

DEVELOPMENT OF A GENERIC DIRECT COMPETITIVE ELISA FOR THE RAPID SCREENING OF QUINOLONES IN RAW MILK AND FOOD ANIMAL EDIBLE TISSUE

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Introduction

Quinolones are synthetic antimicrobials that inhibit the activity of bacterial DNA gyrase enzymes. The earliest members of this group of compounds such as oxolinic acid and nalidixic acid are active against gram-negative bacteria. A second-generation of quinolones are fluoroquinolones such as enrofloxacin, ciprofloxacin, danofloxacin, difloxacin, sarafloxacin, marbofloxacin which have enhanced antibacterial activity against gram-negative bacteria and are also active against gram-positive bacteria. The main application of quinolones has been the treatment of gastrointestinal and respiratory infections both in human and veterinary clinical practices. However, the presence of residues of these compounds in food producing animals is controlled as their widespread use contributes to the appearance of resistant bacteria for these antimicrobials in humans. The European Union has set maximum residue limits (MRLs) for several quinolones including enrofloxacin, difloxacin, sarafloxacin, marbofloxacin and oxolinic acid. The MRLs are set depending on the animal species and matrices. The target tissues are muscle, liver, kidney, fat (or skin and fat) and milk. The MRLs values range from 10 ppb for sarafloxacin in chicken fat to 1,900 ppb for difloxacin in poultry liver. The latter is not to be used in animals from which milk is produced for human consumption^(1,2,3,4).

A variety of methods have been developed for the determination of quinolone residues in different matrices, including microbial screening tests⁽⁵⁾, capillary electrophoresis⁽⁶⁾, high performance liquid chromatography (HPLC)^(7,8), liquid chromatography-tandem mass spectrometry⁽⁹⁾ and immunoassays^(10,11). In the format of the generic direct competitive enzyme-linked immunosorbent assay (ELISA) that we present here, the antibodies directed against quinolones are stable when precoated on the 96-well ready-to-use microtitre plates. The assay is based on the competition between free quinolones present in the sample, and a horseradish peroxidase labelled conjugate. After colorimetric development the absorbance is inversely proportional to the concentration of the analyte. Quinolone determinations for 40 samples are performed in 90 minutes and the method is applicable for the generic screening of quinolone residues below established MRLs.

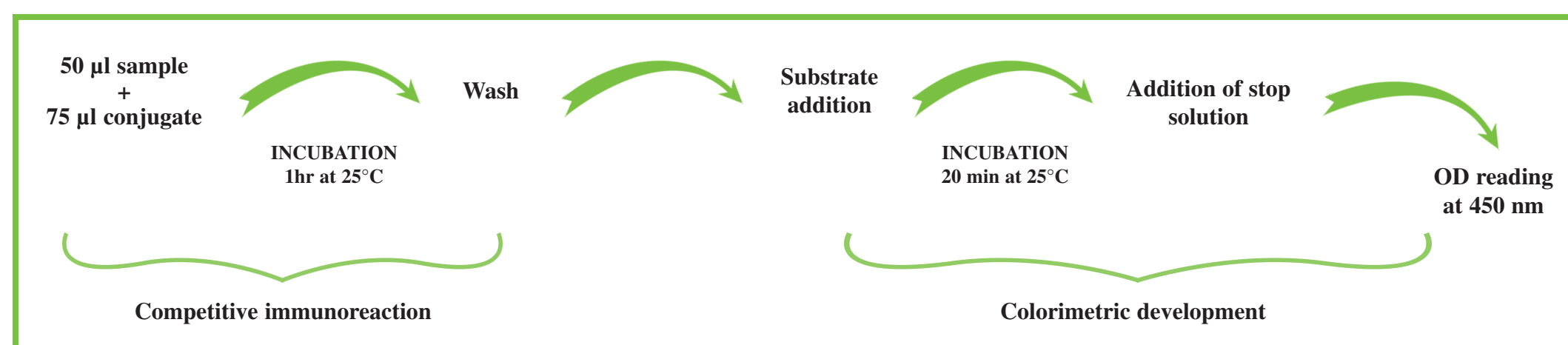
Methodology

The assay is based on the competitive immunoreaction between free quinolones present in the standard/sample and horseradish peroxidase labelled conjugate for binding sites of the in-house generated generic quinolone polyclonal antibodies, stabilised and precoated on a 96-well microtitre plate. Measurement was carried out by reading the absorbance at 450 nm, which was inversely proportional to the concentration of the analyte.

The competitive ELISA was performed as outlined in the flow chart.

Sample preparation

Bovine semi-skimmed and whole milk was centrifuged at 4000 rpm for 15 min and then diluted 10-fold in assay buffer prior to application to the microtitre plate.

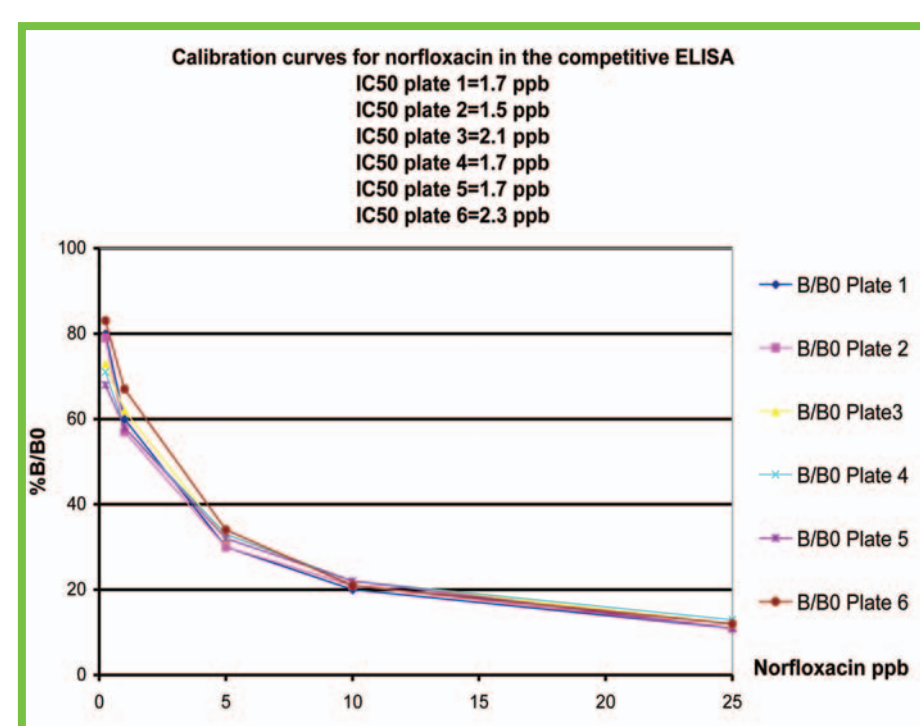


Results

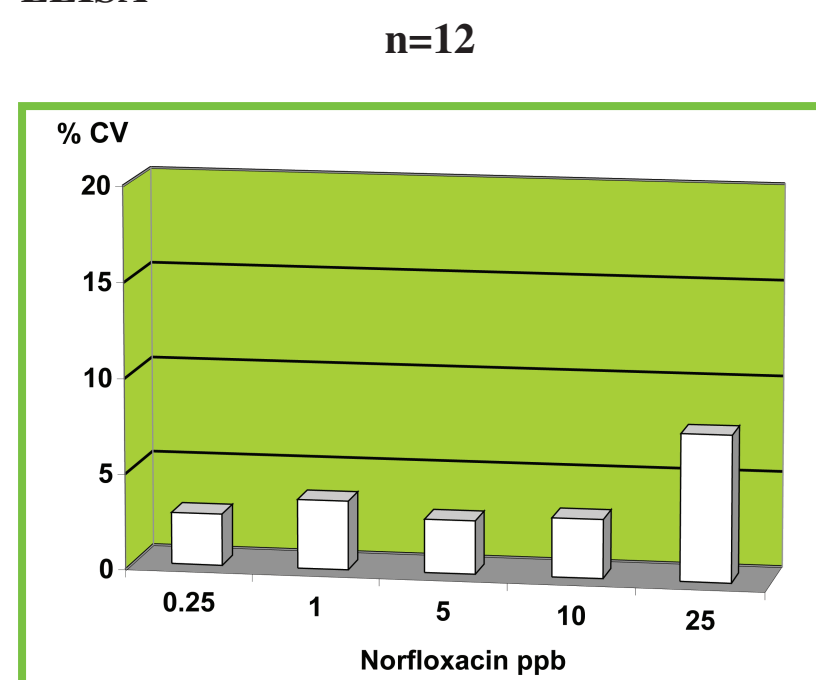
Specificity of the competitive ELISA for different quinolones.

Analyte	% cross-reactivity
Norfloxacin	100
Danofloxacin	299
Enrofloxacin	289
Ofloxacin	111
Enoxacin	74
Sarafloxacin	57
Difloxacin	52
Ciprofloxacin	21

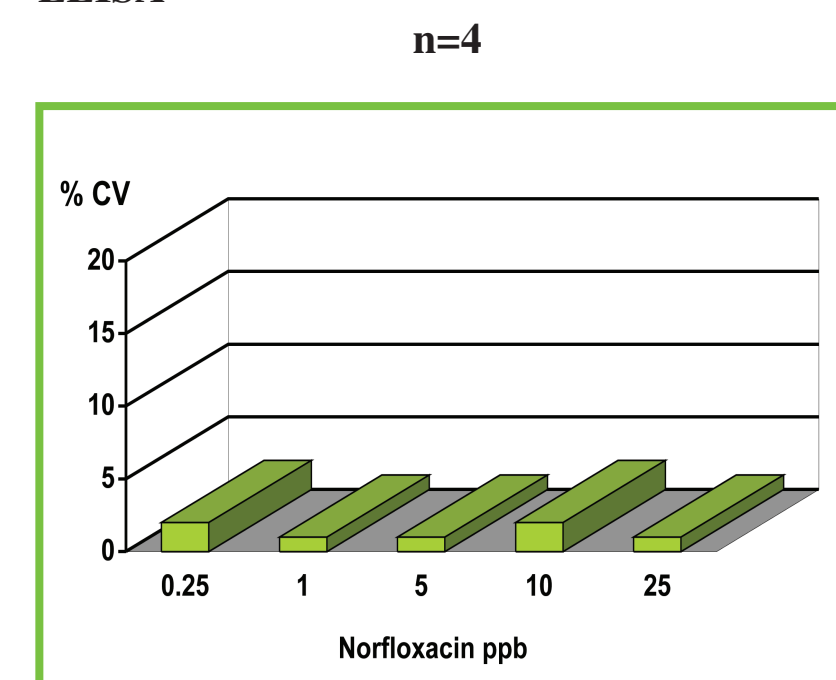
Calibration curve for norfloxacin in the competitive ELISA



Intra-assay precision in the competitive ELISA



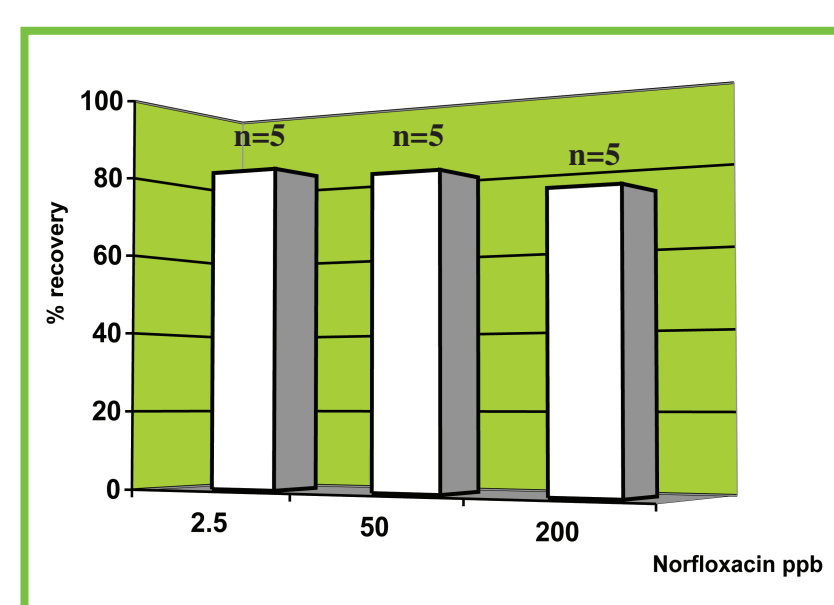
Inter-assay precision in the competitive ELISA



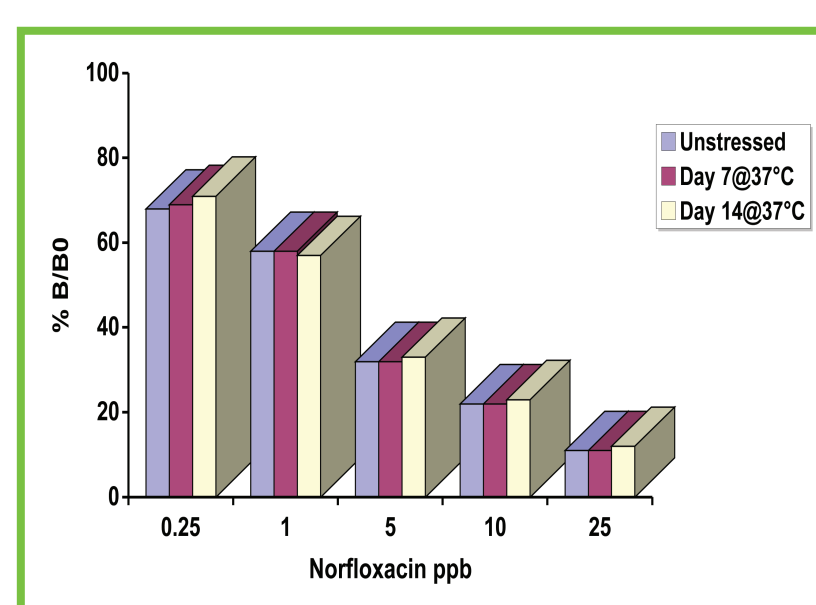
Sensitivity: limit of detection (LOD) in milk samples

In n = 20 blank samples
LOD <0.25 ppb

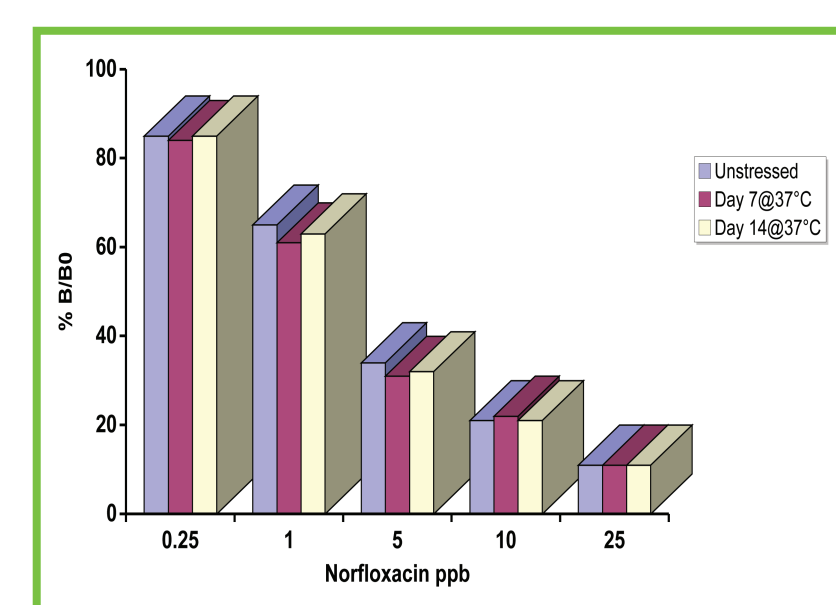
% Recovery in milk for norfloxacin in the competitive ELISA



Accelerated stressing of the ready-to-use conjugate at 37°C



Accelerated stressing of the precoated microtitre plates at 37°C



Conclusion

We present here the development of a generic direct competitive ELISA kit for the detection of quinolone residues below established MRLs as shown by the IC50 values ≤ 2.5 ppb and LOD in milk < 0.25 ppb for the reference antimicrobial norfloxacin. In this ELISA the 96-well ready-to use microtitre plates are precoated with the in-house made polyclonal antibodies directed against quinolones. The precoated plates exhibit a stability of at least one year when stored at $+2^{\circ}\text{C}$ - $+8^{\circ}\text{C}$. The horseradish ready-to-use peroxidase labelled conjugate is stable for at least one year when stored at $+2^{\circ}\text{C}$ - $+8^{\circ}\text{C}$. Generic recognition of quinolones was shown by relative % cross-reactivity values, 299% (danofloxacin), 289% (enrofloxacin), 111% (ofloxacin), 74% (enoxacin), 57% (sarafloxacin), 52% (difloxacin), 21% (ciprofloxacin). Initial precision studies showed %CVs typically $\leq 10\%$ for both intra-assay and inter-assay precision. The recovery rates in milk samples were between 70-130% for three different spiked levels of norfloxacin. Application studies to other matrices are in progress. The method is performed using only 50µl sample solution involving rapid and simple preparation. Results are obtained in 90 min for 40 duplicate samples. This competitive ELISA represents a suitable tool for the rapid generic screening of quinolones when large numbers of samples have to be analysed to monitor legislative compliance.

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