DEVELOPMENT OF LATERAL FLOW IMMUNOASSAY FOR RAPID DETECTION OF OXYTETRACYCLINE IN HONEY SAMPLES

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Abstract: *A lateral flow immunoassay (LFIA) was developed in the competitive reaction format and applied to test residues of the antimicrobial oxyteracycline (OTC) in honey samples. To prepare the assay test, a nitrocellulose membrane was stripped with hapten OTC3 conjugate to ovoalbumin egg protein (OVA-OTC3) and goat anti-rabbit antisera (GAR) as capture and control reagents, respectively. Polyclonal antisera against oxyteracycline was conjugated to colloidal gold nanoparticles and used as the detection reagent. The visual detection limit (cut-off value) of the oxyteracycline LFIA was 20 ng/g, and qualitative results were obtained within 10 min without expensive equipment. The assay was evaluated with OTC spiked honey samples from different geographical origin (n = 25). The results were in good agreement with those obtained from liquid chromatography separation and mass spectroscopy detection (LC-MS), indicating that the LFIA test might be used as a semiquantitative method for the determination of oxyteracycline. The system was also highly specific, showing no cross-reactivity to other chemically similar antibiotics.*

Keywords: *Immunoassay, LFIA, Oxytetracycline, honey*

Introduction

Since their discovery in the mid-1900s, tetracyclines (TCs) have been widely used as therapeutic agents in human and veterinary medicine. These drugs also have particular applications as growth promoters in animal husbandry because of their broad-spectrum activity against a variety of Gram-positive and Gram-negative microorganisms –inhibiting the protein synthesis by binding to the small ribosomal subunit at the A site which binds with the aminoacyl tRNA-, and low cost [1,2].
Honeybees are subject to a number of diseases that affect their brood, with two of the most serious being the larval bacterial diseases American foulbrood (caused by spore-forming Paenibacillus larvae) or European foulbrood (caused by Melissococcus pluton) [3,4]; highly contagious and destructive diseases that affects honeybees [5,6]. In beekeeping, drugs known to be effective against these diseases are oxytetracycline (OTC) and sulfathiazole, in use for a long time.
The use of drug residues to treat bacterial diseases of honeybees cannot be ignored because their presence in the environment is a big concern in many countries. Interference with foodstuffs may lead to the production of food, which is harmful,

having implications in nutrition and safety including the possibility of allergic reactions or resistance. In fact, the widespread overuse of antibiotics in raising animals for food has helped create "superbugs", bacteria resistant to these drugs. These drugs certainly kill bacteria, but in doing so, they drive the process which selects in favour of resistant strains [7].

No maximum residue levels (MRLs) for tetracyclines in honey were set in the European Union, which means that if present, must be below the limit of quantitation (LOQ) of the analytical method used [8]. Since LOQs differ between laboratories, some countries within the European Union have established action limits or tolerated levels, ranging from 20 to 50 ng/g, referring to the sum of all substances within the TC group or 10 ng/g for one, like OTC.

Several methods have been reported for the determination of tetracyclines using a variety of techniques, including chromatography or microbiological growth inhibition [9]. The microbial inhibition tests are cheap and easy to perform, but require 2–3 days for microbe growth, sometimes being non-specific and not sensitive enough for residue monitoring. Among the chromatographic techniques, high performance liquid chromatography (HPLC) is the most used for the determination of multiple TC residues in honey including OTC [10–13]. It is the reference method, being very suitable for confirmation but not for screening purposes. A rapid, sensitive and specific assay would be interesting to detect positive TC samples in routine analysis, which then can be confirmed by reference methods.

Immunoassays can dramatically reduce the number of analyses required to characterize food samples for drug residue contamination. Therefore, during the past decades, a variety of ELISAs have been developed, each for multi-TCs screening in foods [14,15] or specific for an individual TC [16,17].

Nowadays, there is a need for more cost effective, field portable assay systems that can be conducted by users that are as close to the source of contamination as possible. Most of them are basically designed as visual test that require only low-cost instrumentation and offer an advantage of speed, essential to accept or reject goods on-site [18,19].

In the present work, we describe the development and evaluation of a prototype rapid lateral-flow immunochromatographic assay (LFIA) test kit for on-site testing for OTC residues in honey samples, using colloidal gold nanoparticles as the detection reagent.

Materials and methods

Chemicals

Tetracycline hydrate (TC), 7-chlortetracycline monohydrochloride (CTC), oxytetracycline dehydrate (OTC), anhydrotetracycline hydrochloride (ATC), demeclocycline hydrochloride (DMC), doxycycline hyclate (DXC), daunorubicin hydrochloride (DR), methacycline hydrochloride (MC), minocycline hydrochloride (MNC) and rolitetracycline (RTC) were purchased from Fluka–Sigma–Aldrich Química (Madrid, Spain).

Analytical grade solvents were provided by Scharlab (Barcelona, Spain). Goat anti-rabbit immunoglobulins (GAR) were purchased from Sigma (Madrid, Spain). All other reagents used were analytical grade. Nitrocellulose membrane CNPC-SS12 was from Advanced Microdevices Pvt. Ltd. (Ambala Cantt, India). The sample pad, the conjugate release pad and the absorbent pad were from Schleider & Schuell GmbH (Dassel, Germany). Plastic backing was from Estok Plastics (NJ, USA) and the plastic housing was supplied by Acon Biotech (HangZhou, China).

Apparatus

Isoflow reagent dispenser was from Imagene Technology (Hanover, Germany) and CM4000 Guillotine Cutting Module used to prepare test strips was from BioDot Inc. (Irvine, CA, USA). The centrifuge (Hereaus multifuge 3 S-R) was from VWR International Eurolab S.L. (Madrid, Spain). Particle size was carried out by transmission electron microscope S-3700N, from Hitachi High-Technologies Europe GmbH (Krefeld, Germany). The optical density was established spectrophotometrically in a Shimadzu model UV-1063 spectrophotometer from IZASA S.A. (Barcelona, Spain).

Preparation of colloidal gold

Nanometer colloidal gold was prepared according to the procedure described by Frens [20]. Basically, l00 mL of 0.01% chlorauric acid solution (in Milli-Q purified water) was heated to boiling, and then 2.0 mL solution of 1% trisodium citrate was added under constant stirring. Once the colour of the solution changed from blue to dark red within 2 min, approximately, it was boiled for another 15 min. The obtained gold colloidal suspension supplemented with 0.05% (m/v) of sodium azide was stored at 4 ºC in a dark-coloured bottle until use. With the scan between 500 and 600 nm, there is only one maximum absorbent wavelength: 525 nm. The particles obtained were checked by transmission electron microscope, showing that the average diameter of these particles of uniform size was 40 nm.

Gold Labelling of anti-OTC sera

Before conjugation with the colloidal gold, the optimal pH value and antibody concentration were determined to obtain the best sensitivity by checkboard titration. By gentle stirring, 10 mL of colloidal gold solution was adjusted to pH 7.0 with 0.1 M K_2CO_3 or 0.1 M HCl, and then 50 µg of protein A purified polyclonal antisera OTC3-I was added by drop wise. After incubation at room temperature for 15 min, 3 mL of 5% BSA solution was added, and stirring was continued for another 15 min. The mixture was centrifuged at 12000 rpm for 15 min, and the precipitate of the gold-labeled antibodies was resuspended in 5 mL of dilution buffer (0.01 M PBS, containing 1% sucrose and 0.5% Triton-100, pH 7.2) and stored at 4 °C. Gold labelled OTC3-I (detection reagent) was sprayed onto a conjugate pad (0.5 µL per cm^2, glass fiber membrane) and then dried for 1 h at 37 °C.

Immobilization of Capture Reagents

Isoflow reagent dispenser was used to put two lines on the nitrocellulose membrane strips (25 mm width - 88 m large). The dispensed volumes were both 1 µL/cm. After dispensation, the nitrocellulose membrane was dried for 12 h at 37 °C and stored under dry conditions at room temperature until use. The LFIA device for the detection of OTC was a single-antigen direct immunoassay. The device consists of a plastic support to which a nitrocellulose membrane (thickness, 15 ± 1 µm) is mounted. OVA-OTC3 was striped in the “test line” position (0.4 mg/mL), while the goat anti rabbit IgG was striped in the “control line” position (1 mg/mL). Gold particles (40 nm) individually conjugated to OTC3-I was dispensed onto a conjugate pad. The conjugate pad was then affixed to the test strip by overlapping the nitrocellulose membrane at its proximal end; the addition of a sample pad completed the assembly by overlapping onto the conjugate pad (Fig. 1). Devices that could be used to analyze 100 µL of honey solution were produced.

Test procedure

The assay was based on the competitive reaction theory (see Figure 1).

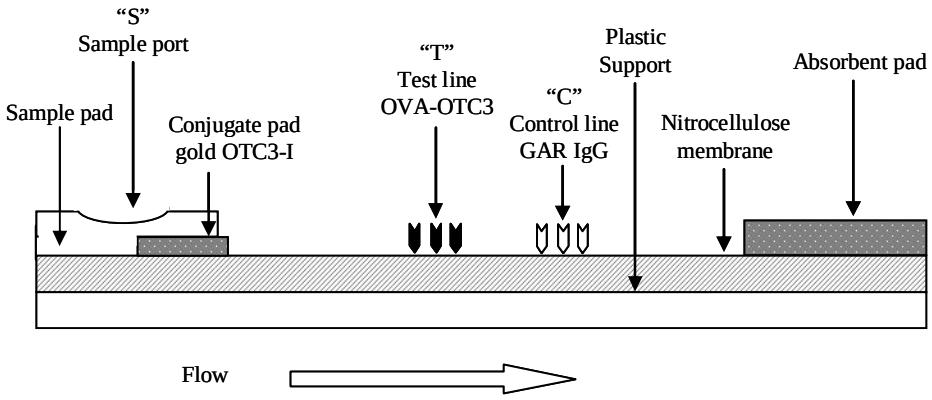

Figure 1. Schematic diagram of lateral-flow immunochromatographic assay

When honey diluted sample (100 µL) is placed in the "sample port" at "S" on the device, it rapidly went through to the conjugate pad, and the gold-conjugated OTC3-I was then solubilised. After that, the gold-conjugated OTC3-I migrates down to the nitrocellulose membrane by capillary action. At the test "T" line, the gold-conjugated OTC3-I binds to immobilized OVA-OTC3, forming a red line. The excess of gold-conjugated OTC3-I migrated farther and Fab region of rabbit polyclonal sera was trapped by the goat anti-rabbit immunoglobulins to form the control line "C". If the sample contains higher amount of oxytetracycline (OTC) -upper than 20 µg/kg-, they bind the gold-conjugated OTC3-I on the conjugate pad, blocking all recognition sites of OTC3-I sera. As a result, the gold-conjugated OTC3-I-OTC does not bind to immobilized OVA-OTC3, obtaining only a red line in C position. When the sample was free of OTC or the concentration was lower than 20 µg/kg, it competed with the immobilized OVA-OTC3 (test line) to bind the limited amount of gold-conjugated OTC3-I. If the concentration of the antibody is sufficient, the gold can be visualized as a red line at "T" position. The intensity of the test line would be inversely proportional to OTC present in the sample. The colour formation for both reactions is complete after 5 to 10 min.

Analysis of honey samples

Twenty five samples from different sources (TC free as assessed by LC-MS) were stored in a dark and dry place. Before analysis, a mix of all honey samples were spiked with OTC or cross related compounds and diluted 1/20 (w/v) in PBS-T at pH 7.5. The solution was vigorously agitated until honey was completely dissolved (around 5 min). Finally, 100 µL of the solution is placed in the sample port of the LFIA using a plastic mini Pasteur pipette.

For confirmation purposes, three grams of honey were exactly weighted into 50 mL polyethylene centrifuge tube. Then, 15 mL of Mcllvaine buffer (pH 4.0) with 0.10 mol/L Na_2EDTA were added and the mixture was vortexed until the honey dissolved completely. Ocatadecyl SPE cartridges (Varian Iberica, Spain) were conditioned with 5 mL of methanol and 5 mL of Mcllvaine buffer (pH 4.0) containing 0.10 mol/L Na_2EDTA. After condition, 5 mL of sample were allowed to pass through the cartridge followed by 2.5 mL of Mcllvaine buffer (pH 4.0): methanol (85:15 v/v) and 2.5 mL of water. The cartridge was dried for 2 min by aspiration and another washing step with 2.5 mL acetonitrile was done. Cartridge was dried again and analytes were eluted with 3.0 mL of ethyl acetate: methanol (75:25 v/v).

The elution mixture was evaporated until dryness under a gentle nitrogen flow in a water bath (30–35 ºC) and the residue was dissolved in 1 mL methanol: water (15:85 v/v). The final extracts were filtered through 0.45 mm syringe filters (Millipore, Spain) and injected onto the HPLC.

All honey samples were also analysed by HPLC-ESI-MS in an Agilent 1100 series LC/MSD Ion Trap (Agilent Technologies, Waldbronn, Germany). The separation was achieved on a Mediterranean C18 (15.0 cm × 2.1 mm I.D., particle size 3.5 microns) purchased from Teknokroma (Barcelona, Spain). A binary mobile phase with gradient elution was used. Ultra-pure water with 0.1% formic acid and acetonitrile were used as solvent A and solvent B, respectively. The gradient starts with 5% B, increases to 90% in 35 min, and then returns to the initial composition for 10 min to condition the column for next injection. The flow-rate was 0.4 mL/min, the column temperature was set to 25 ºC and the injection volume was 20 µL. The mass spectrometer acquired data in quantitative multiple reaction monitoring (MRM) mode. For OTC, two different characteristic fragmentation reactions *m/z* 444 and *m/z* 426 were monitored in the selected reaction monitoring (SRM) mode using a dwell time of 0.1 s

Results and Discussion

Researchers are aware that nanometer colloidal gold particles have been gradually applied in immunoassay, biosensor, bioidentification, gene therapy, arrays and DNA computation [21]. Because immunoglobulins could be labelled with gold instead of enzyme, the substrate is therefore not needed in the reacting system. The strength of colour shows that it is closely related to the size - determined by the amount of trisodium citrate- and quality of the colloidal gold particles. Previous experience in gold assays has shown us that a 40 nm particle is the best for the strip assays. These results have also been described in the literature [22]. Smaller particles give fewer signals because of how the gold scatters light and larger particles tend to migrate slowly, generate a purple blue colour, and are difficult to work with (stability). Also, membranes with a pore size of 15 microns are preferred to for both flow rate and reactivity, since if larger pore sizes are used the flow rate is usually too fast for reactions to take place at low sensitivities and if smaller pore sizes are used a test is difficult to finish in 5-10 minutes.

The concentrations of immunoreagents were optimized to satisfy the following criteria: the appearance of a clear pink colour on the test lines for negative samples within a reasonably short time (up to 10 min); the difference between positive and negative samples could be easily distinguished with the naked eye; minimum immunoreagent consumption. For this purpose, experiments similar to the "checkerboard titration" in ELISA were performed for OTC test. Several dilutions of OVA-OTC3 conjugate coated on the membrane against different amounts of colloidal gold labelled OTC3-I and control line (GAR), from 0.05 mg/mL to 2.5 mg/mL were investigated, using a blank honey sample and samples containing between 5 ng/g OTC and 100 ng/g OTC. Combinations satisfying the above mentioned criteria were selected for further experiments. In accordance with the competitive immunoassay principle, lower amounts of coating conjugates and gold labelled sera resulted in an increase in the assay sensitivity. However, under certain concentrations of the immunoreagents, the colour of the control lines was too faint for visual evaluation. As a result, concentrations of 0.8, 0.4, and 1.0 mg/mL were selected, respectively for gold labelled OTC3-I, OVA-OTC3 and goat anti rabbit.

For the determination of the visual limit of

detection, a control honey, previously shown to have no detectable OTC (LC-MS), was fortified by OTC standard (100 mg/g) at nine concentrations and provided to a panel of 22 trained referees as blind samples, with a protocol assay (as described in analysis of honey samples section) and sufficient number of LFIA, for naked eye evaluation. The results are shown in Table 1.

Table 1.
Detection results of blind samples by LFIA and LC-MS

Sample	OTC added (ng/g)	OTC found (ng/g) LFIA	OTC found (ng/g) LC-MS
1	0	0/66*	n.d.
2	5	0/66	n.d.
3	50	66/66	48.6 ± 4.7
4	15	5/66	17.0 ± 3.6
5	3	0/66	n.d.
6	20	66/66	21.5 ± 3.0
7	35	66/66	32.3 ± 2.4
8	10	15/66	9.8 ± 2.2
9	30	66/66	33.2 ± 4.1

*Visual reading (n positive/n analyzed). All samples were tested by triplicate (n=3).

As can be seen in Figure 2, referees indicated -as positive- the smallest amount of OTC that resulted in no colour development at the test line, which was considered as the cut-off value. In this sense, the visual detection limit (VDL) was selected as the point corresponding to a concentration threshold that ensures 95 % of positive responses [23]. From the data supported by referrers (naked-eye determination), the lateral-flow immunochromatographic assay yielded a positive result at 20 ng/g OTC (100 % of positive responses), while equivocal result was obtained at 10 and 15 ng/g OTC, since 15 test were interpreted as positive and 51 as negative for 10 ng/g (77% of positive responses) and 5 positive versus 61 negative for 15 ng/g (92% of positive responses). The results from the visual evaluation of the lateral-flow tests were in good agreement with the amounts spiked (Table 1) and determined by LC-MS, demonstrating the practical applicability of the developed assay.

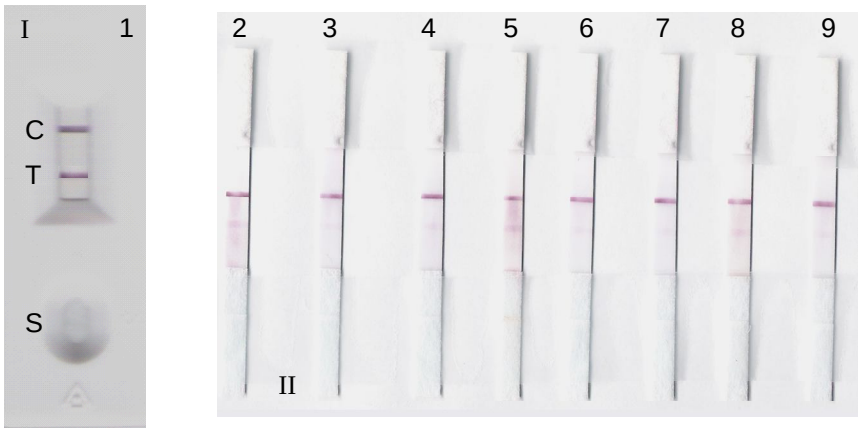

Figure 2. Lateral-flow immunoassay of spiked honey samples. OTC concentrations (from left to right). In panel I: 0 ng/g (S-sample well; T-test line; C-control line); In panel II: 5; 50; 15; 3; 20; 35; 10 and 30 ng/g (strips are out of the plastic housing**). Upper line is the control line; bottom line is the test line.**

The specificity of the OTC method was evaluated in comparison to other analogue compounds: TC, CTC, ATC, DMC, DXC, DR, MC, MND and RTC. Stock solutions of each sulphonamide (100 mg/L) and a mix containing all of them (except OTC), at the same concentration, were prepared in DMSO and stored at 4 ºC and proper diluted with phosphate buffered saline (PBS; 100 mmol/L sodium phosphate, 137 mmol/L NaCl, 2.7 mmol/L KCl, pH 7.5), containing 0.05% Tween 20; yielding concentrations ranging from 0.01 to 50 mg/L. From a control honey free of tetracyiclines (previously tested by LC-MS), different aliquots (1 g) were fortified by adding 20 mL of standards diluted in PBS-T solution of different related compounds. Positive or negative results from the LFIA, three replicates for each honey concentration and cross-reactant were scored, after 100 µL of the corresponding solution was placed in the sample port of the LFIA using a plastic mini Pasteur pipette.

Two clear bands were observed in the test and control lines test strip, even though these compounds were present at a high level. Each analogue compound was found not cross-reacted when tested at concentrations up to 50 mg/g, only tetracycline (TC) have cross reacted at concentrations upper than 70 ng/g. This fact indicates that the developed technique had a high specificity towards OTC.

Few attempts have been made to develop sensitive immunoassays, mainly in ELISA format, for TCs in different matrices such as milk, kidney, fish and honey, with sensitivities ranging from 0.048 to 150 ng/g [24].

A very sensitive LFIA named Tetrasensor, was developed and validated for tetracycline, oxytetracycline, chlortetracycline, and doxycycline in honey with detection limits between 6 and 12 ng/g for each compound [25]. However, the current status of the available immunoassays for a single sulphonamide, such as OTC, is scarce.

In the present work, we describe a LFIA which has a clear limit of detection at 20 ng/g OTC. The assay can be used with small volumes (100 µL) of diluted honey. The assay was shown to have 100% diagnostic sensitivity and good specificity for the detection of OTC in honey. Existing ELISA and other assays for STZ tests are laboratory based, require sample preparation, and are relatively slow compared to the LFIA described here, for the first time.

This result supports the establishment of 20 ng/g as VDL of the LFIA that will be in compliance with the further EU minimum required performance limit (MRPL) of 20 ng/g for OTC in honey, as proposed by the European federation of honey packers and distributors (FEEDM).

The LFIA could be stored at room temperature for at least 6 months or more, and the kit could be delivered and used at ambient temperature (data not shown). Another advantage is cost, where the gold conjugate used is considerably less expensive than enzyme or fluorescent conjugates with easy handling and simple to perform. For the reasons mentioned, the test lends itself better for making rapid large scale screening of honey samples for OTC. The OTC-LFIA has an immediate application as a rapid analytical tool to screen field samples in situ.

On the whole, the LFIA is versatile and can be adapted for fast detection of other analytes of interest, which could be an advantage to take decisions about foods (accept or reject) in a short time. By switching the antibodies or antigen and making small adjustments to the chemistry of the strip format, the same test design may be used for many applications.

Conclusions

A rapid lateral-flow immune-chromatographic device with a colloidal

gold-polyclonal probe was developed and optimized for the detection of oxytetracycline in honey samples. Coupled with a simple and fast sample preparation method, the assay could be accomplished within 10 min without the need of any equipment. The visual detection limit was 20 ng/g, and the developed technique had a high specificity toward OTC, since it recognises TC when is present in honey at concentrations ≥ 70 ng/g. The results from visual evaluations of the lateral-flow tests of spiked honey samples proved the reliability of the assay. This qualitative one-step test based on the visual evaluation of results appears to have practical advantages such as rapidity and simplicity, over existing conventional immunoassay formats.

The proposed analytical system has no equivalence in the market and could be used in the honey sector to carry out on-site screening for OTC at the beginning of the food chain to improve/facility commercial trade.

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