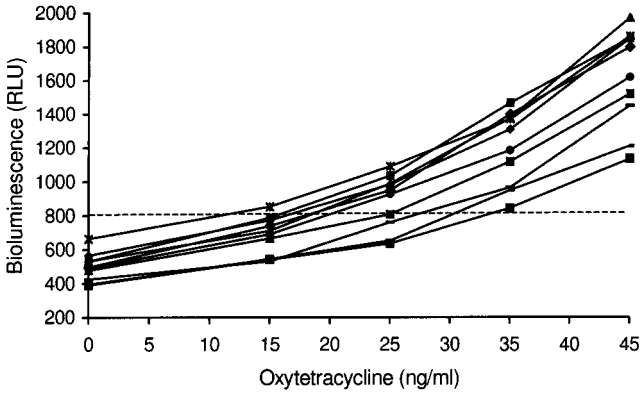

Figure 4. Dose−response curves of *E. coli* K12(pTetLux1) sensor strain for oxytetracycline in 10 different milks. Sensor cells in buffered LB-medium (100 mM MES; pH 6.0) were incubated with milk samples in the presence of 25 mM CDTA for 120 min, after which time the luminescence was measured. Results are presented as relative light units (RLU). Each curve represents one milk spiked with oxytetracycline. The dashed line shows the luminescence signal level used as the definition for the detection limit (average luminescence signal of 10 blank milk samples plus three times the standard deviation). The same data for oxytetracycline is presented as numeric values in Table 1.

There were some important factors that affected the ability of *E. coli* K12(pTetLux1) cells to detect tetracyclines in raw bovine milk. Tetracyclines and divalent cations (in milk mainly Ca^{2+} and Mg^{2+}) form complexes, which are not able to diffuse into the bacterial cells (Yamaguchi et al., 1991; Korpela et al., 1998). Correspondingly, reduction of the concentration of Ca^{2+} and Mg^{2+} ions sensitizes the bacteria to the antibiotic action of tetracyclines (Nanavaty et al., 1998). According to our results, chelation of divalent cations from milk by chelating agents also increased the sensitivity of tetracycline detection significantly (Figure 1). The smaller effect of EGTA to the assay compared to CDTA or EDTA can probably be explained by EGTA's incapability to chelate Mg^{2+} ions. Tetracyclines act by inhibiting protein synthesis of bacteria by binding to ribosomes (Tritton, 1977; Buck and Cooperman, 1990). However, the affinity of tetracyclines is approximately 1000 times higher for the repressor protein that controls the luciferase expression than for ribosomes (Takahashi et al., 1986), which makes the detection of tetracyclines with this sensor system possible. Nevertheless, if the concentration of tetracycline is high enough, luminescence of the sensor cells decreases because of the inhibitory action of tetracyclines. Naturally, in addition to the improvement of the sensitivity of tetracycline detection, the chelating agents also increase the inhibitorial potency of tetracyclines (note that luminescence starts to decrease in a lower concentration of oxytetracycline in the presence of CDTA in Figure 1). Notwithstanding, with all the tetracyclines tested, luminescence did not fall down under the background level in the presence of 25 mM CDTA until at very high concentrations of tetracyclines (1500−3000 ng/mL), which makes the detection range of this sensor system wide and minimizes the occurrence of false negative samples (samples containing very high concentration of tetracycline). Moreover, the dynamic range can be further widened, e.g., by performing the assay with and without chelating agent.

Heat-treatment of the milk samples had a notable effect on the performance of the sensor cells. Heating decreased the variations in the background luminescence signal as well as in the tetracycline-induced luminescence between different milk samples. The plausible reason for this is that heating inactivates some heat-labile components of raw milk, which are inhibitory on the sensor cells (e.g., natural inhibitors like lysozyme and lactoferrin) or may otherwise effect the luminescence of the sensor cells. In microbiological residue assays, in which the milk sample is in direct contact with the test organism, heating of the sample is commonly used to eliminate the effect of natural inhibitors in raw milk (Charm and Ruth, 1993; Suhren and Heeschen, 1993; Nouws et al., 1998).

The complex nature of milk could explain the fact that the composition of bacterial medium (e.g., different buffers etc.) had a minor effect on the assay, probably because proteins, carbohydrates, minerals, and buffering ions present in milk at large quantities overshadow the respective components of the medium. Therefore, extensive testing of different media was not regarded as necessary.

Model analytes from all important antibiotic groups used in the therapy of mastitis and other bacterial infections in dairy cows, as well as commonly known inhibitors of protein synthesis, were selected to investigate the ability of sensor cells to detect tetracyclines in the presence of other antibiotic in the sample. As expected, β-lactams (penicillin G and cefoperazone), which inhibit the cell wall synthesis of bacteria, and antibiotics inhibiting the folate pathway (sulfadiazine, trimethoprim) and DNA replication (norfloxacin) did not interfere with the detection of oxytetracycline at concentrations that could be encountered as residues in raw milk (Table 2). Most importantly, of the protein synthesis inhibitors tested (spiramycin, erythromycin, streptomycin, neomycin, gentamycin, and chloramphenicol), only neomycin and chloramphenicol affected the detection of oxytetracycline by inhibiting oxytetracycline-

induced luminescence − but only at high concentrations (1000−2000 ng/mL). Similar results have been previously reported with the same *E. coli* strain and with a sensor plasmid containing bacterial luciferase gene under the control of heat-inducible promoter/operator region: 50% inhibition of protein synthesis was achieved on the same concentration area of chloramphenicol as reported here (Lampinen et al., 1995). Furthermore, protein synthesis inhibitors in raw milk have been reported to inhibit biosynthesis of β-galactosidase in *E. coli* with almost similar efficiency as presented in this paper (D'Haese et al., 1998). However, even though the maximum inhibition of oxytetracycline-induced luminescence was approximately 50% (e.g., 2000 ng/mL of chloramphenicol 53%), the luminescence signal obtained with 100 ng/mL of oxytetracycline (EU-MRL) was still approximately eight times higher than the background signal level (data not shown). For other tetracyclines, the difference between tetracycline-spiked and blank milk samples would be much higher, because oxytetracycline was found to be clearly the weakest inducer of luminescence. Therefore, other antibiotics present in milk did not significantly disturb the detection of tetracyclines. It is also quite unlikely that other antibiotics at very high concentrations (>1000 ng/mL) would be present in the same raw milk sample with tetracyclines. Furthermore, none of the nontetracycline antibiotics tested induced the luminescence of the sensor cells. This was expected, because the control mechanism of luciferase expression is based on specific recognition of tetracyclines by the TetR repressor protein of the tetA promoter/operator region (Hillen and Berens, 1994).

All measurements were performed by using freeze-dried bacteria, which can be stored at −20 °C at least for a year (our unpublished observations). Lyophilized bacteria are just reconstituted by rehydration, after which they are ready to be used in the measurements almost immediately. From a practical point of view freeze-dried bacteria are similar to any reagent. Furthermore, use of lyophilized sensor bacteria decreases the variations in the assay, because hundreds of homogeneous cell ampules can be obtained from a single batch cultivation of bacteria, and no time-consuming cultivations of bacteria are needed.

Our assay was sensitive enough to detect tetracycline residues in raw bovine milk, the detection limits being below the official limits (European Union Maximum Residue Limits and U.S. Food and Drug Administration Tolerance Limit) for all the seven tetracyclines tested. It provides a fast, specific, simple, and easily automatizable method for qualitative detection of tetracycline residues in raw bovine milk. However, the performance of the assay still needs to be confirmed with milks from cows treated with tetracyclines. We continue to work on getting a comprehensive panel of "naturally contaminated" milk samples for the purpose. Furthermore, the assay calls for collaboratory evaluations and comparisons to officially approved and/or extensively used reference methods.

ABBREVIATIONS USED

CDTA, *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid; CEFO, cefoperazone; CM, chloramphenicol; concn, concentration; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ERY, erythromycin; EtOH, ethanol; EU, European Union; FDA, Food and Drug Administration; GENTA, gentamycin; G-PEN, penicillin G.; HCl, hydrochloride; HPLC, high-performance liquid chromatography; IC, induction coefficient; LB, Luria-Bertani; MES, 2-[*N*-morpholino]ethanesulfonic acid; MRL, maximum residue limit; MS, mass spectrometry; NaCl, sodium chloride; NEO, neomycin; NOR, norfloxacin; RLU, relative light unit; SPIRA, spiramycin; STR, streptomycin; SULFA, sulfadiazine; TRIM, trimethoprim.

ACKNOWLEDGMENT

We express our gratitude to Foss Electric A/S (Hillerød, Denmark) and Maito-Aura dairy (Turku, Finland) for providing the raw milk samples.

LITERATURE CITED

Buck, M. A.; Cooperman, B. S. Single protein omission reconstitution studies of tetracycline binding to the 30S subunit of *Escherichia coli* ribosomes. *Biochemistry* **1990**, *29*, 5374−9.

Charm, S. E.; Ruth, G. P. Advances in Charm technology for antimicrobial residues in milk. *Bull Int. Dairy Fed*. **1993**, *283*, 32−46.

Chopra, I.; Hacker, K.; Misulovin, Z.; Rothstein, D. M. Sensitive biological detection method for tetracyclines using a tetA- lacZ fusion system. *Antimicrob. Agents Chemother*. **1990**, *34*, 111−6.

Chopra, I.; Hawkey, P. M.; Hinton, M. Tetracyclines, molecular and clinical aspects. *J. Antimicrob. Chemother*. **1992**, *29*, 245−77.

D'Haese, E.; Nelis, H. J.; Reybroeck, W. Chemiluminometric beta-galactosidase detection as a basis for a tetracycline screening test in milk. *J. Biolumin Chemilumin* **1998**, *13*, 279−83.

Heeschen, W. H. Residues of antibiotics and sulfonamides in milk: significance and toxicological evaluation, legal situation within the European Community (EEC), and method-related activities of the International Dairy Federation (IDF). *Bull Int. Dairy Fed*. **1993**, *283*, 3−12.

Hillen, W.; Berens, C. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. *Annu. Rev. Microbiol*. **1994**, *48*, 345−69.

Janda, I.; Opekarova, M. Long-term preservation of active luminous bacteria by lyophilization. *J. Biolumin Chemilumin* **1989**, *3*, 27−9.

King, J. M. H.; DiGrazia, P. M.; Applegate, B.; Burlage, R.; Sanseverino, J.; Dunbar, P.; Larimer, F.; Sayler, G. S. Rapid, sensitive bioluminescent reporter technology for naphthalene exposure and biodegradation. *Science* **1990**, *249*, 778−781.

Korpela, M. T.; Kurittu, J. S.; Karvinen, J. T.; Karp, M. T. A recombinant *Escherichia coli* sensor strain for the detection of tetracyclines. *Anal. Chem*. **1998**, *70*, 4457−62.

Lampinen, J.; Virta, M.; Karp, M. Use of controlled luciferase expression to monitor chemicals affecting protein synthesis. *Appl. Environ. Microbiol*. **1995**, *61*, 2981−2989.

Lewis, J. C.; Feltus, A.; Ensor, C. M.; Ramanathan, S.; Daunert, S. Applications of reporter genes. *Anal. Chem*. **1998**, *70*, 579A−585A.

McManus, M. C. Mechanisms of bacterial resistance to antimicrobial agents. *Am. J. Health Syst. Pharm*. **1997**, *54*, 1420−33.

Meighen, E. A. Molecular biology of bacterial bioluminescence. *Microbiol. Rev*. **1991**, *55*, 123−42.

Mäyrä-Mäkinen, A. Technological significance of residues for the dairy industry. In *Symposium on Residues of Antimicrobial Drugs and Other Inhibitors in Milk*; International Dairy Federation: Kiel, Germany, 1995; pp 136−143.

Nanavaty, J.; Mortensen, J. E.; Shryock, T. R. The effects of environmental conditions on the in vitro activity of selected antimicrobial agents against *Escherichia coli*. *Curr. Microbiol.* **1998**, *36*, 212−5.

Nouws, J. F.; Loeffen, G.; Schouten, J.; Van Egmond, H.; Keukens, H.; Stegeman, H. Testing of raw milk for tetracycline residues. *J. Dairy Sci.* **1998**, *81*, 2341−5.

Ramanathan, S.; Shi, W.; Rosen, B. P.; Daunert, S. Sensing antimonite and arsenite at the subattomole level with genetically engineered bioluminescent bacteria. *Anal. Chem.* **1997**, *69*, 3380−4.

Roberts, M. C. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiol. Rev.* **1996**, *19*, 1−24.

Sidyakina, T. M.; Golimbet, V. E. Viability and genetic stability of the bacterium *Escherichia coli* HB101 with the recombinant plasmid during preservation by various methods. *Cryobiology* **1991**, *28*, 251−254.

Standiford, H. C. Tetracycline and chloramphenicol. In *Principles and practise of infectious diseases*; Mandell, G. L., Douglas, R. G., Bennet, J. E., Eds.; Churchill Livingstone: New York, 1995; pp 306−17.

Suhren, G.; Heeschen, W. Improved detection of tetracyclines in milk with a modified microbial receptor assay (Charm test II) and agar diffusion tests. *Milchwissenschaft* **1990**, *45*, 343−347.

Suhren, G.; Heeschen, W. Detection of tetracyclines in milk by a *Bacillus cereus* microtitre test with indicator. *Milchwissenschaft* **1993**, *48*, 259−263.

Takahashi, M.; Altschmied, L.; Hillen, W. Kinetic and equilibrium characterization of the Tet repressor- tetracycline complex by fluorescence measurements. Evidence for divalent metal ion requirement and energy transfer. *J. Mol. Biol.* **1986**, *187*, 341−8.

Tauriainen, S.; Karp, M.; Chang, W.; Virta, M. Luminescent bacterial sensor for cadmium and lead. *Biosens Bioelectron* **1998**, *13*, 931−8.

Tritton, T. R. Ribosome-tetracycline interactions. *Biochemistry* **1977**, *16*, 4133−8.

Virta, M.; Lampinen, J.; Karp, M. A luminescence-based mercury biosensor. *Anal. Chem.* **1995**, *67*, 667−669.

Yamaguchi, A.; Ohmori, H.; Kaneko-Ohdera, M.; Nomura, T.; Sawai, T. Delta pH-dependent accumulation of tetracycline in *Escherichia coli*. *Antimicrob. Agents Chemother.* **1991**, *35*, 53−6.

Received for review November 2, 1999. Revised manuscript received March 30, 2000. Accepted April 26, 2000.

JF9911794