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

asociación argentina de microbiología aam

REVISTA ARGENTINA DE MICROBIOLOGÍA

www.elsevier.es/ram

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