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DEVELOPMENT OF USER-FRIENDLY DIAGNOSTIC KIT FOR DETECTION OF SULFONAMIDE RESIDUES IN BIOLOGICAL FLUIDS

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Lateral flow assays (LFA) are quick residue detection techniques used for screening of biological fluids such as milk, urine or blood and give quick and reliable results. In this study, a rapid and efficient lateral flow assay-based strip has been developed to detect sulfonamide residues in biological fluids. This kit has been designed on competitive format principle of LFA combined with nanotechnology. Primary antibodies were raised successfully in rabbits against hapten (Sulf-BSA) and purified by Octanoic acid-ammonium sulphate (OA-AS) sequential method. Total protein (IgG) content measured by nanodrop spectrophotometer was 8.53 mg/ml. Decreased absorbance values of anti-serum and purified serum preparations at same dilution during direct ELISA indicated that the purified serum antibodies certainly contained antibodies of interest (anti-sulfanilamide antibodies) in concentrated form. Characteristic peak of plasmon resonance of synthesized Gold nanoparticles (GNP; size: 65.19.8 nm and zeta potential:–21.6 mV) was observed at 520 nm with an absorbance of 0.40 followed by their conjugation with primary antibodies. On the nitrocellulose membrane the immunogen (Sulf-Ova) was smeared on the test line and secondary antibodies on the control line. GNP conjugates were impregnated on the conjugate pad. Strips were then run with 50, 100,150 and 200 ppb of sulphanilamide concentrations. At 50 ppb concentration, the sulphanilamide could not be detected whereas at 100, 150 and 200 ppb the sulphanilamide was detected in the samples (Milk and Buffer). The developed assay could detect maximum residue limit values of sulfonamides in milk. The visual detection was achieved by using GNPs. Duration of analysis was 8 to 15 min. These strips are easy to use and can be successfully used for screening of sulfonamides in biological fluids.

Keywords: hapten; antibiotic residue; sulphonamide; BSA; strip; nitrocellulose membrane

INTRODUCTION

Antibiotics are extensively used in veterinary practice in Pakistan. The most important antibiotic families are β-lactams, sulphonamides, amino-glycosides, quinolones, macrolides and tetracyclines (Grave *et al.,* 2006). The improper and prolonged use of these antibiotics can cause the presence of residues in the animal origin products such as milk, meat and eggs (Aarestrup *et al.,* 2008, Bennani *et al.,* 2020). These antibiotic residues have a negative impact on general public health like increased bacterial resistance to antibiotics, drug induced allergies, and disruption of the gut microbiome (Aarestrup *et al.,* 2010). Though all the antibiotics are not found to be toxic at residue levels yet many of them exhibit toxicity and they can trigger intense immune reactions. The presence of antibiotic residues in raw milk has become a major technological hazard for the dairy sector now a day. In addition, food producing animals act as a carrier of resistant bacteria that can cause various diseases (Kim *et al.,* 2015). The World Health Organization (WHO), European Union (EU) and the Food and Agriculture Organization (FAO) have established strict regulations and measures to control antibiotic residues such as maximum residue limits (MRLs) of veterinary drugs that can occur in foods of animal origin (Van Boeckel *et al*., 2015).

Sulphonamides are broadly practiced in food producing animals as therapeutic, prophylactic as well as growth promoters because they act as antibiotic agents against diverse bacterial and protozoal diseases (Zhang and Wang, 2018). Sulphonamides are the most frequent antibiotic contaminants in the feed of animals that generate possibly serious threats like toxic or allergic reactions to human well-being. Some sulphonamides are probably carcinogenic and can lead to hefty debate about food safety (Schwarz and chaslus, 2001; Singh *et al*., 2014). About 5 percent of human patients treated with sulfonamides might show adverse response to these drugs (Tilles *et al*., 2003;Dibbern *et al*., 2008; Mor *et al*., 2012). Thus, the EU and a few other countries like Turkey have legislation regarding MRLs in foods of animal origin (EU regulations, 1990; Food and Drug Regulations, 1991; Turkish Food Codex, 2008). Numerous analytical techniques have already been developed for determining the sulfonamide residues in animal tissues (Genitili *et al.,* 2004). There is an increasing demand for more efficient and exact analytical tools to monitor antibiotic residues in order to avoid negative influence on the food industry and to protect human health. Until now, traditional

microbial inhibition assays (MIA) are widely used because they are found to be simple, quantitative and rapid screening techniques (Babington *et al.,* 2012). They also have some weaknesses like non-quantitative output, long incubation times and lack of specificity (Companyo *et al.,* 2009). Quantitative analysis is done chiefly by Liquid and Gas chromatography in combination with mass spectrometer detectors. These techniques are highly specific, sensitive and reliable (Kantiani *et al*., 2010). However, the advanced analytical equipment relies on qualified and well trained operators and is expensive. In addition, complicated methods for sample pre-treatments, and time consuming lengthy operating procedures make it difficult for general people to use these techniques. Therefore, the advanced analytical technologies are not possible to be used for onsite detection or in resource deficient regions.

Consequently, the point of care (POC) techniques has gained more importance for food safety and clinical analysis. POC testing provides prompt results in less time as compared to complex and time consuming procedures of centralized lab tests. The POC devices based on lateral flow assay (LFA) have become expeditiously growing techniques for both quantitative and qualitative analysis. LFA combines unique features as well as merits of chromatography and bio recognition probes. Lateral flow assay is usually executed on a strip (Moumita *et al.,* 2019). Various strip parts are assembled on a backing card, usually a plastic backing. Four parts of the strip include: a. sample application pad, b. conjugate pad, c. nitrocellulose membrane and d. absorption pad. Nitrocellulose membrane contains a control line and a test line. When the liquid sample flows the reagents become active those were first pre immobilized at various parts of the strip. Various detection formats are available based on the use of bio recognition molecules. Limitations of enzyme linked immunosorbent assay (ELISA) and other conventional clinical techniques could be addressed by LFA processes (Li *et al.,* 2010; Zeng *et al*., 2014). In addition, internationally available strip tests are costly and are not in the access of local farmers and diagnostic laboratories. Therefore, present research was planned to develop user friendly residue testing kits following the basic LFA principles along with the addition of nanotechnology against commonly used sulfonamides in food animals.

MATERIALS AND METHODS

***Materials*:** Sulfanilamide, Ampicillin, Bovine serum albumin (BSA), Ovalbumin, Glutaraldehyde, Methanol, Dioxane, were purchased from bio WORLD chemicals, Dublin, Ohio, USA. Phosphate buffered saline (PBS) and borate buffer were procured from VWR Chemicals BDH®, Radnor, Delaware. Freund's complete adjuvant (FCA) was from InvivoGen Pharmaceutical Co. Ltd. Freund's incomplete adjuvant (FIA)was from ImmunoCruz®, Santa Cruz Biotechnology, Santa Cruz, California, USA.Trisodium citrate and Tetrachloroauric acid (Gold chloride) were purchased from Merck Group, Germany.Caprylic acid, ammonium sulfate, Deionized water, Distilled water, Buffers were obtained from UNI-CHEM, Crnotravska, Beogard, Serbia.Dialysis membrane was MEMBRA-CEL® by Vikase®, Lombard, Illinois, and it was made of cellulose. Goat anti-rabbit Immunoglobulin G (secondary antibodies) and goat anti-rabbit IgG-HRP (secondary antibodies- horseradish peroxidase) were procured from abcam USA. All the required materials for preparation of lateral flow strip, Polyvinyl chloride (PVC) backing card, Polyester fiber for conjugate pad, Glass fiber for sample pad and plastic cassettes were procured from Shanghai Jieyi Biotech, China. Nitrocellulose membrane was purchased from Sartorius biopharma, Germany. All the reagents and buffers used in immune-assay were prepared with deionized or double distilled water.

***Sulfonamide Hapten Preparation*:** Conjugation with bovine serum albumin was achieved via glutaraldehyde reaction according to Hassnoott *et al*. (2000) with slight modifications: Sulphanilamide (16 mg) was dissolved in 1 mL of PBS and 10 mL of ovalbumin solution (1 mg of ovalbumin/ 1 mL of PBS) was mixed in it 8ml of 0.5% glutaraldehyde solution was then added drop wise in the mixture over magnetic stirrer for 3 hours at room temperature. The resulting conjugate was dialyzed against several changes of PBS for 6 days. The conjugate was then lyophilized and stored at - 20ºC until further use. The conjugation was confirmed by comparing the UV spectra of sulfanilamide, BSA and Sulf-BSA by using UV Spectrophotometer (Thermo SCIENTIFIC MULTISKAN GO, USA).

***Production and purification of polyclonal antibodies*:** The Sulf-BSA conjugate was used for production of polyclonal antibodies in rabbits. Pre-immune serum was collected prior to the immunization.

***Immunization protocol*:** The protocol of immunization consisted of the first dose of subcutaneous injection of 500 µg of Sulf-BSA immunogen together with the adjuvant (Freund's complete adjuvant: FCA) in a 1:1 ratio. After the first dose (Immunogen+FCA),5 booster doses (Immunogen+FIA) were given at 40^{th}, 50^{th}, 60^{th}, 70^{th} , and 80^{th} days of study. The animals were decapitated to collect the blood. Blood was centrifuged at 4000 rpm for 10 minutes to collect antiserum. The collected antisera were used to purify polyclonal antibodies by sequential method of octanoic acid and ammonium sulfate precipitation. Purified antibodies were dialyzed with 1X PBS. The concentration of purified antibodies was estimated via nanodrop spectrophotometer (Thermo SCIENTIFIC NANODROP 8000 Spectrophotometer)at the absorbance of 280 nm.

***ELISA for determining quality of pre-immune serum, anti-serum and purified antibodies*:** Sulfanilamide-OVA immunogen was used to coat the wells of microtiter plates. The hapten (5 µg/ml) was dissolved in a 0.05M carbonate

buffer of pH 9.6. 100 µL of conjugate was added to the wells and the plate was incubated at 4ºC for 24 hrs. Unbound fraction of the immunogen was removed by using a washing buffer, 0.01M phosphate buffered saline (pH 7.4), containing Tween 20 (0.1%). Each well was blocked by blocking solution (2% w/v bovine serum albumin in 0.05 M carbonate buffer of pH 9.6) for 2 hours at 37 ºC. Blocked plates were washed with a washing buffer. Diluted sample (100µL)(pre immune serum, antiserum and purified antibodies) in different concentrations (1:100; 1:1000; 1:5000; 1:10000; 1:20000; 1:40000; 1:80000) was added to the wells and incubated at room temperature for half an hour. Wells were washed 3 times before incubation with 1:5000 diluted goat anti-rabbit IgG-HRP at 37ºC for one hour. Wells were washed and 100 µL solution of 3, 3`, 5, 5`-tetramethylbenzidine (TMB) was added. Plates were incubated at 37ºC for 20 minutes. Reaction was stopped by adding 100 µL stop solution (1 N sulphuric acid). Absorbance was checked by ELISA reader at 450nm (Aydin, 2015; Langer *et al.,* 2017; Al-amri and Hashem, 2020).

***Synthesis and Characterization of Gold Nanoparticles (GNPs)*:** Preparation of GNPs was done by citrate reduction procedure as described by Liu and Lu(2006), wherein the reduction of gold chloride was done in the presence of tri-sodium citrate. Confirmation of GNPs preparation was done by measuring spectra in the range of 400 to 700 nm against water as blank using spectrophotometer. Zeta potential and size of prepared GNPs were measured by the method illustrated by Saha *et al*. (2007) by using dynamic light scattering (DLS) method.

***Optimization of conditions for conjugation of GNPs with antibodies*:** Conjugation of antibodies with the gold nanoparticles is a crucial step in the development of lateral flow assay. Therefore, a suitable buffer solution and optimal pH value that was required for stability of gold nano-particles during the conjugation process was determined. 0.75 ml of prepared gold nano-particle solution was taken in 1.5 ml microcentrifuge tube and centrifuged at 16000 rcf for 15min. The supernatant was discarded and pellet was resuspended in 0.75ml of each test buffer (borate and phosphate buffers) at each pH (6.5, 7.5, and 8.5) test value. Finally, the change in colour of gold nano-particles was observed.

***Preparation of antibody conjugates with gold nanoparticles*:** The method described earlier (Lata *et al.,* 2016) was used with some modifications for the conjugation of gold nanoparticles with antibodies. 5ml of gold nanoparticles were centrifuged at 20,000 rcf for 30 min at 4ºC. The supernatant was removed and pellet was resuspended in 2.5 ml of borate buffer (0.1 M, pH 7.5). This phosphate buffer contained the antibodies (50µg/ml). Final solution was mixed over a rotating wheel and incubated for 45 minutes. 100µl of 0.1 M borate buffer (pH=7.5) solution containing10% BSA (100mg BSA/ml of distilled water) was added to block un-reacted sites of gold nanoparticles. Solution was incubated at room temperature for 20 minutes. Resulting colloids were centrifuged at 20,000 rcf at 4ºC for 15 minutes. Supernatant was again removed and pellet was resuspended in 2.5 ml of 0.1 M borate buffer of pH 7.5. The colloids were centrifuged at 20,000 rcf at 4 ºC for 20 minutes. The final pellet containing gold nanoparticles-antibodies conjugates was re-suspended in 500 µl of 0.1 M borate buffer of pH 7.5. Borate buffer also contained 0.1% BSA (1mg of BSA/ 1ml of distilled water). The prepared conjugates were confirmed by spectrophotometric analysis and the shift in the UV absorbance peak of conjugate in the spectra as well as running the lateral flow strip only with the control line at the nitrocellulose membrane. This conjugate was stored at 4ºC.

***Synthesis of Immunogen (Sulf-Ova) as coating agent for test line*:** 7.6 mg of sulfanilamide was diluted in 1 mL of PBS and added to 5 mL of ova solution (1 mg/mL of PBS). Next, 4 mL of glutaraldehyde (0.5%) was added in drops to the mixture, where after the reaction mixture was stirred for 3 h at room temperature (Long *et al.,* 1990). The conjugate was dialyzed against PBS for 3 days and stored at -20 °C. The confirmation of conjugation was done by the comparison of UV spectra of sulfanilamide, ovalbumin and sulf-ova.

***Assembly of lateral flow strip*:** Test strip was constructed on adhesive backing card (6 × 30 CM) of polyvinyl chloride (PVC). It was used as a support for all the main components of the strip. First of all, the central adhesive protecting layer was removed to expose the sticky part and the nitrocellulose membrane (25× 30 CM) was placed in the middle of the adhesive backing card. Then other adhesive protective layers were removed to uncover the sticky backing card and the conjugate pad (21× 30 CM) was placed on the backing card in such a manner that it was overlapping the nitrocellulose membrane by 2mm. The third adhesive protective layer was then peeled off to reveal the sticky portion of PVC backing card and the sample pad (25× 30 CM) was pasted on the backing card in a manner that it overlapped the conjugate pad by 2mm. Finally, the adhesive protective layer from the top most upper portion of the PVC backing card was removed to expose the sticky portion and the absorbent pad (20× 30 CM) was pasted on it in a similar manner that it overlapped the nitrocellulose membrane by 2mm.

Conjugate pad, sample pad and absorbent pad were strongly pressed over the PVC backing card. The overlapping of conjugate pad and absorbent pad on nitrocellulose membrane and the overlapping of sample pad on conjugate pad aids the flow of sample and gold nanoparticle conjugates with antibodies. The antibody-gold nanoparticle conjugates were dispensed on the conjugate pad with the help of micropipette. It was left to dry at room temperature for 3 hours. The immunogen (Sulf-Ova) was applied on the test line and goat-anti-rabbit antibodies (secondary antibodies) were applied on the control line. The applied reagents were left to dry at room temperature for 3 hours.

The final assembled strip on PVC backing card having all the

components of lateral flow strip was cut into uniform 2 mm strips using a paper strip cutter. Each strip was then placed on a separate plastic cassette (4MM ×60MM) containing an open sample port and visible test and control line area.

***Milk, serum, urine and buffer sample preparation for determination of limit of detection (LOD)*:** Stock solutions (10^6 ppb) of sulfanilamide were prepared by dissolving 1000 mg in one liter of buffer, skim milk or whole milk, serum and urine. Further, the required amounts of prepared stock solutions were diluted to get various concentrations (50 ppb, 100 ppb, 150ppb and 200ppb) of sulfanilamide in various samples.

***Specificity and Sensitivity Testing*:** The sensitivity of prepared lateral flow assay strips was examined by using raw milk and borate buffer. After centrifugation of milk sample at 4ºC at 5,000 g the fat layer was removed and the skim milk was collected. Whole milk and skim milk both samples were tested. About 100 µl samples of both milk and buffer were poured at the sample port of the plastic cassette containing the prepared test strip and the absence or presence of control and test lines was monitored. Specificity of the test strip was checked in a cross reactivity checking method with sulfanilamide and ampicillin. The other antibiotic was added in milk and buffer at hundred ppb and a test was carried out to note the results.

***Stability and storage testing of lateral flow test strips*:** The prepared test strips were stored in plastic sealed containers at room temperature. The strips were tested with both the control and spiked milk samples at regular intervals of fifteen days. Absence and presence of the coloured line at test and control line regions was monitored to assess the results.

Statistical Analysis: Mean values and standard deviations of the observations were calculated.

RESULTS

Polyclonal antibodies development against sulphonamides

Accessing the quality of Sulfanilamide-BSA conjugate: It was observed that Sulfanilamide had peak value at 307 nm. BSA had a peak at 278 nm. After conjugation the peak of Sulfanilamide-BSA conjugate was shifted to 292 nm with the increased absorbance value. This indicated successful conjugation of Sulfanilamide with BSA (Fig.1).

Polyclonal antibodies production and purification

Precipitation of serum antibodies (IgG): The antiserum (serum containing antibodies) obtained from rabbit blood was further precipitated to obtain desired antibodies. For this purpose, the sequential method of octanoic acid-ammonium sulphate precipitation was followed, earlier used by Gagnon (2018) and Fishman and Berg (2018). The starting treatment resulted in the precipitation of non-IgG proteins and lipids within an hour of incubation period. The eppendorf tubes containing antiserum were centrifuged and it resulted in the formation of a pellet and supernatant was collected. Further, a clear white pellet of IgG protein resulted in the secondary treatment the precipitate was dissolved in 10ml phosphate buffer saline. The solution was dialyzed against PBS for 48 hours at 4ºC. The purified antibodies were stored at -20ºC.

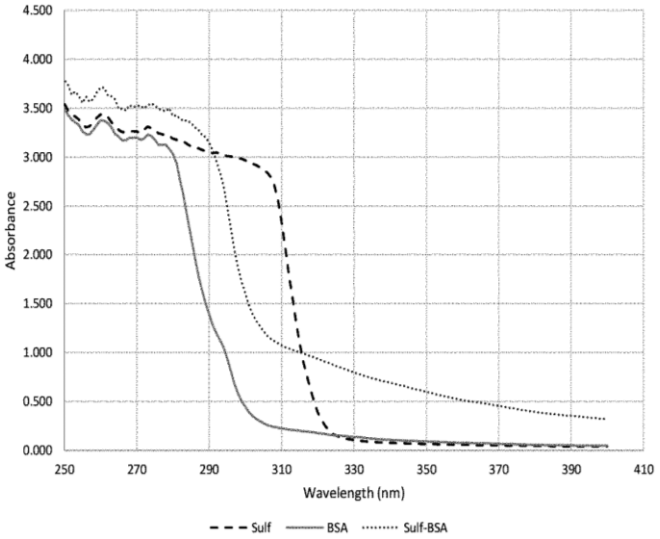

Figure 1. UV spectra of Sulfanilamide, Bovine serum albumin (BSA) and Sulfanilamide-BSA conjugates

***Quantification of serum antibodies (IgG)*:** Nanodrop spectrophotometer (Thermo SCIENTIFIC NANODROP 8000 Spectrophotometer) quantified the antibodies and exhibited the automated results as mg/ml via a computer generated software "ProteinA280". Protein concentration was more than 7 mg/ml (Fig. 2). Maximum concentration of IgG protein was found to be 9. 67 mg/ml while minimum concentration was estimated to be 7.45 in selected samples. The average total protein content in the caprylic acid-ammonium sulphate precipitated samples was 8.53 mg/ml.

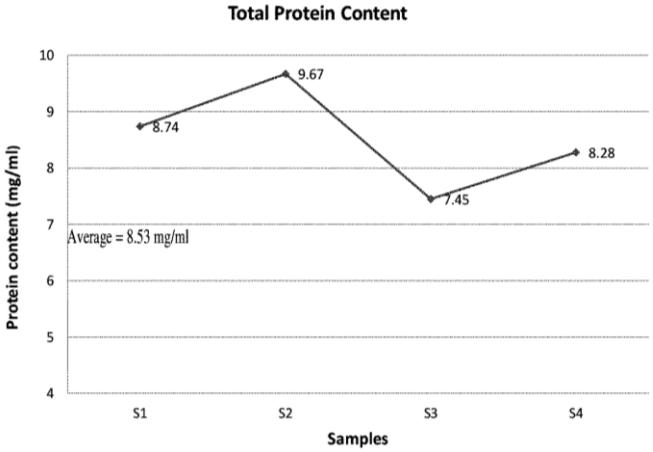

Figure 2. Total protein concentration (IgG) mg/ml in serum samples

***Estimation of the quality of purified antibodies via ELISA method*:** The results of direct ELISA are shown in the Fig. 3. It was evaluated that the pre-immune serum had insufficient activity against the sulphanilamide as the absorbance values were constant with the dilutions. Contrarily, in case of purified antibodies and anti-serum the absorbance values remained around 3.4 and 1.6 respectively at first dilution and it decreased with further dilutions. This determined that the required antibodies in the antiserum as well as in the purified serum have reacted with sulphanilamide which had been coated on the wells of ELISA plate. Decreased absorbance values of anti-serum and purified serum preparations at same dilution indicated that the purified serum antibodies certainly contained antibodies of interest (anti-sulfanilamide antibodies) in concentrated form. Decreased absorbance values of anti-serum and purified serum preparations at same dilution indicated that the purified serum antibodies certainly contained antibodies of interest (anti-sulfanilamide antibodies) in concentrated form.

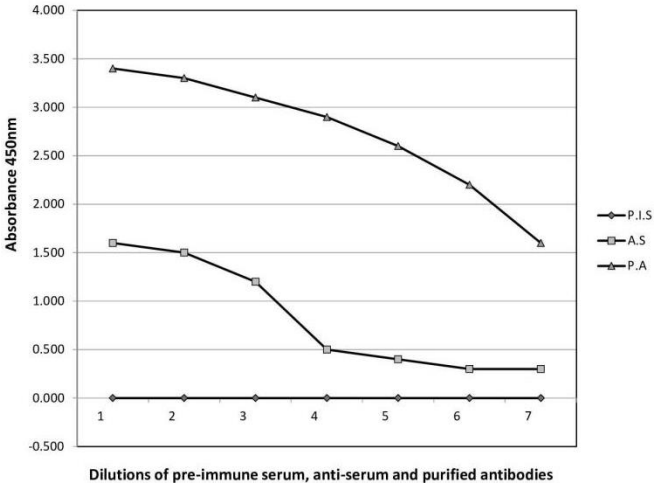

Figure 3. Graph showing the activity of pre-immune serum, anti-serum and purified antibodies against the sulphanilamide

***Measurement of Plasmon Resonance of prepared gold nanoparticles*:** Plasmon resonance attribute of gold nano-particles is due to the robust absorption of electro-magnetic waves in visible region that causes collective oscillations of the conduction electrons of gold nano-particles. In this study the characteristic peak of plasmon resonance was observed at 520 nm with an absorbance of 0.40

***Size distribution and zeta potential of prepared gold nanoparticles*:** The GNPs synthesized by citrate reduction method were analyzed via DLS. The average size of the prepared GNPs was 65.19.8 nm (Fig. 4,). Zeta potential reading of synthesized GNPs was –21.6 mV (Fig.5,) which indicates the presence of negative charge at the surface.

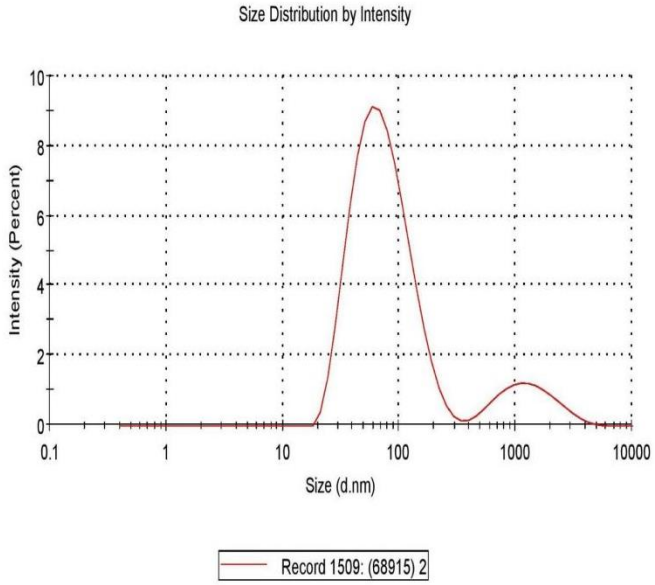

Figure 4. Size distribution of prepared gold nano-particles

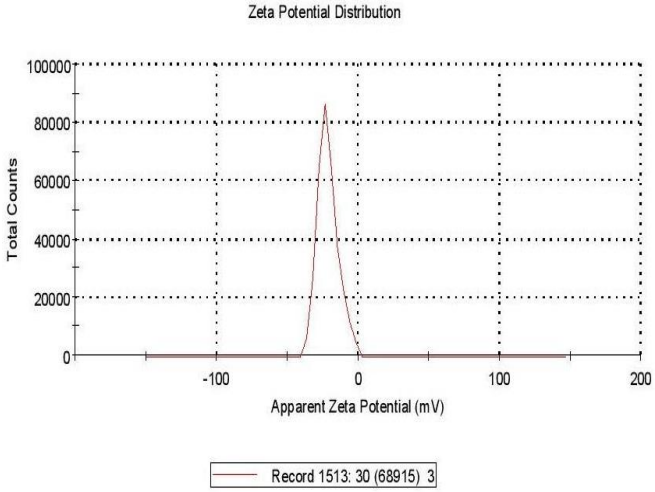

Figure 5. Zeta Potential of prepared gold nano-particles

***Optimization of buffers for conjugation of gold nano-particles with antibodies*:** Gold nano-particles were unstable when suspended in the phosphate buffer of all pH values (6.5, 7.5, and 8.5) (Fig. 6A). It is indicated by the change of bright red colour of gold nano-particles to dark blue colour on suspension in phosphate buffer at each pH value. This colour change occurred due to the aggregation of gold nano-particles. On the other hand, with a borate buffer the blue colour was seen only at 8.5 pH value (Fig. 6B). These observations establish that the 0.1 M borate buffer of pH 7.5 was suitable for conjugation of gold nano-particles with the antibodies.

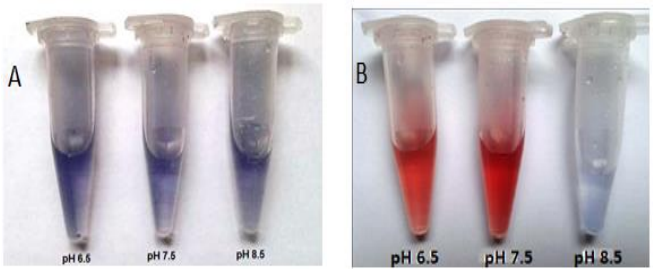

Figure 6. A. Stability of gold nano-particles in phosphate buffer (0.1 M) at different pH values. B. Stability of gold nano-particles in borate buffer (0.1 M) at different pH values

***UV-Spectra of gold nano-particles and conjugate with sulfanilamide*:** Gold nano-particles showed standard peak at 520 nm while after the conjugation with the anti-sulfanilamide antibodies the peak was observed at 532 nm (Fig.7). It confirms the successful conjugation of gold nano-particles with the antibodies.

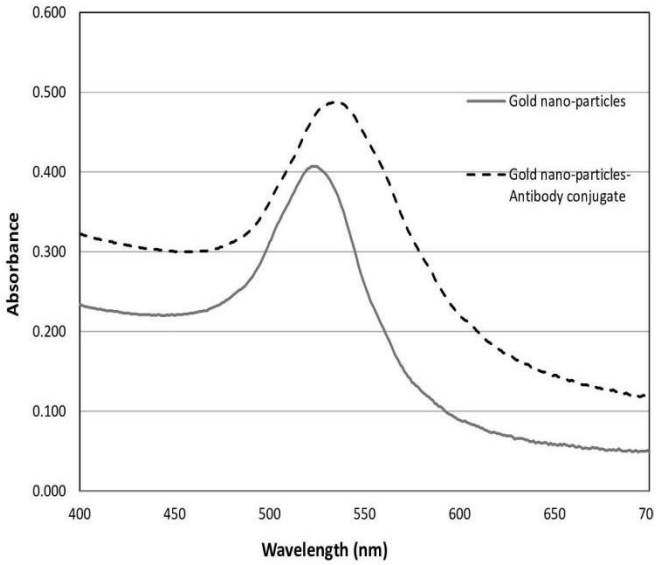

Figure 7. Absorption spectra of gold nano-particles and the conjugates of gold nano-particles with the Sulfanilamide antibodies

***Running lateral flow test strip of Sulfanilamide with only control line*:** The appearance of red line on the control line position pointed out the successful conjugation of gold nano-particles with the anti-sulfanilamide antibodies.

***Assessing the quality of Sulf-Ova Conjugates*:** It was observed that Sulfanilamide had peak value at 307 nm. Ova had peaked at 282 nm. After conjugation the peak of Sulfanilamide-Ova conjugate was shifted to 302 nm with the increased absorbance value. This indicated successful conjugation of Sulfanilamide with Ova (Fig.8).

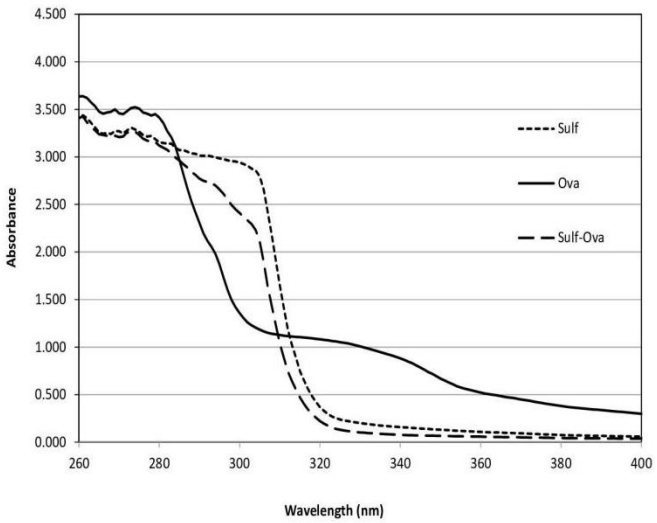

Figure 8. UV spectra of Sulfanilamide, Ovalbumin (Ova) and Sulfanilamide-Ova conjugates

***Test Determination*:** Competitive format was used to detect the antibiotics because these are very small molecules and they have a single antigenic determinant site and cannot bind with both the antibodies at the same time. The free analyte (sulphonamide) in sample and the (Sulf-Ova conjugate coated at test line competed for the limited amount of gold conjugated anti-sulphonamide antibodies. When the analyte was absent in sample the Sulf-Ova conjugate coated at test line bound with the gold conjugated anti-sulphonamide antibodies and thus accumulation of gold nano-particles created a red line at test line position and further moved to control line and got bound with secondary antibodies to make red line indicating negative result (Fig.9.A). When the analyte was present in the sample only one line at control line was seen because the sulphanilamide in the sample bound to the gold labelled anti-sulphonamide antibodies and did not bind to the Sulf-Ova conjugate at the test line indicating positive result (Fig. 9.B).

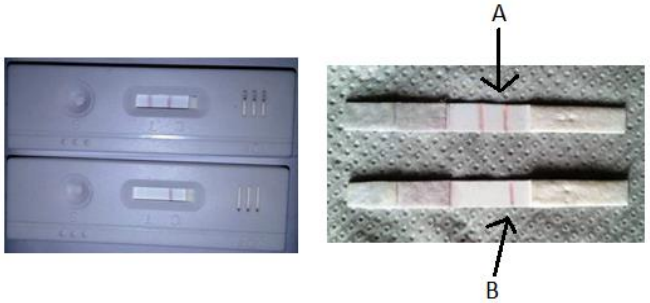

Figure 9. Strips showing negative and positive results of test

Strips were then run with 50, 100,150 and 200 ppb of sulphanilamide concentrations. At 50 ppb the sulphanilamide

was not detected, at 100, 150 and 200 ppb the sulphanilamide was detected in the samples (Milk and Buffer) (Fig. 10).

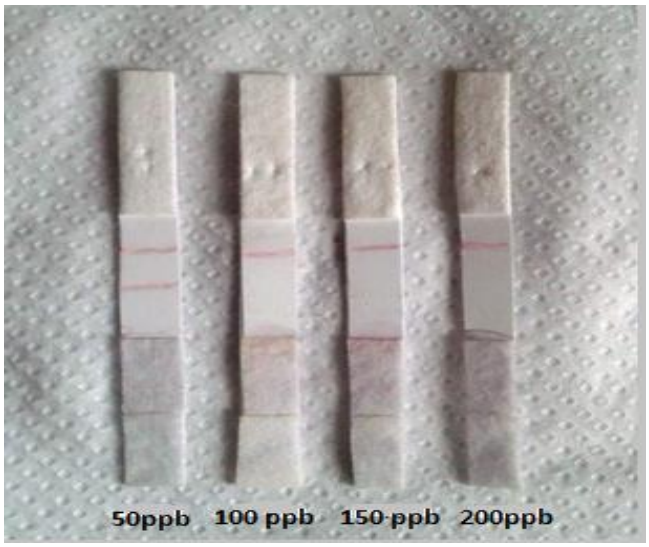

Figure 10. Strips showing negative (at 50 ppb) and positive results at various concentrations (100, 150 and 200 ppb)

For urine and serum samples preparation, 100 microliter of borate buffer (0.1 M, pH 7.5) was added and then the test was performed. Test results were seen in 10 to 15 minutes as compared to the results with skimmed milk where results were seen in 7 to 9 minutes. In the case of a buffer the results were obtained in 4 to 5 minutes.

***Specificity and Sensitivity Testing*:** The sensitivity of prepared lateral flow assay strip was examined by using raw milk and borate buffer containing sulfanilamide at concentrations of 100 ppb. The sensitivity results established proper working of the prepared strips. The specificity of the prepared strip was checked in a cross reactivity checking method with sulfanilamide (100ppb) and ampicillin (100ppb) showed that both the prepared strips were specific for the antibiotics they were prepared for as they did not give positive results with alternate antibiotics.

***Stability and storage testing of prepared LFA strips*:** The prepared strip showed no false positive or false negative results up to one month of preparation. This indicates the stability of the prepared test strips.

DISCUSSION

There is growing demand for more efficient and exact analytical methods to monitor antibiotic residues in order to avoid negative influence on the food industry and to protect human health. Until now, traditional microbial inhibition assays (MIA) are widely used because they are found to be simple, quantitative and rapid screening techniques (Babington *et al.,* 2012). They also have some weaknesses like non-quantitative output, long incubation times and lack of specificity (Companyo *et al.,* 2009). Quantitative analysis is done chiefly by Liquid and Gas chromatography in combination with mass spectrometer detectors. These techniques are highly specific, sensitive and reliable (Kantiani *et al.,* 2010). However, the advanced analytical equipment relies on qualified and well trained operators and it is expensive. In addition, complicated methods for sample pre-treatments, and time consuming lengthy operating procedures make it difficult for general people to use these techniques. Therefore, the advanced analytical technologies are not possible to be used for onsite detection or in resource deficient regions.

Chiefly, the analytical techniques for measuring residues of antibiotics are classified as screening and confirmatory techniques. These confirmatory techniques are laborious. Although these techniques can attain higher detection sensitivity yet they are not able to meet the demand of high through-put on line and on site screening. Consequently, we developed more sensitive, rapid and user friendly analytical strips utilizing lateral flow assay (LFA) principle.

Sulfonamides are very small molecular weight compounds and hence, they might diffuse out of site of injection and remain unable to cause immunogenicity. Therefore, the compounds of small molecular weight are conjugated with carrier protein like bovine serum albumin (BSA), KLH or ovalbumin (OVA). The molecular weight of these carrier proteins is large and the presence of greater number of functional groups facilitates the successful process of conjugation (Lata *et al*., 2017).Sulfanilamide was selected as immunogen for producing group specific polyclonal antibodies because it is the common structural element of all sulfonamides and it does not have a “disturbing” side group. Antibodies produced against sulfanilamide should be group-specific for sulfonamides (Cliquet *et al.,* 2003). Shifts in the UV absorbance peaks of both the conjugates confirmed the success of the process of conjugation. Confirmation of the successful process of conjugation of immunogen with the carrier protein was assessed by observing the shift in the UV absorbance peak of conjugate in the spectra by Peng *et al*. (2011).

The AS-OA method was utilized to purify the serum antibody and results in terms of high yield were very similar to the study done by Perosa *et al*. (1990) and Naveed *et al.* (2019). Ko and Ahn. (2007) indicated that ammonium sulfate precipitation technique is superior to chromatographic technique (cation exchange chromatography) regarding purity and yield of antibodies.

Decreased absorbance values of anti-serum and purified serum preparations at same dilution indicated that the purified serum antibodies certainly contained antibodies of interest (anti-sulfanilamide antibodies) in concentrated form. Lata *et al.* (2017) utilized direct ELISA method to determine quality

of purified antibodies and our results were supported by these research studies.

Conclusion: An LFA using polyclonal antibody to detect sulfonamides in milk has been established and this developed assay can detect MRL values in milk. Total time required for completion of test analysis is 8 to 15 minutes. Prepared LFA test strips were established to be stable at room temperature up to 1-month duration. No complex instruments, long time periods, and sophisticated laboratory equipment are required when utilizing these test strips for screening purposes in milk or other biological fluids. The optic detection was attained due to the bright red color of Gnps and this assay was found to be specific. Suggested method for producing Gnps was established to be authentic and simple for LFA. Present assay is user friendly, quick, and cost effective.

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