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Article

Development of a Lateral-Flow Immunochromatographic Strip for the Detection of Oxytetracycline Residues in Biological Fluids

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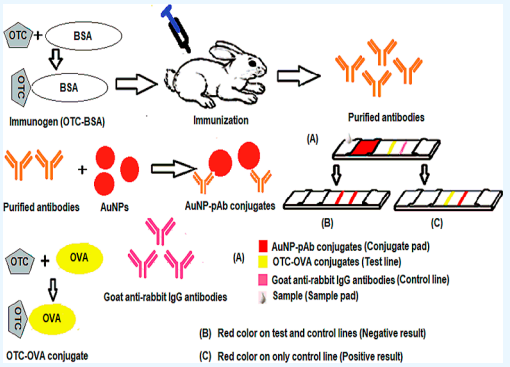
ABSTRACT: Oxytetracycline (OTC) is extensively used in veterinary medicine and for growth promotion around the globe. The indiscriminate use of OTC in food-producing animals leaves residues in animal products. The presence of these residues in animal products causes economic losses and harmful effects on consumers. Different regulatory bodies set maximum residue limits (MRLs) for different tetracyclines. To avoid harmful effects, there is a need for a simple, fast, and economical method for the screening of animal products. In this study, a fast, economical, and user-friendly lateral-flow immunochromatographic (LFIC) assay based on gold nanoparticles (AuNPs) was developed to detect the presence of OTC residues in biological fluids. AuNPs provided visual results as red lines in 6−15 min. Polyclonal rabbit IgG antibodies were produced using the immunogen of OTC. These antibodies were purified by the combined ammonium sulfate-octanoic acid precipitation method. Antibodies were conjugated to AuNPs as recognition biomolecules. A LFIC strip was optimized using borate buffer spiked with different concentrations of the OTC. The visual limit of detection (LOD) in different biological samples (milk, serum, and urine) was determined using samples spiked with OTC. The LOD was found to be 15 μg/L, which is very low from the MRL (100 μg/L) set by different regulatory authorities. This LFIC strip can be used to detect OTC residues in biological fluids for point-of-care testing (POCT). These strips are easy to use, cost-effective, and portable and provide quick results without the use of laboratory instruments.



1. INTRODUCTION

Antibiotics are commonly used in humans and animals. According to a previous study, antibiotics used in animals are approximately 70−75% of the total antibiotics used around the globe in 2017.[1] Tetracyclines are widely used antibiotics for the treatment of different diseases in poultry, swine, cattle, and sheep. Due to the increase in human population, there is increased demand for animal production for meat and milk to fulfill the demand. Specifically, OTC, doxycycline, and chlortetracycline are being employed for the promotion of growth by improving feed efficiency and weight gain worldwide.[2]

OTC is produced by *Streptomyces* spp., which is bactericidal in action and belongs to the group of broad-spectrum antibiotics. OTC inhibits protein synthesis during the translation of mRNA by binding to the 30S ribosomal units of 70S ribosomes of bacterial cells. OTC is widely used in tetracycline around the globe due to its broad antimicrobial activity, good therapeutic activity, easy access, and affordability.[3] OTC is being used in different animals for the treatment of gastrointestinal and respiratory bacterial infections. It is majorly used against aerobic bacteria including different Gram-positive and Gram-negative bacteria, *Rickettsia*, *Mycoplasma*, and *Chlamydia* species. OTC is being manufactured in different formulations that can be administered through oral and parenteral routes. OTC has shown wide distribution throughout the body, causing the accumulation of OTC residues in the brain, lungs, spleen, eyes, synovial fluid, liver, and kidney, as well as in meat, eggs, and milk. OTC is mainly metabolized in the liver and excreted majorly through bile and urine.[4]

As the OTC treatment improves the health of animals and reduces the death rate, it results in decreased economic loss. On the other hand, extra-label use and improper observation of withdrawal time for OTC may cause residues in different animal products and urine. OTC residues in milk may inhibit the growth of bacteria present in milk, which are responsible for the production of yogurt and cheese, causing economic losses. The use of animal food products containing OTC residues by humans may also cause health hazards to humans

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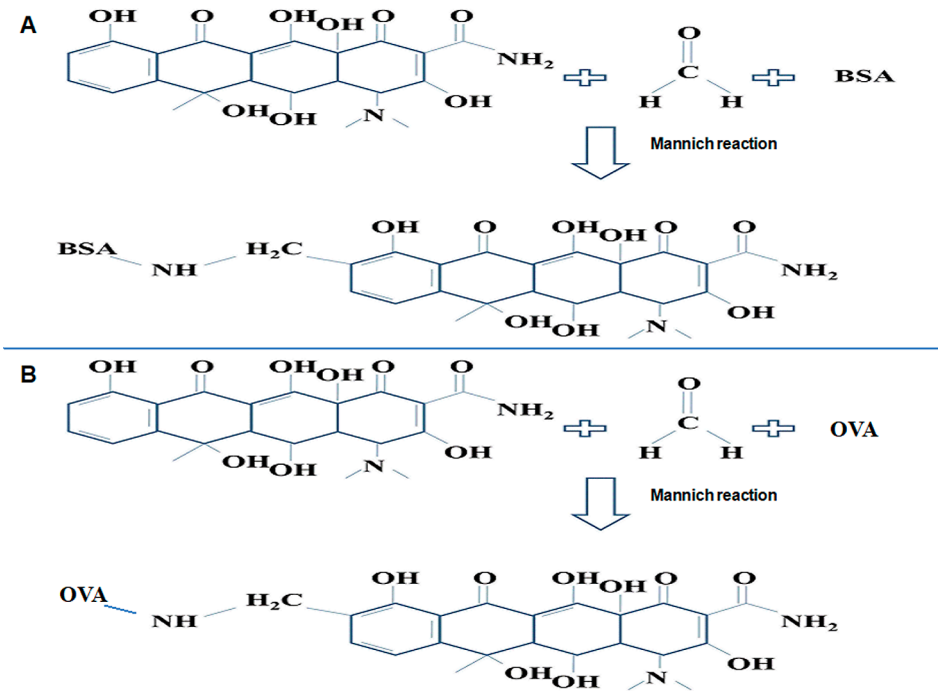

Figure 1. Mannich reactions of OTC with BSA and OVA: (A) Mannich reaction of OTC and BSA and (B) Mannich reaction of OTC and OVA.

such as the development of antibiotic-resistant bacteria, allergic reactions, and toxicity.[5,6]

To avoid economic losses and health hazards, different regulatory authorities set the maximum residue limit (MRL) for OTC residues in milk. For OTC, the European Union (EU) set the MRL value at 0.1 mg/L (100 μg/L).[7] In the recent era, many screening and confirmatory assays like microbiological assays, enzyme-linked immunosorbent assays (ELISA), different chromatographic assays coupled with mass spectrometry, and fluorescent detection methods based on copper nanoclusters, poly(adenine) (poly A) DNA-templated Au nanoclusters, and carbon dots have been developed for the detection and quantification of OTC in milk.[8−12] Although these methods are highly specific and sensitive and have high throughput, they require expensive chemicals and instruments and highly skilled personnel for the operation and maintenance of instruments. Moreover, these methods also require sample pretreatment and a long time for the analysis of a single sample. These attributes make these methods unsuitable for multiple samples and point-of-care testing (POCT) in remote areas in a short duration. So, there is a need for the development of a screening method that should be highly specific, easy to use, cost-effective, time-efficient, and suitable for POCT.

Lateral-flow immunochromatographic (LFIC) assay is a paper-based assay that is easy to perform, cost-effective, time-saving, and portable; requires no sample pretreatment; and provides the results on-site. This assay can be carried out without expertise and expensive instruments. LFIC assay is a semiquantitative method involving chromatography and antigen−antibody interaction. Antigen−antibody interaction makes it sensitive and specific. LFIC is being used to detect different analytes such as drugs, infectious agents, proteins, and pathogens.[13,14]

The most important part of the LFIC assay is the signaling label. Many types of nanoparticles are used as signaling labels in LFIC assays. The application of nanoparticles provides cost-efficient, fast diagnosis, and simplified analysis procedures with enhanced sensitivity and selectivity of the LFIC assay. Nanoparticles that have been used in LFIC assays include colored nanoparticles (gold, selenium, and carbon nanoparticles), magnetic nanoparticles (silver nanoparticles), quantum dots, upconverting phosphor nanoparticles, and latex beads.[15,16] AuNPs are widely used as signaling labels in LFIC assays due to their various advantages over other signaling labels. AuNPs have unique optical characteristics, as they have an intense red color and provide visual results as red lines for the on-site detection of different antigens without using the aid of any other instrument. AuNPs are biocompatible and easily conjugated with different recognition elements (DNA, aptamer, and antibodies).[17]

Although there are many LFIC assays being used for the detection of antibiotic residues in milk, no LFIC assay has been developed for the detection of OTC antibiotic residues in different biological fluids of animals. In this study, a AuNP-based LFIC assay was developed to detect OTC in different biological fluids. i.e., milk, serum, and urine. For the development of the LFIC assay, polyclonal rabbit IgG antibodies were produced and purified. Polyclonal antibodies (pAbs) were conjugated with AuNPs as a recognition element for the antigen (OTC). These LFIC assay-based strips were optimized and used to detect OTC in biological fluids.

2. RESULTS AND DISCUSSION

2.1. Confirmation of Immunogen and Antigen. OTC has a molecular mass of 460.4 Da. Due to its smaller size, OTC is not immunogenic. So, OTC was conjugated to bovine serum albumin (BSA) or ovalbumin (OVA) via the Mannich reaction to produce immunogen (OTC-BSA) and coating antigen (OTC-OVA) for the immunochromatographic strip, as shown in Figure 1A,B.

UV−visible spectroscopy was performed at wavelengths of 250−450 nm for the confirmation of the presence of OTC-BSA and OTC-OVA. OTC showed two maximum absorbance

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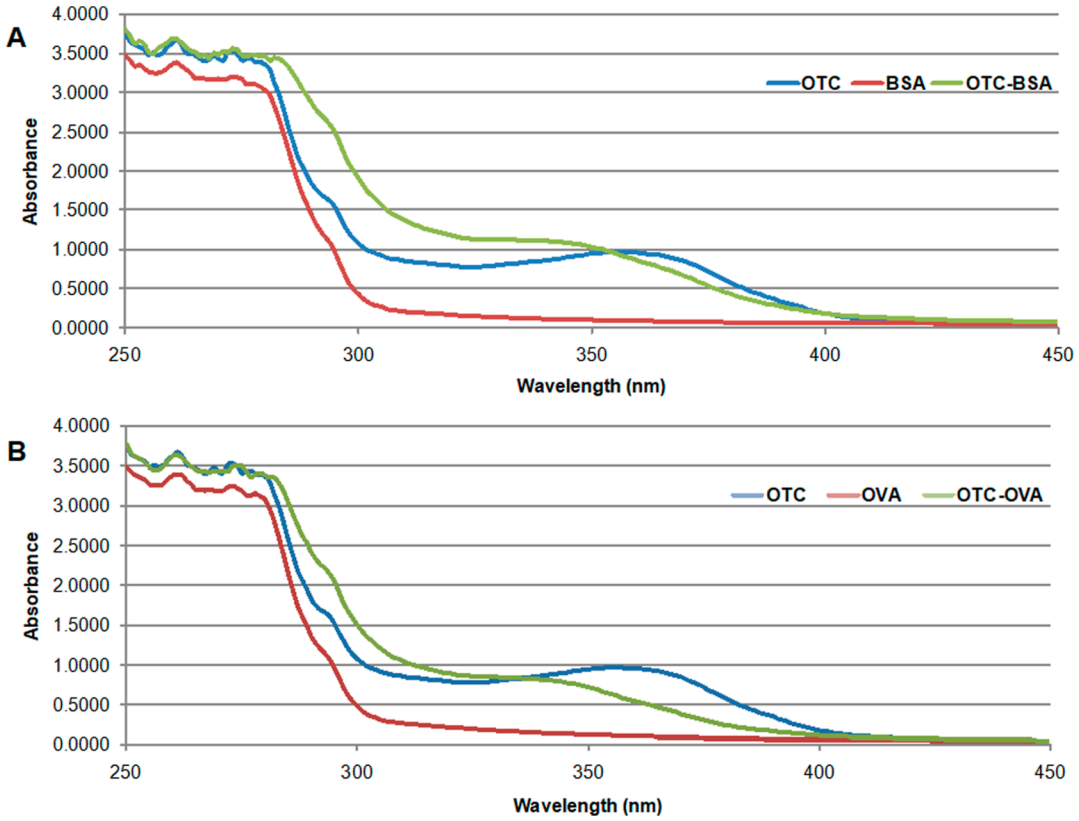

Figure 2. UV−visible spectra: (A) UV−visible spectra of OTC, BSA, and OTC-BSA, and (B) UV−visible spectra of OTC, OVA, and OTC-OVA.

peaks at wavelengths 286 and 356 nm and BSA showed maximum absorption at a wavelength of 278 nm, while OTC-BSA exhibited absorbance peaks at 289 and 337 nm, as shown in Figure 2A. This shift in absorbance peaks confirmed the presence of OTC-BSA. OVA showed an absorbance peak at a wavelength of 278 nm, while OTC-OVA showed an absorbance peak at 290 nm, as shown in Figure 2B. This shift of the maximum absorbance peak suggested the successful formation of OTC-OVA. This method of confirmation was used in previous studies.[18,19]

2.2. Quantification of Polyclonal Rabbit IgG Antibodies. The immunogen (OTC-BSA) alone is not capable of producing antibodies. So, the immunogen was emulsified with Freund's adjuvants (FAs) for the production of antibodies. Antibodies were purified by the combined ammonium sulfate-octanoic acid (AS-OA) precipitation method. Purified antibodies were quantified by a nanodrop quantification technique. The maximum concentration of antibodies was found in sample O2 i.e. 6.45 mg/mL. The minimum concentration of antibodies was found in sample O4 i.e. 5.22 mg/mL. The average concentration of polyclonal rabbit IgG antibodies was found to be 5.825 mg/mL, as shown in Figure 3. This quantity is comparable with the results of a previous study.[20]

2.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to check the purity of the purified antibodies. SDS-PAGE showed only two bands at 50 and 25 kDa, as shown in Figure 4. This indicated that antibodies were successfully purified using the combined AS-OA precipitation method. This result is in line with the previous study.[21]

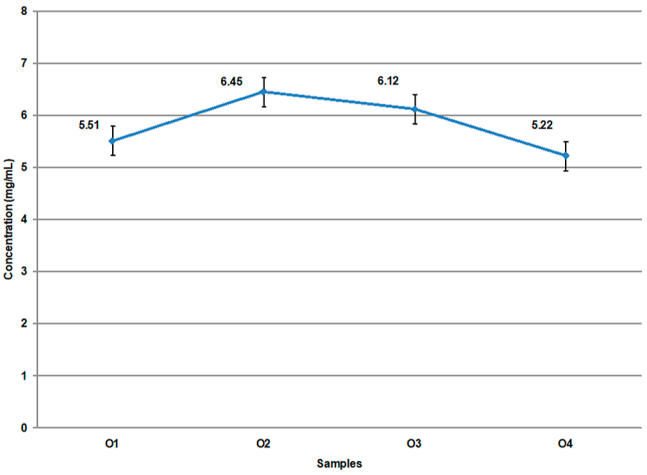

Figure 3. Quantification of polyclonal rabbit IgG antibodies purified by a combined AS-OA precipitation method.

2.4. Confirmation of Gold-Labeled Polyclonal Antibodies. AuNPs were conjugated with purified antibodies for dispensing on the conjugate pad in the manufacture of immunochromatographic strips. For the confirmation of gold-labeled pAb, UV−visible spectroscopy was performed at wavelengths of 400−600 nm. AuNPs showed maximum absorbance at a wavelength of 520 nm, while the gold-labeled pAb showed the maximum absorbance at a wavelength of 533 nm, as shown in Figure 5A. This shift of the maximum absorbance peak and increased absorbance confirmed the formation of gold-labeled pAb. This method was used to confirm the conjugation in a previous study.[22] Gold-labeled pAb was confirmed by running a lateral-flow immunochroma-

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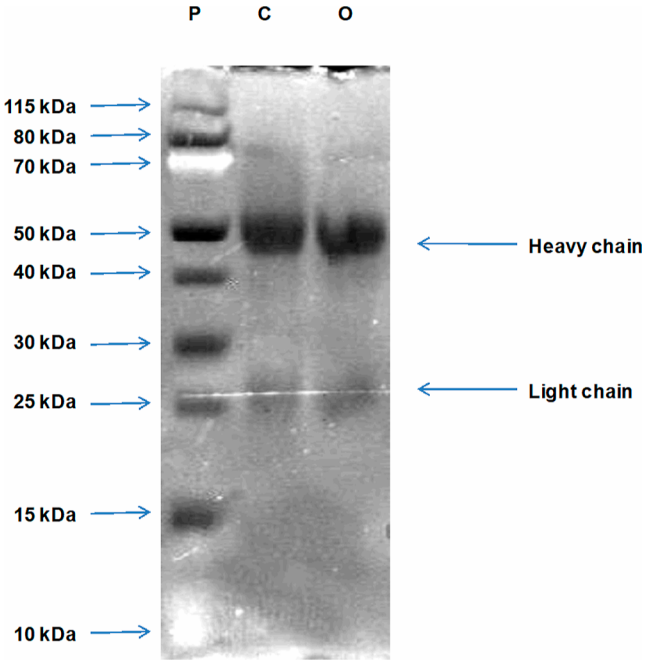

Figure 4. Separating gel containing different bands where "P": prestained protein ladder, "C": polyclonal anti-CFT IgG antibodies (part of other work), and "O": polyclonal anti-OTC IgG antibodies.

tographic strip with only gold-labeled pAb on the conjugate pad and goat anti-rabbit IgG antibodies on the control line. The formation of red color on the control line indicated the successful formation of gold-labeled pAb, as shown in Figure 5B. This result is in line with previous work.[23]

2.5. Limit of Detection of LFIC Strips. A schematic diagram of the competitive format is shown in Figure 6A. A competitive format was reported in previous literature.[24] For the determination of the LOD of this method, the LFIC strips were run with borate buffer samples spiked with different concentrations of the OTC and analyzed. The LFIC strips were run with milk, serum, and urine samples spiked with different concentrations of OTC and analyzed. It is obvious from Figure 6B that the visual LOD for the borate buffer sample is 15 μg/L. This visual LOD is the same for milk, serum, and urine samples, which is obvious from Figures 6C–E. So, these strips can be used to detect OTC in biological fluids. This LOD for milk is far below the MRL of the OTC in milk set by different regulatory authorities. This visual LOD by the LFIC assay is comparable with the previous studies where the authors reported visual LODs of 10 and 35 μg/L of tetracycline antibiotics in milk and serum, respectively.[25,26]

3. CONCLUSIONS

A competitive LFIC strip was developed for the detection of OTC residues in biological fluids, i.e., milk, serum, and urine. This developed LFIC strip is sensitive, fast, user-friendly, cost-effective, time-efficient, and portable compared to other conventional methods. pAbs were used as recognition elements for the development of the LFIC assay. These pAbs were labeled with AuNPs, which provided visual results as the red line(s). It took an average time of 6–15 min for assay. LOD for the strip was found to be 15 μg/L which is too low compared to the MRL of the OTC for milk prescribed by different regulatory authorities. This strip can be used to screen milk for the safety of consumers, as well as this strip can detect the OTC in serum for diagnostic purposes and in urine for environmental safety.

The developed method, however, provides only preliminary qualitative results. Samples showing positive results should be

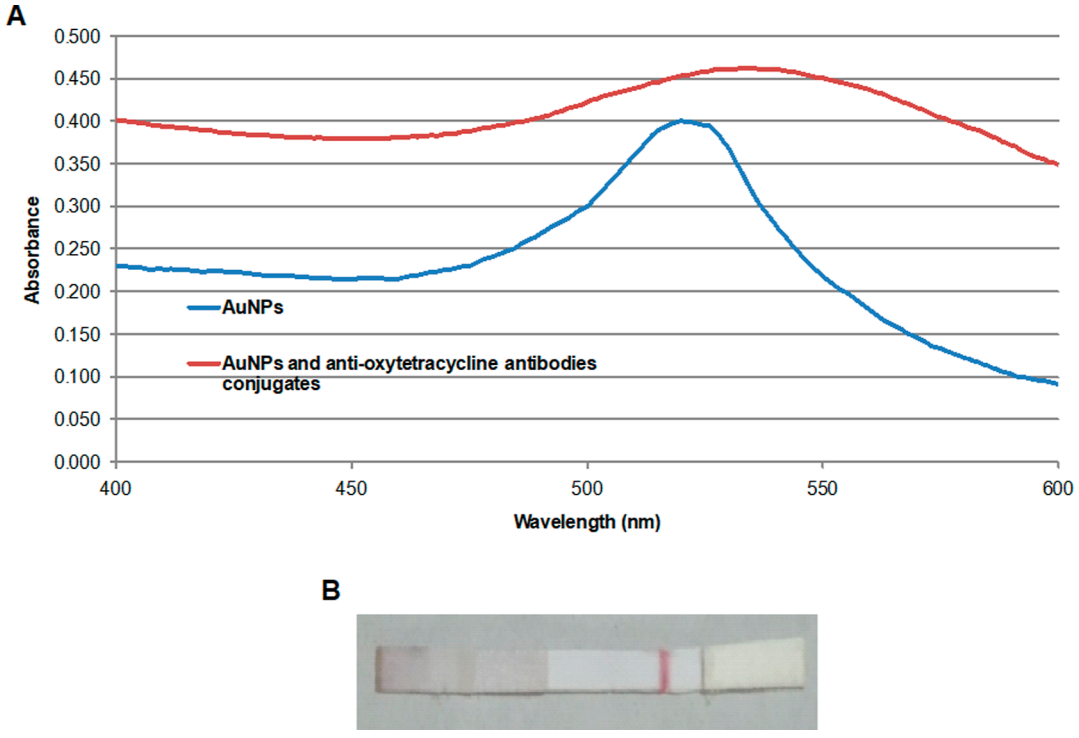

Figure 5. Confirmation of gold-labeled pAb: (A) UV–visible spectra of AuNPs and gold-labeled pAb and (B) red line on the control line showing the formation of gold-labeled pAb.

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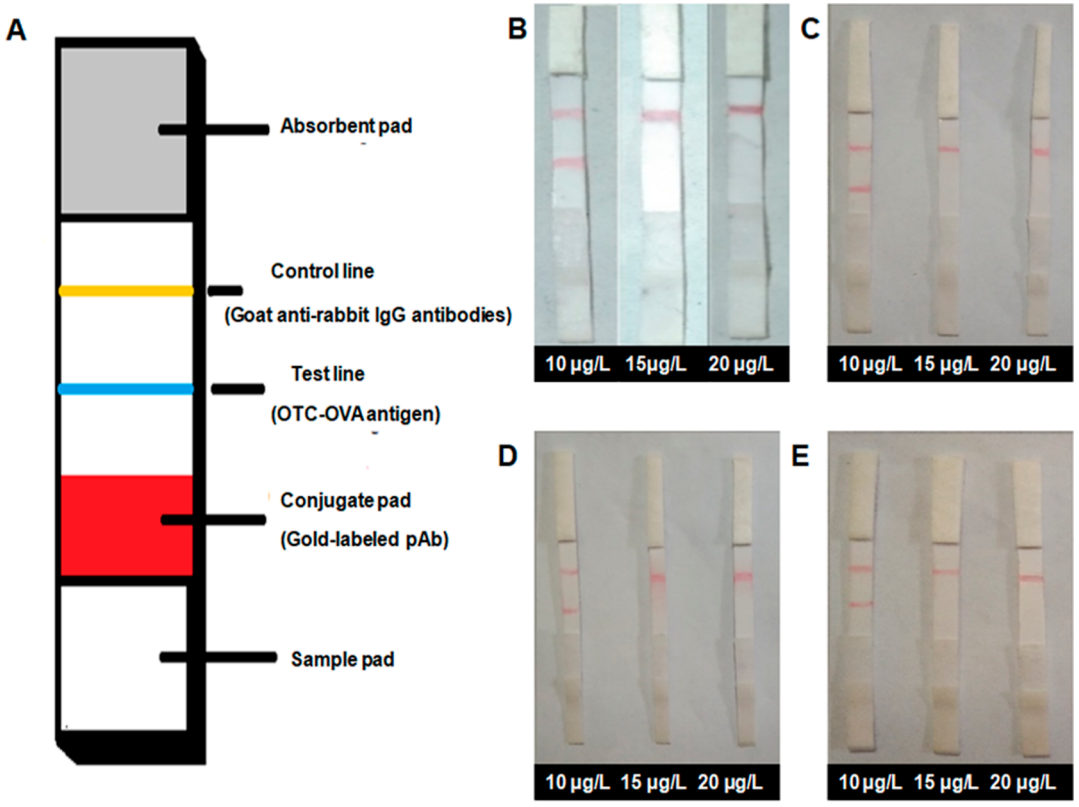

Figure 6. (A) Schematic diagram of the LFIC strip. (B) Strips run with borate buffer spiked with OTC, (C) strips run with milk samples spiked with OTC, (D) strips run with serum samples spiked with OTC, and (E) strips run with urine samples spiked with OTC.

further confirmed by one of the highly sensitive instrumental methods like HPLC and LC−MS/MS, etc.

4. MATERIALS AND METHODS

4.1. Chemicals and Instruments. OTC donated by Aptly Pharmaceutical Pvt Ltd. (Faisalabad, Pakistan). BSA, OVA, and methanol were purchased from bioWORLD (Dublin, Ohio, USA). Formaldehyde (37%) was purchased from Merck KGaA (Germany). Phosphate buffer saline (PBS) and borate buffer were procured from VWR Chemicals BDH (Radnor, Delaware). Freund's complete adjuvant (FCA) was purchased from InvivoGen (San Diego, USA) and Freund's incomplete adjuvant (FICA) (ImmunoCruz) was procured from Santa Cruz Biotechnology, Inc. (Santa Cruz, California). AS was purchased from Flinn Scientific Inc. (Illinois). Monohydrate 2-(*N*-morpholino)ethanesulfonic acid (MES) and glycine were procured from Phyto Technology Laboratories (USA). Ethylenediaminetetraacetic acid (EDTA), β-mercaptoethanol, acrylamide, glycerol, and ammonium persulfate (APS) were purchased from Biochem Chemopharma (France). Bromophenol blue and bis-acrylamide were purchased from ACTGene (USA). Tris was procured from Amresco (USA), and Page Ruler prestained protein ladder was procured from Thermo Scientific (Lithuania). Tetramethylethylenediamine (TEMED) and sodium dodecyl sulfate (SDS) were procured from Carl Roth GmbH + Co. KG (Germany). Goat anti-rabbit IgG antibodies were purchased from Elabscience (China). OA and AuNPs were purchased from Sigma-Aldrich (USA). Dialysis membrane (MEMBRA-CEL) was procured from Viskase (Lombard, Illinois). Polyvinyl chloride (PVC) backing card, conjugate pad, and sample pad were procured from Shanghai Jieyi Biotechnology Co. Ltd. (China). Nitrocellulose (NC) membrane was purchased from Sartorius Biopharma (Germany). A Multiskan GO microplate spectrophotometer was purchased from Thermo Fisher Scientific (Finland). An EBA 200 centrifuge was purchased from Andreas Hettich GmbH & Co. KG (Germany). A Z 216 M-K temperature-controlled centrifuge was purchased from HERMLE Labortechnik GmbH (Germany). A hot plate and a magnetic stirrer (M Top MS 300 HS) were procured from Misung Scientific Co. Ltd. (Korea). A nanodrop spectrophotometer (NAS-99) was purchased from ACTGene (USA). A Christ ALPHA 1-4 LDplus freeze-dryer was purchased from Martin Christ Gefriertrocknungsanlagen GmbH (Germany). An incubator was procured from Memmert GmbH + Co. KG (Germany).

4.2. Preparation and Confirmation of Immunogen (OTC-BSA) and Antigen (OTC-OVA). OTC and BSA were conjugated via the Mannich reaction according to the method of ref 27 with some modifications. OTC (30 mg) was dissolved in 1 mL of methanol and BSA (100 mg) was dissolved in 5 mL of distilled water and mixed with the methanolic solution of OTC. Formaldehyde (1.5 mL) was added dropwise with constant stirring. The mixture was kept for 24 h at RT for complete reaction. Filtration was done five times in a falcon tube at 875*g* for 20 min in a centrifugation machine. The same protocol was used for the preparation of the OTC-OVA by only replacing BSA with OVA. The confirmation of the presence of OTC-BSA and OTC-OVA was done by UV−visible spectroscopy.

4.3. Animals. Four rabbits (n = 4) weighing 1000−1200 g were purchased from Jhang Bazar, Faisalabad, and kept in the animal house of the Institute of Microbiology, Faculty of Veterinary Science, University of Agriculture, Faisalabad. Conditions of the temperature and light−dark cycles were

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optimized. Rabbits were fed seasonal food and water at regular intervals of time. The in vivo part of this study was conducted according to the guidelines of National Biosafety Rules 2005, Punjab Biosafety Rules 2014, Punjab Animal Health Act 2019, and Bioethics Protocol recommended by the bioethical committee of the University of Agriculture, Faisalabad, in draft no. 1928.

4.4. Immunization of Rabbits with Immunogen. Preimmune serum was prepared before injecting before the immunization and stored at −20 °C until further use. Rabbits were immunized according to the method described by ref 23.

Immunization doses were prepared by dissolving 500 μg of the OTC-BSA conjugate (Immunogen) in 500 μL of normal saline and emulsified in an equal volume of FCA. The immunization doses were given subcutaneously at 0 days. Booster doses were prepared by dissolving 500 μg of the OTC-BSA conjugate in 500 μL of normal saline and emulsified with an equal volume of FICA. Five booster doses of immunogen were given subcutaneously on the 15th, 25th, 35th, 45th, and 55th day, as shown in Table 1. On the 65th day, rabbits were slaughtered. Antisera were prepared and stored at −20 °C until further use.

Table 1. Schedule for Immunization of Rabbits with the OTC-BSA Immunogen

doses	days	OTC-BSA/adjuvant (1:1)
immunization dose	0	OTC-BSA/FCA[a]
1st booster dose	15th	OTC-BSA/FICA[b]
2nd booster dose	25th	OTC-BSA/FICA
3rd booster dose	35th	OTC-BSA/FICA
4th booster dose	45th	OTC-BSA/FICA
5th booster dose	55th	OTC-BSA/FICA

[a]FCA = Freund's complete adjuvant. [b]FICA = Freund's incomplete adjuvant.

4.5. Purification, Quantification, and Characterization of Polyclonal Antibodies. The purification of pAbs was done by the combined AS-OA precipitation method described in ref 28 with some modifications. Serum (5 mL) was taken and diluted with an acetate buffer in a 1:3 ratio. OA (25 μL/mL) was added dropwise, and the mixture was stirred for 30 min. After that, it was centrifuged for 20 min at 11,200*g*, and the supernatant was separated. The supernatant was diluted with an equal volume of PBS. The pH was set at 7.4, and the temperature was reduced to 4 °C. AS (1 mL) was added, and the mixture was allowed to stand overnight at 4 °C. Fractionated serum was centrifuged at 2800*g* for 20 min, and the supernatant was discarded. Pellets were resuspended in an equal volume of PBS and dialyzed against PBS for 48 h. Purified antibodies were stored at −20 °C until further use. Purified pAbs were quantified using the nanodrop quantification technique.[29] To determine the purity of pAbs, SDS-PAGE was done according to the method described by ref 30.

4.6. Preparation and Confirmation of Gold-Labeled Polyclonal Antibodies. Purified pAbs and AuNPs were conjugated according to the method described by ref 31 with some modifications. Purified pAbs were mixed with 10 mM Tris buffer (pH 8.5). A AuNP solution was added with a solution of potassium carbonate (1 M) to adjust the pH to around 8.5−9.0. Purified pAbs (1 mL) were mixed with AuNPs (1 mL) with an orbital shaker, and the mixture was incubated at 25 °C for 60 min. BSA (10%) was added to block unreacted sites of AuNPs and mixed on an orbital shaker for 10 min. The mixture was incubated again at RT for 30 min and centrifuged at 4 °C for 20 min at 16,000*g* to collect the pellets of gold-labeled pAbs. These pellets were dispersed in TBSA (10 mM, pH 8.5 Tris buffer containing 1% BSA and 1% sucrose). This colloid of gold-labeled pAbs was stored at 4 °C until further use. Confirmation of gold-labeled pAb bioconjugates was done using UV−visible spectroscopy and running the strip impregnated with gold-labeled pAbs on the conjugate pad and secondary antibodies on the control line.

4.7. Fabrication of the LFIC Strip. A backing card, sample pad, conjugate pad, NC membrane, and absorbent pad were used in the assembly of the strip. The backing card acted like the backbone of the strip as it supported all the other four components. The NC membrane was placed in the middle portion. The absorbent pad was pasted on the upper portion, and the conjugate pad was pasted on the lower part in such a way that they overlapped NC by 2−3 mm from each side. At last, the sample pad was pasted on the lower part as it covered 2−3 mm of the conjugate pad. All the components were pressed gently for proper fixation, and the strip was cut into desired dimensions of 3.5 × 60 mm. Gold-labeled pAb was dispensed on the conjugate pad. Test and control lines were impregnated with antigen (OTC-OVA) and goat anti-rabbit IgG antibodies, respectively. Strips were dried for 2−3 h at RT for proper fixation.

4.8. Principle and LOD of the LFIC Strip. A stock solution (10^6 μg/L) of OTC was prepared in a borate buffer. This stock solution was diluted to obtain various concentrations of OTC. Dilutions (100 μL) were placed on the sample pad. The buffer solution was moved to the conjugate pad, where it was mixed with gold-labeled pAbs. Then, it was moved to the NC membrane where antigen (OTC-OVA) was coated on the test line (T line) and goat anti-rabbit IgG antibodies on the control line (C line). When the analyte (OTC) was insufficient or absent, gold-labeled pAbs were combined with antigen (OTC-OVA) coated on the test line and the remaining were moved forward to the control line where they were combined with goat anti-rabbit IgG antibodies showing a red color on test and control lines (negative result). When the analyte (OTC) was present in sufficient concentration, gold-labeled pAbs were occupied by analyte (OTC) and did not react with antigen (OTC-OVA) and moved forward to the control line where they got captured by goat anti-rabbit IgG antibodies showing red color on control line (positive result). The completion of the reaction and formation of red lines took 6−10 min. The intensity of the red color on the test line was inversely proportional to the concentration of the analyte (OTC) in the buffer.

4.9. Analysis of Milk, Serum, and Urine Samples Spiked with OTC. Milk was centrifuged at 5000*g* for 5 min. The liquid portion was mixed with a known concentration of the OTC to make a stock solution and diluted to obtain various concentrations. Blood was centrifuged at 1500*g* for 10 min to get serum. Serum and urine were also spiked with known concentrations of the OTC to make stock solutions. These stock solutions were diluted to get various concentrations. These different dilutions (100 μL) were used to run the LFIC strips, and the results were analyzed.

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Author Contributions

The study conception and design were done by B.A. and F.M. Material preparation, data collection, and experimental work were conducted by A.A. Data analysis was performed by A.S.Q. and S.R. The first draft of the manuscript was written by A.A. and B. A. All authors have read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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