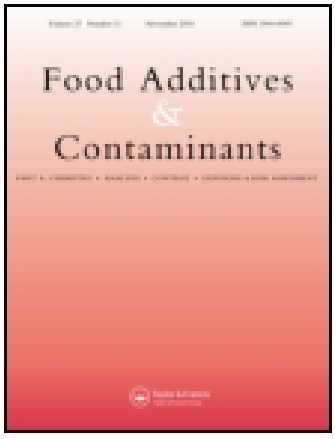


Food Additives & Contaminants: Part A
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tfac20

# Application of a luminescent bacterial biosensor for the detection of tetracyclines in routine analysis of poultry muscle samples

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Published online: 20 May 2010.

To cite this article: M.G. Pikkemaat, M.L.B.A. Rapallini, M.T. Karp & J.W.A. Elferink (2010) Application of a luminescent bacterial biosensor for the detection of tetracyclines in routine analysis of poultry muscle samples, Food Additives & Contaminants: Part A, 27:8, 1112-1117, DOI: 10.1080/19440041003794866

To link to this article: http://dx.doi.org/10.1080/19440041003794866

*Food Additives and Contaminants*
Vol. 27, No. 8, August 2010, 1112–1117

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# Application of a luminescent bacterial biosensor for the detection of tetracyclines in routine analysis of poultry muscle samples

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(*Received 16 February 2010; final version received 20 March 2010*)

Tetracyclines are extensively used in veterinary medicine. For the detection of tetracycline residues in animal products, a broad array of methods is available. Luminescent bacterial biosensors represent an attractive inexpensive, simple and fast method for screening large numbers of samples. A previously developed cell-biosensor method was subjected to an evaluation study using over 300 routine poultry samples and the results were compared with a microbial inhibition test. The cell-biosensor assay yielded many more suspect samples, 10.2% versus 2% with the inhibition test, which all could be confirmed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Only one sample contained a concentration above the maximum residue limit (MRL) of 100 $\mu g\,kg^{-1}$, while residue levels in most of the suspect samples were very low (<10 $\mu g\,kg^{-1}$). The method appeared to be specific and robust. Using an experimental set-up comprising the analysis of a series of three sample dilutions allowed an appropriate cut-off for confirmatory analysis, limiting the number of samples and requiring further analysis to a minimum.

**Keywords:** bioassay; method validation; microbial screening; veterinary drug residues; antibiotics; oxytetracyclin; tetracycline; meat



## Introduction

Veterinary drug use is under major public debate. There is great concern about resistance development against antibiotics, and subsequently the possibility of transfer from animal pathogens or commensals ultimately to human pathogens. This has elicited a reconsideration of the application of certain antibiotics for non-human use, which has, for example, led to the withdrawal of enrofloxacin for use in poultry in the United States (US Food and Drug Administration (USFDA) 2005). The World Health Organization (WHO) has defined a list of antibiotics considered critically important for human treatment (WHO 2005) that should preferably not be used for animal purposes. In Europe, concerns about antibiotic resistance resulted in a ban on the use of antibiotics for growth-promoting purposes (European Commission 2003).

Compared with the resistance threat, the toxicological risks of veterinary drug residues in food products of animal origin, can be considered relatively minor. Nevertheless, to protect the consumer from being exposed to harmful levels of residues, the European Union has set procedures for determining maximum residue limits (MRLs) for antibiotics in food products of animal origin (European Commission 2009). To enforce residue legislation, national monitoring programmes are statutorily required under European Union Directive 96/23 (European Commission 1996). Apart from basic surveillance to safeguard consumers from unwanted levels of residues in their food, effective residue monitoring also serves as an instrument to establish prudent use of antibiotics. Fast and inexpensive high-throughput methodology is a prerequisite for effective enforcement.

Tetracyclines are in many countries the most used veterinary drug group. They are inexpensive broad-spectrum drugs, widely available, and their residues are frequently found in food commodities of animal origin (Okerman et al. 1998; Myllyniemi et al. 1999; Schneider et al. 2009; Pikkemaat et al. 2009). The detection of tetracycline residues in animal matrices can be achieved using a variety of methods. On one end of the spectrum are the physical chemical liquid chromatography-mass spectrometry (LC-MS)-based methods (Oka et al. 2000). These require sophisticated equipment and specialized technicians to operate it, and are generally considered too expensive to be applied for initial screening. At the other end of the

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ISSN 0265–203X print/ISSN 1464–5122 online

DOI: 10.1080/19440041003794866
http://www.informaworld.com

spectrum are the microbiological assays (Pikkemaat 2009). These tests rely on growth inhibition of a sensitive test bacterium. They are very cheap and can be applied with minimal training and equipment. For detection of tetracyclines *Bacillus cereus* ATCC 11778 is the most commonly used bacterium (Bovee and Pikkemaat 2009). The inhibition zones can be considered a semiquantitative result. A major drawback of this type of test is the lengthy incubation (overnight) before the result is obtained.

Recently we reported the development of an assay for the detection of tetracyclines in poultry based on a bioluminescent bacterial sensor as an alternative for 'classical' microbial screening methods (Virolainen et al. 2008). The sensor bacterium is an *Escherichia coli* strain harbouring a plasmid containing a bacterial luciferase operon under the control of a tetracycline sensitive repressor (Korpela et al. 1998). The induction of the *lux* operon, and subsequently the production of a bioluminescent signal, only occurs in the presence of tetracycline. The test is much faster than microbial inhibition tests, as results can be obtained after 3 h, and very inexpensive since it does not require any additional reagents. So far the sensor had been tested on several animal food products, but it had only been tested using spiked materials (Kurittu et al. 2000; Pellinen et al. 2002; Virolainen et al. 2008). To determine the practical applicability in routine monitoring conditions, a large-scale evaluation using field samples was performed, running the test in parallel with the conventional microbial inhibition test.

## Materials and methods

### *Sample material*

A total of 337 poultry samples (chicken, turkey and guinea fowl) originating from a national monitoring programme and other surveillance programmes were analysed. For sample preparation, muscle tissue was chopped in a kitchen blender and transferred to a 15 ml centrifuge tube. After incubating the tube for 20 min at 65°C, it was centrifuged for 10 min at 27,000 *g*. The supernatant (muscle fluid/extract) was transferred to a fresh tube and kept at −20°C until further use.

### *Microbial inhibition test*

The microbial inhibition test for tetracycline is based on growth inhibition of *Bacillus cereus* ATCC 11778. The test plate contained 31.4 g $l^{-1}$ Iso-sensitest agar (Oxoid, Basingstoke, UK) adjusted to pH 6.0. After sterilization the agar was cooled to 48°C, supplemented with 500 µg $l^{-1}$ chloramphenicol and inoculated with approximately $10^5$ colony-forming units (CFU) $ml^{-1}$of the test organism. The inoculated agar was poured in square Petri dishes as a 2.2 mm layer. After solidifying, holes with a diameter of 14 mm were punched in the agar and samples of 250 µl muscle extract were applied. The samples were supplemented with 40 µl of a 1 M phosphate buffer, pH 6.0, containing 12.5 µg $ml^{-1}$ chloramphenicol. After 16–18 h incubation at 30°C the presence of tetracyclines became visible as a zone of growth inhibition around the punch hole. Muscle extract from a sample spiked with 100 µg $kg^{-1}$ oxytetracycline was included in each test plate as a reference.

### *Cell-biosensor assay*

The tetracycline cell-biosensor assay was performed as described by Virolainen et al. (2008). In short, lyophilized sensor cells were reconstituted in Luria broth containing 0.5 µg $ml^{-1}$ polymyxin B for 1 h at room temperature. The reconstituted cells were diluted fourfold in Luria broth containing a 100 mM, pH 6, phosphate buffer and 0.5 µg $ml^{-1}$ polymyxin B. Aliquots of 50 µl were dispensed in white 96-well flat bottom microtitre plates (Greiner Bio-One GmbH, Frickenhausen, Germany). Additionally, the assay contained 150 µl of poultry meat fluid and 25 µl of 225 mM ethylenediamine tetra-acetic acid (EDTA) solution to obtain a final EDTA concentration 25 mM. Each sample was analysed in triplicate. The microtitre plates were incubated at 30°C and 300 rpm. At time points zero and 3 h bioluminescence was read using a Synergy™ HT Multi-detection Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). Because the absolute bioluminescence signal strongly depends on the type of instrument used, results are reported as the ratio between the signal of induced and the non-induced cells. This induction coefficient (IC) was calculated by dividing the average signal after 3 h of incubation by a blank control, a poultry meat fluid sample which previously was found to be free of tetracyclines. Negative samples typically yielded IC values between 0.5 and 1.5, so a sample was considered positive if $IC > 2.0$.

### *Chemical confirmation*

Quantification and identification of the suspect samples from the microbial inhibition test were performed using an in-house optimized and according to Commission Decision 657/2002/EC validated method. Samples were extracted with an EDTA-McIlvain buffer (0.1 M, pH 4.0). The primary extract was transferred to an OASIS™ HLB solid-phase column (Waters, Milford, MA, USA) preconditioned with methanol and buffer. After a washing step with water and buffer, the analytes were extracted from the column using methanol. The methanol was evaporated under a stream of nitrogen. The residues were

reconstituted in acetonitrile/water solution (2/98, v/v). The analytes were separated from the matrix components and detected by using liquid chromatography (LC) in combination with triple quadrupole mass spectrometric detection, using electrospray ionization in the positive mode (LC-ESI(+)QqQ-MS). The LC separation was on a Symmetry® C18 column 5 µm 3.0 × 150 mm (Waters) using a gradient of (solvent A, water 1 mM ammonium acetate pH 2.6; solvent B, 10 mM ammonium acetate pH 2.6/acetonitrile (10/90, v/v): 0–1 min, 0% B; 1–10 min, linear increase to 50% B; 10–14 min, linear increase to 100% B with a final hold of 4 min. For identification the selective reaction monitoring (SRM) mode was used for the specific detection of each analyte of interest. The ions monitored for tetracyclines were $m/z$ 445 > 410 and $m/z$ 445 > 154; for oxytetracycline $m/z$ 461 > 337 and $m/z$ 461 > 201; for doxycycline $m/z$ 445 > 428 and $m/z$ 445 > 154; for chlorotetracycline $m/z$ 479 > 153 and $m/z$ 479 > 444 and for the internal standard demeclocycline $m/z$ 465 > 154. For quantification, a 'detector response' – peak area ratios IS/standard tetracycline – versus a 'tetracycline concentration' plot was constructed. To this end, blank samples were fortified with different concentrations of the tetracyclines (0–250 µg kg$^{-1}$). The collected samples were analysed together with the calibration samples (Matrix Match Standard – MMS); concentrations were calculated using the least-squares linear regression method.

## Results and discussion

### *Microbial inhibition test*

All 337 samples were initially analysed using a microbial inhibition test as part of the routine procedure. Out of the 337 samples, seven (2%) tested positive on the *B. cereus* test plate (Table 1). After LC-MS/MS analysis, the presence of doxycycline was confirmed in all samples. One non-compliant result was found (195 µg kg$^{-1}$); the remainder of the suspect samples contained concentrations between 26 and 50 µg kg$^{-1}$ doxycycline.

In practice, the result of the microbial inhibition test is compared with a quality control (QC) reference sample to visualize day-to-day variation in sensitivity of the test. This control sample is obtained from poultry muscle spiked with 100 µg kg$^{-1}$ oxytetracycline (the MRL). Oxytetracycline is used because it is the least detectable tetracycline residue, so if a sample shows an inhibition zone smaller than the control, it is assumed to be compliant. The positive samples in this study provide an opportunity to evaluate how well spiked samples represent incurred material. Figure 1 shows the concentration of the samples plotted against the inhibition zone (corrected for the QC inhibition), and the result of a concentration series of doxycycline

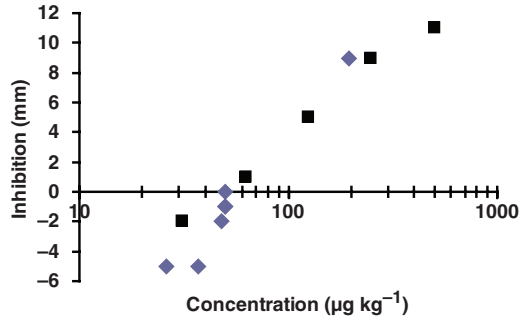


Figure 1. Inhibition zones obtained from the microbial inhibition assay plotted against the concentration of doxycycline. Squares indicate a series of spiked poultry samples; and diamonds represent the incurred samples obtained in this study. The inhibition zone was compared with a reference obtained from a sample spiked with 100 µg kg$^{-1}$ oxytetracycline.

spiked samples. From Figure 1 it is clear that differences between spiked and incurred materials are almost negligible. Since the trend line crosses the $x$-axis approximately at 50 µg kg$^{-1}$, it can also be concluded that doxycycline can be detected approximately twofold more sensitively compared with oxytetracycline, which is the reference compound.

### *Cell-biosensor assay*

Without knowledge about the outcome of the initial microbial screening, all 337 samples were additionally tested using the bacterial biosensor. The assay is based on the induction of a luminescence signal upon the presence of tetracyclines. In previous studies (Kurittu et al. 2000; Pellinen et al. 2002; Virolainen et al. 2008) the assay proved to be sensitive to all veterinary relevant tetracyclines (tetracycline, doxycycline, chlortetracycline and oxytetracycline). Typical dose–response (induction coefficient – IC) curves of the assay show a parabolic behaviour, since high concentrations will inhibit protein production (and ultimately kill the test organism), decreasing the signal upon increasing concentrations. High tetracycline concentrations therefore theoretically could remain unnoticed. To circumvent this problem, the assay was performed using three subsequent dilutions of the same sample, so if a sample contains high concentrations of tetracycline, the diluted samples will yield an increased IC. Sample analysis was performed on nine individual days, and each series included an oxytetracycline calibration line (Figure 2).

Out of the 337 samples, 36 (10.7%) showed a positive cell-biosensor result (IC > 2) (Table 1). These samples included the seven samples that were also identified with the microbial inhibition assays. The additionally identified samples were analysed by LC-MS/MS (except for three samples for which no

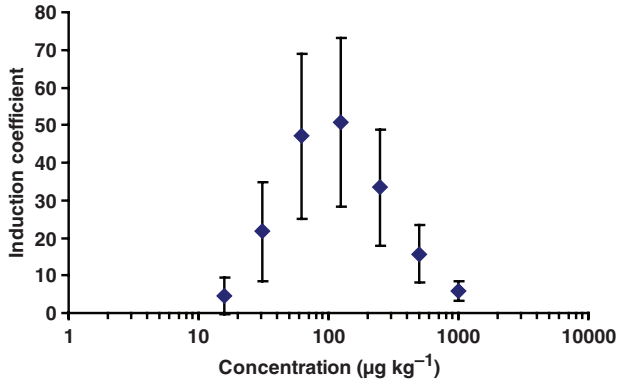


Figure 2. Induction coefficient (IC) plotted against the concentration of oxytetracycline in spiked samples. The results were obtained from nine individual experiments.



material was remaining). In all positive samples the presence tetracyclines was confirmed, while two negative samples, which were included as a (blind) control, tested negative. The concentrations appeared to be very low; two samples contained concentrations between 10 and 50 µg kg$^{-1}$, but most samples were even below the limit of quantification (10 µg kg$^{-1}$).

The majority of the samples contained doxycycline. Oxytetracycline was found in one sample, while three samples contained chlortetracycline, of which one additionally contained a similar amount of tetracycline. Remarkably, in almost all the doxycycline-positive samples tetracycline was also found. Since tetracycline itself in the Netherlands is not registered for use in poultry, this could indicate that the applied doxycycline product contained a substantial percentage of tetracycline.

The absolute as well as the relative (compared with the oxytetracycline calibration line) IC value appeared to have very limited predictive value, which becomes evident from the oxytetracycline calibration data (Figure 2), showing a large standard deviation. The peak of the curve, on the other hand, always occurred at 125 µg kg$^{-1}$, which is similar to former results (Virolainen et al. 2008). Although the lowest oxytetracycline spike (15 µg kg$^{-1}$) in one experiment failed to produce a positive result, the single incurred sample obtained in this study showed that the detection limit for oxytetracycline probably lies around the limit of quantification of the chemical confirmation (10 µg kg$^{-1}$).

One explanation for the variations in the IC is the presence of varying levels of haemoglobin. The bioluminescence spectrum overlaps with the absorption spectrum of haemoglobin, attenuating the bioluminescence intensity (Luker and Luker 2008). In practice, the colour of the extracted fluid from chicken muscle varied between almost colourless to pink. This phenomenon became very obvious when the assay was subjected to guinea fowl, which has a very dark red colour. The samples initially showed an unusual low luminescence signal, and when they were fortified with doxycycline no measurable signal could be produced. Another source of variation in the induction factors may be introduced by the use of reconstituted lyophilized cells. Batches of cells may not be identical with respect to the number of cells and thus may yield batch dependent ICs. This variation could probably be minimized by standardizing (by optical identity measurement) the cell suspension to be used in the assay.

As mentioned above, to prevent false-negative results, the experimental set-up comprised three subsequent dilutions of the same sample. In contrast to the IC value itself, the pattern of the IC values generated by such a series of samples did appear to be a suitable predictor for the concentration. The seven samples showing a response in the microbial inhibition assay all showed an increased signal upon twofold dilution. Six additional samples that did not show microbial growth inhibition also showed this pattern. Of these 13 samples, only the MRL violation and one sample confirmed at 47 µg kg$^{-1}$doxycycline showed an increasing signal in the fourfold dilution as well. Using this pattern as a cut-off leads to a two-third reduction of samples requiring additional chemical confirmation.

### *Effect of other antibiotics (specificity)*

Since the assay requires active bacterial metabolism, other antibiotics could potentially affect the assay. Only one of the 337 samples appeared to contain antibiotic residues other than tetracycline; the presence of 16 µg kg$^{-1}$ enrofloxacin was confirmed in a sample yielding a suspect result with a quinolone specific microbial inhibition assay (Pikkemaat et al. 2007). This sample did not show any aberrant behaviour. Sensitivity to other antibiotics was further tested by exposing the tetracycline cell-biosensor to several veterinary antibiotics (erythromycin, neomycin, enrofloxacin, flumequin, lincomycin, amoxicillin, penicillin and a combination of sulfadiazine and trimethoprim) in concentrations ranging from 0.5 to four times their respective MRL in poultry muscle, but none of these was capable of inducing a luminescence signal. To ensure that the absence of an induction signal was not caused by growth inhibition of the sensor cells or by inhibition of the luminescence signal, the same experiment was also carried out using an *Escherichia coli* strain that constitutively expresses the luciferase operon (*E. coli* XL-1 plus pCGLS-11; Frackman et al. 1990). The concentrations mentioned above did not show an effect on the luminescence signal, while doxycycline control started to inhibit luminescence at 250 µg kg$^{-1}$ (2.5 × MRL). Additional testing showed

Table 1. Overview of the results.

| Total number of samples | Suspect inhibition assay | Positive biosensor assay | Maximum residue limit (MRL) violation |
|---|---|---|---|
| 337 | 7 (2%) | 36 (10.7%) | 1 (0.3%) |

Table 2. Comparison of the performance and requirements of the methods.

| | Microbiological assay | Cell-biosensor |
|---|---|---|
| Results | Qualitative | Qualitative |
| Sensitivity ($\mu g\,kg^{-1}$) | 50–100 | 10 |
| Specificity | Tetracyclines | Tetracyclines |
| Sample preparation | Isolation of muscle fluid | Isolation of muscle fluid |
| Measurement time (h) | 12–16 | 3 |
| Instruments required | Incubator | Incubator; bioluminescence reader |
| Applicability | Laboratory and on-site | Restricted to the laboratory |
| Market price per sample (€) | 15.00 | 7.50 |

that at least $3000\,\mu g\,kg^{-1}$ ($30 \times$ MRL) enrofloxacin is required to influence the assay negatively.

## Conclusions

It is very important to review the performance of a method using incurred samples and routine operating conditions. Too often, methods are only validated using spiked materials in a limited experimental set-up. This may lead to an overestimation of the sensitivity, and sample matrix variations will be only limited.

To the best of our knowledge, this study represents the first example of the application of a bacterial biosensor assay in large-scale routine analysis. According to European Union Commission Decision 2002/657/EC on the performance criteria for analytical methods, a screening method should have a false compliant rate of $<5\%$ at the level of interest (European Commission 2002). Considering the fact that the level of interest, or maximum residue limit (MRL), for tetracycline residues in poultry is $100\,\mu g\,kg^{-1}$, it can be concluded that this method easily fulfils this requirement. The assay requires minimal sample preparation, is easy to perform, and is much faster than traditional microbial inhibition tests (a comparison of the performance and requirements of the methods is given in Table 2). It appeared to be able to detect tetracycline levels far below the MRL, and by applying a test format using a series of three sample dilutions, an appropriate cut-off for further confirmatory analysis can be made. The assay appeared to be very specific and robust, with the limitation that it is not suitable for the analysis of guinea fowl and most likely also not for other 'red' poultry species, though this probably could be solved by some additional sample preparation.

The result of this study furthermore shows that application of tetracyclines in poultry is widely spread, but withdrawal times are generally obliged, since the concentrations that were encountered were generally far below the MRL.

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