Rev Argent Microbiol. 2016;48(2):143–146

asociación argentina de microbiología aam

REVISTA ARGENTINA DE MICROBIOLOGÍA

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BRIEF REPORT

Novel bioassay using *Bacillus megaterium* to detect tetracycline in milk

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Received 23 November 2015; accepted 23 February 2016
Available online 27 April 2016

KEYWORDS
Tetracyclines;
Milk;
Bacillus megaterium;
Antibiotics;
Detection;
Bioassay

Abstract Tetracyclines are used for the prevention and control of dairy cattle diseases. Residues of these drugs can be excreted into milk. Thus, the aim of this study was to develop a microbiological method using *Bacillus megaterium* to detect tetracyclines (chlortetracycline, oxytetracycline and tetracycline) in milk. In order to approximate the limits of detection of the bioassay to the Maximum Residue Limit (100 μg/l) for milk tetracycline, different concentrations of chloramphenicol (0, 1000, 1500 and 2000 μg/l) were tested. The detection limits calculated were similar to the Maximum Residue Limits when a bioassay using *B. megaterium* ATCC 9885 spores (2.8×10^8 spores/ml) and chloramphenicol (2000 μg/l) was utilized. This bioassay detects 105 μg/l of chlortetracycline, 100 μg/l of oxytetracycline and 134 μg/l of tetracycline in 5 h. Therefore, this method is suitable to be incorporated into a microbiological multi-residue system for the identification of tetracyclines in milk.

PALABRAS CLAVE
Tetraciclinas;
Leche;
Bacillus megaterium;
Antibióticos;
Detección;
Bioensayo

Novedoso bioensayo con *Bacillus megaterium* para detectar tetraciclina en leche

Resumen Las tetraciclinas son utilizadas para la prevención y el control de las enfermedades del ganado lechero; los residuos de estos medicamentos pueden ser excretados en la leche. El objetivo de este estudio fue desarrollar un método microbiológico con esporas de *Bacillus megaterium* para detectar las tetraciclinas en la leche. Con el propósito de aproximar los límites de detección del bioensayo al límite máximo de residuo permitido para tetraciclinas en leche (100 μg/l), se analizaron diferentes concentraciones de cloranfenicol (0, 1.000, 1.500 y

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http://dx.doi.org/10.1016/j.ram.2016.02.001

2.000 μg/l). Los límites de detección son similares a sus respectivos límites máximos de residuos cuando se utiliza un bioensayo con esporas de *Bacillus megaterium* ATCC 9885 (2,8 x 10^8 esporas/ml) y cloranfenicol (2.000 μg/l). Este bioensayo detectó 105 μg/l de clortetraciclina, 100 μg/l de oxitetraciclina y 134 μg/l de tetraciclina en 5 h. Por lo tanto, este método es adecuado para ser incorporado en un sistema microbiológico multirresiduo para la identificación de tetraciclinas en leche.

Tetracyclines (TCs) are antibiotics used for the prevention and control of a variety of infectious diseases. These compounds are active against both gram-negative and gram-positive bacteria[11]. In dairy cattle, TCs are used for the treatment of bacterial enteritis, infectious metritis, colibacillary mastitis and keratoconjunctivitis.

Cows metabolize about 25–50%[13] of tetracyclines administered, and an appreciable amount of these drugs can be excreted into milk. TC residues can cause effects on consumers, such as allergic reactions, liver damage, yellowing of teeth and gastrointestinal disorders[5]. In the dairy industry, TC residues produce changes in the organoleptic characteristics of fermented products[10].

For this reason, control authorities such as the European Union[4] and Codex Alimentarius[3] have recommended a Maximum Residue Level (MRL) of 100 μg/l for chlortetracycline, oxytetracycline and tetracycline in milk.

Antibiotics in milk are widely evaluated using microbiological inhibition methods. Some authors propose the use of *Bacillus cereus* ATCC 11778 in a Petri dish to detect TC residues in milk[2,6,9,12]. These microbiological methods are highly sensitive to TCs but require trained personnel and a prolonged incubation time to measure their response (18–24 h).

In order to decrease the response time of these microbiological methods, Nagel et al.[8] and Tumini et al.[15] recommend the use of bioassays in microtiter plates containing *B. cereus* and *Bacillus pumilus* spores, which reduces the response time (5–6 h). However, it should be noted that *B. cereus* spores present risks for operators because they produce toxins that cause gastrointestinal disturbances[1]. Furthermore, the bioassay developed by Tumini et al.[15] requires the use of a photometric reader to interpret the results.

Therefore, the aim of this work was to design a microbiological inhibition bioassay in microtiter plates using *Bacillus megaterium* with a dichotomous response (positive–negative) indicated by a change in the color of the redox indicator present in the culture medium. This bioassay is economical and easy to implement in a laboratory for the control of residues in milk.

For the bioassay elaboration, Mueller Hinton Agar culture medium (38 g/l, Biokar®, Ref. 10272, France) was fortified with glucose (10 g/l, Sigma Aldrich®, Ref. G8270, St. Louis, MO, USA), brilliant black (200 μg/l Sigma Aldrich®, Ref. 211842, St. Louis, MO, USA) and toluidine blue (10 μg/l of Sigma Aldrich®, Ref. 89640, St. Louis, MO, USA) indicators[15] and *B. megaterium* ATCC 9885 spores (2.8×10^8 spores/ml) at pH 8.5 ± 0.1. These concentrations were obtained by diluting a stock spore suspension of *B. megaterium* (5.6×10^{10} spores/ml) determined by counting with Petrifilm ™ plates (3M, St Paul, MN, USA). The media was fractionated into four aliquots and a chloramphenicol (CAP) solution was added to obtain concentrations of 0, 1000, 1500 and 2000 μg CAP/l in the culture medium. Subsequently, 100 μl of the preparation was added to each microplate well using an electronic dispenser (Eppendorf Research® Pro, Hamburg, Germany). Bioassay plates were sealed and conserved at 4 °C until use. Next, sixteen replicates of twelve concentrations of chlortetracycline (CTC, Sigma C-4881), oxytetracycline (OTC, Sigma O-5750) and tetracycline (TC, Sigma T-3258) were analyzed (0, 40, 60, 80, 100, 120, 140, 160, 180, 200, 300, 500 μg/l), with the aim of obtaining at least two negative results in the lowest concentrations and two positive results at the highest levels. Subsequently, 50 μl of solution containing milk and the corresponding antibiotic concentration was added to each microplate well and left to diffuse into the agar medium for 1 h. The microplate was washed several times with distilled water and incubated in a water floating bath (Dalvo, Santa Fe, Argentina) at 45 ± 1 °C until the color of the negative controls changed (from black to yellow). The visual interpretation was carried out by 3 qualified people, and the test results were evaluated as ''negative'' or ''positive''. ''Ambiguous'' qualifications were considered ''positive''. Since the visual evaluation of the bioassay is an ordinal variable with two dichotomous responses (''negative'' and ''positive''), it is appropriate to use a logistic model to evaluate the data. The results were analyzed using stepwise logistic regression in SAS[14]. The logistic regression model used was the following:

$$L_{ijk} = \text{Logit}[P_{ijk}] = \beta_0 + \beta_1[\text{TCs}]_i + \beta_2[\text{CAP}]_j + \beta_{12}([\text{TCs}] * [\text{CAP}])_{ij} + \varepsilon ijk \quad (1)$$

where L_{ijk} = the dependent or response variable of the linear logistic model; $[P_{ijk}] = [P_p/(1 - P_p)]$ or the ratio of the probability of a ''positive'' response/the probability of a ''negative'' response; $[\text{TCs}]_i$ = effect of tetracycline concentration ($i = 1, 2, \ldots 12$ levels), $[\text{CAP}]_j$ = effect of chloramphenicol concentrations ($j = 0$, 1000, 1500 or 2000 μg/l), $([\text{TCs}]*[\text{CAP}])_{ij}$ = effect of interaction between tetracycline and chloramphenicol concentrations; β_0, β_1, β_2, and β_{12} = coefficients estimated for intercept terms, tetracycline, chloramphenicol and interaction

Table 1 Logistic regression models representing TC and CAP effects on the bioassay response

TCs	$L = \log[P] = \beta_0 + \beta_1[\text{TCs}] + \beta_2[\text{CAP}]$	*C*%
Chlortetracycline	$L = -12.436 + 0.0534^*[\text{CTC}] + 0.0049^*[\text{CAP}]$	88.5
Oxytetracycline	$L = -16.111 + 0.0730^*[\text{OTC}] + 0.0058^*[\text{CAP}]$	93.3
Tetracycline	$L = -12.137 + 0.0570^*[\text{TC}] + 0.0037^*[\text{CAP}]$	90.8

TCs: tetracyclines; CAP: chloramphenicol; *C*%: concordance correlation coefficient.

between tetracycline and chloramphenicol, respectively; and ε_{ijk} = residual error. The detection limits of the bioassay were calculated as the concentration of antibiotic that produces 95% of the positive frequency[7].

The results show that the [CAP] and [TCs] terms were significant for the TCs analyzed ($p < 0.05$); however, their interaction [CAP]*[TCs] was not significant ($p > 0.05$), indicating that CAP produces an antimicrobial effect in the bioassay[8,15]. High ''χ^2'' values for CAP ($\chi^2_{CTC} = 199.02$; $\chi^2_{OTC} = 204.68$; $\chi^2_{TC} = 134.23$) showed that CAP incorporation into the culture medium improves bioassay sensitivity for detecting TCs in milk. The coefficients calculated for the factors found to be statistically significant using the logistics regression model are reported in Table 1. Concordance percentages were adequate (CTC = 88.5%; OTC = 93.3%; TC = 89.8%) and showed good fit to the model. The ''β_1'' coefficient indicates that the increase in the frequency of positive results rise with the TC concentration in milk. These coefficients showed that *B. megaterium* has similar sensitivity to all three antibiotics in milk, since their ''β_1'' values were equivalent ($\beta_{1CTC} = 0.0534$; $\beta_{1OTC} = 0.0730$; $\beta_{1TC} = 0.0570$). The ''β_1'' coefficients evidence the antimicrobial effect of CAP; the values obtained were similar ($\beta_{2CTC} = 0.0049$; $\beta_{2OTC} = 0.0058$; $\beta_{2TC} = 0.0037$), indicating that the CAP's antimicrobial activity acted in a similar manner. Figure 1 represents the dose–response curves elaborated with the coefficients calculated by the logistic regression model (β_0, β_1 and β_2). It depicts the effect of [TC] and [CAP] on the relative frequency of positive results in this bioassay. The frequency of positive results increases as the concentration of antibiotics in the milk increases. The addition of CAP to the culture medium displaces dose–response curves to a lower detection level[8,15]. The detection limits of the bioassay for each tetracycline and different CAP levels (Table 2) were calculated by applying the logistic regression model, using the 95% relative frequency of positive results. Additionally, Table 2 shows the MRLs established by the European Union. Chloramphenicol incorporation into the culture medium (0–2000 μg/l) decreases the TC detection limits of the bioassay (CTC: from 290 to 105 μg/l; OTC: from 260 to 100 μg/l; TC: from 268 to 134 μg/l). The levels obtained are similar to the MRLs established by the previously mentioned legislation (100 μg/l). The traditional microbiological methods developed in Petri dishes require an incubation period of between 18 and 24 h. Using these methods, Nouws et al.[9] report sensitivities of 100 μg/l of TC, 100 μg/l of OTC and 15 μg/l of OTC when using *B. cereus*. In a similar study, Raspor Lainscek et al.[12] determine 100 μg/l for tetracycline, 100 μg/l for oxytetracycline, 80 μg/l for chlortetracycline in milk when using *B. cereus* ATCC 11778 in the STAR protocol. In addition, Gaudin et al.[6] detected higher concentrations for OTC (250 μg/l) and TC (250 μg/l) and good sensitivity

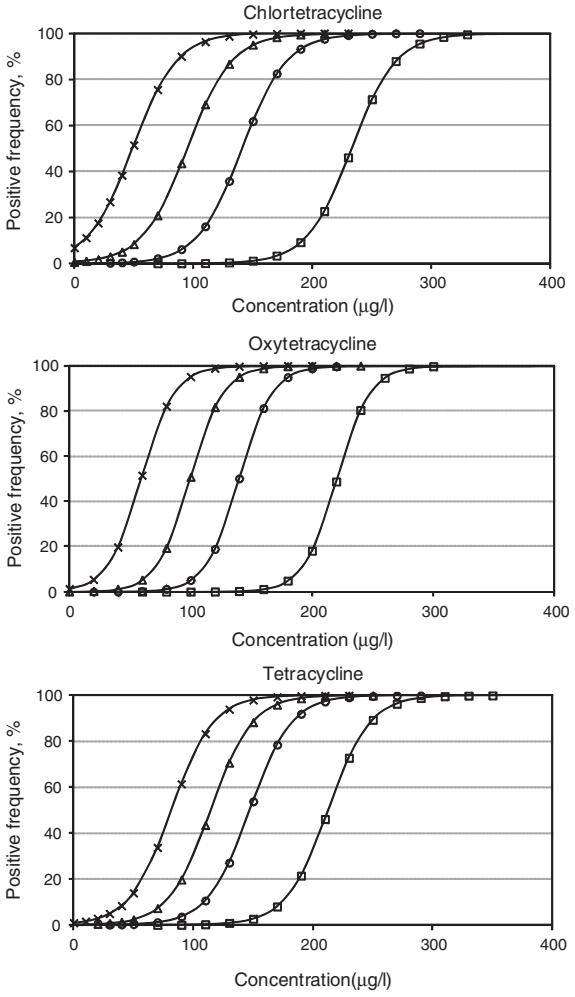

Figure 1 Tetracyclines dose–response curves for different chloramphenicol concentrations (□ CAP: 0 μg/l; ○ CAP: 1000 μg/l; △ CAP: 1500 μg/l; × CAP: 2000 μg/l).

for CTC (50 μg/l). In sheep milk, Althaus et al.[2] obtained low detection limits of tetracycline residues in a Petri dish when using *B. cereus* (DL_{CTC}: 25 μg/l; DL_{OTC}: 75 μg/l; DL_{TC}: 85 μg/l). Subsequently, Nagel et al.[8] optimized a bioassay in microtiter plates using the same bacteria test with 470 μg CAP/l. These authors detected 100 μg/l of OTC and 109 μg/l of TC, but did not detect levels close to the MRL of CTC (300 μg/l). In contrast, the bioassay using *B. megaterium* developed in this work has better sensitivity for the detection of chlortetracycline residues in milk (105 μg/l). Additionally, the detection limits calculated using visual readings of the bioassay developed in this work (105 μg/l of CTC, 100 μg/l of OTC and 134 μg/l of TC) are similar

Table 2 Effect of chloramphenicol on the detection limits (μg/l) of tetracyclines in milk

Tetracyclines	Concentration CAP (μg/l)				MRLs
	0	1000	1500	2000	
Chlortetracycline	290	198	154	105	100
Oxytetracycline	260	182	140	100	100
Tetracycline	268	199	167	134	100

CAP: chloramphenicol; MRLs: Maximum Residue Limits (μg/l).

to those calculated by Tumini et al.[15] when using a photometric reader to interpret the results of a bioassay in microtiter plates using *B. pumilus* spores (DL_{CTC}: 117 μg/l; DL_{OTC}: 142 μg/l; DL_{TC}: 105 μg/l). This microbiological inhibition bioassay using *B. megaterium* spores and 2000 μg/l of chloranphenicol detects adequate levels of tetracycline residues in milk with a 5 h response time. Furthermore, this method provides a dichotomous response that facilitates interpretation of the results. Moreover, this bioassay can be incorporated into a microbiological multi-residue system for the identification of tetracyclines in milk in order to select samples for subsequent unequivocal confirmation of these molecules in high resolution chromatographic techniques such as HPLC-MS–MS.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare that they have no conflicts of interest.

Acknowledgements

This research work has been carried out as part of the CAI + D'11 Projects (PI 501 201101 00575 LI, H.C.D. Resol 205/13 Universidad Nacional del Litoral, Santa Fe, Argentina) and PICT 2011-368 (Res. N° 140/12, Agencia Nacional de Promoción Científica y Tecnológica).

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