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Effect of Chloramphenicol on a Bioassay Response for the Detection of Tetracycline Residues in Milk

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ABSTRACT

The tetracyclines are commonly used in veterinary medicine, and yet the residues are not always detected by microbiological inhibitor tests using *Geobacillus stearothermophilus* subsp. *calidolactis* C-953 at their Maximum Residue Limit levels. In order to improve the sensitivity of these methods, a bioassay was evaluated to study the effect produced by the incorporation of different chloramphenicol (CAP) concentrations in the culture medium. The specificity and detection limits of six tetracyclines in milk were determined. As the levels of CAP increased, a decrease in specificity, from 97.9% (for 0, 50, 200 and 400 μg/kg of CAP) to 88.0% (for 600 μg/kg of CAP) was observed. The logistic regression model indicates a significant effect of the CAP concentration. However, the tetracycline-CAP interaction was not significant, and thus, a synergetic effect can not be considered between the two antimicrobials, with only a simple sum of their antimicrobial effects. When the CAP concentration is increased from 0 to 400 μg/kg, the detection limits of chlortetracycline (590 μg/kg-316 μg/kg), doxycycline (115 μg/kg-62 μg/kg), meclocycline (105 μg/kg-52 μg/kg), oxytetracycline (446 μg/kg-273 μg/kg), rolitetracycline (191 μg/kg-134 μg/kg) and tetracycline (302 μg/kg-158 μg/kg) are observed to decrease.

Key words: tetracycline, chloramphenicol, milk, bioassay, microbiological inhibitor test

INTRODUCTION

Tetracyclines (TCs) are broad-spectrum agents, exhibiting activity against infections caused by both Gram–positive and Gram–negative bacteria, as well as chlamydia, mycoplasmas, rickettsiae, and protozoan parasites.

Oxytetracycline (OTC), chlortetracycline (CTC) and tetracycline (TC) are widely used in the field of veterinary medicine for treating mastitis and metritis in cows. OTC and TC are also frequently added at subtherapeutic concentrations to cattle feeds, primarily, for collective prophylaxis. Other TCs, such as doxycycline (DC), meclocycline (MC) and rolitetracycline (RTC) are not currently used, but may be available commercially[(1)].

Either the inappropriate use of these drugs or the inadequate withdrawal period of treated animals can reveal TCs residues[(1-2)]. One of the most worrying problems associated with TCs residues is the idiosyncratic reactions, especially in hypersensitive consumers[(3-5)]. Like other antibiotics, TCs may be agents for the selection of antibiotic-resistant organisms[(6)]. In addition, some residues can interfere with the starter cultures used in processed milk products[(7)]. Thus, Mäyrä-Mäkinen[(8)] has reported that TC of 0.3 μg/mL and 0.7 μg/mL modified the organoleptic properties of Edam and Emmenthal cheeses, respectively.

To prevent these problems, the European Union (EU)[(9)] and the Codex Alimentarius[(10)] have set a Maximum Residue Limit (MRL) for OTC, CTC and TC (100 μg/kg) in foods of animal origin such as meat, milk, and eggs.

An analytical method for the routine monitoring of TCs residues in milk must be rapid, precise and economical in cost and time[(11)]. The bioassay techniques are widely used as screening methods[(12)], since they are capable of analyzing a large number of samples. In later stages, confirmation and quantification methods are required[(13-16)].

A wide variety of screening methods elaborated by

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different laboratories is available on the market[17-19]. Microbiological inhibition methods using *Geobacillus stearothermophilus* subsp. *calidolactis* C-953 as a biological sensor permit relatively quick responses are significantly remarkable since responses are obtained (about 2.5 to 3.5 hr) due to high temperature incubation (64°C). These microbiological inhibitor tests show tetracycline detection limits above the MRLs, which does not make them suitable for the analysis of this antibiotic group[20-22].

In order to improve the sensitivity of the microbiological methods for the detection of TCs, some authors recommend the use of chelating agents in the culture medium such as glycol-bis (2-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA)[23] or salts of oxalic acid[24-25], as these substances capture the bivalent ions (Ca^{+2}, Mg^{+2}) present in milk. The TCs have a high affinity for these bivalent ions and chelating agent incorporation improve the availability of TCs, thus reducing the detection limits of these microbiological methods.

Adding CAP to the culture medium is another way of improving the sensitivity, since the combined action with TCs produces a more pronounced inhibition of protein synthesis in bacteria. In fact, the TCs interfere with the binding of aa- tRNA with A site of 30S ribosomal subunit[26], which inhibits the elongation of the peptidic chain. In a similar way the CAP interfere tRNA binding to 50S ribosomal subunit[27].

Nouws *et al*.[28] incorporated 100 µg/kg CAP in order to improve the TCs detection limits on the plate in which *Bacillus cereus* is used. Unlike *G. stearothermophilus*, when *B. cereus* is used, the response time is extended to 18-24 hours. Thus, this bioassay is not practical to be implemented as a routine method.

Nevertheless, there are no available studies to investigate the effect of CAP on the sensitivity of *G. stearothermophilus* to TCs in a microtiter-plate test with dichotomous response. Due to this, the aim of this work was to evaluate the incorporation of CAP into the culture medium of a bioassay to determine its antimicrobial action.

MATERIALS AND METHODS

I. *Preparation of the microplates*

The culture medium, made of casein peptone (5 g/L), yeast extract (2.5 g/L), glucose (1 g/L) and agar (15 g/L), was sterilized to 121°C for 15 min, cooled to 50 ± 1°C and the pH was adjusted to 7.0±0.1.

The culture medium was inoculated with a spore suspension of *G. stearothermophilus* subsp. *calidolactis* C-953 (1×10^7 spores/mL, Merck®, Ref. 1.11499) and the bromocresol purple indicator (0.05 mg/mL) was added. The culture medium was divided into aliquots that were fortified with the CAP solution to obtain the following concentrations:

Specificity of bioassay: CAP = 0, 50, 200, 400 and 600 µg/kg. There were four plates for each level of CAP (20 microplates).

Detection limits: CAP = 0, 50, 200 and 400 µg/kg. There were twelve plates for each level of CAP (48 microplates).

For each aliquot, every well of the ELISA microplates was filled with 100 µL of culture medium, sealed with adhesive bands and conserved at 4°C until use.

II. *Specificity of bioassay*

The animals came from cattle herds in Las Colonias (Santa Fe, Argentina). For the specificity study, 192 milk samples taken from drug-free animals were analyzed.

The milk samples selected had a chemical composition and pH values considered as habitual for bovine milk, low somatic cell counts (SCC < 400000 cells/mL) and a bacterial count acceptable for cow milk (CFU < 100000 cfu/mL).

The milk samples were analysed in duplicate on ELISA microplates fortified with the five previously detailed CAP levels (CAP= 0, 50, 200, 400 and 600 µg/kg).

For this purpose, 50 µL of milk samples were added in each individual well of the microplates. It was left at 4°C for one hour to allow the diffusion of the natural inhibitors of the milk to take place. Next, each microplate was washed several times with distilled water, floated in a water bath and then incubated at 64 ± 1°C for 3.5 hours.

Each sample of milk was also analysed in duplicate using the Copan® test (CH ATK) microplate P&S (Chr Hansen, Hoersholm, Denmark), so that their results can be compared with the specificity of the bioassay fortified with different levels of CAP.

A visual interpretation was carried out by 3 qualified people and evaluated as "negative" or "positive". "Doubtful" qualifications were considered "positive"[29].

The specificity of the bioassay was calculated as follows:

Specificity = "negative/total samples"

III. *Detection Limits of Bioassay*

Milk samples were fortified with six TCs: chlortetracycline (CTC), doxycycline (DC), meclocycline (MC), oxytetracycline (OTC), rolitetracycline (RTC) and tetracycline (TC), as detailed in Table 1.

These drugs were stored and handled according to the manufacturers' instructions before use. All dilutions were prepared in 10 mL volumetric flasks at the moment when the analyses were carried out, in order to avoid any possible inconvenience due to solution instability.

The antimicrobial solutions were prepared in one step only from the respective stock solution using antimicrobial-free milk (Copan® test microplate P&S, Chr Hansen, Hoersholm, Denmark). The final drug concentrations in milk (µg/kg) were reached after serial dilutions, in such a way that the volume of the antimicrobial

Table 1. Tetracycline concentrations used in the experiment

TCs	Product number	Concentrations (μg/kg)
CTC	Sigma C-4881	0, 100, 150, 200, 250, 300, 350, 400, 450, 500, 600, 800
DC	Sigma D-9891	0, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140
MC	Sigma M-1388	0, 20, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140
OTC	Sigma O-5750	0, 25, 50, 75, 100, 125, 150, 175, 200, 300, 400, 500
RTC	Sigma R-2253	0, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220
TC	Sigma T-3258	0, 25, 50, 75, 100, 125, 150, 175, 200, 300, 400, 500

CTC: Chlortetracycline, DC: Doxycycline, MC: Meclocycline,
OTC: Oxytetracycline, RTC: Rolitetracycline, TC: Tetracycline.

agent solution did not exceed 1% of the volume of the final solution to be analysed[30-31].

For each tetracycline and CAP level, 16 replicates from each of the twelve concentrations detailed in Table 1 were analysed (2 microplates for each level of CAP, 8 microplates for each tetracycline).

A volume of 50 μL of each solution was used for the bioassay. The temperatures, diffusion and incubation time detailed in the specificity study were used. The visual interpretation was also carried out by 3 qualified people, and the test results were evaluated as "negative" or "positive".

IV. *Statistical Analysis*

As the visual evaluation of the bioassay is an ordinal variable at two dichotomous levels ("negative" and "positive"), it is appropriate to use a logistic model to treat the data[32].

To analyze the results, the stepwise procedure was applied to the logistic regression option of SAS[33], taking the model of those variables that presented a value of Chi-squared ≥ 3.94 to be relevant. The logistic regression model was the following:

$$L_{ijk} = \text{logit}\ [P_{ijk}] = \beta_0 + \beta_1\ [T]_i + \beta_2\ [CAP]_j + \beta_{12}\ ([T][CAP])_{ij} + \varepsilon_{ijk}$$

Where: L_{ijk} = the dependent or response variable of the linear logistic model; $[\ P_{ijk}\ ] = [P_p / (1\text{-}P_p)]$ or the ratio of the probability of "positive" response/the probability of "negative" response; $[T]_i$ = effect of tetracycline concentration (i = 1, 2, . . 12 levels, Table 1), $[CAP]_j$ = effect of CAP concentrations (j = 0, 50, 200 or 400 μg/kg), $([T][CAP])_{ij}$ = effect of interaction between tetracycline and CAP concentrations; β_0, β_1, β_2, and β_{12} = coefficients estimated for terms of intercept, tetracycline, CAP and interaction between tetracycline and CAP, respectively; and ε_{ijk} = residual error.

The concordance coefficient[33] was applied as the rank correlation between the observed responses and predicted probabilities.

The detection limit of the visual interpretation of the bioassay was estimated as the concentration that produces 95% of the "positive" results[29,31].

RESULTS AND DISCUSSION

I. *Specificity of Bioassay*

The results obtained from the analysis of 192 antibiotic-free milk samples are shown in Table 2. It is shown that the addition of 400 μg/kg of CAP in the culture medium does not produce an increase in the frequency of positive results (specificity = 97.9%), obtaining the same specificity as the CH-ATK® Test.

However, if compared with the microplates prepared with 400 μg/kg (23 *vs.* 4), the incorporation of 600 μg/kg of CAP does produce an increase in the frequency of positive results to the bioassay accompanied by an appreciable decrease in their specificity (88.0%). For this reason, the assay of the detection limits of TCs in milk samples was carried out with 400 μg/kg of CAP.

It should be noted that the specificity (97.9%) of

Table 2. Effect of the levels of CAP on the specificity of the bioassay

CAP	Sample number	Negative	Positive	Specificity (%)
CH-ATK® Test	192	188	4	97.9
0 μg/kg	192	188	4	97.9
50 μg/kg	192	188	4	97.9
200 μg/kg	192	188	4	97.9
400 μg/kg	192	188	4	97.9
600 μg/kg	192	169	23	88.0

Specificity: "negative/total samples".

Table 3. Significance of the effects calculated by means of the logistic regression model

Tetracyclines	Factors	"χ^2" value	"p" value
	[CTC]	148.3518	0.0001
Chlortetracycline	[CAP]	97.3408	0.0001
	[CTC]*[CAP]	0.0531	0.8177
	[DC]	104.8903	0.0001
Doxycycline	[CAP]	59.4681	0.0001
	[DC]*[CAP]	0.9314	0.3345
	[MC]	94.4473	0.0001
Meclocycline	[CAP]	58.6459	0.0001
	[MC]*[CAP]	0.9857	0.3208
	[OTC]	149.6728	0.0001
Oxytetracycline	[CAP]	75.8476	0.0001
	[OTC]*[CAP]	0.2845	0.5937
	[RTC]	149.0186	0.0001
Rolitetracycline	[CAP]	63.3352	0.0001
	[RTC]*[CAP]	0.0025	0.9604
	[TC]	113.0115	0.0001
Tetracycline	[CAP]	100.0742	0.0001
	[TC]*[CAP]	0.0637	0.8008

CTC: Chlortetracycline, DC: Doxycycline, MC: Meclocycline, OTC: Oxytetracycline, RTC: Rolitetracycline, TC: Tetracycline, CAP: Chloramphenicol.

microplates fortified with 400 μg/kg of CAP was similar to the values of 98%[34] and 95%[35] reached when the Delvotest® test in cow milk samples.

II. *Detection Limits of Bioassay*

Table 3 summarises the results by applying the logistic regression model. It is shown that the levels of CAP in the culture medium significantly affected ($p < 0.0001$) the response of the bioassay for the six TCs analysed. The interaction of the different TCs with CAP, however, was not significant ($p > 0.05$) in the six studied cases.

This fact indicates that CAP produces an antimicrobial effect in the bioassay, but there is no synergetic action between the two antimicrobial agents, although both antimicrobials participate at tRNA level and impede the synthesis of proteins.

In fact, statistical model points to a simple additive antimicrobial effects of CAP and TCs, as opposed to a synergetic effect, such as, sulphonamides and antifolates (e.g. trimetoprim, tetroxoprim, pirimetamine).

The coefficients obtained for six TCs by means of a logistic regression model are shown in Table 4. The values of "β_1" coefficients indicate the increase in the frequency of positive results as the TC concentration increases in the milk samples. It can be observed that DC ($\beta_1 = 0.0960$) and MC ($\beta_1 = 0.1057$) possess higher values of this coefficient compared with CTC ($\beta_1 = 0.0216$) and OTC ($\beta_1 = 0.0249$). The higher "β_1" coefficient values indicate a smaller increment in their concentrations to produce 100% positive results, while TCs have lower values of the "β_1" coefficients, thereby requiring greater concentrations in order to reach 100% positive results. Similarly, the "β_2" coefficients represent the antimicrobial action caused by the CAP in the culture medium. The fact that the values of these coefficients (Table 4) for the six analysed TCs fell between $\beta_2 = 0.0108$ (OTC) and $\beta_2 = 0.0142$ (MC), indicating that the CAP's antimicrobial action performed in a similar way.

It may be observed in Table 4 that the concordance coefficients were high, ranging between 97.3% (RTC) and 99.0% (TC), which demonstrate the close fit of the model.

Figure 1 shows the effect of the concentrations of TCs and CAP on the relative frequency of positive results as well as the curves constructed by means of the logistic regression model ("β_0", "β_1" and "β_2" coefficients, Table 4). An increase in CAP concentration produces a displacement of the dose-response curve to lower concentrations, improving the sensitivity of the bioassay.

Table 4. Summary of logistic regression model coefficients

Tetracyclines	β_0	β_1	β_2	β_{12}	C
Chlortetracycline	-9.8056	0.0216	0.0148	-	98.2 %
Doxycycline	-8.1365	0.0960	0.0128	-	98.3 %
Meclocycline	-8.1833	0.1057	0.0142	-	98.5 %
Oxytetracycline	-8.1687	0.0249	0.0108	-	98.6 %
Rolitetracycline	-10.1647	0.0685	0.00983	-	97.3 %
Tetracycline	-10.2874	0.0438	0.0158	-	99.0 %

C: concordance coefficients.

Table 5. Effect of the concentration of CAP on the detection limits (μg/kg) of tetracyclines in milk

Tetracyclines	Concentration CAP (mg/kg)				MRLs*
	0	50	200	400	
Chlortetracycline	590	556	453	316	100
Doxycicline	115	109	89	62	-
Meclocycline	105	99	78	52	-
Oxytetracycline	446	425	360	273	100
Rolitetracycline	191	184	163	134	-
Tetracycline	302	284	230	158	100

*MRLs (μg/kg), EU maximum residue limits, CAP: Chloramphenicol.

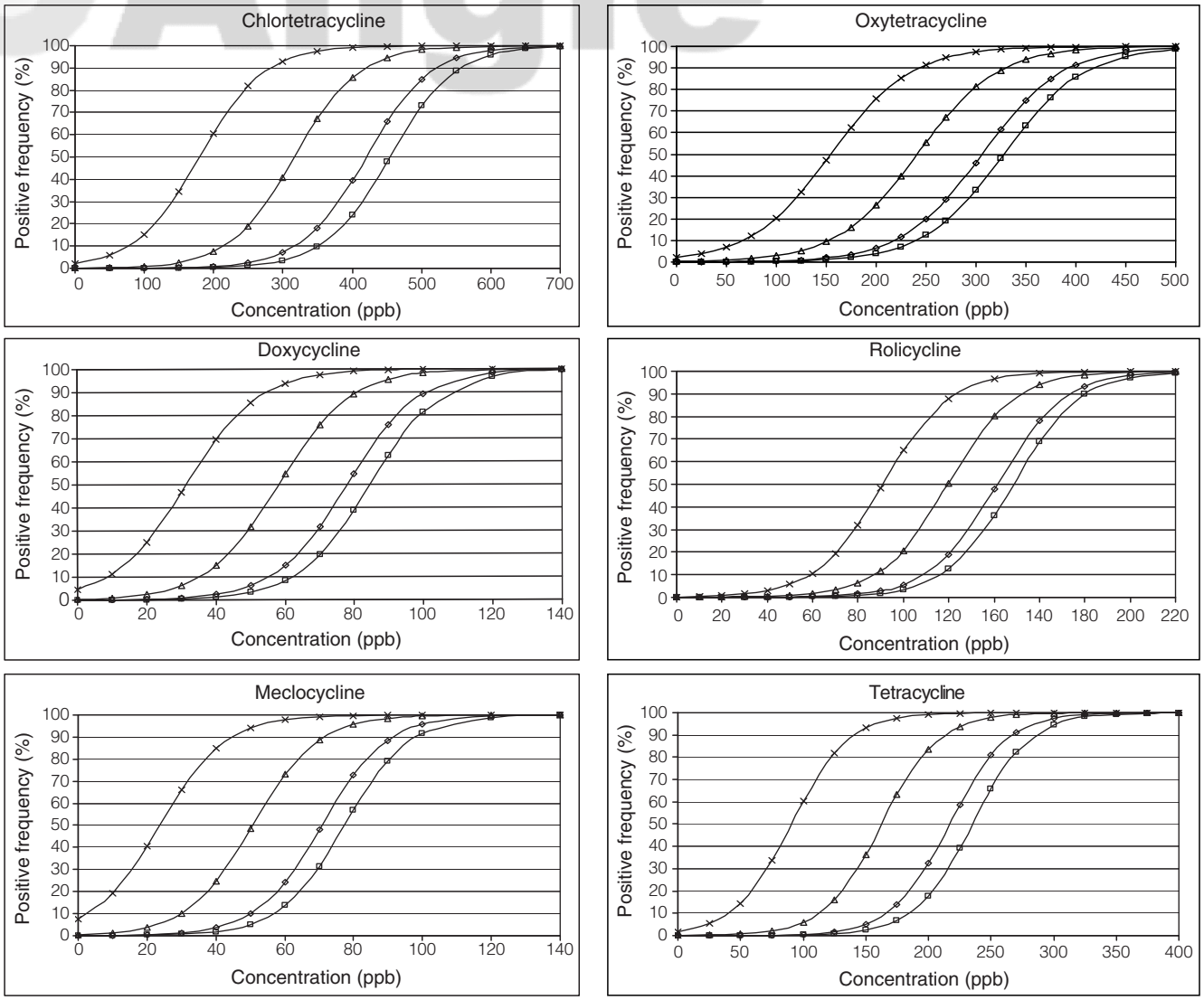

Figure 1. Tetracycline dose-response curves for different chloramphenicol concentrations (□ CAP: 0 μg/mL, ◊ CAP: 50 μg/mL, Δ CAP: 200 μg/mL, X CAP: 400 μg/mL).

The detection limits of the bioassay calculated by means of the logistic model and the 95% relative frequency of positive results[21] are summarised in Table 5, along with the values of the Maximum Residue Limits set out by the European Union (EU-MRLs). The incorporation of CAP into the culture medium decrease the detection limits of six TCs, although this decrease does not reach the values of EU-LMRs.

The detection limits of OTC (273 μg/kg) and TC (158 μg/kg) calculated in the bioassay containing 400 μg/kg of CAP (Table 5) are lower than the 400 μg/kg[36] or 500 μg/kg[37] of OTC and 600 μg/kg[38] of TC determined in the Delvotest® SP microbiological inhibition test using *G. stearothermophilus*.

The BRT® AiM test, wich uses a redox indicator (Brilliant Black) in bioassay response, presents very high detection limits. Frank[39] obtained values of about 5000 μg/kg for OTC and TC residues in cow milk samples.

It should be mentioned that TCs possess a tendency to form chelate complexes with ions of bivalent metals[40]. However, TCs can be displaced from these complexes by the addition of stronger chelating agents such as EGTA[23]. In order to improve the sensitivity of *G. stearothermophilus* to TCs, further studies should be carried out by means of the incorporation of CAP and chelating agents of calcium so that the detection limits could approach those of the Maximum Residue Limits.

CONCLUSIONS

The incorporation of 400 μg/kg of CAP into the culture medium of a bioassay that contains *G. stearothermophilus* diminishes the detection limits of the TCs in

milk without affecting the specificity. Also, such CAP concentrations allow us to achieve detection limits which are near those of the Maximum Residue Limits.

This decrease in the detection limits is due to a sum of the antimicrobial effects of the CAP and TCs, rather than a synergetic effect of the two antimicrobial agents.

In order to approach the Maximum Residue Limits of TCs in milk, further studies should be carried out that contemplate the combined incorporation of CAP and chelating agents into the culture medium of the bioassay.

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