Production of Polyclonal Antibody for Oxytetracycline and their use in Lateral Flow Assay

Laxmana Naik\textsuperscript{a}, Kiran Lata\textsuperscript{a}, Rajan Sharma\textsuperscript{a}, Bimlesh Mann\textsuperscript{a}, and Rajput, Y.S.\textsuperscript{b}

\textsuperscript{a}Dairy Chemistry Division, ICAR-National Dairy Research Institute, Karnal-132001, India.
\textsuperscript{b}Animal Biochemistry Division, ICAR-National Dairy Research Institute, Karnal-132001, India.

*Corresponding Author: Laxmana Naik  Email: laxmandenaik@gmail.com

Received: 21/12/2014  Revised: 27/12/2014  Accepted: 28/12/2014

Abstract

A new synthesis way of haptens for oxytetracycline (OTC) with Keyhole limpet hemocyanin (KLH) was demonstrated. This hapten conjugate (OTC-KLH) were used to successfully raise polyclonal antibodies against OTC. Utilizing this antibodies a rapid, user friendly and sensitive lateral flow assay (LFA) was designed to detect the OTC in milk system. From this assay results could be accomplished within 5 min. without the need of any equipment. The visual detection limit was 30 μg/kg. Proposed concept of OTC antibody production found to be a simple and reliable method for LFA. Thus, the developed assay could be used in the milk system to carry out onsite screening for OTC, from the beginning of the food chain to improve commercial trade and to uphold the consumer safety.

Key words: Oxytetracycline, Milk, Antibiotic residue, Lateral flow assay.

1. Introduction

In the animal husbandry practice; milk producers basically rely on the use of antibiotics and medicinal drugs to treat mastitis and other infectious diseases. Antibiotics are essentially chemicals substances produced by or derived from microorganism (mold or bacterium) which have capacity to inhibit the growth of or to kill other microorganisms (generally bacteria) and cure infections. Some antibiotics can be used to treat a wide range of infections and are known as broad-spectrum antibiotics; while other antibiotics are effective against a few types of bacteria and are called narrow-spectrum antibiotics. Tetracyclines (TCs) antibiotics rank among the antimicrobial substances as most frequently and most extensively used in the animal food production (Hernandez et al., 2000; Okerman et al., 2004; Mojica et al., 2011; Mu et al., 2011), because of their broad-spectrum activity against gram-positive as well as gram-negative bacteria. They are also used for promoting growth in cattle and poultry (Kaae et al., 2008). Of the eight commercially available TCs; Oxytetracycline (OTC) is most commonly applied to food-producing animals and are approved by the European Union (EU) and the United States Food and Drug Administration (US FDA) for therapeautic use in cattle, pigs, sheep and poultries (Rodriguez et al., 2009). To prevent any harmful health effects on consumers, many organizations have established the maximum residual limit (MRL) for OTC in milk (Table 1).

If appropriate measures are not taken, indiscriminate use of antibiotics in dairy animals results in high risk of contamination of the milk supply (Beltran et al., 2014). Implications of the presence of antibiotic residues in milk as a result of veterinary treatments have been documented, including their negative effects on consumers’ health, such as allergies, development of antibiotic resistance (Dewdney et al., 1991; Oliver et al., 2011). Many investigations revealed that antibiotics residues adversely affects the fermented milk industry, to list some of them; increase in rennet clotting time, decrease in diacetyl production, inhibition of lactic acid production (Ramakrishna et al., 1985; Ram et al., 2000; Basanta, 2001), delay in cheese ripening process and interference with the alkaline phosphatase activity (Manolkidis and Alichanidis, 1971) and in some cases false positive alkaline phosphatase test due to microbial produced heat-resistant phosphatase (Murthy and Cox, 1988). From the regulatory point of view; to sustain in the international trade, milk and milk products have to comply with the international standards. The milk products consignments meant for export tested for antibiotics residual parameters as per the requirements of the importing country (EIC, 2014). Exporting country has to assure that the product shall meet the standard and if not the importing country imposes ban.
Table 1: Maximum Residual Limits for Oxytetracycline in Milk.

<table>
<thead>
<tr>
<th>Organization</th>
<th>MRL (ppb)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAO / WHO</td>
<td>100</td>
<td>FAO (2003)</td>
</tr>
<tr>
<td>Codex Alimentarius Commission</td>
<td>100</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>Mercosur</td>
<td>100</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>Australia</td>
<td>100</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>EU</td>
<td>100</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>USA</td>
<td>300*</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>New Zealand</td>
<td>100*</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>Japan</td>
<td>100*</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>South Africa</td>
<td>100*</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>Taiwan</td>
<td>100*</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>Switzerland</td>
<td>100*</td>
<td>Diserens et al. (2010)</td>
</tr>
<tr>
<td>FSSAI</td>
<td>100 (Imported products)</td>
<td>EIC (2014)</td>
</tr>
</tbody>
</table>

Note: 1.* Includes for all TCs (Safe level).
2. ppb = parts per billion = μg/L = μg/Kg = ng/g = ng/ml = pg/mg = 10^-9

the trade may hamper. Hence, to screen the presence of antibiotics in milk is of great significance.

To prevent any harmful health effects on consumers, many organizations have established the maximum residual limit (MRL) of OTC in milk. Codex, WHO and EU set the MRL at 100 ppb, while the US FDA MRL is 300 ppb (Diserens et al., 2010). A number of quantitative test methods are available for detection of antibiotics in milk, but they require sophisticated instrumentation and lab facilities, these test methods are costly and laborious. At present few commercial test kits are available in the market, they are expensive, are qualitative in nature. We have attempted to develop a rapid test for the OTC detection in milk using lateral flow assay (LFA).

LFA is traditionally an antibody-based immunochromatographic assay. This method employs basic principles from the subjects of biology, chemistry, physics and engineering fields (Lata et al., 2013). The fundamental principle underlying in LFA is the movement of few drops of (liquid) sample containing the target analyte(s) along a strip of polymeric material by the action of capillary flow across various reactive zones, where the reorganization molecules have been attached, that exert more or less specific interactions with the analytes (O’Farrell, 2009; Posthuma-Trumpie et al., 2009; Anfossi et al., 2013). This test device is functionally divided into different segments; sample pad, conjugate pad, testing zone (capture zone), and wicking pad. Typically, all these segments are made up from different porous and flexible membranes; which serve one or more functions. These different parts are overlap onto one another and are pasted on a backing card using a pressure sensitive adhesive. LFA can be used as point of care (POC) testing device, and has become the most famous way of diagnosis (Lata et al., 2013; Sajid et al., 2014).

Performance of the lateral flow assay is mainly based on antibody raised against the target analyte. It works as a heart of the LFA system, it will be targeting a particular analyte and mostly screened for the highest affinity and specificity. Generation of antibody against target analyte or antigen is a very important step. It can be developed according to the requirement. When the target analyte is of large molecular weight, then as such it is injected into the host animal for antibody development. Hence, there is no requirement of preparation of immunogen conjugate (hapten). But if it is of small molecular weight then it should be conjugated with carrier protein, using either bovine serum albumin (BSA) or ovalbumin (OVA) or keyhole limpet hemocyanin (KLH) or by dextrans. Pastor-Navarro et al. (2007) proposed a method for preparation of hapten conjugate for OTC, using Keyhole Limpet Hemocyanin (KLH) as a carrier protein. Recently, Noraini and Azura, (2011) proposed a method for production of hapten for tetracycline (TC), to generate the polyclonal antibody. They used KLH as carrier protein and a TC-KLH conjugate was prepared and this was utilized as immunogen.

2. Materials and Methods

Oxytetracycline hydrochlorides (OTC-HCl), bovine serum albumin (BSA), Folin and Ciocalteau’s phenol reagent, 3, 3′, 5′-tetramethylbenzidine (TMB) liquid substrate for ELISA were purchased from M/s. Sigma Aldrich. Copper sulphate, sodium potassium tartrate, sodium carbonate and sodium hydroxide were purchased from M/s. Merck chemicals, Mumbai. Polyclonal antibodies against OTC-HCl were raised in rabbit; it was purified by protein-A affinity column.
Naik et al…Production of Polyclonal Antibody for Oxytetracycline and Their Use in Lateral Flow Assay

Shoppers from M/s. Bangalore Genei (Merck Millipore). Goat-ant Rabbit IgG (affinity purified) and HRP conjugated Goat-ant Rabbit IgG were procured from M/s. Bangalore Genei (Merck Millipore). Keyhole Limpet Hemocyanin (KLH) procured from Thermo Scientific. All the reagents used were of ‘AR’ grade unless otherwise specified.

Cellulose dialysis membrane (Fisherbrand; 3500 Da cut off) was purchased from Fisher Scientific. Whatman filter papers - No. 1 was purchased from GE Da cut off) was purchased from Fisher Scientific. Grade unless otherwise specified.

Thermo Scientific. All the reagents used were of ‘AR’ grade unless otherwise specified.

HRP conjugated Goat-anti Rabbit IgG were procured from M/s. Bangalore Genei (Merck Millipore). Milli-Q water (resistivity 18.2 MΩ/cm) was prepared fresh as and when required.

Disodium hydrogen phosphate (Na2HPO4) in 800 ml water. Then pH was adjusted to 7.4 and volume was made up to 1000 ml.

Magnetic Stirrer (Spinot- digital, Tarsons), ELISA Reader (Tecan I-Control, Infinite F200Pro) were used in this experiment.

Reagents

a. Copper sulphate solution (1%, w/v): Prepared by dissolving 1.0 g copper sulphate in distilled water and then final volume was made up to 100 ml.

b. Sodium potassium tartrate solution (2%, w/v): Prepared by dissolving 2.0 g sodium potassium tartrate in distilled water and then final volume was made up to 100 ml.

c. Sodium hydroxide solution (0.2 M): Prepared by dissolving 8.0 g sodium hydroxide pellets in distilled water and then final volume was made up to one liter.

d. Sodium carbonate solution (4%, w/v): Prepared by dissolving 4.0 g sodium carbonate in distilled water and then final volume was made up to 100 ml.

e. Alkaline reagent: Prepared by mixing of 49 ml of reagent ‘c’ and 49 ml of reagent ‘d’. Then 1 ml of reagent ‘a’ was added followed by 1 ml of reagent ‘b’. This reagent was prepared fresh as and when required.

f. Folin’s reagent (1 N): Prepared by mixing of 5 ml Folin and Ciocalteau’s phenol reagent to 5 ml distilled water. This dilution was done immediately before use.

g. OTC Stock solution: 10 mg OTC was dissolved in 100 ml PBS.

h. OTC Working solution: 125 μl of the solution ‘g’ was diluted to 10 ml with PBS.

i. Coating antigen Samples: OTC-BSA conjugate (0.5 μg/100 μl) was prepared in 0.05 M carbonate-bicarbonate buffer, pH 9.6.

OTC-KLH conjugate was also prepared in same manner (for immunization).

j. Coating antibody Samples: OTC antiserum and purified antibody

k. Blocking solution (2%, w/v): Prepared by dissolving 2 g BSA in 10 ml of carbonate buffer (0.06 M, pH 9.6).

l. Washing buffer solution (PBST, 0.1% Tween-20): Prepared by dissolving 1 g Tween-20 in 100 ml of PBS solution.

m. Chromogen Substrate: 3, 3’-5, 5’-Tetramethylbenzidine (TMB) liquid substrate.

n. Labeling Substrate: Diluted HRP goat anti-rabbit IgG (100 μl per well)

o. Stop solution (1N H2SO4): Prepared by dissolving 11.11 ml concentrated H2SO4 to 100 ml distilled water.

p. Phosphate Buffered Saline (PBS) (pH 7.4): Prepared by dissolving 8 g NaCl, 0.2 g KCl, 0.24 g KH2PO4 and 1.44 g Na2HPO4 in 800 ml water, then pH was adjusted to 7.4 and volume was made up to 1000 ml.

q. Coating Buffer-Carbonate buffer (0.06 M, pH 9.6): Prepared by mixing 16 ml sodium carbonate solution (prepared by dissolving 3.2 g sodium carbonate in 500 ml distilled water) and 34 ml sodium bicarbonate solution (prepared by dissolving 2.52 g sodium bicarbonate in 500 ml distilled water). Finally pH of the solution was adjusted to pH 9.6 using 0.1 N NaOH solution.

r. Milk sample preparation: Fresh milk sample was collected from Cattle Yard, National Dairy Research Institute, Karnal. Milk was skimmed and heated at 70°C and cooled. Stock solution (10 mg/100 ml) of OTC was prepared in milk. To prepare working solution, took 125 μl of stock solution and final volume was made up to 25 ml with milk. The working solution was diluted appropriately with milk to obtain different OTC concentration in milk (ppb). An amount of 50 μl of prepared milk sample was used for the test.

s. Buffer: Phosphate-buffered saline (PBS) pH 7.4: Prepared by mixing 8 g NaCl, 0.2 g potassium chloride (KCl), 0.24 g potassium dihydrogen phosphate (KH2PO4) and 1.44 g disodium hydrogen phosphate (Na2HPO4) in 800 ml of water. pH was adjusted to 7.4 with 0.1 N NaOH solution and volume was made up to 1000 ml.

Note: The stock solution, working solutions of OTC and milk samples spiked with OTC were stored in amber colour containers at 4°C.
2.1 Synthesis of Hapten to Oxytetracycline

OTC is low molecular weight substances; hence it is not Immunogen. To make it immunogen; hapten conjugate was prepared using OTC and a carrier protein (KLH) by the method of Pastor-Navarro et al. (2007) with some modification. This reaction was carried out under light-protected condition. In this protocol, 2 mg KLH and 10 mg OTC were suspended in phosphate-buffered saline (PBS). This mixture was incubated for overnight at 4°C under constant stirring. The conjugate was dialyzed against 1xPBS. This hapten conjugate was used for immunization in rabbit. Confirmation of conjugation was done by spectroscopic observations and by estimating protein (i.e. KLH) in conjugate preparation.

2.2 Accessing the Quality of OTC Conjugates

Conjugates were diluted 10 times with PBS buffer. OTC (10 mg/100 ml) and KLH (10 mg/100 ml) stock solution were prepared in PBS buffer and diluted to 5 times prior to taking spectra in UV-spectrophotometer (Shimadzu UV-2550). Spectrum observation was recorded from 200 nm to 350 nm.

Protein content in the conjugate was determined according to the method of Lowry et al. (1951) with slight modifications as given below:

a. To 0.2 ml of diluted conjugate sample (protein concentration ≤ 500 μg/ml), added 2.0 ml of alkaline reagent (e). Mixed the contents rapidly and allowed to stand for 10 min at room temperature.

b. Thereafter, 0.2 ml of 1 N Folin’s reagent (f) was added, and mixed immediately and allowed to stand at room temperature for 30 min.

c. The blue colour developed was measured by taking absorbance at 750 nm in a 96 well Microplate reader (Tecan, Model infinite 200, Austria). Double distilled water was used as blank in place of conjugate and was processed under identical conditions as that of sample.

d. A calibration curve of bovine serum albumin (BSA) from the concentration of 25 to 250 μg/ml was prepared. The results were expressed as mg protein/ml of conjugate.

2.3 Development and Purification of Antibody

Development of polyclonal antibody against OTC was outsourced to Merck Millipore, Bangalore Genei, (Service No.: 30582, Lot No.: 131073). For this appropriate quantity of OTC-KLH hapten conjugate (immunogen) was provided to the firm. The immunization schedule detailed below was followed:

a. A rabbit was selected (New Zealand white) for polyclonal antibody development and pre-immune serum was collected prior to the start of service.

b. Intramuscular injection of approximately 500 μg of immunogen was injected along with 1:1 ratio of Freund’s complete adjuvant (FCA) on first day.

c. After 15th, 25th and 35th days respectively 1st, 2nd and 3rd booster doses were given with 200 μg of immunogen with Freund’s incomplete adjuvant (FIA).

d. Subsequently on 45th and 55th days 4th and 5th booster doses were given with 100 μg of immunogen with FIA.

e. On 65th and 75th days, animal was bled, blood was collected from ear vein and antiserum was prepared.

f. On completion of service, pre-immune serum (2 ml), antiserum, (25 ml) and purified antibody (25 mg) was provided to us by the firm. Antibody was purified using 2 ml of Protein-A agarose beads from 5 ml of serum. Purified antibody was dialyzed against 1xPBS. Purity of antibody was confirmed on SDS-PAGE. The concentration of purified antibody estimated by absorbance method was 8 mg/ml. All these products were stored at -20°C in small aliquots until use. Repeated thawing and freezing was avoided.

2.4 ELISA Titer for Antiserum and Purified Antibody

The procedure described by Chen et al. (2009) was followed with little modification as outlined below: Competitive indirect ELISA format was followed. All the analysis was performed in triplicates. The results were expressed in percentages of the maximum absorbance (B/Bo %) using the following equation:

\[
\frac{\text{Maximum absorbance (B)}}{\text{Maximum absorbance (Bo)}} \times 100
\]

a. Micro-titer (96 wells) plates were coated with 100 μl of antigen conjugate.

b. The coated plates were incubated overnight at 4°C.

c. The wells were washed three times with washing buffer solution to remove unbound conjugate.

d. Blocking solution (150 μl per well) was added to each well to block additional binding sites on the plate surface and incubated for 2 h at...
room temperature and then three times washing was given after incubation.
e. Various concentrations of diluted antiserum (1/100, 1/1000, 1/5000, 1/10000, 1/20000, 1/40000 and 1/80000) / purified antibody (0.09, 0.19, 0.38, 0.75, 1.5, 3.0 and 6.0 (µg/100 µl) were added to each well at 100 µl and then the plate was incubated again for 2 h at room temperature.
f. Excess of antibody/antiserum was removed by 3 times washing with washing buffer. Labeling was done by addition of 100 µl of goat-anti-rabbit IgG-HRP to each well (diluted in 0.01 M PBS, 1:5000) and Incubated for another 1 h at 37ºC.
g. In dark condition 100 µl of TMB solution added to each well and incubated for 15 min at room temperature.
h. Reaction was terminated by addition of stopping solution: 1 N H₂SO₄ (50 µl/well).
i. The absorbance was taken at 450 nm in TECAN ELISA reader infinite F200 PRO, Austria.

2.5 Application of OTC Antibody in Lateral Flow Assay

Lateral flow strips were designed as described by Lata et al. (2013), membrane accessories were pasted on the backing card. OTC-BSA conjugate and goat-anti rabbit IgG-antibody were applied as a thin line on the test line and control line of nitrocellulose membrane respectively. At our laboratory colloidal gold nanoparticles (GNP) were prepared. A method for conjugation of GNP with this OTC antibody was optimized (Note: this section is communicated elsewhere for publication). This GNP-OTC conjugates were placed over the conjugate pad. Membranes were dried and then cut in to 5 mm size width strips. The assay was validated by appearance of red lines within 5 minute on the nitrocellulose membrane. Presence of control line indicates that test strips were working or not. Appearance of two lines (i.e. at control as well as test line) indicated that the OTC analyte in sample is below detection limit or absent. Appearance of one line (i.e. at control line) indicated that the OTC analyte was above the detection limit (positive).

3. Results and Discussion

3.1 Antibody Development against Oxytetracycline

A new technique of hapten preparation for OTC was demonstrated based on the formation of the carboxamido and diazo-derivatives; this method maintains the OTC nucleus and possesses an aromatic or aliphatic spacer arm in order to create a degree of heterology in the chemical structure. The hapten was then used to successfully to raise polyclonal antibodies. Quality of antibody was accessed using ELISA technique and finally these antibodies were used in LFA.

OTC is a small molecule with a molecular size of 460,434 Da. Therefore, OTC itself is non-immunogenic and not able to elicit immune response in animals for producing anti-OTC antibody. To make it immunogenic, it was conjugated to a carrier protein before immunization (Siti and Nur, 2011). The design of specific hapten should mimic the original compound in the structure electronic and hydrophobic properties. Also, the hapten would present suitable functional groups (–COOH; –NH₂, –OH, –SH, etc.) to link the molecule to a carrier protein (Pastor-Navarro et al., 2007). Taking this into account, in the present study, OTC was conjugated with Keyhole Limpet Hemocyanin (KLH) as a carrier protein to generate immune response and produce polyclonal antibody. KLH is a complex high molecular weight protein used as a carrier protein in antibody production because of its excellent immunogenicity and it confers to attach antigens (Nuria et al., 2007; Zhang et al., 2007).

3.2 Accessing the Quality of OTC Conjugates

Conjugation is very crucial for success of immunization, so confirmation of conjugation was attempted with various techniques such as spectroscopic observations, Lowry method and polyacrylamide gel electrophoresis (PAGE). To obtain confirmation of successful conjugation, UV absorbances was recorded for OTC, KLH and OTC-KLH conjugate from 220 to 420 nm, as shown in Fig 1. It was observed that OTC had two peaks at 278 nm and 358 nm, KLH had one peak at 279.5 nm. After conjugation peak of OTC-KLH conjugate was shifted to 278 nm with increased absorbance value, there was minute shift on the second peak at 358 nm was also observed, indicating that successful conjugation between OTC and KLH (Fig 1-A). Absorbances of OTC-BSA conjugate was compared with OTC and BSA, as recorded in UV spectrophotometer. The peak of OTC was 276.12 nm, for BSA it was 278 nm, and for OTC-BSA conjugate peak was shifted to 277.19 nm (Fig 1-B). Shift in peaks of conjugate vis-à-vis OTC and BSA showed the successful conjugation. Peng et al. (2011) also have confirmed that the conjugation of hapten and carrier protein by noting the shift in UV absorbance peak of conjugate in the spectra. According to Lynn et al. (1998), UV absorbance for proteins changed during conjugation reaction conditions and the UV absorbance for hapten also changed when coupled
Fig 1: UV Spectra of OTC, KLH and OTC-KLH Conjugates (A), OTC, BSA and OTC-BSA Conjugates (B).

to proteins. The technique also has been used to ascertain the conjugation of tetracycline to KLH wherein it was observed that tetracycline had two peaks at 276 nm and 358 nm, KLH had peak at 278 nm whereas tetracycline-KLH conjugate peak shifted to 275 nm and 349 nm (Siti and Nur, 2011).

The protein content of conjugate can be used to confirm the conjugation. Hence, in the present study Lowry method was employed. Since, it was expected that pure OTC will not react with Lowry reagent, the reaction of Lowry reagent in conjugate would confirm the presence of protein (i.e. KLH) and thus ascertain the conjugation. In the present study the protein content was 2.29 mg/ml determined. This confirms the presence of protein in the conjugate and hence successful conjugation also. Earlier, Usleber et al. (1998) have employed Lowry method to ascertain the formation of immunogen (ampicillin-BSA) prepared by glutaraldehyde reaction wherein 3.3 mg/ml protein content has been reported in the preparation.

In native PAGE electrophoresis most proteins separate by charge to mass ratio. The conjugation of KLH with OTC would change the molecular weight of KLH and thus, it was envisaged that native KLH could be differentiated from conjugate KLH (OTC-KLH) on the gel after native PAGE. However, results showed that bands of KLH and OTC-KLH could not be differentiated and thus this technique was not suitable to ascertain the conjugate formation. This may be due to minute difference in the molecular weight of KLH and OTC-KLH conjugate. Molecular weight of KLH and OTC have been reported 350,000 and 390,000 Da respectively. The combined molecular weight of OTC and KLH (conjugate) increased to a very less extent (350,365.4 Da or 390,365.4 Da) and it may be possible that this small change in molecular weight was not perceptible on the gel. Other reason may be that all carboxylic group of OTC was used in conjugation with KLH so no carboxylic group was free (Pastor-Navarro et al., 2007), so negative charge was absent. Positive charge was created due to amine group present in OTC. Hence, in the gel migration of conjugate was not properly seen with reference to the KLH.

3.3 Production of Polyclonal Antibody and Its Purification

Polyclonal antibody was raised in New Zealand white rabbit. Prior to start of service pre-immune serum was collected and immunization schedule was followed as described. The OTC-KLH conjugates were injected with the addition of Freund’s adjuvant which was an inexpensive strategy for polyclonal production. Freund’s adjuvant, which is paraffin oil based, had been used for stimulation of the immune system by Mycobacterium in Complete Freund’s adjuvant to generate high antibody titers (Trott et al., 2008). Not only this adjuvant activated the immune system, it also retained the antigen to be released slowly into the injection site (Bollen et al., 1996). The booster dose injections given to the rabbit during immunization period stimulates the body to produce more specific IgG against target, as previously reported by Faridah (2010). Serum was precipitated using saturated ammonium sulphate to precipitate antibody. Partially purified antibody was then run through Protein-A affinity column to obtain pure IgG antibody against OTC. Concentration of antibody was found to be 8.6 mg/ml. The purity of antibody was found to be ~90% on 12% SDS PAGE (Fig 2). The two bands on the
SDS-PAGE indicate the two chains (heavy and light chain) of immunoglobulin. This suggested that the immunization and purification method used were successful.

Fig 2: SDS-PAGE for Purified Antibody against Oxytetracycline.

Note: (Lane1- Marker protein, Lane2- Purified Antibody, 20 μg and Lane3- Purified Antibody, 10 μg).

3.4 Determination of the Quality of Antibody Using ELISA Technique

Quality of purified antibody and antiserum was ascertained by direct ELISA. Since OTC may not properly bind to the micro plate wells, OTC was conjugated to BSA and this conjugate was used as coating antigen. Since the purified antibodies as well as antiserum are expected to contain anti-KLH antibodies also (as KLH was used for conjugation with OTC for immunization), BSA was conjugated with OTC and used as coating antigen to avoid cross-reaction. In this experiment after each incubation, wells were thoroughly washed with washing buffer solution to remove unbound materials. Addition of blocking solution was made to block additional binding sites on the plate surface. Different concentrations of diluted antiserum and purified antibody were added and then incubated. This second incubation ensured that immobilized conjugate could bind any uninhibited antibody/antiserum. Excess of antibody/antiserum was removed by washing and labeling was done by addition of goat-anti-rabbit IgG-HRP and then incubated to develop the colour. Under dark condition, chromogen (TMB solution) was added and reaction was terminated by addition of stopping solution and then absorbance was recorded in ELISA plate reader.

The results of direct ELISA are presented in (Fig 3). From the graph it is clear that; upon dilution of antibody, antiserum and preimmune serum at same factor, the over absorbance values in preimmune serum and antiserum vis-à-vis purified antibody preparation at the same dilution indicated that purified antibody preparation indeed contained the antibody of interest in the concentrated form. Concentration corresponding to the absorbance value of 1.2-1.5 μm was sufficient to detect the target analyte and hence considered as titer value. In the present case antibody concentration at 1.5 μg/100 ml was found to be optimum concentration. Based on the antibody titer result, KLH can be considered as a good carrier protein for a small hapten molecule such as tetracycline (Siti and Nur, 2011). The higher antibody titer indicated the good quality of antibody produced. The results indicated that the antibody produced against OTC was successful compared to the pre-immune antiserum.

3.5 Application of OTC Antibody in Lateral Flow Assay

LFA is based on antigen antibody interaction. Since antibodies are protein, these antibodies will bind to nitrocellulose membrane by various non-covalent forces. The assay is a type of competitive reaction, wherein free analyte in sample and analyte coated at test line (OTC-BSA conjugate) compete for limited amount of colloidal gold-labeled OTC antibody. If analyte is absent in the sample or present below detection limit, the OTC-BSA conjugate coated at test line would bind gold labelled OTC antibodies and thus accumulation of gold will create a red line. The intensity of the red color at test line is inversely proportional to the concentration of target analyte in the sample i.e. the more analyte in the test sample, the weaker the test signal and vice-versa. If the presence of OTC concentration in the sample is above the detection limit, whole of colloidal gold-labeled OTC antibody would bind to the OTC which is present in the test sample and thus would not be available for OTC-BSA at test line and thus no colour line is formed (Fig 4). In such type of competitive formats of lateral flow assay, the amount of gold labelled OTC antibody deposited at conjugation pad is very crucial and should be same in all strips, at least in a batch. In this study, it was observed that 10 μl of gold labelled OTC antibody conjugate was sufficient and throughout the study, the similar conditions were maintained. The appearance of control line indicates the proper working of strip. If no line is visible in the control line, the test is invalid. Results of lateral flow strip can be obtained within 5 min with naked eyes and for this no additional interface is required (strip reader), so these strips are user-friendly. Our test results are comparable with the findings of Isabel et al. (2011), this team developed -
Fig 3: ELISA titer graph of antiserum and purified antibody generated against oxytetracycline.

Fig 4: Principle of lateral flow assay for detection of oxytetracycline.

Fig 5: Limit of detection of oxytetracycline in milk (Concentrations in ppb)
LFA to detect OTC in honey sample and the visual LOD was 20 μg/kg. In our findings the LOD was 30 μg/kg for milk system (Fig 5), this value is below the MRL.

4. Conclusion
This work was aimed to design a rapid, user friendly and sensitive lateral-flow assay for the detection of oxytetracycline in milk. A commercial successful LFA may require significant amounts of antibody; hence, in this assay we proposed a simple and cost effective strategy for the development of hapten to raise the polyclonal antibody against OTC. To further develop sensitive and specific immunoassays, these antibodies were conjugated with colloidal GNP and used for the design of lateral flow strips. From this assay results could be accomplished within 5 min without the need of any equipment. The visual detection limit was 30 μg/kg. Thus, the developed assay is potentially effective tool for the direct and routine analysis/screening of OTC residues in milk system.

Acknowledgements
The authors are thankful for the financial support for this research work in the form of fellowships, which was awarded by University Grant Commission, Govt. of India, and to the Indian Council of Agricultural Research Institute, New Delhi.

References


Noraini SB and Azura NMS (2011). Production of polyclonal antibody against tetracycline using KLH as a carrier

Naik et al…Production of Polyclonal Antibody for Oxytetracycline and Their Use in Lateral Flow Assay

Journal of Microbiology, Immunology and Biotechnology | Year-2014 | Volume 1 | Pages 08-17
© 2014 Jakraya Publications (P) Ltd


