



Revista Argentina de Microbiología

ISSN: 0325-7541

ram@aam.org.ar

Asociación Argentina de Microbiología  
Argentina

Tumini, Melisa; Nagel, Orlando G.; Molina, Pilar; Althaus, Rafael L.  
Novel bioassay using *Bacillus megaterium* to detect tetracycline in milk  
Revista Argentina de Microbiología, vol. 48, núm. 2, abril-junio, 2016, pp. 143-146  
Asociación Argentina de Microbiología  
Buenos Aires, Argentina

Available in: <http://www.redalyc.org/articulo.oa?id=213046439010>

- How to cite
- Complete issue
- More information about this article
- Journal's homepage in [redalyc.org](http://redalyc.org)

redalyc.org

Scientific Information System

Network of Scientific Journals from Latin America, the Caribbean, Spain and Portugal

Non-profit academic project, developed under the open access initiative



BRIEF REPORT

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



Melisa Tumini<sup>a</sup>, Orlando G. Nagel<sup>a</sup>, Pilar Molina<sup>b</sup>, Rafael L. Althaus<sup>a,\*</sup>

<sup>a</sup> Cátedra de Biofísica, Departamento de Ciencias Básicas, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, R.P.L. Kreder 2804, 3080 Esperanza, Argentina

<sup>b</sup> Instituto de Ciencia y Tecnología Animal, Universidad Politécnica de Valencia, Camino de Vera 14, 46071 Valencia, Spain

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 ( $2.8 \times 10^8$  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.

© 2016 Asociación Argentina de Microbiología. Published by Elsevier España, S.L.U. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

### 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

\* Corresponding author.

E-mail address: ralthaus@fcv.unl.edu.ar (R.L. Althaus).

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 ( $2,8 \times 10^8$  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 multiresiduo para la identificación de tetraciclinas en leche.

© 2016 Asociación Argentina de Microbiología. Publicado por Elsevier España, S.L.U. Este es un artículo Open Access bajo la CC BY-NC-ND licencia (<http://creativecommons.org/licencias/by-nc-nd/4.0/>).

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<sup>11</sup>. In dairy cattle, TCs are used for the treatment of bacterial enteritis, infectious metritis, colibacillary mastitis and keratoconjunctivitis.

Cows metabolize about 25–50%<sup>13</sup> 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<sup>5</sup>. In the dairy industry, TC residues produce changes in the organoleptic characteristics of fermented products<sup>10</sup>.

For this reason, control authorities such as the European Union<sup>4</sup> and Codex Alimentarius<sup>3</sup> 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<sup>2,6,9,12</sup>. 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.<sup>8</sup> and Tumini et al.<sup>15</sup> 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<sup>1</sup>. Furthermore, the bioassay developed by Tumini et al.<sup>15</sup> 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<sup>®</sup>, Ref. 10272, France) was fortified with glucose (10 g/l, Sigma Aldrich<sup>®</sup>, Ref. G8270, St. Louis, MO, USA), brilliant black (200 µg/l Sigma Aldrich<sup>®</sup>, Ref. 211842, St. Louis, MO, USA) and toluidine blue (10 µg/l of Sigma Aldrich<sup>®</sup>, Ref. 89640, St. Louis, MO, USA) indicators<sup>15</sup> and *B. megaterium* ATCC 9885 spores

( $2.8 \times 10^8$  spores/ml) at pH  $8.5 \pm 0.1$ . These concentrations were obtained by diluting a stock spore suspension of *B. megaterium* ( $5.6 \times 10^{10}$  spores/ml) determined by counting with Petrifilm<sup>™</sup> 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<sup>®</sup> 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  $45 \pm 1$  °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<sup>14</sup>. 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 ( $i = 1, 2, \dots, 12$  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;  $\beta_0$ ,  $\beta_1$ ,  $\beta_2$ , and  $\beta_{12}$  = coefficients estimated for intercept terms, tetracycline, chloramphenicol and interaction

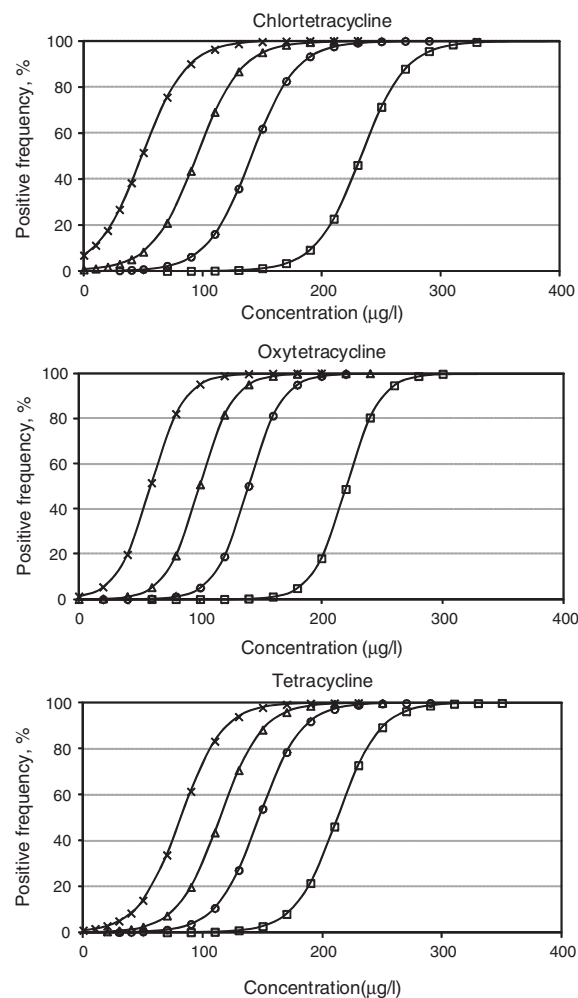
**Table 1** Logistic regression models representing TC and CAP effects on the bioassay response

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

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

between tetracycline and chloramphenicol, respectively; and  $\varepsilon_{ijk}$  = residual error. The detection limits of the bioassay were calculated as the concentration of antibiotic that produces 95% of the positive frequency<sup>7</sup>.

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<sup>8,15</sup>. High " $\chi^2$ " 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 " $\beta_1$ " 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 " $\beta_1$ " values were equivalent ( $\beta_{1CTC} = 0.0534$ ;  $\beta_{1OTC} = 0.0730$ ;  $\beta_{1TC} = 0.0570$ ). The " $\beta_1$ " 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 ( $\beta_0$ ,  $\beta_1$  and  $\beta_2$ ). 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<sup>8,15</sup>. 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  $\mu\text{g/l}$ ) decreases the TC detection limits of the bioassay (CTC: from 290 to 105  $\mu\text{g/l}$ ; OTC: from 260 to 100  $\mu\text{g/l}$ ; TC: from 268 to 134  $\mu\text{g/l}$ ). The levels obtained are similar to the MRLs established by the previously mentioned legislation (100  $\mu\text{g/l}$ ). The traditional microbiological methods developed in Petri dishes require an incubation period of between 18 and 24 h. Using these methods, Nouws et al.<sup>9</sup> report sensitivities of 100  $\mu\text{g/l}$  of TC, 100  $\mu\text{g/l}$  of OTC and 15  $\mu\text{g/l}$  of OTC when using *B. cereus*. In a similar study, Raspor Lainscek et al.<sup>12</sup> determine 100  $\mu\text{g/l}$  for tetracycline, 100  $\mu\text{g/l}$  for oxytetracycline, 80  $\mu\text{g/l}$  for chlortetracycline in milk when using *B. cereus* ATCC 11778 in the STAR protocol. In addition, Gaudin et al.<sup>6</sup> detected higher concentrations for OTC (250  $\mu\text{g/l}$ ) and TC (250  $\mu\text{g/l}$ ) and good sensitivity



**Figure 1** Tetracyclines dose-response curves for different chloramphenicol concentrations ( $\square$  CAP: 0  $\mu\text{g/l}$ ;  $\circ$  CAP: 1000  $\mu\text{g/l}$ ;  $\triangle$  CAP: 1500  $\mu\text{g/l}$ ;  $\times$  CAP: 2000  $\mu\text{g/l}$ ).

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

**Table 2** Effect of chloramphenicol on the detection limits ( $\mu\text{g/l}$ ) of tetracyclines in milk

Tetracyclines	Concentration CAP ( $\mu\text{g/l}$ )				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 ( $\mu\text{g/l}$ ).

to those calculated by Tumini et al.<sup>15</sup> when using a photometric reader to interpret the results of a bioassay in microtiter plates using *B. pumilus* spores ( $DL_{CTC}$ :  $117 \mu\text{g/l}$ ;  $DL_{OTC}$ :  $142 \mu\text{g/l}$ ;  $DL_{TC}$ :  $105 \mu\text{g/l}$ ). This microbiological inhibition bioassay using *B. megaterium* spores and  $2000 \mu\text{g/l}$  of chloramphenicol 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).

## References

1. Abee T, Groot MN, Tempelaars M, Zwietering M, Moezelaar R, Van Der Voort M. Germination and outgrowth of spores of *Bacillus cereus* group members: diversity and role of germinant receptors. *Food Microbiol.* 2011;28:199–208.
2. Althaus R, Berruga M, Montero A, Roca M, Molina M. Evaluation of a microbiological multi-residue system on the detection of antibacterial substances in ewe milk. *Anal Chim Acta.* 2009;632:156–62.
3. Codex Alimentarius, Available online: [ftp://ftp.fao.org/codex/ccrvdf19/rv19\\_06e.pdf](ftp://ftp.fao.org/codex/ccrvdf19/rv19_06e.pdf) Codex committee on residues of veterinary drugs in foods. 9th session of the 30 August–3 September 2010. Discussion paper on methods of analysis for residues of veterinary drugs in foods (CX/RVDF 10/19/6). Vermont, USA; 2010.
4. Council Regulation. Council Directive n° 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Off J Eur Union.* 2009;15:1–72.
5. Fritz JW, Zuo Y. Simultaneous determination of tetracycline, oxytetracycline, and 4-epitetracycline in milk by high-performance liquid chromatography. *Food Chem.* 2007;129:7–1301.
6. Gaudin V, Maris P, Fuselier J, Ribouchon N, Cadieu P, Rault A. Validation of a microbiological method: the STAR protocol, a five-plate test for the screening of antibiotic residues in milk. *Food Addit Contam.* 2004;21:422–33.
7. International Dairy Federation. Guidance for the standardized evaluation of microbial inhibitor test. IDF Standard N° 183. Brussels, Belgium: IDF; 1999.
8. Nagel OG, Molina MP, Althaus RL. Optimization of bioassay for tetracycline detection in milk by means of chemometric techniques. *Lett Appl Microbiol.* 2011;52:245–52.
9. Nouws J, Van Egmond H, Shulders I, Loeffen G, Schouten J, Stegeman H. A microbiological assay system for assessment of raw milk exceeding EU maximum residue level. *Int Dairy J.* 1999;9:85–90.
10. Packham W, Broome M, Limsowtin G, Roginski H. Limitations of standard antibiotic screening assays when applied to milk for cheesemaking. *Aust J Dairy Technol.* 2001;56:15–8.
11. Pastor Navarro N, Morais S, Maquieira A, Puchades R. Review on immunoanalytical determination of tetracycline and sulfonamide residues in edible products. *Anal Chim Acta.* 2009;395:907–20.
12. Raspor Lainscek P, Biasizzo M, Henigman U. Implementation of the *Bacillus cereus* microbiological plate used for the screening of tetracyclines in raw milk samples with STAR protocol – the problem with false-negative results solved. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess.* 2014;31:1840–9.
13. Rysz M, Alvarez P. Amplification and attenuation of tetracycline resistance in soil bacteria: aquifer column experiments. *Water Res.* 2004;38:3705–12.
14. SAS® Institute Inc. SAS users guide: statistics version 9.1. Cary, NC; 2001.
15. Tumini M, Nagel O, Althaus R. Microbiological bioassay using *Bacillus pumilus* to detect tetracycline in milk. *J Dairy Res.* 2015;82:248–55.