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Development and validation of a rapid lateral flow test for the detection of fluoroquinolones in meat and blood

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ABSTRACT

Fluoroquinolones are antibiotics of considerable relevance in veterinary therapy. If they fail to be properly controlled, they can eventually reach animal-derived food products. As most of the biologically based screening tests used in surveillance plans are not able to detect fluoroquinolones, it would be highly convenient to devise a complementary test designed to detect them specifically. Therefore, a new lateral flow immunoassay test was developed for the *ante* and *post mortem* detection of fluoroquinolones in blood and meat samples, respectively, of the main food-producing species. In both matrixes, the test was able to detect most of the European Union-authorized fluoroquinolones at the Maximum Residue Limits set in the European Union for meat; as an instance, the limit of detection was 50 μg/kg for enrofloxacin, 200 μg/kg for flumequine, and 100 μg/kg for marbofloxacin. Hence, this new technique is proposed as an automatized, sensitive, specific, rapid, and robust tool for the management of fluoroquinolone residues in all stages of meat production: no only as a *post morten* method, in meat samples, but also as an *ante mortem* method, in blood samples from living animals.

1. Introduction

The persistence of antibiotic residues in foodstuffs has become a problem on a worldwide scale, not only for technological reasons (such as the inhibition of fermentative processes in the milk industry) or for analytical reasons (including interferences in pathogen analyses), but also due to the effects of antibiotic residues on human health, such as direct hepatic and renal toxicity or the emergence of antibacterial-resistant microorganisms (Palma et al., 2020). This is a situation of major concern worldwide (WHO, 2021), and the reason is no other than the loss of antibiotic effectiveness against common illnesses.

Food-producing animals have traditionally been the main consumers of antibiotics (EFSA, 2017). Although global sales of antibiotics are currently decreasing in animals (EFSA, 2021), antibiotics are still widely used in veterinary medicine. To control the authorization of new veterinary medicines, a restrictive legal framework has been established (Regulation 2004/726/EC), along with maximum residue limits in foods of animal origin (Commission Regulation No 37/2010) in conjunction with national surveillance plans (Council Directive 96/23/EC) and specific requirements to be applied in official control campaigns (EU Regulation 2021/808).

In Europe, methods for the official control are categorized into two main groups, which are traditionally applied sequentially: screening and confirmatory methods (Regulation (EU) 2021/808). Screening methods are implemented as a first step and focus on separating negative samples from presumptive non-compliant samples, which should be confirmed via a suitable validated method, usually a chromatographic technique (Moga et al., 2021). Nonetheless, screening methods might not be able to detect all the antimicrobial families currently in use in veterinary medicine. For instance, the traditional EU four-plate test (Bogaerts & Wolf, 1980) does not include a test plate reasonably sensitive to fluoroquinolones (FQ). Likewise, tests based on *G. steaorthermophilus* growth are not able to detect antibiotics of the FQ family at regulatory levels (Mata et al., 2014).

A further difficulty lies in the fact that a significant proportion of the antibacterial medicines authorized for livestock contain molecules of the

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FQ family (mainly enrofloxacin). For instance, more than 40% of the antibacterial medicines commercially authorized for animal use by the Spanish Agency of Medicines and Medical Devices (AEMPS) contain FQ (CIMAVET, 2020). Although their consumption has strongly decreased over the last 10 years, they still represent 9% of all sales of antibiotics in Europe (EMA, 2021). Moreover, certain studies have revealed amounts that are even more worrisome, as the presence of FQ in almost 25% (Yang et al., 2020) or even nearly 50% of meat samples under study (Verma et al., 2020).

Hence, the problems derived from FQ are not over once they have been administered, as the human intake of food containing FQ residues may cause allergic reactions and dysbiosis in the microbiota that inhabit our gut (Shen et al., 2019). Moreover, most FQ are also regarded as last resort antibiotics in human therapy; their use in livestock must thus necessarily remain restricted (European Medicines Agency EMA, 2020) The Maximum Residue Limits (MRLs) for FQ in animal muscle intended for human consumption are regulated by the European Commission (Commission Regulation (EU) 37/2010) and presented in Table 1.

In view of this situation, the development of new methods designed to specifically detect FQ could be of great relevance to serve as a complement to current broad-spectrum screening tests. At present, several methods are available for this purpose, such as microbial-based analysis (Appicciafuoco et al., 2015; Sanz et al., 2011), chromatographic techniques (Lu et al., 2019), and biosensors (Gaudin, 2017b; Aymard et al., 2022). However, immunoassays, especially LFIA (Lateral Flow Immunoassay), stand out for their prolonged shelf-lives (usually without refrigeration), the fast result obtaining (5–15 min), their sensitivity, specificity, and simplicity of use, their ability to be integrated with reader systems, and the fact that they are money- and time-saving (Ahmed et al., 2020). Nonetheless, until now, tests developed for FQ detection have occasionally presented a series of disadvantages such as detection profiles for specific FQ (Chen et al., 2012; Lei et al., 2022; Mukunzi et al., 2018; Sheng et al., 2017; Yang et al. 2019; Yu et al., 2019; Zhang & Cheng, 2017) or unspecific detection profiles that include compounds from other antibacterial families (Chen et al., 2016). Although there are immunoassays for the simultaneous detection of several FQ in meat, they do not cover all the FQ with an established EU-MRL (Suryoprabowo et al., 2014). At any rate, no rapid test has hither to been described for the joint detection of the majority of FQ molecules with an established EU-MRL.

On the other hand, when antibacterial residues lying over the EU-MRL are detected in a meat sample, its entrance into the food chain should be avoided by seizing the carcasses, which implies important economical loses and a strong environmental footprint. In this context, the *ante mortem* detection of antibacterial residues can prevent unnecessary slaughters: once a positive animal is found, the extension of the withdrawal period until the acquisition of negative results would be enough to assure its aptitude to enter in subsequent stages of the food chain. A method for the *in vivo* detection of antibacterial residues, based on the growth inhibition of *G. stearothermophilus*, has recently been developed to detect antibacterial residues in blood (Serrano et al., 2021), a matrix which accurately reflects the amount of antibiotics present in muscle (Serrano et al., 2020). However, this kind of test fails to detect FQ at the regulatory levels. A method designed to specifically detect FQ in blood would allow for a more comprehensive *ante mortem* control of antibacterial residues. Although such a method would substantially improve antimicrobial screening in food chain, we are not aware that any procedure of this kind has been developed to date.

Hence, our study's aim was to develop a rapid LFIA strip for the detection of a broad-spectrum of molecules of the FQ family, and which would be capable of detecting FQ not only in meat/muscle (after slaughter, as a common control tool linked to subsequent corrective measures) but also in blood (as an autocontrol tool, prior to slaughter, hence linked to preventive measures) thus being likewise applicable to living animals. Reading of results could be adapted to an automatic electronic system to easy the performance and interpretation of results.

Table 1
Detection capability (*CCβ*) of the test for fluoroquinolones (FQ) in meat (μg/kg). Test Line/Control Line (TL/CL) ratio obtained from the Lateral Flow Immunoassay analyses performed are indicated, as well as the standard deviation (SD) and the number of meat samples (n) and the animal species tested. The European Union-Maximum Residues Limits (EU-MRL) (μg/kg) for each FQ is included for comparative purposes.

	EU-MRL	*CCβ*	TL/CL	SD	n	Species[a]
Enrofloxacin	100	50	0.69	0.21	33	B/P/C
Ciprofloxacin	100	40	0.74	0.12	20	B/P/C
Flumequine	200–400	200	0,77	0.18	60	B/P/C
Marbofloxacin	150	100	0.85	0.13	40	B/P/C
Difloxacin	400	>400	3.40	0.33	10	B/P/C
Oxolinic acid	100	50	0.26	0.04	20	B/P/C
Danofloxacin	100–200	50	0.45	0.07	20	B/P/C

[a] **B: Beef, P: Pork, C: Chicken.**

2. Materials and methods

2.1. Chemicals and reagents

FQ molecules, complete Freund's adjuvant (FCA), incomplete Freund's adjuvant (FIA), enzyme immunoassay-grade horseradish peroxidase-labeled goat anti-mouse immunoglobulin, bovine serum albumin (BSA), and keyhole limpet hemocyanin (KLH) were purchased from Sigma-Aldrich (Madrid, Spain). FQ and other antibiotics used during the validation of the new method are listed in Table S1. Other reagents and chemicals were obtained from local providers; all of them were of analytical grade.

2.2. Preparation of bioconjugates

Bioconjugates of FQ to different carrier proteins (KLH, BSA) were prepared as immunogens to obtain specific antibodies and as membrane capture reagent. The carbodiimide chemistry method was applied as described by Hermanson (2013). When possible, conjugates were characterized by MALDI-TOF and by spectrophotometry (Genesys 150 UV–Visible spectrophotometer, Thermofisher). Bioconjugates were stored at −20 °C until use.

2.3. Preparation of monoclonal antibodies

Monoclonal antibodies preparation was outsourced to Inycom Biotech (Zaragoza, Spain), a specialized firm. Briefly, female BALB/c mice were subcutaneously immunized with several FQ–KLH conjugates. For the first immunization, conjugates were emulsified with FCA; FIA was used in the subsequent boost injections. Ten days after the third immunization, blood samples from each immunized mouse were measured by indirect ELISA. Mice with the highest titer of antibodies were sacrificed, and spleen cells were fused with Sp 2/0 murine myeloma cells by using polyethylene glycol (PEG). Supernatant of hybridoma cell growth in HAT medium was screened using an indirect ELISA. The suitable clones were selected for limiting dilution, then expanded and subcloned. Four antibodies were pre-selected and purified from culture medium by Protein A affinity chromatography, then precipitated with ammonium sulphate, and stored at 4 °C until use.

2.4. Preparation of gold nanoparticules (GNP) functionalized with antibodies

GNP (20 nm, BBI Solutions) were functionalized with the four pre-selected antibodies. Each antibody (0.1 mg) was independently added dropwise to 20 mL of the GNP suspension, mixed, and then incubated for 2 h. The resulting suspension was blocked with 0.1% BSA, and the mixture was incubated at room temperature for 2 h. GNP suspension was

centrifuged for 15 min at 20,000×*g* and supernatant was discarded (Sigma 3-18 KS, Germany). Functionalized GNP were resuspended in a solution of 1% BSA and 2% sucrose. Size and aggregation of GNP were checked by Dinamic Light Scattering (Zetasizer Nano S, Malvern, USA) and absorbance measurement at 520 nm (Genesys 150 UV–Visible spectrophotometer, Thermofisher). GNP were dispensed in tubes (200 μL) and freeze-dried to achieve long-term stability.

2.5. Preparation of lateral flow immunochromatography strips

Eight FQ-BSA conjugates (Table S2) were tested as capture reagent for the test line, and goat anti-mouse IgG were used as the capture reagent for the control line. The FQ-BSA conjugates and goat anti-mouse IgG lines were sprayed onto the nitrocellulose membrane (Unisart CN95, Sartorius, Germany) at 1 μL/cm using a dispenser (BioDot ZX1010, USA). The nitrocellulose membrane coated with capture reagents was laminated together with the sample and absorbent pads onto a 30-cm-long plastic backing card as support. As a final step, the card was cut into 4-mm-wide strips using a strip cutter (BioDot, Irvine, USA).

2.6. Sample preparation

As one of this test's applications is to serve as a complement to microbial screening tests that do not properly detect FQ, sample preparation should be as easy and analogous as possible, so that samples can be analyzed by the two methods simultaneously, thus simplifying the procedure. Hence, the sample preparation procedure for the analysis of FQ in meat was adapted from that described by Mata et al. (2014) for the screening of antibiotics in meat using microbial inhibition tests. Briefly, a piece of meat (3 ± 0.5 g) without adipose or conjunctive tissue was cut and placed in a polypropylene heat-resistant plastic tube. The tube was closed (but not sealed) and heated in a water bath at 100 °C for 4–5 min. The meat was then pressed and removed with forceps, and the obtained fluid was collected and centrifuged. Figure S1 shows an example of the piece of meat before extraction and the juice obtained with the described procedure.

For the analysis of FQ in blood, it was necessary to obtain blood serum following the method described by Serrano et al. (2020). Briefly, blood was coagulated for at least 1 h at room temperature (20–25 °C). The coagulum was then removed, serum was centrifuged at 3000×*g* for 10 min at 4 °C, and supernatant was collected. Both meat juice and blood serum were diluted in buffer before being subjected to the test, in order to standardize the sample characteristics, to adapt the sensitivity to the current EU-MRL set for FQ in muscle, and to improve the detection signal. Before selecting the best performance, several buffers were tested (phosphate, carbonate, borate, CHES [Cyclohexyl-2-amino ethanesulfonic acid], MES [2-(N-morpholino) ethanesulfonic acid], and PBS [phosphate buffer saline]), as well as conditions such as buffer concentration, pH, and additives (NaCl, $CaCl_2$, or Tween supplementation). Results were visually read and using the IRIS reader (Zeulab, Zaragoza, Spain).

2.7. Validation of the test

2.7.1. Detection capability for screening (CCβ)

According to Commission Implementing Regulation (EU) 2021/808, the detection capability for screening (*CCβ*) is the smallest content of the analyte that can be detected or quantified in a sample with an error probability lower than or equal to 5% (*β error*).

CCβ values were determined by fortifying meat fluid or blood sera with several FQ at different levels. In the preparation of the fortified samples, at least 2 different standard solutions were used for each substance. To calculate the *CCβ*, each antimicrobial was initially analyzed at 2–4 levels around the EU-MRL established for muscle, or at a level matching the limit of detection (LoD) expected for that compound. When the test did not detect an expected concentration as positive, a higher level was included in the study. Detection capability was determined according to a Community Reference Laboratories (CRL) guideline for the validation of screening methods for veterinary medicine residues (CRL, 2010). The guideline stipulates that the number of replicates to be evaluated depends on the detection capability's degree of closeness to the EU-MRL (in a range varying between 20 and 60 analyses on different days).

Since no specific guidelines have been set forth for the validation of screening methods for the detection of antibiotics in blood, a representative number of samples were analyzed in order to demonstrate the method's applicability for testing blood samples by checking their correspondence with muscle. Although Regulation 2021/808 contemplates the adaptation of these methods to different matrixes, it does not stipulate the number of samples to be analyzed. Gaudin (2017a) suggests performing 10 replicates; hence, more than 10 replicates for the most representative molecules were performed (enrofloxacin and marbofloxacin: 14 repetitions), and further analyses beyond 10 replicates were performed for other FQ as well.

2.7.2. Selectivity/specificity

To determine the test's selectivity/specificity, several approaches were possible. Firstly, to determine its specificity, molecules from antimicrobial families other than the FQ family were analyzed by fortifying muscle juice at a high concentration (10–100 times the corresponding EU- MRL). Samples were tested in duplicate.

A second way to determine selectivity/specificity is to verify the false-positive rate in negative samples. Thus was achieved by testing 180 meat samples from eight different species (beef, lamb, pork, rabbit, chicken, turkey, duck, and quail) obtained from local markets. In addition, 190 blood samples from pigs grown in antibiotic-free farming conditions were tested. Results lying below the established cut-off value (TL (test line)/CL (control line) ratio ≤1.0) would indicate a false-positive result and would require additional confirmation.

2.7.3. Robustness

The robustness of the assay was evaluated by introducing changes in the standard procedure and determining their effects on the results. Sample volume, assay temperature, and assay time were selected as the main critical factors and evaluated their influence on the false-positive and false-negative rates. To evaluate the false-negative rate, four different meat samples were fortified with enrofloxacin at 50 μg/kg, as this compound is considered a representative substance, and were tested at several levels for each critical factor.

2.7.4. Validation on meat and blood samples containing in-vivo-administered antibiotics

In general, to simplify the validation of a new method, blank matrices fortified with specific molecules are usually prepared and immediately tested to verify the method's performance; however, it is likewise recommended to test a number of naturally incurred samples. Meat and blood samples tainted with enrofloxacin were obtained from a sample bank stemming from treated pigs (Serrano et al., 2020). Samples were analyzed with the new test and results were compared to those obtained by LC-MS/MS (Liquid Chromatography Tandem Mass Spectrometry) as described by Serrano et al. (2020).

2.8. Data processing and representation

The PRISM® program was used for data processing and representation (GraphPad Software, Inc., San Diego, CA, USA).

3. Results and discussion

There are several techniques for detecting FQ in meat, but most of them present disadvantages compared to LFIA tests but, to our knowledge, there are no comprehensive LFIA tests for the *ante* and *post mortem*

detection of all FQ family molecules in husbandry. Hence, a new broad-spectrum LFIA test for the detection of FQ in food-producing animal samples would be of great convenience.

3.1. Characterization of bioconjugates

Bioconjugates of FQs to KLH and BSA were prepared as immunogens and capture reagent, respectively. KLH bioconjugates were used for immunization without characterization due to the difficulties to obtain adequate spectra by MALDI-TOF. Eight FQ-BSA bioconjugates were prepared to optimize the immunochromatographic assay. Table S2 shows the hapten to BSA concentration and molar ratio determined by MALDI-TOF and spectrophotometry for each one of the bioconjugates after the conjugation reaction.

3.2. Development of the anti-FQ antibody

Four clones (Y-005, Y-006, Y-007, and Y-014) were selected from the first screening and further evaluated. Initially, the ability to bind eight different haptens was tested by direct ELISA. Best results were obtained with BSA-enrofloxacin, BSA-difloxacin and BSA-marbofloxacin bioconjugates and accordingly were selected for further evaluation. Table S3 shows the ability of eight free FQ to compete against the immobilized haptens (BSA-enrofloxacin, BSA-difloxacin, and BSA-marbofloxacin) to bind the corresponding antibody. Results are displayed as the percentage of binding in presence of FQ in a sample. As a reference, 100% of binding was obtained in absence of FQ.

In general, Y-014 antibody showed the highest capacity to bind free FQ and therefore the best binding inhibition to the three immobilized haptens. Seven out of eight FQ were able to efficiently compete against the immobilized haptens, with inhibitions higher than 80%. Therefore, Y-014 antibody was selected to optimize the new LFIA test.

3.3. Optimization of the LFIA test

GNP were functionalized with Y-014 antibody at a concentration of 5 μg/mL. Particles were then characterized by measuring their size (Z-value) and polydispersity index with mean values of 83 nm and 0.261, respectively, which indicate that no aggregates are present and that the particles are of the expected size.

Apart from physical characterization, functionalized GNP should be evaluated in order to select optimal conditions for LFIA assays. Thus, functionalized GNP was tested against a battery of FQ bioconjugates that had been previously immobilized on a nitrocellulose membrane. A series of different buffers, pH conditions, and additives was also evaluated. Table S4 shows the values of the intensity signal obtained for each tested condition in absence of competing antibiotics. A higher signal thus indicates an improved interaction between the antibody and the immobilized hapten. The antibody was only able to bind enrofloxacin, ciprofloxacin, and marbofloxacin bioconjugates, obtaining the best interaction at pH 8.0. The obtained signals could even be improved by adding Tween 20.

The 3 FQ bioconjugates with capacity to bind the antibody were tested against free FQ in order to select that with the best competition properties. Five FQ for which a regulatory level in meat has been established in the EU were tested at low concentrations (below the EU-MRL). In absence of free FQ, all antibodies are available to bind the immobilized FQ-bioconjugate, then inhibition is zero. When free FQ are in the sample they compete to bind the antibody and then an inhibition of the FQ-bioconjugate binding is displayed. Very poor inhibition rates (0–9%) were obtained with danofloxacin and flumequine when using enrofloxacin and ciprofloxacin bioconjugates. The best inhibition profile was obtained with the marbofloxacin bioconjugate, which showed inhibition rates ranging between 29 and 59% for the five FQ (Table S5). Higher inhibition rates indicate that free FQ may compete more favorably to bind antibody and therefore higher sensitivity could be achieved in the final assay.

Therefore, the combination of Y-014 antibody-GNP and BSA-marbofloxacin bioconjugate was selected to prepare the prototype of a new LFIA test for the detection of FQ in muscle and blood.

3.4. Test description and procedure

The assay consists of 2 main components as displayed in Fig. 1.

1) Detection particles that are composed of GNP functionalized with a specific antibody against FQ. These are firstly put in contact with the sample (and with the analyte if present).
2) A strip with 2 capture lines, one specifically for FQ (test line, TL) and the other as control (CL, control line).

In a first step, the freeze-dried detection GNP are placed in contact with the sample and re-hydrated while mixing. Immediately afterwards, the strip with the capture reagents is introduced in the tube. Assay is performed at 40 °C for 6 min, after which the results are read either visually or with a strip reader (IRIS). Fig. 1 shows the principle of the assay and the interpretation of results.

When the intensity of the TL is higher than that of the CL, the result of the assay is considered negative. Conversely, when intensity of the CL is higher than that of the TL, the result is positive. If the intensity of both lines is equal, the result is at the LoD and it is considered weak positive. To facilitate interpretation and obtain an objective measure value, results are preferably read with a strip reader. For this purpose, the ratio between the intensity of the two lines is calculated. Thus, when no analyte is present in the sample, the TL/CL ratio is considerably higher than 1.0 (Fig. 1b). However, when FQ are present in the sample, the analyte competes to bind the specific antibody in GNP, and low or even no signal is displayed in the TL. When a low quantity of antibiotic is present in the sample, the competition takes place, but at levels that are not sufficiently elevated to achieve a complete inhibition of the TL. Since the cut-off is set at 1.0, samples with a TL/CL ratio equal to or lower than 1.0 are considered positive.

3.5. Effect of the sample preparation over the test performance

In order to obtain a sample adapted to LFIA analysis, meat juice was procured. Nevertheless, it could not be directly tested due to its low pH (5.4–5.6), which would have affected the test's performance. For this reason, it was necessary to dilute the meat juice in buffer to obtain optimal assay pH and to standardize differences among samples. Several buffers and dilutions were evaluated, always seeking to avoid an excessive loss of sensitivity.

Fig. 2a shows the results obtained from the analyses performed on blank meat juice or juice spiked with enrofloxacin at 100 μg/L and 200 μg/L, as well as on several dilutions in different buffers. Results are presented as the ratio between the intensity of the test line and that of the control line (TL/CL). Meat juice without dilution had a ratio of 1.5 (average value) for negative samples, a value that is difficult to interpret with the naked eye, and which is more thoroughly exposed to environmental and sample variations. Although the cut-off value for the discrimination of positive samples was set as equal to or lower than 1.0 to facilitate visual interpretation of the tests, it is recommended to obtain ratio values greater than 2.0 with blank or negative samples.

Dilution in phosphate buffer at pH 6.0 or 8.0 and phosphate saline buffer (PBS) yielded results with TL/CL ratios over 2.0. Since PBS is a conventional buffer, it was also tested at several dilutions (1/5 to 1/20), with good results for all of them. Results with carbonate, borate, CHES, and MES buffers were not as satisfactory. Hence, their use was discarded. Finally, dilutions in PBS at 1/10 and 1/20 in presence of enrofloxacin were also tested (Fig. 2b), to verify whether the buffer might affect the interaction of free FQ with antibodies. Good inhibition was observed with a TL/CL ratio under 1.0, thereby indicating that the

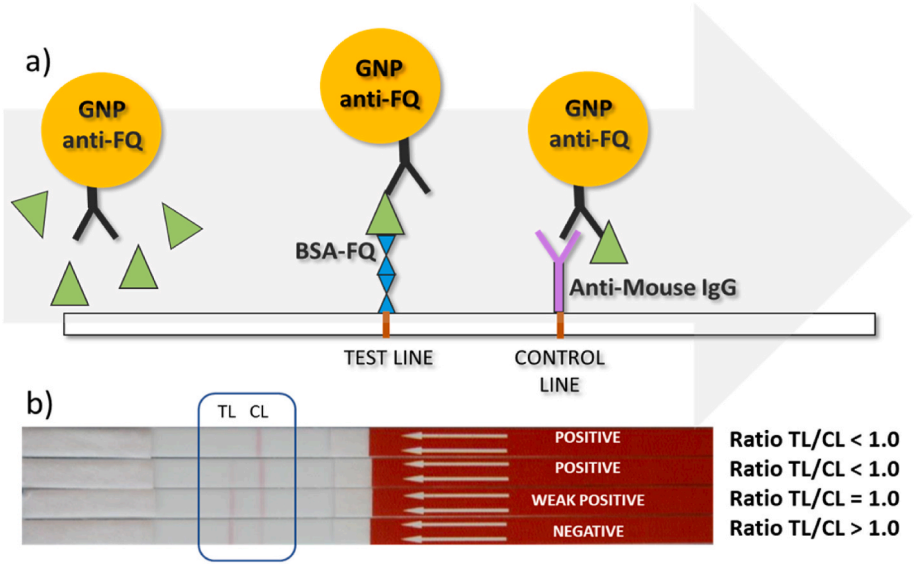

Fig. 1. Fig. 1a. Principle of the immunochromatographic technique. Fig. 1b. Lateral Flow Immunoassay (LFIA) strip results.

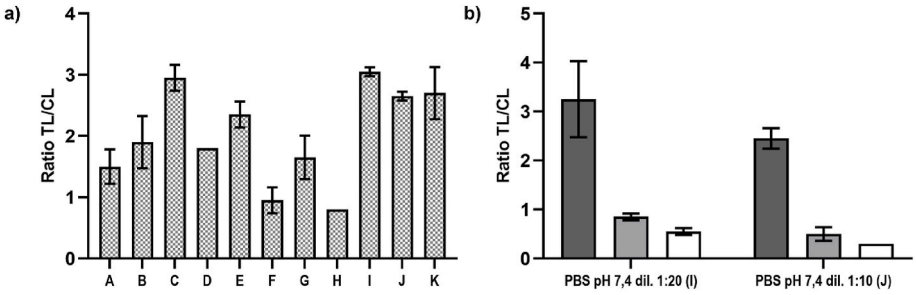

Fig. 2. Fig. 2a. Test Line/Control Line (TL/CL) ratio obtained from the Lateral Flow Immunoassay analyses performed in duplicate on blank meat juice diluted in different buffers at different concentrations: A) Without dilution; B) Phosphate 10 mM pH 7,4 dil. 1:20; C) Phosphate 20 mM pH 8,0 dil. 1:20; D) Carbonate 20 mM pH 10,0 dil. 1:20; E) Phosphate 20 mM pH 6,0 dil. 1:20; F) CHES 20 mM pH 10,0 dil. 1:20; G) Borate 20 mM pH 8,5 dil. 1:20; H) MES 20 mM pH 6,6 dil. 1:20; I) PBS pH 7,4 dil. 1:20; J) PBS pH 7,4 dil. 1:10; K) PBS pH 7,4 dil. 1:5. Results are presented as the average ratio between the intensity of the test line and the control line (TL/CL), resulting from 2 analyses per condition. Fig. 2b. TL/CL ratio obtained from the analyses performed in duplicate on blank meat juice (dark grey bars) or juice spiked with enrofloxacin at 100 μg/L (light grey bars) and 200 μg/L (white bars) diluted 1:20 in PBS pH 7.4 (condition I from Fig. 2a) or 1:10 (condition J from Fig. 2a).

performance with PBS buffer could perfectly detect FQ molecules.

Blood could not be directly analyzed with the LFIA technique, as the nitrocellulose membrane became clogged due to the blood's composition; moreover, it became stained with a deep red color, thereby preventing interpretation of results. Therefore, serum from blood was obtained and applied at the same buffers and dilutions previously used for meat juice, obtaining similarly satisfactory results as above.

3.6. Validation of the test

The validation of the new test was performed following the guidelines set out by Community Reference Laboratories (CRL, 2010) and Commission Implementing Regulation (EU) 808/2021.

3.6.1. Detection capability for screening (CCβ)

CCβ values were determined by fortifying meat juices or blood sera with FQ at different levels. The CRL guidelines (CRL 2010) stipulate that each substance must be tested 20, 40, or 60 times depending on the closeness of the detection capability to the EU-MRL. In the present study, the expected LoDs for enrofloxacin, ciprofloxacin, danofloxacin, and oxolinic acid were equal to or less than half the EU-MRL. Therefore, according to the CRL guidelines, a minimum of 20 samples per substance and level were to be tested, whilst 40 and 60 samples, respectively, in the case of marbofloxacin and flumequine, since the expected LoDs lay between half the EU-MRL and the EU-MRL level. To verify the test's applicability to muscle from different species, samples from beef, pork, and chicken were included in the study. Table 1 summarizes the test's detection capability for the FQ for which a regulatory level has been established in the European Union (EU-MRL). Following the CRL guidelines, the *CCβ* is set at a concentration at which at least 95% of the tested samples yield a positive result. Except for difloxacin, all the substances were detected at the expected level: equal to or lower than the EU-MRL.

Apart from the detection capability values, TL/CL average ratios are likewise shown in Table 1: values ≤ 1.0 are considered positive. With this criterion, other molecules of the FQ family that are unauthorized in the European Union (and thus for which no EU-MRL has been established) were also tested to determine the corresponding LoD in muscle (Table 2).

Since no specific guidelines have been established for the validation of screening methods for the detection of antibiotics in blood, a significant number of samples spiked with two representative FQ molecules (enrofloxacin and marbofloxacin) were analyzed to demonstrate the method's applicability for testing blood and verifying its correspondence with muscle. The number of samples was chosen in accordance with the review by Gaudin (2017b), who suggest an amount of 10–20

Table 2
Limit of detection (LoD, μg/kg) of the test for FQ with no European Union-Maximum Residues Limits (EU-MRL) (μg/kg). Test Line/Control Line (TL/CL) ratio obtained from the Lateral Flow Immunoassay analyses performed are indicated, as well as the standard deviation (SD) and *n* represents the number of meat samples analyzed.

	EU-MRL	LoD	TL/CL	SD	n
Norfloxacin	–	100	0.23	0.02	2
Lomefloxacin	–	10	0.71	0.00	2
Perfloxacin	–	50	0.46	0.11	2
Fleroxacin	–	50	0.71	0.04	2
Enoxacin	–	50	0.58	0.03	2

samples for applicability studies. In addition, LoD for other FQ in blood were also determined with four samples. For that purpose, blank blood sera obtained from pigs bred in antibiotic-free conditions were spiked with FQ levels equal to or slightly lower than the LoDs described for muscle. Table 3 summarizes the LoDs obtained for six FQ in blood serum.

The LoDs obtained for blood serum were quite similar to the *CCβ* determined in muscle. A few differences were found for flumequine (200 μg/kg in muscle vs 100 μg/L in blood) as well as for marbofoxacin (100 μg/kg in muscle vs 75 μg/L in blood). Nevertheless, as the LoDs were lower in blood and the test is intended for screening, the correlation between FQ concentrations in muscle and blood is quite close (Serrano et al., 2020). This slight difference even provides additional consumer health protection, as the *ante mortem* test is capable of detecting even lower concentrations. With the exception of difloxacin, all molecules were detected at or below the EU-MRL for meat. This situation is not a cause for concern, as difloxacin is not present in commercial drugs intended for animal use according to the EMA database of medicines authorized for animal use in the European Union (EMA, 2020).

Results thus prove that LoDs for FQ in blood and meat are similar. This new test is therefore a thoroughly suitable tool for the analysis of FQ in blood, and can be used as an effective *ante mortem* technique in farms or slaughterhouses not only with the aim of preventing unnecessary slaughter of contaminated animals, but also to preclude the presence of FQ along the food chain.

3.6.2. Selectivity/specificity

To determine the new test's specificity to exclusively detect FQ, molecules from other antimicrobial families were analyzed by spiking pork muscle juice at a high concentration (10–100 times the corresponding EU-MRL). Samples were tested in duplicate. As is shown in Table S6, no antibacterial compounds from other families gave positive results, even at the highest level tested (100 times the EU-MRL), a finding that proves that the test is specific for FQ.

With the aim of identifying other possible interferences, a high number of presumptive negative samples were evaluated. False-positive rate was determined by testing 180 meat samples from eight different species (beef, lamb, pork, rabbit, chicken, turkey, duck, and quail). Table S7 summarizes the number of meat samples that were analyzed with the new LFIA test and the average ratio by species. Among the 180 analyzed samples, no false-positive results were detected. In addition, 190 blood serum samples from pigs grown in antibiotic-free farming conditions were tested. Again, no false-positive results were found, as all the analyzed samples displayed ratio values above the established cut-off value of TL/CL 1.0. These results underscore the test's high specificity and confirm its accurate performance on both matrixes tested.

Table 3
Performance of the test for the detection of fluoroquinolones (FQ) in blood at concentrations equal to or slightly lower than the European Union-Maximum Residues Limits (EU-MRL) (μg/kg) described for muscle. Test Line/Control Line (TL/CL) ratio obtained from the Lateral Flow Immunoassay analyses performed are indicated, as well as the standard deviation (SD) and *n* represents the number of blood samples analyzed.

	LoD (μg/kg)	TL/CL	SD	n
Enrofloxacin	50	0.53	0.07	14
Ciprofloxacin	50	0.41	0.02	4
Flumequine	100	0.86	0.04	4
Marbofloxacin	75	0.69	0.10	14
Oxolinic acid	50	0.09	0.01	4
Danofloxacin	50	0.17	0.04	4

3.6.3. Robustness

The robustness of the assay was evaluated by introducing changes in the procedure and determining their effects on results. Sample volume, assay temperature and assay time were selected as the main critical factors, and their influence on the false-positive and false-negative rates was evaluated. For each factor, four different pork muscle juices were used as negative samples and were also spiked with 50 μg/kg of enrofloxacin. Samples were tested, and visual and instrumental reading were performed. Results are shown in Table S8 as the number of positive or negative replicates with visual reading and the mean value obtained with the IRIS reader. In order to illustrate variations associated with modifications in performance, maximum and minimum values for each condition are also indicated.

As shown in Table S8, neither false-positive nor false-negative results were observed within the range of changes under the conditions introduced in the procedure. Sample volume fluctuations from 150 to 250 μL, assay time ranging between 4 and 8 min, or assay temperature ranging between 38 and 42 °C did not have an impact on the test's result. Hence, this procedure can be regarded as a robust method for the detection of FQ in samples coming from animals intended for human consumption.

3.6.4. Validation with meat and blood samples containing in-vivo-administered antibiotics

In general, to simplify the validation of a new method, blank matrices fortified with specific molecules are usually prepared and immediately tested to verify the method's performance. However, analytes incurred in real samples can have suffered modifications by *in vivo* or *post-mortem* metabolism, or even degradation. Thus, an exclusive validation with fortified samples does not provide a faithful reflection of the test's performance under real conditions. Hence, in order to verify a method's performance, it is also recommended to test a certain number of naturally incurred samples. Hence, in the present study, meat and blood samples naturally containing different levels of enrofloxacin/ciprofloxacin were obtained from the sample bank built by Serrano et al. (2020). In that sample bank, ciprofloxacin was also measured in enrofloxacin-treated pigs due to the partial transformation of enrofloxacin to ciprofloxacin once administered. Samples were analyzed with the new test and results were compared to those obtained by LC-MS/MS (Table 4).

As is shown in Table 4, all the muscle samples containing enrofloxacin/ciprofloxacin at levels equal to or above the EU-MRL were detected by the LFIA test with TL/CL ratios between 0.1 and 0.7. Two samples with levels of enrofloxacin below the EU-MRL (44 and 83 μg/kg) were also detected with TL/CL ratios of 1.0 and 0.9, thereby indicating that those results were close to the method's LoD. Blood obtained from the same animals was also analyzed. All animals whose muscle gave a positive result with the test were also positive when blood was analyzed with the same method (Table 4).

These results confirm that there is a strong correlation between the results of the new test in blood and muscle from animals administered *in vivo* with enrofloxacin; moreover, they reassert the correspondence previously shown among spiked samples of meat juice and blood serum. Hence, the newly developed LFIA test can be regarded as a suitable tool for the analysis of FQ, not only in muscle but also in blood. It is thus an accurate *ante mortem* tool for the detection of animals with unauthorized FQ levels at farms or slaughterhouses, prior to slaughter. As a

Table 4
Performance (Test Line/Control Line (TL/CL)) of the new Lateral Flow Immunoassay (LFIA) test for the detection of fluoroquinolones (FQ) in incurred meat and blood samples coming from pigs administered with enrofloxacin and characterized by LC-MS/MS. Each sample corresponds to an individual, and samples are divided into three different administration batches (L1, L2, L3). The Table also includes as a control the result of a blank sample coming from an animal raised in antibiotic-free conditions (B4). Results: + (positive); +/− (weak positive); - (negative).

	Muscle				Blood			
	LFIA Test		LC-MS/MS		LFIA Test		LC-MS/MS	
Sample	TL/CL Ratio	Qual.[a]	Quant.[a]	Qual.[a]	TL/CL Ratio	Qual.[a]	Quant.[a]	Qual.[a]
L1E1	0.3	+	381	+	0.4	+	449	+
L1E2	0.2	+	1042	+	0.2	+	489	+
L1E3	1.0	+/−	44	+/−	0.4	+	50	+
L1E4	2.3	–	<LoQ	–	2.1	–	10	–
L1E5	2.3	–	<LoQ	–	1.6	–	17	–
L1E6	2.9	–	<LoQ	–	2.2	–	<LoQ	–
L1E7	1.8	–	<LoQ	–	1.8	–	<LoQ	–
L1E8	2.7	–	<LoQ	–	2	–	<LoQ	–
L1E9	2.2	–	<LoQ	–	2.3	–	<LoQ	–
L1E10	3.6	–	<LoQ	–	2.7	–	<LoQ	–
L2E1	0.2	+	742	+	0.3	+	415	+
L2E2	0.3	+	428	+	0.3	+	322	+
L2E3	0.3	+	344	+	0.6	+	150	+
L2E4	0.6	+	199	+	0.6	+	83	+
L2E5	1.4	–	30	–	1.3	–	28	–
L2E6	1.3	–	38	–	1.5	–	25	–
L3E1	0.1	+	1858	+	0.2	+	152	+
L3E2	0.3	+	468	+	0.4	+	150	+
L3E3	0.4	+	295	+	0.5	+	98	+
L3E4	0.7	+	152	+	0.6	+	60	+
L3E5	0.9	+	83	+	0.7	+	36	+
L3E6	2.2	–	18	–	1.3	–	22	–
B4	2.5	–	<LoQ	–	2.7	–	<LoQ	–

LFIA cut-off: Pos: ≤1.0. LC-MS/MS LoQ (Limit of Quantification) =10 μg/Kg.
[a] **Quant.: Quantitative result. Qual.: Qualitative result.**

consequence, the withdrawal period will be extended until the new LFIA test scores negative results, thereby preventing the entrance of these compounds in the food chain. Moreover, data obtained in muscle prove its suitability for detecting FQ after slaughter, hence for its implementation in common surveillance plans.

4. Conclusions

The new LFIA method developed for the detection of molecules from the fluoroquinolone family proved to be an adequate technique for tracking them in animal samples *ante* and *post mortem*. On the one hand, it was capable of specifically detecting FQ in meat samples from several species, which demonstrates and underscores its usefulness as a holistic technique for detection of FQ in slaughterhouses. Moreover, this method was also able to detect FQ in blood, which allows for its use in husbandry, thereby avoiding not only the entrance of antibiotics into the food chain, but also unnecessary animal slaughter. For both purposes, the limits of detection are in agreement with those established by European legislation; this is hence a convenient tool that can serve as a complement for screening tests that are unable to detect FQ. Therefore, this groundbreaking LFIA technique is proposed as a specific, rapid, and sensitive tool for fluoroquinolone residue management in all stages of meat production, from farm to fork, complying with the One Health approach.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare no conflcts of interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2023.110116.

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