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Diagnosis of Brucellosis under Field Conditions Using Gold Nanoparticle-Based Lateral Flow Technology and Compared with Other Serological Tests

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Abstract

The annual prevalence of brucellosis was determined in the buffalo population residing in and around the Hyderabad district of Telangana, India. A total of 204 vaginal samples of buffaloes collected from private dairy farms, were subjected to PCR assay, ELISA and developed kit on lateral flow technology, to detect *Brucella* organisms. Out of the total samples screened, 113(55.4%), 92(45.1%) and 87 (42.64%) were found to be positive, respectively. Considering PCR assay as the gold standard method, the laboratory developed kit based on lateral flow technology, has shown 76.99% and 100% sensitivity and specificity, respectively.

Keywords: Brucellosis, PCR, ELISA, Lateral flow technology, Gold Nanoparticles

Introduction:

Brucellosis is an endemic zoonosis with infection predominantly occurring in Middle East, Mediterranean rim (Portugal, Spain, Greece), Asia, Africa, South and Central America where the intake of dairy products is high, and insufficient animal health facilities (Rubach et al., 2013). Brucellosis is highly contagious and a major public health problem worldwide, causing abortion and infertility in domestic and wild animals (Lapaque et al., 2005). The disease has zoonotic importance caused by different species of the genus *Brucella*; they are Gram-negative, non-motile, non-spore-forming, coccobacilli facultative anaerobe, intracellular bacteria causing active and progressing chronic symptoms affecting both animal and human population. In animals, brucellosis mainly affects reproduction and fertility, reduces the survival of newborns, and diminishes milk yield. In human beings, the disease symptoms are weakness, joint and muscle pain, headache, undulant fever, hepatomegaly, splenomegaly, night sweats and chills, marked asthenia and anorexia (Hugh-Jones et al., 2008).

Numerous diagnostic methods, including molecular assays (PCR), serological, Viz RBPT, Agar Gel Immunodiffusion Test (AGID), ELISA, Lateral flow technology (LFT) etc. and culture methods are available to determine the prevalence of brucellosis. The enormous popularity of PCR as a technique is due to its automation, sensitivity and specificity but requires sophisticated laboratory instruments, experienced technical personnel and more time to accomplish which are the major drawbacks on implementation at field level.

Serological tests like RBPT can be implemented at field level, However, there are certain drawbacks to RBPT, such as, it cannot distinguish antibodies of vaccinated and infected animals. Another milestone in the diagnosis of brucellosis is the development of ELISA in which various forms of ELISA like sandwich, competitive and Indirect are available. These tests are specific to identify *Brucella* antibodies in samples, but these are laborious, time-consuming, costly and require a technical person for which cannot be implemented at the field level. AGID test is also reliable and simple to detect the presence of antibodies in the sample (Serum) but cannot be implemented at the field level. Lateral flow is a promising technique that enables testing at the field level without the need of specialized equipment and personnel. It is also user-friendly and simple enough that even people without formal education can determine whether an infection is present or not.

An attempt was made to develop the LFT-based kit using Gold Nanoparticles which can identify *Brucella* spp. in vaginal swabs.

Materials and Methods:

The study was conducted for a period of 12 months from May 2019 to June 2020 in the Department of Veterinary Public Health and Epidemiology, College of Veterinary Science, PVNRTVU, Rajendranagar, Hyderabad, Telangana, India.

A) Collection of samples

All samples were collected from *Brucella* suspected buffaloes and handled according to the guidelines of OIE/WOAH. Sterile vaginal swabs were inserted into the vagina and once secretions adhered, the swab was removed and placed in a tube. Each swab tube was aseptically packed, properly labelled and transported to the laboratory within 2 hours at 4^0C. Vaginal swabs were collected from 204 buffaloes from Kachiguda slaughterhouse and private dairy farms located in and around the Greater Hyderabad Municipal Corporation.

B) Immunization of Rabbits and Poultry

Male Rabbits (8 weeks aged) and Female poultry (8 weeks aged) were immunized with 1 ml adjuvant mixed heat-killed *Brucella* strain 19 vaccine (having 0.6ml Freund's adjuvant and 0.33ml heat-killed *Brucella* strain 19 vaccine having $5X10^8$ CFU/ml Concentration) was given periodically in a scheduled manner (0th, 14th, 30th, 60th and 90th day) through subcutaneous and intra muscular routes respectively. Institutional Animal Ethical Committee (IAEC) approval (07/28/ CVSc, Hyd. IAEC) was obtained through the Animal Ethical Committee of PVNRTVU., Hyderabad.

C) Purification of polyclonal antibodies from Rabbit (IgG) and Poultry (IgY)

Rabbit serum and Poultry eggs were collected periodically on the 21st, 60th, 90th and 120th day after 1st dose of immunization and polyclonal antibodies were purified with the ammonium sulphate precipitation method and sodium chloride precipitation method respectively then dialyzed and protein quantification was done by Bradford method

D) Indirect ELISA Test to Determine IgG and IgY Antibody Titer

Qualitative and quantitative analysis of *Brucella* antibodies produced in rabbits and poultry was done by indirect ELISA test. The OD values were taken at 605nm wavelength (Spectrophotometer) for serial dilutions from 1:500 to 1:128000 for serum of rabbit and egg of poultry yolk.

E) Sandwich ELISA Test to Determine the Presence of Brucella Organisms in Field Samples

Sandwich ELISA was followed as per the protocol described by Chin (1983). A volume of 100µl of poultry antibodies (IgY) diluted in coating buffer (1:500) was added to each well and kept at 37^0C for 45 minutes in the incubator. After washing, ELISA plate wells were blocked by adding a blocking agent. Standards were prepared by adding 100µl of heat-killed *Brucella* vaccine (concentration 10^9 CFU per 2ml) to the 1st well of Column A and serial dilution was done from the 1st well to the 8th well of the column.

A volume of 100µl of the vaginal swab (field) samples were added to wells (B to H) from the 2nd column to the 12th column and kept at 37^0C for 45 minutes in the incubator. After washing, the antibodies (rabbit IgG antibodies, 1:5000) and kept for incubation. For each well 100µl of commercially available HRP (Horseradish peroxidase) Conjugated anti-rabbit secondary antibodies (Sigma Company) in 1:5000 dilutions were added to each well after incubation and washing. Stop solution was added i.e. TMB (Tetra methylene Benzidine) and observed for colour development and OD values were measured at 605 nm wavelength in UV Spectrophotometer for further analysis.

F) Polymerase Chain Reaction

By adopting snap and chilling method, 100µl of the buffaloe vaginal swab liquid was centrifuged at 2,348 g for 5 minutes and processed according to standard protocol.

A set of oligo nucleotide (B4/B5) primers derived from *Bcsp31* genes (Bailey et al*.,* 1992) synthesized by a commercial firm (Saha Gene, Hyderabad) was used for PCR amplification of *Brucella* organism up to the genus level. PCR conditions have been optimized before sample run. Different conditions were standardized for annealing temperature, denaturation time and number of cycles to be completed to get optimum product. The targeted PCR amplicon was processed and further visualized as single compact band under UV light and documented with Gel Documentation System.

G) Development of Kit based on Lateral flow technology to detect Brucella in vaginal samples

Preparation of gold nano particles and p^H adjustment

The gold nano solution was prepared as per the protocol of Turkevitch et al. (1951) with little modifications wherever necessary. The prepared gold nanocrystalline solution underwent a continuous stirring process using a magnetic stirrer at a constant speed. The colour of the solution underwent a transformation, transitioning from pale yellow to black, and ultimately to a deep cherry red (Figure 1). Subsequently, the solution was scanned using a Spectrophotometer (Cary 5000, Varian, UK) from a wavelength of 400 nm to 700 nm. The analysis revealed a single peak at 520 nm (Figure 2), which confirmed the formation of a monodispersed gold nanocrystalline solution. The pH of the gold nanoparticle solution was adjusted to 8.5 by gradually adding 130 µl (for 10 ml of gold nanoparticles) of 0.2 M sodium hydroxide to facilitate proper conjugation. The path length was set to 10 mm, the scan speed was set to 12 nm/s, and the bandwidth was set to 1 nm. A graph was subsequently

obtained, which further confirmed the formation of nanoparticles.

Determination of gold nanoparticle size and Zeta potential

The prepared gold nanoparticles solution was subjected to Zeta analyzer (Malvern, U.S.) and the average size of gold nanoparticles was 40.54 nm and a single peak showed that the prepared gold nanoparticles were mono dispersion colloid. Zeta potential of the nanoparticles was analysed and measured i.e. - 8.13mv (Figure 3)

Stability checking of gold nanoparticles in different buffers at different pHs

A 1 ml of pH-adjusted gold-nano particle solution was centrifuged at 13,800 rpm for 20 minutes at 40 degrees Celsius. The pellet was collected and added to 0.05 M borate, phosphate and citrate buffers, each with a different pH of 6.5, 7.5 and 8.5. No change in colour was observed in the gold nano solution at pH 7.5 and 8.5 of the buffers, indicating its stability. Conversely, at pH 6.5, the solution exhibited a blue/black colour change in all the buffers, indicating its instability.

Optimization of gold nano solution concentration to antibody concentration

Two ml of gold nanoparticles solution (8.5 pH) was centrifuged at 13800 rpm for 20 min at 4^0C. Pellet was collected and dissolved in 1ml of 0.05 M phosphate buffer having 8.5 pH. One twenty µl of the above solution was added to each Eppendorf tube starting from 1 to 7 from right side to left side. A volume of 20µl (having 200µg) rabbit antibodies was added to 1st Eppendorf and serial dilution was made up to 7th Eppendorf (3.125µgm) and kept at 37^0C for 20 min on vortex for 20 minutes. After 20 min, 15µl of NaCl (10% i.e. 1gm NaCl in 10ml of Milli Q water) was added to each Eppendorf tube and observed for the change in colour.

Conjugation of Rabbit Antibodies to Gold Nano Particles

Four ml of gold nanoparticles solution was added in Eppendorf tubes and centrifuged at 13800 RPM for 15 min at 4^0 C, supernatant was discarded and the pellet was collected. Two ml of 0.05 mM phosphate buffer (pH 8.5) and 40µl (40µg of antibody) of rabbit antibody was added. Then it was vortexed at 20 rpm for 20 min at the room temperature. A volume of 200µl of 10% bovine serum albumin (BSA), 200µl 10% glycerol anhydrous ($C_3H_8O_3$ Mol. Wt 92.1) and 200µl 10% PVP (polyvinyl pyrrolidine PVP K 30, Mol. Wt 40000 $(C_6H_9NO)_x$) was added slowly one after another to above solution while mixing the contents slowly with hand (Lata et al., 2015).

Development of kit

Gold antibody (rabbit) conjugate was sprayed on conjugation pad, poultry antibodies (IgY) were adhered at test line and antirabbit antibodies were adhered at control line on nitrocellulose membrane. Sample pad, conjugation pad and absorbent pad was adhered at both the ends of nitrocellulose membrane which was supported by polyvinyl chloride (PVC). It was cut into pieces and placed in plastic cassettes which serve as kit for diagnosis of brucellosis.

Results and Discussion:

Inactivation of Brucella vaccine

Heat-inactivated *Brucella abortus* strain 19 vaccine (10^9CFU/2ml) was used to raise the hyperimmune sera in rabbits and Poultry to standardize the serological test like Sandwich ELISA and Developed LAT with gold nanoparticles.

Extraction of Brucella Cell Membrane Proteins

A total of 23.6 mg cell membrane protein was extracted from vaccine containing 10^9 CFU/2 ml concentration using cell disruption method.

Quantification of Protein In extracted Brucella Cell membrane, hyper immune serum and Egg Yolk

Hyperimmune serum and egg yolk of poultry were purified to concentrate *Brucella* antibodies by ammonium and sodium chloride precipitation methods respectively. *Brucella* cell membrane protein purified hyper immune serum from rabbit and egg yolk from poultry were quantified by Bradford method.

The OD values for *Brucella* cell membrane protein, rabbit (serum) and poultry (egg yolk) proteins were taken at 595 nm wavelength (Spectrophotometer) compared with bovine serum albumin known concentration standards and converted in to concentrations (mg/ml).

Quantification of Total Protein

Whole *Brucella* cell membrane protein, hyperimmune sera in rabbits and in poultry were quantified and purified to 1.18mg/ml, 47.8 and 13.8 mg/ml respectively.

Serological tests

Indirect ELISA Test to Determine IgG and IgY Antibody Titer

It was observed that rabbit antibodies could react at 1 in 5000 dilution and poultry antibodies were capable of reacting at 1 in 2500 dilution.

PCR Assay

Standardization of PCR Assay

Specific amplification of 223bp fragment without any spurious product (Figure 1). The field samples were analyzed is represented in Figure 2.

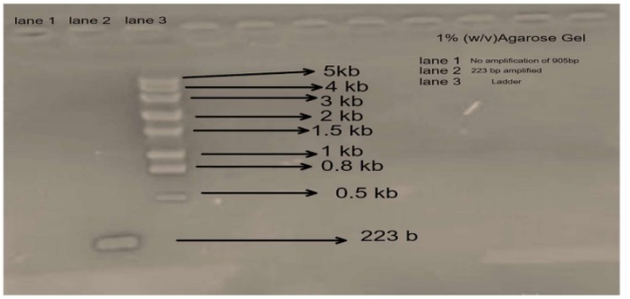

Figure 1: Optimization of PCR assay for *Brucella* spp. (Lane 1: Negative control, Lane 2: Amplification of 223 bp gene and Lane 3: DNA Ladder

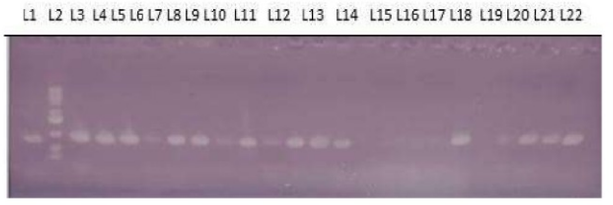

Figure 2: PCR assay results of 20 field samples.
L1: Positive control
L2: Ladder
L3 to L22: Field samples
Positive samples are- 15: L3, L4, L5, L7, L8, L9, L10, L11, L12, L13, L14, L18, L20, L21, L22)
Negative samples are- 5: L6, L15, L16, L17, L19

Lateral Flow Technology

Lateral flow kit was formulated based on lateral flow technology by conjugating the gold nano particles.

Preparation of Gold Nano Particles and Testing for its Presence

Gold nanoparticles were synthesized by citrate reduction method, tested for the presence of nano particles by physical appearance and UV Spectroscopy method.

Physical Appearance

Initially gold solution was pale yellow in colour when sodium citrate was added then colour changed to white, black and finally became cherry red in colour indicating formation of nano particles (Figure 3)

Figure 3: Cherry red colour of Gold Nano Solution

UV Spectroscopy Method

The cherry red coloured gold nano solution was subjected to UV visible spectroscopy at 300nm to 700nm wave length; surface absorption band with maximum absorption at 520nm wavelength with a single peak confirms the mono dispersion of nano particles. UV spec scanning result showing single peak was given

Determination of Gold Nano Particles Size

Prepared gold nano particles solution was subjected to Zeta potential analyzer (Malvern) and the average size of gold nano particles was 40.54 nm (Figure 4).

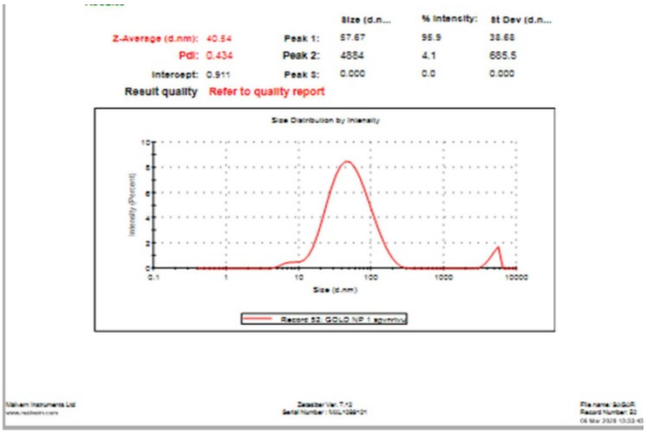

Figure 4: Report showing size of the GNP by Size (40.54nm) in zeta potential analyzer.

Determination of Zeta Potentials of Gold Nano Particles

The zeta potential of gold nano particles was measured by Zeta Potential analyzer and found to be -8.13mv.

Stability Checking of Gold Nano Particles in Different Buffers at Different pH

Gold nano particle solution pH was adjusted to 8.5. Then added to borate phosphate and citrate buffers having 3 different pH of 6.5, 7.5 and 8.5. No change of colour in gold nano solution at 7.5 and 8.5pH of borate, phosphate

and citrate buffer indicated its stability and at 6.5 pH, colour changed to blue/black in all the buffers indicated its instability.

Optimization of Antibodies to Gold Nano Particles for Conjugation

It was observed from Eppendorf tubes that change of colour from cherry red to blue/ black and formation of clumps in 6th and 7th Eppendorf tube indicating that conjugation was improper and from 1st to 5th Eppendorf tube there is no change of colour and consistency indicating in all the 5 tubes conjugation was complete.

Checking for the Conjugation by UV SPEC Method

Rabbit antibodies were conjugated to gold colloidal solution by surface absorption method. When observed under UV spectrophotometer the absorption peak (λMax) shifted from 520nm to 525nm indicating the proper conjugation of antibodies to surface of gold nano particles (Figure 5).

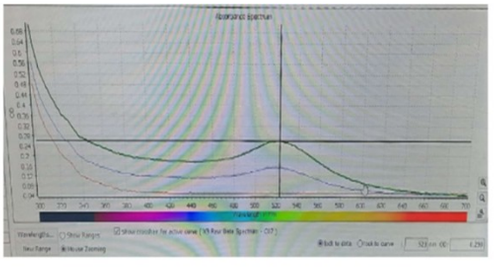

Figure 5: UV SPEC Method of finding the conjugate showing the shift in absorption peaks

Construction of diagnostic kit based on lateral flow technology

Diagnostic kit was developed by pasting poultry (IgY) antibodies as test line, anti-antibodies of rabbit at control line by an instrument (Eazy Print) on nitrocellulose paper and conjugated gold nano particles (with rabbit polyclonal antibodies) on conjugation pad. The absorbent pad and sample pad was adhered to poly vinyl chloride backing material on opposite sides and these are cut into 1.5cm wide strips and placed in plastic cassette. (Figure 6, Figure 7). The developed strips are used for optimization trials (Figure 8).

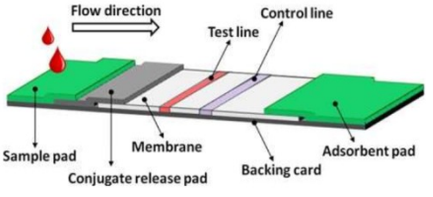

Figure 6: Typical configuration of a lateral flow immunoassay test strip

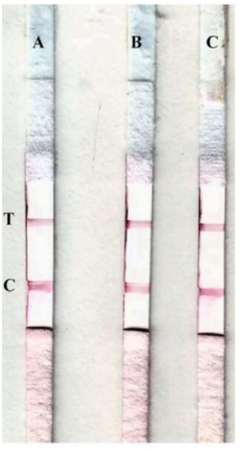

Figure 7: Developed Strip based on LFT

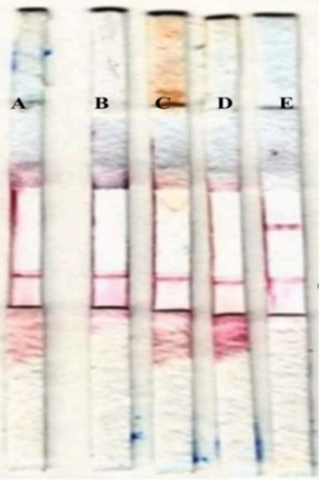

Figure 8: Developed strips to test field samples showing positive for known positive controls

Sensitivity and specificity of developed kit compared with PCR assay and ELISA

Out of 204 vaginal samples PCR assay has given 113 positives whereas the developed kit has given 87 positives. The entire samples positive by the developed kit were positive by PCR. The sensitivity of the developed kit compared to PCR assay was 76.99%.

By the developed kit 117 samples were negative whereas 91 samples were negative by PCR assay. All the PCR assay negatives were negative by developed kit. Hence the specificity of developed kit was 100%.

Table 1. Sensitivity and specificity of developed kit compared with PCR assay

Type of Assay	Number of Positive Samples	Number of Negative samples	Sensitivity of developed kit based on LFT	Specificity of developed kit based on LFT
PCR Assay	113	91	NA	NA
LFT kit	87	117	87/113=76.99 %	91/91= 100%

The sensitivity of developed kit based on lateral flow technology compared to ELISA was 94.56%. All the 112 samples negative by ELISA were negative by developed kit hence the specificity of the developed kit is 100%.

Table: 2 Sensitivity and specificity of developed kit assay compared with ELISA

	Number of Samples	Number of positive samples	Number of negative samples	Sensitivity	Specificity
ELISA	204	92	112	NA	NA
LFT KIT	204	87	117	87/92=94.56%	112/112= 100%

Bovine brucellosis is widespread in India and appears to increase in recent times, due to increased trade and rapid movement of livestock. For effective control and prevention, a standard diagnostic method which can be applied at field level is still under incubation. There are so many kits available, but all of them identify the *Brucella* antibodies from serum samples only, but none of them works for antigen detection from vaginal swabs. Hence identification of *Brucella* organisms in vaginal swabs is very important in early stages before formation of antibodies.

In recent past, the kits based on lateral flow technology has gained importance since its several merits, viz. ease of application, cost effectiveness, less time consumption, noninvolvement of technical person, early detection, accuracy and long shelf life. Hence the present study was undertaken to develop a new diagnostic kit based on Lateral flow technology, and to study the efficacy of the developed kit on field samples

Indirect ELISA test was performed to know the efficacy of antibodies present in serum of rabbit and egg yolk. In the present study, rabbit antibodies reacted at 1:5000 dilution and poultry antibodies reacted at 1:2500 dilution whereas Chin, (1983) reported rabbit poly clonal antibodies have reacted in 1:6400 dilutions which has given higher efficient antibodies than the present study

Standardization of PCR conditions was done with slight changes in number of cycles, Time-temperature combinations based on preliminary studies. Development of kit includes preparation of good quality nanoparticles, conjugation of antibodies to nanoparticles, adhering of antibodies at control and test lines on nitrocellulose membrane, spraying of gold antibody conjugate on conjugation pad, cutting into small strips and finally assembling it into plastic cassettes.

In the present study, the gold nanoparticle solution was prepared by the Citrate reduction method with little modifications wherever necessary recommended by Turkevitch et al. (1951). The Gold nano particle solution was subjected to physical appearance, and UV spec to confirm the formation of nano particles. The size and stability were estimated by Zeta analyzer. Nara et al., 2010 and Lata et al. (2015) also used the same procedure and observed similar colour changes while preparing gold nano particles solution

The cherry red coloured gold nano solution was subjected to UV visible spectroscopy at 300 nm to 700 nm wavelength surface absorption band with maximum absorption at 520 nm wave length with a single peak was observed confirming the mono dispersion of Nano particles.

Lata et al. (2015) also observed Maximum peak at 520 nm wave length that indicating the smaller size of the nano particles. Ngo et al. (2015) and Seol et al. (2011) observed gold nanoparticles peak at the wavelength of 521nm which was slightly higher than the present study.

Average size of gold nano particles in the present study was 40.54 nm with a single peak showing mono dispersion colloid with good stability, better handling during conjugation and line detectability on nitrocellulose paper (Kimling et al., 2006). Lata et al. (2015) prepared gold nano particles with an average size of 34.8 nm which is slightly less than the size of

Nano particles in the present study. Jans et al. (2010) prepared gold nano particles with an average size of 110.0 ± 4.5 nm, which is much bigger than in the present study. Lower size of the gold nano particles (20 nm) was reported by Jagirani, et al. (2022). Oh et al. (2010) used poly ethyleneglycol (PEG) as reducing agent that resulted in 18nm sized gold nano particle.

Gold nano solution was subjected to zeta potential analyzer which has given a value of -8.13mv. Zeta potential values of -17.66 mV, -43.2 mV and -44.1 mV were reported by Memon et al. (2022), Lata et al. (2015) and Nara et al. (2010), respectively which are less than the values observed in the present study. The lower value of the zeta potential provides stability to nanoparticles against agglomeration, which was probably due to capping of the gold nano particles by active molecules such as sodium citrate (Nara et al., 2010).

Phosphate, borate and citrate buffers having different pH (6.5, 7.5 and 8.5) with 0.05mM concentration were evaluated to determine the suitable buffer required for conjugation of gold nano particles to antibodies.

As there is no colour change at 7. 5 and 8.5 pH in all the buffers indicating their suitability for conjugation whereas all the buffers at 6.5 pH change of colour to blue/black was observed indicating non suitability for conjugation at the pH.

Male et al. (2008) tested the effects of aggregation by citrate (pH 4.5), acetate (pH 5.5) and borate (pH 9) buffer, with 35mM molarity and reported that in all the buffers nano particles were unstable, whereas in present study at pH 7.5 and 8.5 gold nano particles were stable in borate, phosphate and citrate buffer having 0.05mM molarity.

Based on a theoretical model and experimental findings, sodium citrate was shown to play a significant influence in the pH of the solution and the size of the nanoparticle (Yang et al., 2007; Liu et al., 2007 and Kumar et al., 2007).

After conjugation of gold nano particles to antibodies the colour of the solution changes from cherry red to bluish red (Jazayeri et al., 2016) and in UV SPEC the absorption peak (ʎMax) shifts to higher wavelength (Khaing et al., 2008).

In the present study, after conjugation the colour has changed from cherry red to Bluish red and Peak was shifted from 520nm to 525nm in UV SPEC, indicating satisfactory conjugation of antibodies to gold nano particles has taken place.

Lata et al. (2015) also observe shift of maximum peak from 520 nm to 524nm, and Seol et al. (2011) observed change of peak from 519 nm to 524nm after conjugation which was almost like the present study. Kimling et al. (2016) observed shift of maximum peak from 518 nm to 526 nm whereas Ngo et al. (2015) observed that change of peak from 521 nm to 526nm after conjugation, which were slightly higher than the peak observed in the present study.

For preparation of kit, the procedure followed by Tel et al. (2022) for diagnosis of *Francisella tularensis* was followed. Gold antibody (rabbit) conjugate was sprayed on conjugation pad, poultry antibodies (IgY) were adhered at test line and anti-rabbit antibodies were adhered at control line on nitrocellulose membrane and sample pad, conjugation pad and absorbent pad was adhered at both the ends of nitrocellulose membrane which was supported by poly vinyl chloride (PVC) and cut in to pieces and placed in plastic cassettes which serves as kit for diagnosis of brucellosis.

Out of 204 vaginal swabs tested for PCR assay, ELISA and by laboratory developed kit 113,92 and 87 samples were positive for *Brucella,* taking PCR assay as gold standard test, the sensitivity and the specificity of the developed kit was 76.99% and 100%, respectively, whereas the sensitivity and specificity of ELISA were 81.4% and 100%, respectively. The sensitivity of the developed kit was less than the sensitivity of ELISA whereas the specificity of the developed kit and ELISA was the same.

Lower sensitives of 25% and 53.8% of lateral flow assay considering PCR assay as gold standard test was reported by Pfukenyi et al. (2020) and Hasaniet al. (2020), respectively than the sensitivity observed in the present study by kit developed based on lateral flow assay. The sensitivity of ELISA test (52.5%) compared to PCR assay reported by Pfukenyi et al. (2020 was less than the sensitivity of ELISA (81.4%) observed in the present study.

Prakash et al. (2019) developed lateral flow technology kit with sensitivity and specificity of 80.0% and 94.0%, respectively using PCR as reference diagnostic tests, which are higher than the findings in the present study.

Manasa et al. (2019) reported that lateral flow assay kit showed 89% and 99% sensitivity and specificity, respectively, when compared with competitive ELISA as the gold standard.

Gusi et al. (2019) developed lateral flow assay kit and reported sensitivity and specificity of 96.6% and 98.8%, respectively compared to RBPT which were higher than the values observed in the present study.

Ashraf et al. (2014) reported that sensitivity and specificity of 87% and 97%, respectively. Rahman et al. (2011) reported sensitivity and specificity of 84.6% and

93.7% of lateral flow assay compared to Tube agglutination test, respectively which were higher than the values observe in the present study.

In conclusion, since all the kits available for diagnosis of brucellosis at field conditions detect anti-*Brucella* antibodies in blood with no active brucellosis, one lateral flow technology based kit has been developed that detects antigen from vaginal swab and hence active brucellosis.

Conclusion:

The case demonstrates prompt clinical recovery of a severe case of an acute hemorrhagic diarrhoea syndrome adding oral spiramycin and racecadotril where traditional line of treatment using fluids and supportive antibiotics were not successful. This is the first documented case of treating canine haemorrhagic gastroenteritis (HGE) with a combination of spiramycin and racecadotril administered orally for 5 days and this line of treatment may be considered in dealing with difficult cases of this nature.

Conflicts of interest:

The authors declare that there is no conflict of interest.

Authors' contribution:

All authors contributed equally in writing of this article.

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