DEVELOPMENT OF GOLD BASED RAPID ASSAY FOR DIAGNOSIS OF BLUETONGUE IN SMALL RUMINANTS

Thesis

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IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Master of Veterinary Science
(Animal Biochemistry)

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Dedicated to.....

My Beloved Father

&

Mother

भारतीय पशु चिकित्सा अनुसंधान संस्थान
(सम विश्वविद्यालय)
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Dated: 27/6/ **2014**

Certificate

This is to be certified that the research work embodied in this thesis entitled "Development of gold based rapid assay for diagnosis of bluetongue in small ruminants" *submitted by* Dr. Pravas Ranjan Sahoo, Roll No. 5148, *for the award of* Master of Veterinary Science *Degree in* Animal Biochemistry *at Indian Veterinary Research Institute, Izatnagar, is the original work carried out by the candidate himself under my supervision and guidance.*

It is further certified that Dr. Pravas Ranjan Sahoo, Roll No. 5148, *has worked for more than 21 months in the Institute and has put in more than 150 days attendance under me from the date of registration for the* Master of Veterinary Science *Degree in this Deemed University, as required under the relevant ordinance.*

Praveen Singh
(PRAVEEN SINGH)
Chairman
Advisory Committee

Certificate

We the undersigned members of Advisory Committee of **Dr. Pravas Ranjan Sahoo, Roll No. 5148,** *a candidate for the degree of* **Master of Veterinary Science** *with the major discipline in* **Animal Biochemistry**, *agree that the thesis entitled* **"Development of gold based rapid assay for diagnosis of bluetongue in small ruminants"** *may be submitted in partial fulfillment of the requirement for the degree.*

We have gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented for the award of **Master of Veterinary Science** *Degree of this Institute.*

It is further certified that the candidate has completed all the prescribed requirements governing the award of **Master of Veterinary Science** *Degree of the Deemed University, Indian Veterinary Research Institute, Izatnagar.*

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Pravas Ranjan Sahoo

Date: 29/7/2014
Place: *IVRI, Izatnagar*

(Pravas Ranjan Sahoo)

CONTENTS

ABBREVIATIONS

µg	:	Microgram
µl	:	Microliter
BSA	:	Bovine Serum Albumin
BTV	:	Bluetongue virus
GNP	:	Gold Nanoparticle
$HAuCl_4$	:	Tetra chloro auric acid.
KH_2PO_4	:	Potassium dihydrogen phosphate
KV	:	Kilovolt
mg	:	Milligram
ml	:	Milliliter
mM	:	Millimole
MW	:	Molecular weight
NaCl	:	Sodium chloride
NaN_3	:	Sodium azide
ng	:	Nano gram
nm	:	Nanometer
PBS	:	Phosphate buffered saline
PEG	:	Poly ethylene glycol
RPM	:	Rotation per minutes
SPR	:	Surface Plasmon Resonance
TEM	:	Transmission electron microscopy.
Tris-HCl	:	Hydroxy methyl amminomethane hydrochloric acid
W/V	:	Weight per volume

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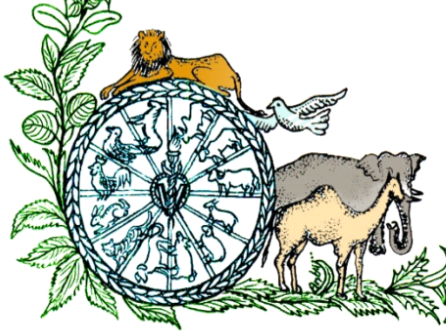
Introduction



1 INTRODUCTION

Bluetongue is a non-contagious, insect borne viral disease of domestic and wild ruminants caused by *orbivirus* of family reoviridae, which is transmitted by culicoides miting midges. Sheep is the main principal host. It is characterized by incubation period of four to eight days. (Tweedle and Mellor, 2002) followed by fever, hyperemia of the lips and nostrils with excessive salivation, oral ulceration with swollen, protruding, cyanotic tongue At the end of the pyrexia stage, affected sheep may develop coronitis, laminitis, painful hooves, paresis and necrosis of striated muscles ultimately comes to lameness. Severity of disease varies with the breed of sheep, virus strain and environmental stress. The morbidity rate can be as high as 100% and mortality rate is very low of about 0-30%.

Bluetongue is placed in the Office International des Epizooties list of animal diseases. Bluetongue was first seen in South Africa at the end of the 18th century. In India, it was first reported in Maharashtra in 1964. The manifestations of bluetongue range from an in apparent to a fatal outcome depending on the serotype of the virus, the species, breed and age of the infected animal. Twenty six serotypes of Bluetongue virus are available till today. This virus is a nonenvolvped 90 nm size with 10 segmented double standard RNA genome expressing 7 structural proteins (VP1 to VP7) & 4 non structural protein (NS1 to NS3 and NS3A) .Out of these ,this VP7 protein is conserved across all serotypes. So this VP7 protein acts as epitope for different immunological tests. The worldwide economic losses due to bluetongue have not been expressed in exact numbers, but the estimate is 3 billion US$ a year (Tabachnick *et al*., 1996). The losses are both direct (death, abortions, weight loss or reduced milk yield and meat efficiency) and, what is more important, indirect as a result of export restrictions for live

animals, their semen and some products such as fetal bovine serum. The costs of preventive and control measures should also be taken into account. In cases of a wider spread of bluetongue, these measures could have a serious impact on the quantity of meat and animal products available for the consumer market; therefore, bluetongue is considered a potential biological weapon.

Therefore, Proper Diagnosis of Bluetongue is very important to minimize the economic loss to our country. For this, different conventional diagnostic methods like virus isolation, different types of ELISA (sandwich, indirect, competitive), molecular techniques like RT-PCR, molecular beacon fluorescent probe assay were already developed. But these methods are time consuming, costly, needs skilled personals and not applicable to the field levels. So a very fast, simple, low cost and sensitive diagnostic systems need to be developed for rapid diagnosis of bluetongue. Gold based Rapid Assay which I am now going to develop, may fulfill the above requirements. Not only of its rapidness, low cost and simplicity, it is also one of the most sensitive techniques which can detect the pathogen in naked eye in very minute quantity. This assay may be of two types. One, SPR based Biosensor Assay and second, Lateral Flow Assay. SPR based biosensor Assay is one of the modern label free technique which can monitor the protein-protein interaction, protein-DNA interaction in real time with high specificity and sensitivity. But the lateral flow assay is the pen side test which can detect the minute quantity of analyte with quick time. It is a field oriented assay. Now this assay becomes popular not only in the medical sector but also in the veterinary field because of its simplicity and its low cost. Lateral flow device has already developed for different animal diseases (FMD, PPR, Rotavirus, and JE), different antibiotic residues (Streptomycin, ciprofloxacin) but there are still new possibilities to exploit it. The variability of the lateral flow assay is mainly dependent upon the target analytes to be diagnosed.

Gold nanoparticle is one of the extensively used labels in lateral flow assays due to its easy preparedness, non toxicity, small particle size and also its high surface plasmon properties, the evaluation of result can be made by visual assessment because its surface plasmon resonance falls in the visible spectrum. Therefore, gold nanoparticle based immunochromatographic assay has provided attractive means for devoloping different diagnosis kits against different pathogens. Gold nanoparticle based diagnostic kits already have been commercialized for pregnancy

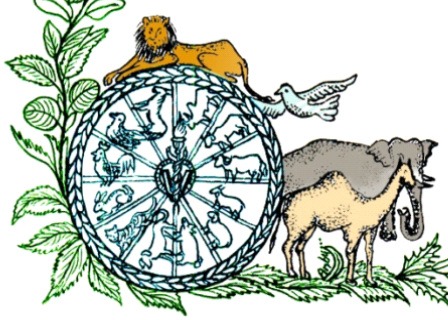
diagnosis in biomedical field. Because of its stability and it's easy to use, gold nanoparticle has given more preference over the conventional label like latex beads, flour chrome and enzymatic labels. Moreover, nanoscale surfaces of gold nanoparticle are appropriate for accelerating the antigen antibody reaction, which enhances the immunoassay signals (Richars *et al*., 1996) After antigen antibody reaction, the label gold nanoparticle accumulates at that location, appears on the membrane and gives red color. (Yoshiki *et al*.,2001). For quick diagnosis and control measures of diseases, the development of gold biosensors chip is spreading in various field including medial, environmental and forensic applications. The lateral flow assay, using nitrocellulose membrane as the immunosorbent, provides a unique analytical platform that permits a one step, rapid and low cost analysis (Xiulan *et al*., 2005). The immunochromatographic assay system has devised in order to determine the concentration of the target analyte simply and rapidly. Furthermore, we can monitor the level of pathogen on real time with naked eye. Because of these advantages, the immunochromatographic assay may be developed against bluetongue antibody. So we can easily diagnose the bluetongue in population in quick time. The economic loss of the country due to morbidity will be minimized.

Keeping these facts in view current research programmed entitled **"Development of Gold Based Rapid Assay for Diagnosis of Bluetongue in Small Ruminants"** for the detection of blue tongue antibody by incorporation of recombinant VP7 protein will be able to detect serum antibody against bluetongue virus from the field serum sample. This test will be invaluable for future eradication programs and also seroprevalence of bluetongue in our country. This assay must help the proper diagnosis of bluetongue disease at field level at very low cost price.

Objectives:

1. **Synthesis & characterization of gold nanoparticles**
2. **Purification & Concentration of IgG from Bluetongue hyperimmune sera using affinity chromatography method**
3. **Development of gold based lateral flow device using purified recombinant VP7 protein for diagnosis of BTV antibody in small ruminants.**

Review of Literature



2 REVIEW OF LITERATURE

2.1 History and Distribution of the Bluetongue

Bluetongue was first recorded at the end of the 18th century in South Africa after an import of fine-wool sheep from Europe. It was first referred to as fever, malarial catarrhal fever of sheep or epizootic malignant catarrhal fever of sheep. In 1933 it was first diagnosed in cattle and, because its clinical signs were similar to those of foot-and-mouth disease, it was called pseudo-foot-and-mouth disease, seer beck or sore-mouth (Vellema, 2008). "Bluetongue", the name used today, has been derived from the African "bloutong", which was coined by South-African farmers who noticed tongue cyanosis in seriously diseased animals (MacLachlan *et al.*, 2009). In 1906, Theiler was the first to report that the infecting agent was a filtrable virus (Mehlhorn, 2008). Bluetongue can develop and spread when susceptible hosts, BTV and competent insect vectors are all present at the same time. Traditionally, the virus was present in a geographic band between the latitudes 40°N and 35°S where its vectors, certain species of biting midges, were living (Rodriguez-Sanchez *et al*., 2008; Vellema, 2008; Wilson and Mellor, 2009). In North America and China the virus spread even further, up to 50°N (Mellor *et al*., 2000). Before the 1940s the occurrence of bluetongue was limited to South Africa (MacLachlan *et al*., 2009). The first well recorded epidemic beyond the African continent dates back to 1943 in sheep in Cyprus (MacLachlan, 2004), but there are indications that bluetongue had been there since 1924 (Rodriguez-Sanchez et *al*., 2008). In 1943–1944 bluetongue was found in Israel (Shimshony, 2004). In 1948 it was reported in Texas, USA, between 1956 and 1957.A large epidemic broke out on the Iberian peninsula, and subsequently blue-tongue was also found in the Middle East, Asia and Southern European countries (Mellor

and Wittmann, 2002; MacLachlan, 2004) In Australia, it first appeared in 1977, and in South America it was found in the 1980s. Bluetongue is also present in Central America and Mexico, Papua New Guinea, Thailand, China, Japan, the Indian subcontinent, Mediterranean countries (Greece, Spain, Italy, Corsica), Portugal, Bulgaria and other countries (Baylis and Mellor, 2001; Mellor and Wittmann, 2002; Tweedle and Mellor, 2002; Anonymous, 2007; Mehlhorn *et al*., 2007; Mellor *et al.*, 2008).

2.2 Epidemiology

2.2.1. Southern Europe

During the 20th century, only short outbreaks of bluetongue were occasionally recorded in Southern European countries (Spain, Portugal, Greece and Cyprus) (Hendrick, 2009). However, since 1998, BTV has been present in Southern European and Mediterranean countries from which it has gradually spread to areas previously free of the virus. In 1998, the disease was recorded in Greece, in 1999 in Turkey and Bulgaria, then also in Serbia, Montenegro, Kosovo and Macedonia. In 2000, bluetongue was found in Sardinia, Sicily, mainland Italy, and in Corsica, Menorca and Mallorca. In 2001, it was first found in Croatia and the following year in Bosnia and Albania. Up to now serotypes 1, 2, 4, 9 and 16 have been isolated in the region of Southern Europe (Mellor and Wittmann, 2002; Anonymous, 2007; Saegerman *et al.*, 2008).

2.2.2. Northern Europe

In August 2006, bluetongue epidemics broke out suddenly and unexpectedly in Northern Europe, first in the Netherlands and shortly afterwards in Belgium, Germany and the north of France (Wilson and Mellor, 2009). They were caused by the BTV serotype 8 (Elbers *et al.,* 2008a; Mintiens *et al.*, 2008) that is common in South African Republic, India (Prasad et al., 1992b), Central America and the Caribbean. This serotype had never been reported in Europe before 2006 (Mehlhorn *et al.*, 2007; Mintiens *et al*., 2008) and up to now its origin has not been traced (Mintiens *et al., 2*008; Wilson and Mellor, 2009). There are several theories explaining its introduction, for instance, import of animals at a viraemic stage of the disease, infected semen or embryos, inadvertent shipment of infected biting midges together

with animals or plants, or the introduction of biting midges either through their own flight over long distances or with the aid of wind (Saegerman *et al.,* 2008).

By the end of 2006, BTV-8 had further been discovered in Luxembourg (Carpenter *et al*., 2009; Wilson and Mellor, 2009). Owing to survival over the winter, the BTV-8 appeared in these countries again the next year and even spread to Great Britain, Switzerland, Denmark and the Czech Republic (Saegerman *et al*., 2008; Schwartz-Cornil *et al*., 2008; Hendrick, 2009). New outbreaks of bluetongue were reported in Hungary, Austria and Sweden in 2008 (Carpenter *et al*., 2009; Agren *et al*., 2010; Lewerin *et al*., 2010). The BTV-8 strain that has invaded Northern Europe is highly virulent not only for sheep, but also cattle or South American camelids and, in addition, it can cross the placenta, which was not typical of the field strains of BTV in the past (MacLachlan, 2010). The BTV-8 epidemic in Northern Europe has probably caused greater economic damage than any previous single serotype bluetongue outbreak.

In 2008, BTV-6 was identified in the Netherlands and Germany and BTV-11 in Belgium. Both viruses were derived from the vaccine strains (Eschbaumer *et al.,* 2010) most likely introduced to Europe through the illegal use of attenuated vaccines (Mac Lachlan, 2010).

A new virus, similar to BTV, and infecting goats was discovered in Switzerland in early 2008. It was named *Toggenburg orbivirus,* and is a so far unknown orbivirus with low pathogenicity and a potential BTV serotype 25. And similarly in the Arab countries, in 2010, a potential serotype 26 of BTV was identified in sheep and goats in Kuwaitfor (Maan *et al.*, 2011).

2.2.3. USA, Australia, Israel

As in Southern Europe, new BTV serotypes were identified in the USA, Australia and Israel during the last decade. In the USA, in addition to the serotypes 2, 10, 11, 13 and 17 found in enzootic outbreaks before 1999, BTV serotypes 1, 3, 5, 6, 9, 12, 14, 19, 22 and 24 were newly isolated. In Australia serotypes 2 and 7 were newly identified in 2007 and 2008. The situation was similar in Israel where, in addition to the previously recorded BTV serotypes 2, 4, 6, 10 and 16, serotypes 8, 15 and 24 were discovered after 2006 (Mac Lachlan, 2010).

2.2.4. Bluetongue in India

The first outbreak of bluetongue in India was recorded in 1964 among sheep and goats in Maharashtra State, on the basis of clinical signs and detection of BTV antibodies in the sera of animals which had recovered (Prasad *et a*l., 1992b). Bluetongue virus infection is widely distributed in domestic ruminants. Evidence for the existence in India of 18 of the 24 known serotypes of BTV has been reported on the basis of presence of serum neutralizing antibodies from different states in the country. Bluetongue disease is mainly present in the Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana, Uttar Pradesh, Rajasthan, Gujarat, Madhya Pradesh, Maharashtra, Orissa, Andhra Pradesh, Karnataka and Tamil Nadu in sheep and goat. This disease is mainly present in Jammu and Kashmir, Himachal Pradesh, Punjab, Haryana Uttar Pradesh, Rajasthan, Gujarat, Madhya Pradesh, Maharashtra, Orissa, Andhra Pradesh, Karnataka, and Tamil Nadu for buffalo and cattle in India. Although BTV has been isolated from *Culicoides,* the species has not been identified. Outbreaks of BT occur in sheep and cause heavy mortality and morbidity. Clinical BT has not been observed in cattle and buffalo. BT vaccine is not available in the country; hence vaccination against this disease is not practiced. In order to combat the impact of this disease on the Indian livestock industry, implementation of a clear cut national policy is imperative.

2.3. Bluetongue Virus

Bluetongue virus is a member of the genus *Orbivirus* in the family *Reoviridae*. It is similar in morphology to other orbiviruses, such as epizootic hemorrhagic disease virus, African horse sickness virus or equine encephalitis virus. So far 24 BTV serotypes have been identified worldwide (Roy and Noad, 2006; Schwartz-Cornil *et al*., 2008) with the already mentioned potential serotypes 25 and 26 recently isolated in Switzerland and Kuwait, respectively (Maan *et al*., 2011). Bluetongue virus is a nonenveloped virus, 90 nm in diameter, with a triple layered icosahedral protein capsid (Prasad *et al*., 1992a; Roy and Noad, 2006). Its genome consists of ten double-stranded (ds) RNA segments coding for seven structural proteins (VP1–VP7) (Biswas, 2005) and four non-structural proteins (NS1–NS3 and NS3A) (Kar *et al*., 2007; Roy, 2008). BTV remains stable in the presence of proteins and can survive for years, for

instance, in blood stored at 20 °C. It is sensitive to 3% NaOH, organic iodine complex, phenol and b-propiolactone.

2.4. Transmission

2.4.1 Culicoides biting midges

Bluetongue is almost always transmitted by biting midges of the genus *Culicoides* (Diptera: *Ceratopogonidae*) and therefore outbreaks depend on the concomitant presence of competent insect vectors and susceptible ruminants. The genus *Culicoides* at present includes 1300 to 1400 species (Mellor *et al*., 2000), but only about 30 of them are BTV vectors (Meiswinkel, 2004). Although biting midges are ubiquitous (Mellor *et al*., 2000), they are most frequently present in warm, damp and muddy areas which are rich in organic matter and plentiful in animal hosts they can feed on. They are most active from about one hour before sunset until one hour after sunrise (Mellor *et al*., 2000).

2.5. Host range

All ruminants are susceptible to infection with bluetongue, but clinical disease is most often manifested in sheep, a serious disease also develops in white-tailed deer (*Odocoileus virgin-ianus*) (Johnson *et al*., 2006). In cattle, which play an important role in the epidemiology of BTV mainly because of prolonged viraemia, the disease has in the past mostly been reported to have a subclinical course (Tweedle and Mellor, 2002). However, in the epidemics caused by BTV-8 in Western and Central Europe, even cattle showed clinical disease (Elbers *et al*., 2008a). Under natural conditions the disease may also be present in wapiti (*Cervus elaphus canaden-sis*), proghorn *(Antilocapra americana)*, African antelopes and other wild ruminants, but it can also affect camelids (Henrich *et a*l., 2007; Meyer *et al*., 2009).

2.6. Pathogenesis

After introduction through the bite of an infected midge, the virus is transported by the host dendritic cells from the skin to the local lymph nodes (the sites of initial virus replication) (MacLachlan, 2004). Subsequently, it spreads to the blood circulation inducing a primary viraemia which seeds secondary organs, i.e., lymph nodes, spleen and lungs (Sanchez-Cordon

et al., 2010). The virus replicates in vascular endothelial cells, macrophages and lymphocyte. In early viraemia virus is associated with all blood elements, while at later stages of viraemia it exclusively associates with erythrocytes. Virus particles appear to be sequestered in invaginations of the erythrocyte membrane (MacLachlan, 2004), allowing prolonged viraemia in the presence of neutralizing antibodies.

2.7. Viraemia and immune response

Viraemia in infected animals has a prolonged course, but is not persistent. Its duration depends on the longevity of erythrocytes to which virus is bound, in contrast to the other blood cells, even at the late stage of infection (MacLachlan *et al*., 2009). It is also related to the species and breed of the infected animal. Viraemia lasts 14 to 54 days in sheep and 19 to 54 days in goats. In cattle, viraemia may last as long as 60 or, even 100 days which makes this animal an important host from the epidemiological point of view. The infected animals react to BTV with interferon production and humoral and cell-mediated immune responses. Serotype specific neutralising antibodies against the VP2 protein confer protection against homologous strain reinfection (Schwartz-Cornil *et al*., 2008). Neutralising antibodies are also induced, to a lesser degree, by the VP5 protein. The sera of infected ruminants also contain sero group specific antibodies induced by the VP7 protein (Biswas, 2005), as well as antibodies against other structural and non-structural proteins (MacLachlan, 2004).

2.8. Clinical signs

Bluetongue in sheep is manifested as an acute, chronic or subclinical condition; fine wool breeds are most susceptible. An incubation period of four to eight days (Tweedle and Mellor, 2002) is followed by fever, apathy, tachypnea, and hyperemia of the lips and nostrils with excessive salivation and serous nasal discharge that is initially clear, then becomes mucopurulent and upon drying may form a crust around the nostrils. Edema of the tongue, lips, submandibulum and sometimes ears appears, petechiae develop on the conjunctiva and ulcers on the oral mucosa. Cyanotic tongues are found in rare cases. In some cases, dyspnoe, profuse hemorrhagic diarrhea or vomiting that can cause aspiration pneumonia is recorded. At the end of the pyrexia stage, affected sheep may have coronitis, laminitis or paresis and necrosis of

striated muscles and, as a result, stand with an arched back and are reluctant to move. Torticollis, dermatitis and breaks in the wool may also develop (Tweedle and Mellor, 2002; Anonymous, 2004; Elbers *et al.*, 2008a ; Kirschvink *et al.*, 2009). Infection in pregnant ewes may lead to abortion, foetal mummification and the birth of weak calves with potential congenital defects (hydrocephalus, cerebral cysts, retinal dysplasia, etc. (Tweedle and Mellor, 2002; Saegerman *et al*., 2011). Chronically affected sheep may succumb to other diseases such as bacte-rial pneumonia (MacLachlan and Gard, 2009).

2.9. Conventional method avaible for diagnosis of bluetongue

A preliminary diagnosis based on clinical signs, post mortem findings and epidemiological assessment should be confirmed by laboratory examination. Samples to be examined in the laboratory should include non-coagulated blood (EDTA or heparin is preferred), blood serum, post-mortem tissue samples of spleen, lymph nodes, lungs, liver, bone marrow and, when indicated, heart and skeletal muscles; in addition, brain tissue is collected in fetuses (Tweedle and Mellor, 2002). For transport, blood serum samples should be frozen at –20°C and the other samples should be kept on ice (Tweedle and Mellor, 2002). Full blood samples can be stored at + 4°C for a long time; isolated blood cells in 10% dimethyl sulphoxide require storage at a temperature of – 70°C.

2.9.1. Bluetongue virus isolation:

Bluetongue virus can be propagated in embryonated chicken eggs, cell cultures or in sheep. Embryonated eggs, nine to 12 days old, are used for BTV isolation (Anonymous, 2004) and intravenously inoculated with the material examined. This method is 100 to 1000 fold more sensitive than yolk sac inoculation, but is demanding in terms of technical skills and experience (Clavijo *et al*., 2000). The material obtained from embryonated eggs can either be fur-ther propagated in cell culture or directly examined using molecular methods (PCR or *in situ* hybridization) (Clavijo *et al*., 2000). Bluetongue virus can be detected by polyclonal antibody based sandwich ELISA (Chand *et al*., 2009).

2.9.2. Antigen identification

A direct identification of BTV in blood or tissue samples is possible with use of the reverse transcriptionpolymerase chain reaction (RT-PCR) method that allows for serotyping

and can detect BTV RNA in samples as late as six months after infection. A quantitative assessment of RNA in an examined sample is possible by real time RT-PCR (Shaw *et al*., 2007; Toussaint *et al*., 2007). The identification of a BTV serotype is carried out in the virus neutralization test. Other available diagnostic methods include antigen capture ELISA, immunospot and immunofluorescence tests (Anonymous, 2004), but they are rarely used.

2.9.3. Antibody identification

Serogroup specific antibodies against BTV can be detected by a competitive ELISA test targeted to the VP7 protein. This is a rapid method permitting determination of serum or plasma antibody as early as the 6th post infection day (Koumbati *et al*., 1999). BTV antibody can be detected by based on VP7 by using indirect ELISA (De *et al.,* 2009). There are other commercial ELISA kits developed recently by which early antibodies or antibodies against BTV in individual or bulk milk samples can be detected (Mars *et al.,* 2010). In addition, sero group specific antibodies can be identified by an agar gel immunodiffusion test, which, however, may produce cross reactions with other orbiviruses, a complement fixation test and a haemagglutination inhibition test (Anonymous, 2004). The serum neutralization test has the highest specificity and sensitivity of all the tests, but is also most expensive and time consuming (Hamblin, 2004).

2.9.4. Differential diagnosis

The clinical signs of bluetongue can easily be mistaken for those of other ruminant diseases such as orf (contagious pustular dermatitis), foot and mouth disease, acute photosensitization, acute haemonchosis (with depression and submandibular oedema), facial eczema, *Oestrus ovis* infestation, pneumonia, plant poisoning, salmonellosis, sheep pox, peste des petits ruminants (Tweedle and Mellor, 2002), malignant catarrhal fever, pododermatitis, rinderpest, infectious bovine rhinotracheitis, bovine viral diarrhoea, bovine popular stomatitis, bovine herpes mamillitis and epizootic haemorrhagic disease (Mehlhorn *et al*., 2008; Williamson *et al*., 2008; Savini *et al*., 2011).

2.10. Gaps in knowledge

These all conventional diagnostic methods have these following disadvantages.

- Time consuming, costly and requires skilled personnel.
- Can't detect pathogen real time.
- Great loss to the economy of our country due to high morbidity and mortality.
- Not applicable to field level.

In order to overcome these disadvantages, Rapid assay can be developed for diagnosis of blue tongue.

2.11. Rapid assay

2.11.1. Lateral Flow Assay

Lateral flow assay is traditionally an immunochromatographic method which is being utilized in detection of different analytes. The advantages of lateral flow assays are that they are easy to use, low cost and rapid diagnostic tools. Due to these properties these types of assays are of interest. Products based on lateral flow are already on the market but due to their potential in many different fields their development continues.

2.11.2. Basic Principle

The basic lateral flow assay consists of six different parts. First, the sample is added to the sample pad from which it continues to flow to the conjugate pad, depending on the assay type the labeled antibody or antigen is immobilized. Then the solution is moving in the membrane by capillary forces. When the solution reaches the test line, the antibodies there attach to antigens. Usually after the test line there is also a control line, where excess antibodies can bind with other antibodies. The function of this site is to control that the test and the capillary flow are functional. In the opposite end of the strip when compared to the sample pad is the sixth part, the absorbent pad which absorbs excess liquid. The principle and construction of lateral flow assay is presented in Figure 1.

Even though the principle of this assay is straight forward, the properties of the parts are of high importance. Nevertheless, the specificity of antibody to antigen determines whether the test is functional at all. If the antibodies also attach easily to other than the wanted antigen, the assay is not good even though the other parts have been optimized. On the other hand, the

properties of the parts can also cause some major errors in the test even when the antibodies are highly specific to antigen.

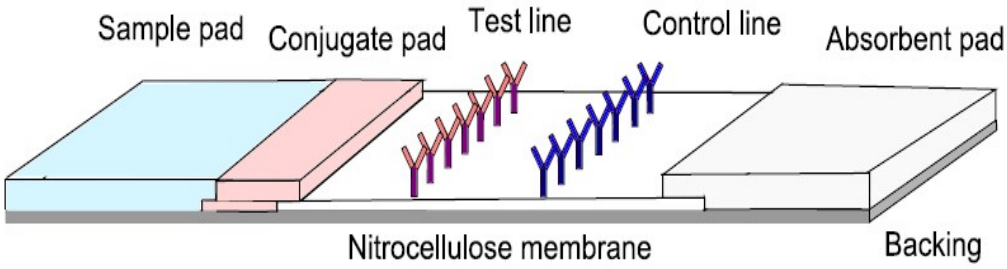


Fig 1: components of immunochromatographic strip

2.11.3. Membrane Properties

The membrane is the most critical part of the lateral flow assay. It has to be able to meet many requirements, such as immobilize molecules firmly, allow short and long term storage and let the solution phase interact with the immobilized phase without affecting the chemistry. The materials which are studied contain cellulose, cellulose acetate, polyether sulfide and nylon, but nitrocellulose is often used. All these materials have thickness of approximately 100 µm and average pore size ranging from 0.05 to 15 µm in diameter and they can attach molecules at least on some level. However, nitrocellulose has established its place over the others due to its higher capacity to immobilize antibodies over other cellulose based membranes. It is also easier to block than nylon membranes (Tonkinson *et al*., 2002).

The history of nitrocellulose as a binding membrane started in the 1960s. Since then nitrocellulose has been widely used in many applications from blotting to protein arrays and immunoassays. In blotting procedures, it has been used after gel electrophoresis to transfer molecules out of the gel to membrane by capillary or electrotransfer (Tonkinson *et al*., 2002).

Nitrocellulose is derived from cellulose by substituting hydroxyl moieties on each sugar unit by nitrate groups. The thin film is formed when dry nitrocellulose is dissolved in organic solvents and solvents are evaporated away. The pores are formed if non solvent such as water is added to the solution containing organic solvents. The resulting nitrocellulose film has a three dimensional and hydrophobic structure. When considering the use of nitrocellulose in lateral flow assays, it has a few favorable properties. Firstly, its porosity and pore size can be easily

controlled during manufacturing, and secondly, proteins can be immobilized easily in its surface. In lateral flow assays the membrane has important functions. It serves as stable binding surface to capture antibodies on the test and control lines but also controls the diffusive and capillary flow of the mobile phase. Membranes are usually defined through parameters such as pore size, capillary flow rate, porosity and thickness. Pore size is the measure of how large hard particles can flow through the membrane. However, pore size distribution determines more accurately how large particle can flow in the membrane without clogging it. Due to the fact that pore size is quite difficult to measure reliably, capillary flow rate is the measure that is used more often. It is not constant because it decreases exponentially as the liquid moves further from the origin but the capillary flow time is inversely related to the capillary flow rate and easy to measure. It is defined as the time required for liquid to move along and completely fill a membrane of defined length, and the unit of measure is seconds per centimeter. Even though the flow rate is an important characteristic when optimizing the time required for a test, too high a flow rate also decreases sensitivity. The capture antibodies are more likely to bind with antigens in the mobile phase if they pass them slowly. Due to the significant decrease in the flow rate over distance, the location of the test line is an important factor when optimizing the test. Porosity expresses how much air is inside the membrane. Along with the thickness of the membrane and the structural characteristics of the used polymer, porosity influences the surface area available for protein binding. The more binding sites the polymer has inside, the more easily immobilized proteins find suitable places to attach. Pore size also has an effect on the binding of the proteins. As pore size decreases, the membrane surface area increases and more proteins can attach to the membrane. However, if the pores are too small, the molecules do not fit in it anymore. The proteins can be attached to the membrane by drying in room temperature. However, the exact mechanism behind the attachment is not fully understood. The possible forces vary from hydrophobic interaction, hydrogen bonding to covalent and electrostatic interactions (Jones *et al*., 1999) and studies suggest that the binding is through non-covalent, hydrophobic interactions (Oehler *et al*., 1999). The hydrophobic nature of nitrocellulose may cause a problem when the antibody coated label molecules are flowing through it. To prevent this unspecific binding, the membrane has to be blocked. The commonly

used reagents are bovine serum albumin (BSA) with some surfactants such as polyvinylpyrrolidone (PVP), Tween or Triton. The membrane structure is important to the functioning of the membrane. It defines in which direction the flow can proceed. In the assay the most important direction is lateral orientation but usually the sample is applied in the membrane from vertical direction (Jones *et al*., 1999). Nitrocellulose structure is quite brittle and the surface is scratched easily. The membrane is also irreversibly compressive. These aspects affect the continuity of membrane and complicate the handling of the membrane. Usually, backing with nonporous films enhances the handling properties.

The handling of the membrane also depends on the thickness of the membrane and the 100 µm is practically the lower limit of the unbaked membrane which can be processed. Thickness is also one parameter for defining how much liquid the membrane can absorb. Particularly, the volume after the test line determines how many labeled molecules reach the detecting antibodies. Additionally, the width of the test and control lines is partly controlled by the membrane thickness. If the membrane is very thin, the same amount of solution spreads over a wider area because the distance of vertical flow is shorter and then the liquid starts to flow laterally. Moreover, thickness also influences the visibility of the test and control lines. The more antibodies attach to molecules in the surface of the membrane, the better the line seems to be. Only the first 10 µm from the surface are visible to the eye (Wang *et al*., 2009). If most of the antibodies are attached inside the membrane rather than on the surface, most of the labeled, bind molecules are hidden from visual inspection.

2.11.4. Test Line

In addition to the specificity of antibodies, other properties of the test line influence the quality of the test. In many cases the result from the test can be read with the naked eye and then the line has to be sharp and clear. If the line is unclear the result is unclear. In order to achieve a readable line, some factors need to be recognized. If the test uses a control line, the same aspects are also valid for it.

Firstly, in test line amount of protein bound to the membrane has to be high enough. Otherwise the line is weak and the sensitivity is reduced. However, if too much protein is

applied, the membrane binding sites can be saturated and the line spreads. The saturation limit for immunoglobulin G (IgG), the most frequently used antibody, is approximately 5 mg/ml. Secondly, the procedure to apply the antibodies to the membrane has to be optimized. Otherwise the amount of protein cannot be controlled.

Thirdly, the binding of capture antibodies to the membrane has to be strong enough to keep the antibodies attached even though the flow is applied. If the attachment is insufficient the antibodies will unbound which results in a weak and diffusive capture line. The weak line may also be a result of diffusion of capture antibody solution when applied in the membrane. The fast lateral flow rate can also enhance the diffusion (Jones *et al.*, 1999). However, the liquid spreads faster than proteins due to the fact that proteins usually attach eagerly with membrane and migration happens only if the membrane is already saturated or proteins are chemically masked. Even though the capillary flow rate will affect the spreading, a more critical factor is the contact angle between the liquid stream and the membrane surface. Also, if the solution is too viscous, it will penetrate to the membrane too slowly and then evaporation can influence the protein attachment and result in varying line quality.

The binding properties can be also enhanced by the right humidity and optimal application buffer. The proteins should be soluble in the buffer and ionic strength, acidity and coprecipitating agents should help the protein be in an energy state which enhances the binding with the membrane. Even though the binding rate should be last enough to prevent the diffusion, too rapid binding can cause a too thin test line. Also, if the area being covered with capture antibodies is too small, the result is the same. In low humidity the membrane collects a static charge which attracts dirt and dust easily. However, water is electronegative and this can cause repulsive forces which diminish the protein binding.

2.11.5. Control line

It is the line after test line in the strip at which specific captured molecule is spotted which may be dependant or may be independent to the control line. This line must come whether the analyte present or absent in sample to be tested failing which may be called as invalid to the test.

2.11.6. Sample Pad

The function of a sample pad is to ensure even distribution of the sample solution to the conjugate pad. It can also be designed to prevent flooding by controlling the rate at which liquid enters the conjugate pad. A sample pad can be made of woven meshes or cellulose filter and in it can be processed to contain components which can increase the viscosity of the sample, help the sample to combine with antibodies, to solubilise the detector reagent or standardize the acidity of the sample. Blocking agents can also be put in the sample pad.

2.11.7. Conjugate pad

It is usually made of non woven filters such as glass fibers. They have low non specific binding, consistent flow characteristics and bed volume. Because the detector reagents, such as label antibody complexes, are placed and dried on the conjugate pad, the reagents have to be able to lift off from it. Moreover, the conjugate pad has an important role in leading the solution to the membrane and it cannot contain any extra free particles which may block the membrane capillaries.

2.11.8. Absorbent Pad

The function of an absorbent pad is to increase the total volume of the sample which passes through the test and control line. If an absorbent pad is not used, the dimension of the membrane affects the sensitivity of the test. Then the area after the test line determines how large a part of the sample reaches the capture antibodies. Basically, the absorbent pad increases the volume by as much as the pad can absorb liquid. This also makes it possible to wash unbound detector particles away from the test and control lines which decrease the background and the detection limit. The material of the absorbent pad is not as critical as in other parts of the test but it has to absorb liquid efficiently. For example, cellulose filters can be used. In some applications end of assay indicators are used but their use is not possible if an absorbent pad is used.

2.11.9. Other Important Properties

In order to produce valid lateral flow tests, additional properties have to be noticed. The material and composition of adhesives, possible effects of lamination as well as the procedure

of cutting the membrane in suitable pieces influences to the functioning of the test. Adhesives are used to keep plastic backing in place. They are also needed in lamination. The composition of glue may influence the chemistry of the test and it has to be selected carefully. In addition to adhesives used in lamination, the plastic coating has an impact on how the test strip will dry and how the liquid moves in the membrane during the drying. The coating secures continuous flow between sample pad, conjugate pad and membrane. Cutting the test trip membrane may also be a problem due to the fact that the membrane is very weak compared to the plastic backing. The membrane may also compress during the cutting and as a result, labels and conjugates may be blocked in places not intended.

2.12. Labels in lateral flow assay

Labels are the essential part of the test, because the attachment of the antibody and antigen is difficult to visualize without them. Colloidal gold is the most used label in lateral flow assays (Posthuma-Trumpie *et al*., 2009) but colored latex particles (Danks and Barker, 2000) and other coloured particles such as carbon (Posthuma-Trumpie *et al*., 2012) and selenium (Lou *et al*., 1993) are also used. Fluorescence (Song and Knotts, 2008) and chemiluminescence emitting dyes (Mirasoli *et al*., 2012) along with the enzyme labels (Fung *et al*., 2009) are also tested. The use of paramagnetic particles is a relatively new invention. Even though the label molecules are different from their molecular properties they all have to be able to attach to the wanted antigen or antibody in order to produce some detectable signal correlating with antigen concentration. The conjugated labels also have to be stable in the test conditions.

Lateral flow as an assay type requires some extra properties from the label. The label has to remain stable in varying moisture conditions as well as in close proximity to membrane molecules. It also has to be small enough to fit to flow in the membrane structure. Because, lateral flow tests need to be rapid and inexpensive diagnostic tools, the label has to be simple to detect and simple to use.

2.12.1. Gold Nanoparticle

Colloidal gold is gold in nanoparticle size which is dispersed evenly throughout the solution and it differs in many ways from bulk metal. The most distinguishable feature is its

color change. Bulk gold appears yellow but spherical colloidal particles with a diameter around 10 nm are red in aqueous solutions. If the size of the particles increases to nearly 100 nm or the shape is changed, the color also changes, for example, rod like the colloidal dispersion appears bluish (Sharma *et al*., 2009). This kind of behavior is explained by absorption of light. In the surface of the metal there is a conduction band where electrons are moving freely. When the diameter of metal particles is reduced to nanoscale, the conduction band electrons of different particles exhibit collective oscillating on a frequency that is suitable for absorbing visible light. This effect is called the surface Plasmon absorption (Burda *et al*., 2005). Another feature related to this work is the behavior of colloidal dispersions. Similar to fog and mist, the solution of gold nanoparticles is colloidal dispersion, where the dispersed, solid phase is kept suspended, due to the thermal or Brownian motion (Sharma *et al*., 2009).

Both the presented properties are important when applied to the lateral flow test. The red color forms the recognizable line when interpreting the results and it also shows that after the conjugation with antibodies the colloidal gold particle solution is still stable. If the gold particles form larger aggregates or settle, the flow in the membrane can be disturbed.

Reviews of the methods of preparing colloidal gold are numerous in literature (Daniel and Astruc, 2004; Eustis and Sayed, 2006; Nguyen *et al.*, 2011) the production of nanoparticles can be approached by different means. Bulk gold can be decomposed, by ion irradiation (Birtcher *et al.,* 2004) or colloidal gold can be produced by using chemical processes. The disadvantage of bulk dismantling is that the size distribution and the shape of the particles are difficult to control. Gold nanoparticle can be produced by citrate reduction method which was originally introduced in 1951 by Turkevitch (Turkevich *et al*., 1951), Frens in 1973 (Frens, 1973) reported that the size of colloids can be controlled by differing the amount of gold compared to the other substances. Due to the simplicity of the method, it is still used today. Gold particles can also be reduced by sodium borohydride ($NaBH_4$) in the presence of stabilizing thiols, other sulfur ligands or other molecules such as anine, for instance.

2.12.2. Colloidal Gold in Lateral Flow Assay

Colloidal gold is used as a label in lateral flow assay without any complex signal amplification procedure. The reasons for its popularity are that it is easy to use and the procedure

has only one phase before the signal can be detected. Additionally, the results can be read without a reading device. The assay time is also short and colloidal gold conjugates are stable in many climates. It is also reasonably inexpensive and it does not require highly educated personnel to conduct the test. Colloidal gold as a label has also been compared to conventional ELISA and a good agreement has been found (Chan *et al*., 2003).

2.13. Different Formats and Detection limit in Lateral Flow Assay

Lateral flow assay can be performed in different formats. It may be of competitive assay and sandwich assay. The detection limits achieved with visual detection vary from 0.1 ng/ml to 1 μg/ml. Cortisol was detected by lateral flow assay with a detection limit of 30 ng/ml (Nara *et al*., 2010) and 17-α-hydroxy progesterone, a marker for congenital adrenal hyperplasia, in a serum was detected with a detection limit of 2.5 ng/ml (Tripathi *et al*., 2012) by using competitive assay. Similar results have also been measured with sandwich assay. Human heart type fatty acid binding protein was detected with a detection limit of 2.8 ng/ml (Chan, 2003) and prostate acid phosphatase was detected with a detection limit of 0.25 ng/ml (Fang *et al.,* 2011). Insecticide carbonyl has been tested with a detection limit of 0.1 μg/ml (Wang *et al*., 2009). The differences in detection limits may result from differences in the procedure of producing the test and due to the different materials used. Also every researcher has developed their own standards for the limit of a detectable line. However, visual detection and the properties of colloidal gold affect the detection limit on at least some level, no detection limit is below 100 pg/ml.

2.14. Conjugation of gold nanoparticle with biomolecule

Conjugation of gold nanoparticle with biomolecule is an important step for devolpment of lateral flow assay. Bioconjugation of gold nanoparticle can be done by 1. Physical adsorption method and 2. Covalent method. Physical adsorption method is a pH dependant method but covalent method can be done by carbodiimide chemistry. The most common route is to use amine coupling, whereby carboxyl groups in the matrix form covalent amide bonds with primary amine groups in proteins (ligands). However, this process does not occur spontaneously, the carboxyl groups need to be activated. Activation is performed with a 1:1 mixture of N-Ethyl

N'-(3-Dimethylaminopropyl) Carbodiimide (EDC) and N-Hydroxyl-succinimide (NHS). EDC reacts with the carboxyl group and forms a reactive intermediate which in turn reacts with NHS to form an active NHS ester. As biomolecule is passed over the gold nanoparticle, the NHS-moiety (which is a good leaving group) reacts spontaneously with a primary amine group in the ligand and covalent bond between ligand and matrix is formed. The process is illustrated in Fig. 2. Most proteins contain several primary amines, and thus, immobilization can be achieved without seriously affecting the ligand's biological activity.

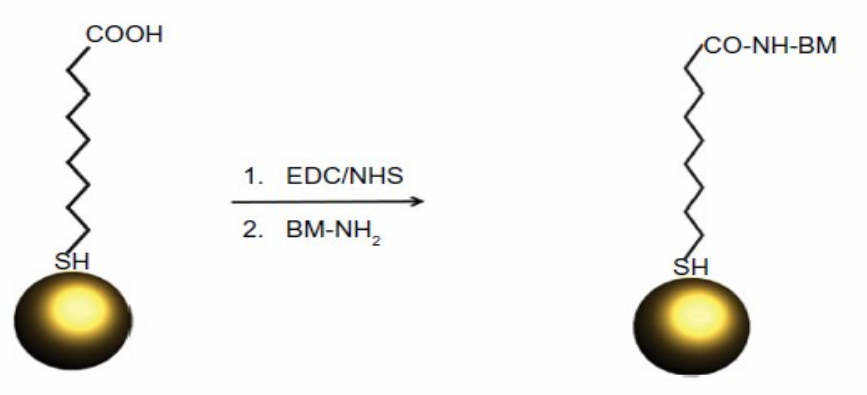


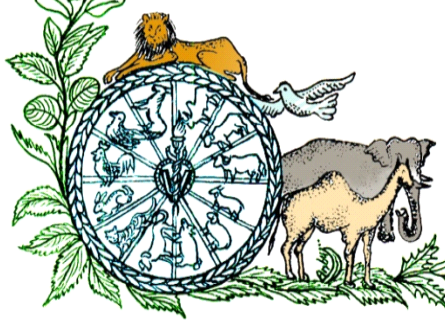
Fig 2: Conjugation of Gold Nanoparticles with ligand.

2.15. Advantages of the rapid assay over the conventional diagnostic tests for the diagnosis of bluetongue

Rapid assay is one of the most advanced, near future diagnostic procedure for eradication of bluetongue virus. The rapid assay has the following characteristics

1. Cheaper diagnostic system
2. Extremely small sample volume required.
3. High specificity and sensitivity.
4. A more rapid evaluation of multiple samples in a single platform.
5. Applicable to field level.

Materials and Methods



3 MATERIALS AND METHODS

The study reported was undertaken at IVRI, Izatnagar with an aim to develop a rapid assay for Bluetongue disease of small ruminants. The details of reagent, buffer and solutions used in the present study have been presented in Appendix or described at appropriate places.

A lateral flow assay using bluetongue VP7 protein has been developed. The test uses gold conjugated secondary antibody to capture field serum sample and VP7 protein. Captured antibody from clinical serum samples are detected by binding effects on test line of lateral flow strip. Details on materials and procedures used are given in subsequent sections.

3.1 Chemicals and Reagents used in development of Lateral Flow Assay

The chemicals used in this study were mostly dried and were prepared according to requirement. The auric chloride ($HAuCl_4$) was from CDH (INDIA). Sodium citrate tribasic was from Sigma (USA). Bovine serum albumin (BSA) was from Amresco (USA).Polyethylene glycol (PEG) was from Merck (INDIA). Sodium azide was from Spectrochem (INDIA). Potassium dihydrogene phosphate ($KH_2 PO_4$) was from Loba chemie (INDIA). Bluetongue VP7 protein and its hyper immune sera was procured from IVRI, Mukteswar. Protein G, protein A, anti sheep and anti rabbit antibody was from Sigma (INDIA).Nitrocellulose membrane, absorption pad, sample pad and conjugate pad were from MDI (INDIA).tween20, gelatin, standard +ve serum, standard –ve serum, skim milk powder, Bradford regent, Bicinchoninic acid (G Biosciences), Cu blue solution (G Biosciences).Indirect ELISA kit (IVRI, Mukteswar).

3.2 Equipments

1) Centrifuge with swing bucket rotor-Thermo Scientific
2) IgG purification kit Protein A Based, Gravity Flow -HIMEDIA
3) Transmission electron microscope - Philips CM 10
4) Zetasizer-Melvorn
5) Strip spotting machine-Easy printer LPM -02
6) Eppendropff centrifuge-Spinwin
7) ELISA reader-Spectromax
8) Sonicator-IMECO
9) pHmeter –Systronics
10) Amicon Ultra-0.5 Centrifugal Filter Devices-M
11) UV/VIS spectrophotometer-Systronics

3.3 Procedure for purification of IgG from hyperimmunosera

Antibody purification is performed to concentrate and enrich antigen specific antibodies and lower the back ground signal by removing any non specific proteins. This step is performed when highly purified antibodies are required. Affinity purification of IgG commonly uses protein A column. The Himedia antibody purification kit protein A agarose spin columns designed for simple and rapid antibody purification from bluetongue hyper immune sera.

Antibody purification steps

1) Elimination of preservative
2) Equilibration of spin column
3) Application of sample
4) Washing
5) Elution
6) Neutralization
7) Regeneration and storage of column.
8) Desalting and concentration

1. Elimination of the preservative

Equilibrate the column and buffers to room temperature. Remove the lower cap of the column and place it in the 2ml collection tube. Centrifuge at 500X g for 1 min to allow elimination of the preservative.

2. Equilibrium of the spin column

Equilibrate the spin column with 0.4ml of binding buffer and mix manually. Centrifuge at 500X g for 1 minute and discard the flow through. Repeat this step once. Do not let the resin bed dry.

3. Application of the sample

Close spin column outlet with cap. Add up to 0.5 ml of the hyper immune sera (containing the IgG against VP7 protein) through the top of the spin column. Close the lid and keep sera and resin in contact for at least 30 minutes before removing the bottom cap. Mix manually inverting the spin column. Centrifuge at 500X g for 1 minute and collect the flow through.

4. Washing

Transfer the spin column to a new collection tube. Add 0.4 ml of Binding Buffer through the top to eliminate all the proteins that have not been retained in the column. Mix manually inverting the spin column. Centrifuge at 500 X g for 1 minute and discard the flow through. Repeat the washing step twice for a total of three washes.

Wash the spin column with binding buffer until the OD 280 nm of the washes reach the base line.

Keep all washes if required.

5. Elution of pure IgG

Transfer the spin column to a new collection tube and close the column out let with cap. Add 0.4ml of elution buffer and close the lid, mix thourghly for 10 minutes before removing the bottom cap. Centrifuge at 500 X g for 1 minute, collect the elute and label it. Repeat the elution step twice for a total of three individual elutes.

6. Neutralization of eluents

Each 0.4 ml of eluted fraction can be neutralized by the addition of 40 ul of neutralization buffer. Assay protein concentration by measuring the absorbance at 280 nm and combines the fractions with highest absorbance.

7. Regeneration and storage of the column

Regenerate the immobilized protein a column by washing at least 3 times with 0.4 ml of elution buffer. Columns may be generated for at least 3-4 times without significant loss in binding capacity. For storage, wash the column with 10 ml of distilled water and store it upright in 0.4 ml of 1X PBS at 2-8^{0}C

8. Desalting and concentration

Desalting and concentration purified IgG from hyper immune sera against VP7 protein is done by Amnicon ultra -0.5 Centrifugal filter devices. Add up the 400 ul of sample into ultra filter device and cap it. Placed capped filter device into the centrifuge rotor, spin the device at 14,000x g for approximately 10-30 minutes. Remove the assembled device from the centrifuge and separate the amnicon ultra filter device from the micro centrifuge tube. Recover the concentrated sample by upside down in a clean micro centrifuge tube and spin for 2 minutes at 1000xg to transfer the concentrated IgG to the tube. The ultra filtrate was collected and add 0.25%w/v sodium azide if antibodies are to be stored in freeze at -20^{0}C for further use.

3.4 Standard Protocol for Bicinchoninic acid (BCA) Protein Assay

1. Pipette 50µl of each standard and protein samples into an appropriately labeled tube.
2. Add 1ml Working Solution to each tube, seal and vortex to mix.
3. Incubate the assays at 37°C for 30 minutes or room temperature for 2 hours. For the enhanced protocol, incubate at 60°C for 60 minutes. We recommend a water bath for even heat transfer.
4. Cool the tubes to room temperature and transfer 1ml sample to a cuvette.
5. Set a spectrophotometer to 562nm and blank with water. Read all the samples.
6. Subtract the average absorbance of the blank standard from the samples and then prepare a standard curve to determine protein concentrations.

3.5 Quantification of purified IgG by Bradford Method

Bradford assay is a very fast and simple method used to estimate total protein concentration in different sample. Procedure is given below.

1. Into four separate microcentrifuges, added 5,10,15,20 µl etc of .5mg/ml BSA solution. Brought the volume of each to 100 ul with 0.15 N NaCl.
2. 100 µl of 0.15 N NaCl was added into one tube, which serve as a blank
3. 1 ml Bradford reagent was added to each tube and was vertexed. Allowed to stand at room temperature for 2 minutes.
4. Absorbance at 595nm was determined using 1ml micro cuvette. A standard curve was generated by plotting absorbance at 595nm versus protein concentration.
5. Repeated steps 1to 4 for the unknown sample, using the unknown in place of BSA. The standard curve was plotted to determine the concentration of unknown sample.

3.6 Synthesis of gold nanoparticles

Synthesis of gold nanoparticle was done by sodium citrate method (Turkevrich *et al*., 1951). A series of nanoparticles were synthesized using different dilutions.3 different concentration of 10 mM, 15mM, 20 mM solutions of auric chloride were prepared in our house by taking 200 µl, 300ul and 400 µl of 1% tetrachloroauric acid with 50 ml distilled water. 2 ml Sodium citrate (1.5%) was prepared by taking 30 mg of sodium citrate with 2 ml distilled water. First the flask containg gold solution was boiled on magnetic stirrer heating plate and previously prepared sodium citrate was added by drop wise manner by syringe. Fig.3a showing the procedure for synthesis of gold nanoparticle. First red color solution appeared after 3-5 mins of boiling, the solution was converted to dark red color after 10-15 mins. The solution was cooled down and adjusted to 50 ml by adding required HPLC water. The three different concentrations of gold nanoparticle solutions were stored at 4^{o}C for further use. The color variation of three different concentration of gold nanoparticle solution was shown in Fig. 3b.

3.7. Characterization of gold nanoparticle

Characterization of gold nanoparticle was done to determine its size, charge, voltage potential and λ_{max}. It was done by transmission electron microscopy, dynamic light scattering

and UV-VIS spectrophotometry. The transmission electron microscopic measurement was done on Phillips CM10 at All Indian institute of medical sciences, New Delhi. The sample of 10 mM, 15mM and 20mM of gold nanoparticle samples were first sonicated for 10 minutes to prevent the particle aggregation. A drop of each sample was put on the carbon coated copper grid. The film was allowed to dry for 15 minutes and excess solution was removed using blotting paper. The size of each nanoparticles were measured from enlarged images of TEM by taking different counts for each angles.

The dynamic light scattering of gold nanoparticles was done at zeta sizing instrument Malvern, UK at Institute of Genomics and Integrative Biology, New Delhi. The charge and zeta potential of three different concentration of gold nanoparticles were measured in automatic three runs of average 100 times in cuvette type zeta sizing instrument.

The optical properties of gold nanoparticles were performed by u/v spectrometer (Spectromax) at central instrumentation facility, IVRI, Izatnagar. The spectrum of 10mM, 15mM and 20mM gold nanoparticle was performed from 350 nm to 750 nm wavelength.

3.8. Procedure for gold-secondary antibody conjugation

Gold conjugated antibody was made by simply physical adsorption method. This method of conjugation is pH dependant method. First secondary antibody (anti sheep and anti rabbit IgG at 50ug /ml was prepared by diluting it with 5mM KH_2PO_4 (pH 7.5) to a final volume of 100ul.i.e 20 µl of antibody was diluted with 80 µl of KH_2PO_4 to make it 100ul in 1 ml eppendoff tube. 900 µl of previously prepared gold nanoparticle was added to it and kept it for incubation at room temperature for 15 mins to 30 mins. This time allow the gold nanoparticles for conjugation. After incubation 50 µl of 1% (w/v) PEG which was dissolved in 50mM KH_2PO_4 solution (pH 7.5), and 100ul of 10% (w/v) BSA, which was also dissolved in 50mM KH_2PO_4 (pH 9), were added to block the non coated gold nanoparticle surfaces and this solution was kept for 10 mins. After immobilization and blocking procedures, gold nanoparticle conjugated secondary antibody was pelleted by centrifuging 13000 RPM for 20 mins in refrigerated centrifuge machine. After centrifugation, the pellet was collected after discarding the supernatant. Then the pellet was dissolved by sonicating it for 15 secs and 1 ml

Fig. 3a : Synthesis of gold nanoparticles by citrate reduction method on magnetic stirror with hotplate

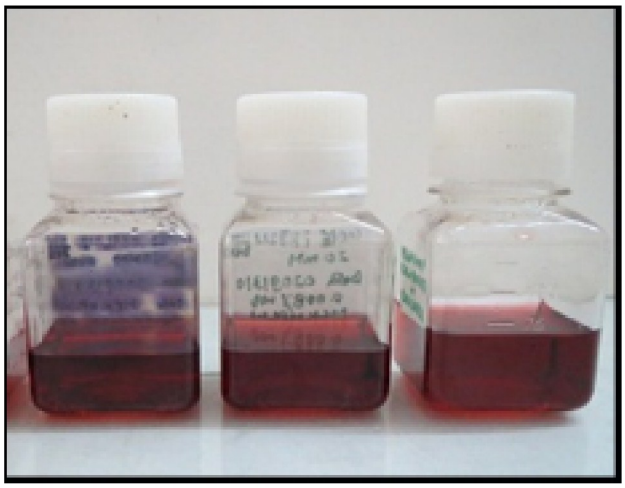


Fig. 3b : Color variation of different concentration of GNP i.e from left 20 mM, 15 mM and 10mM, respectively

preserving solution was added to the pellet to dissolve it properly. It was again gone for centrifugation at 13000RPM for 15mins. After centrifugation, the pellet was collected after discarding the supernatant. The pellet containing gold conjugated secondary antibody was sonicated for 15secs, vertexed in 50 µl of preserving solution and stored at 4^{o}C for further use.

3.9. Preparation of lateral flow device for detection for bluetongue antibody

Lateral flow device was developed against bluetongue disease of small ruminants mainly requires the following

1. Nitrocellulose membrane
2. Sample pad
3. Conjugate pad
4. Absorption pad
5. Plastic cassette with sample and test window.
6. Strip printer.
7. Vacuumed dryer

In this study, we have used three types of nitrocellulose membrane of different sizes of 8µ, 10µ and 15µ. All the nitrocellulose membranes are shown in Fig. 4a. First the nitrocellulose membrane with plastic lamellate was spotted by easy strip printing machine for the test and control line. The expressed VP7 protein (480ug/ml) and protein G (1mg/ml) were stripped at test and control lines respectively to the nitrocellulose membrane by fine needle. The spotting procedure by easy printer is shown in Fig. 4b. Thereafter, the strip was dried by air dryer and marked with T and C for further known. The sample pad, the conjugate pad and the absorption pad were taken properly and assembled to the membrane at their appropriate places. Structure of fully prepared strip after assemblance is shown in Fig. 5a. Then this plastic lamellate with membrane was kept for 1 hour at room temperature for adequate drying and was cut into number of 5 mm sizes individual immunochromatographic strip. The conjugate pad was either normally vacuums dried with previously prepared conjugate or left as such. Then the individual strip was inserted into the plastic cassette with windows carefully. Now our lateral flow device is ready for use to detect of BTV IgG at the field level. The lateral flow device which is ready for testing was shown in Fig. 5b.

3.10. Procedure for the testing the purified hyper immune sera with the lateral flow device

The lateral flow device was first tested with the purified IgG from hyper immune sera. The purified IgG was diluted with PBS in serial 2 fold dilution upto 128 and then tested with the strip. The 0.3 µl of previous prepared secondary antibody with gold conjugate was pippetted to the conjugate pad and 0.6 µl of diluted purified IgG was dispensed to the sample pad, kept it for few seconds for reaction. The secondary antibody is mainly bind to the F_c region of the primary antibody. After some time, 20 ul of PBS-T was flown to the strip. After reaching the binding complexes to the test line, they must bind to the VP7 protein. So a strong, red, demarcating line was appeared at the test line and also at the control line, as the binding complexes also bind to protein G. The controlled line was also appeared strongly. The unbinding complex, debris, conjugates, PBS and hyper immune sera were absorbed at the absorption pad, and then the strip was kept for 3mins. Two clear cut demarcating lines were appeared after sometimes. A serial dilution of hyperimmunosera was done with PBS buffer and tested with the lateral flow device for the analysis of limit of detection. The principle and construction of this assay is presented in Fig. 6a. The mechanisms of this assay in case of positive and negative sample are shown in Fig. 6b and Fig. 6c respectively.

3.11. Testing of the field sera with the lateral flow device

The 156 unknown clinical sheep sera were collected from the affected sheep from the field. The serum was tested with indirect ELISA by using VP7 protein.

3.11.1. Procedure for Indirect ELISA

50ul of diluted VP7 antigen was dispensed to all wells except the antigen blank wells) in an ELISA plate and was incubated the plate at 37^{0}C on an ELISA plate shaker for 1 hr. The plate was washed three times with washing buffer(PBS-T).100ul of blocking buffer was added to all wells and was incubated at 37^{0}C on ELISA plate shaker for 1 hr. The plate was washed three times with washing buffer (PBS –T). 50ul of diluted standard known positive serum, standard known negative serum and test sera were dispensed to the respective wells according to the preplanned test format and the plate was incubated at 37^{0}C on an ELISA plate shaker

Fig. 4a : Different types of nitrocellulose membranes for lateral flow device

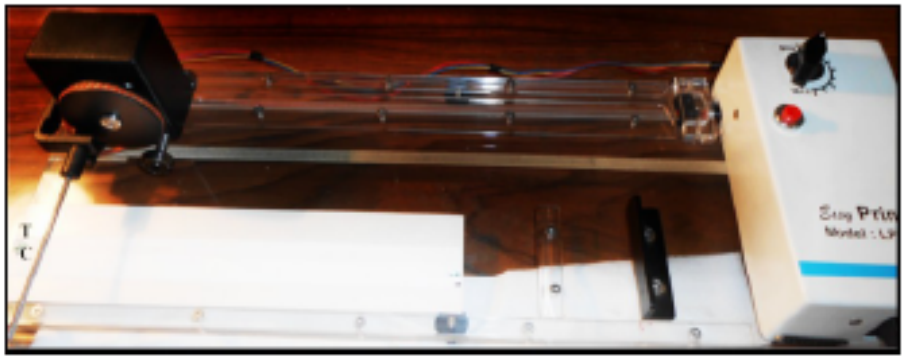


Fig. 4b : Spotting of VP7 and protein G on the strip by easy printer

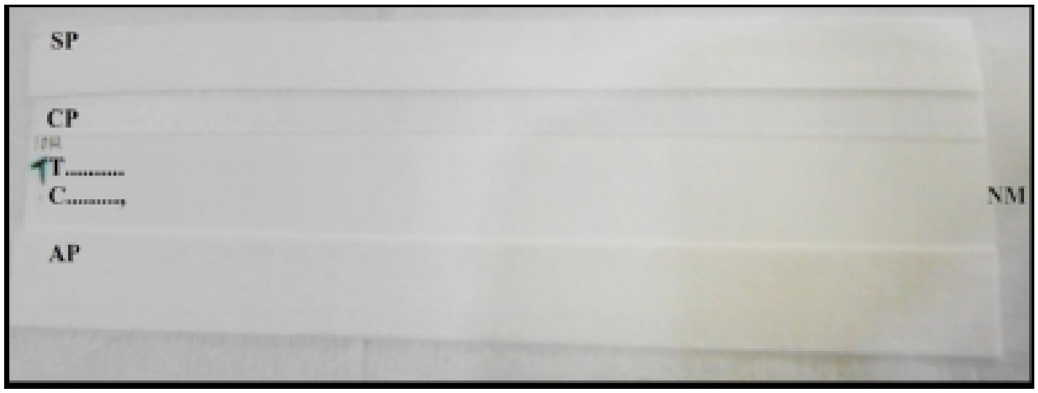


Fig. 5a : Structure of fully prepared strip after assembling with sample, conjugate and absorption pad at appropriate places
SP : Sample pad, **CP :** Conjugate pad, **T:** Test line, **C:** Control line, **NM:** Nitrocellulose membrane, **AP:** Absorption pad

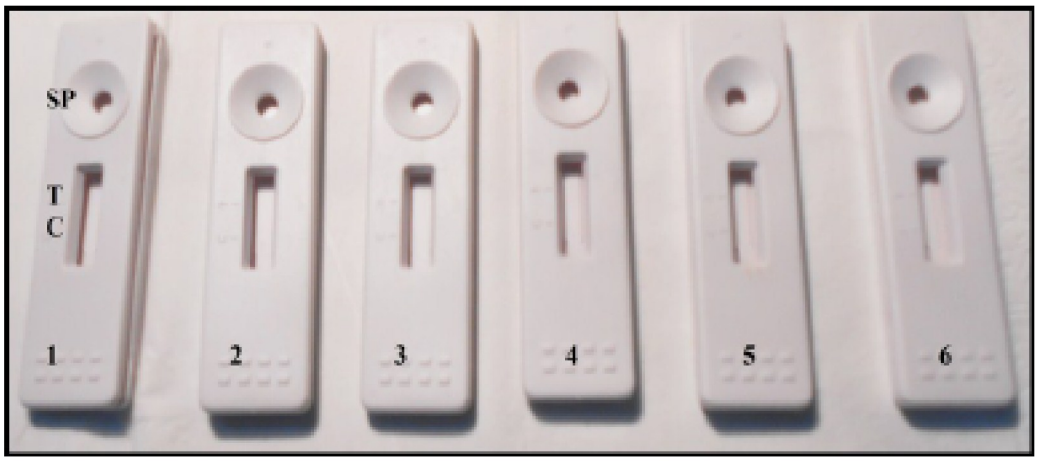


Fig. 5b : Structure of lateral flow device (1-6) after inserting the nitrocellulose membrane ready for testing

for 1 hr. The plate was washed three times with washing buffer. (PBS –T). 50 µl of diluted 1:5000 in blocking buffer) donkey anti sheep HRPO conjugate to all the wells and the plate was incubated at 37^0C on an ELISA plate shaker for 1 hr. The plate was washed three times with washing buffer.50 µl of freshly prepared chromogen substrate solution (OPD) to all the wells and the plate was incubated at 37^0C for 10 to 20 minutes in dark till color devolps. 50 ul of stopping solution was added to all wells and the plate was tapped gently. The absorbance of the colored solution of all wells was recorded at 492 nm in an ELISA reader.

The test serum showing OD_{492} value double or more than double the mean of negative serum control was considered as positive for bluetongue virus antibody.

3.11.2. Procedure for testing the field sera with the lateral flow device

The 156 clinical sera of affected sheeps were tested with lateral flow device. The result was interpreted accordingly by observing the control and test lines.

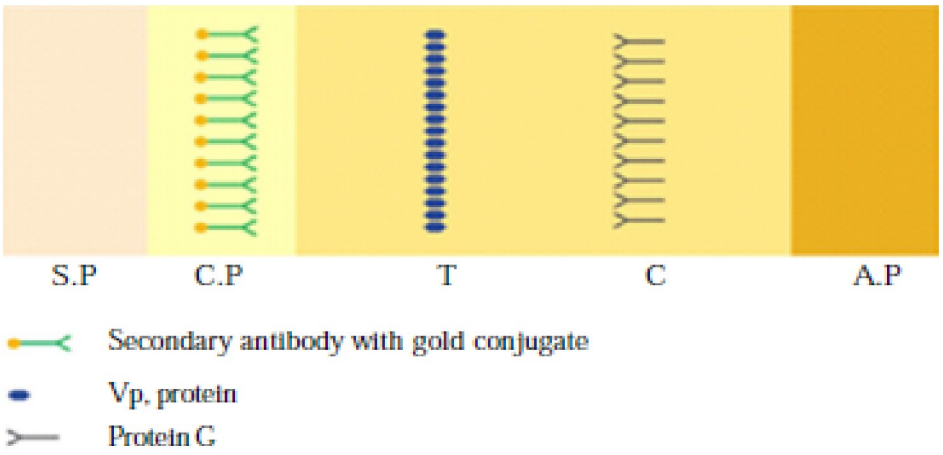


Fig. 6a : Showing overview structure of Lateral flow strip for BTV Ab detection

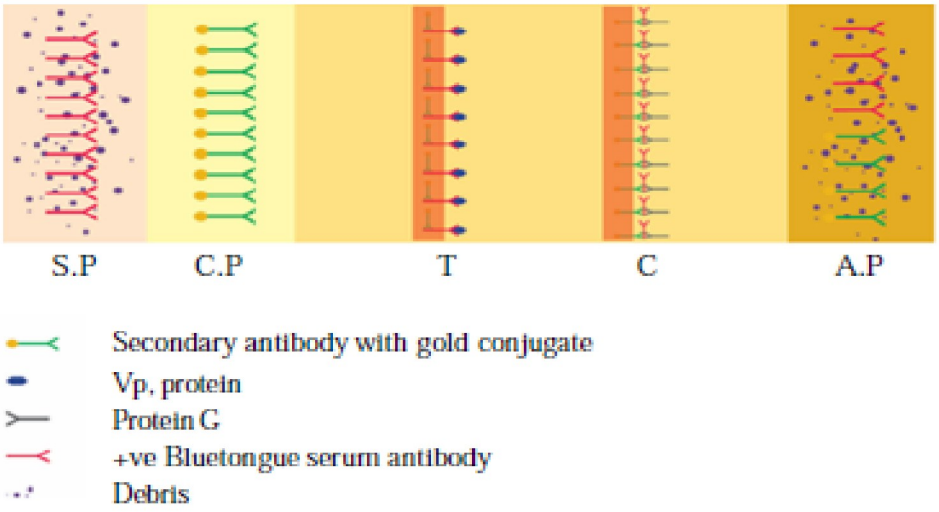


Fig. 6b : Showing the mechanism in case of positive sample

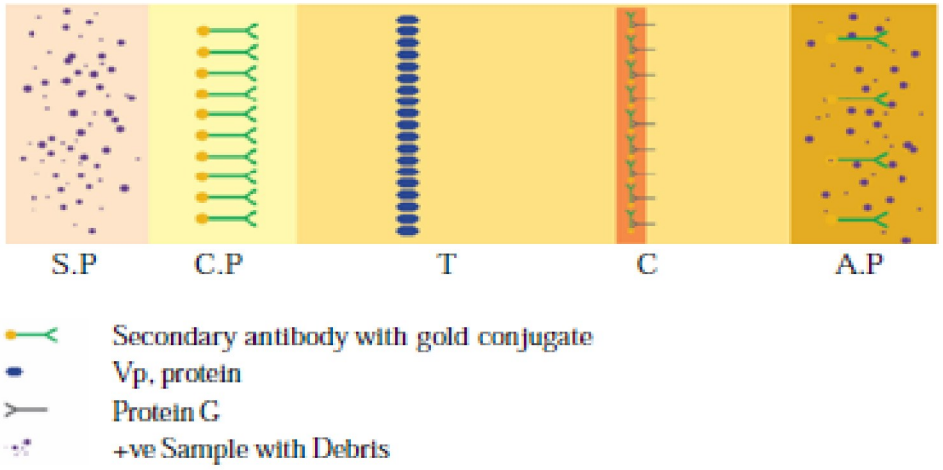
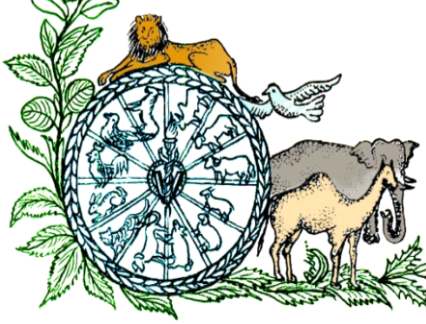


Fig. 6c : Showing the mechanism in case of negative sample

Results and Discussion



4 RESULTS AND DISCUSSION

Now, the bluetongue is serious disease of concern affecting all small ruminants in all states of India as well as all countries of gulf. Another concern of this disease is variations of its serotypes. Around 26 serotypes already exist in the world. So it is very difficult to diagnose the proper serotype affecting the small ruminants. The bluetongue virus remains in the red blood cells so the virus is not present in the body secretion like saliva, nasal mucous and in tears. So it is very difficult to diagnose the bluetongue virus, because it is not in significant quantity in the body fluids. So serum IgG detection for bluetongue is not only the solution for diagnosis of this disease but also helps the seroprevelance of disease.

The diagnostic technique should be simple, convenient, rapid and cost effective. The immunochromatographic strip test based on VP7 protein described here is efficient technique for diagnosis of bluetongue under field conditions. The avaible conventional techniques such as indirect ELISA, RT-PCR, haemagglutination inhibition test and counter immune electrophoresis etc have many problems in terms of requirement of high skilled personels and also time. On other hand, all such techniques cannot be practiced routinely under field conditions, due to inadequate laboratory facilities prevailing in the developing countries. Presently described lateral flow assay may be substitute for all conventional diagnostic techniques due to its rapid, cost effective, field oriented diagnosis of bluetongue. The assay may serve as a new diagnostic method for seroprevelance of bluetongue.

The VP7 protein of bluetongue virus is conserved along all 26 serotypes. VP7 is a group specific antigen and has been used often for establishment of immunoassays for BTV

antibody detection (Afshar *et al*., 1992; Reddington *et al*., 1991). For this, VP7 protein acts as epitope for binding of virus with antibody. So the lateral flow device was developed by using VP7 protein. Seropositivity can indicate previous exposure to BTV as antibody can be detected 7–9 days post infection in sheep and cattle by c-ELISA (Batten *et al*., 2008). So the clinical serum at the 8th day of infection was best sample for the detection IgG of this disease.

The efficiency of the bluetongue IgG detection on lateral flow device depends on numerous factors including membrane flow rate, membrane pore size, concentration of VP7 protein at test line, concentration of protein A at control line, amount of gold conjugate and the concentration of sample tested. Here, this rapid assay employs a phenomenon i.e. nothing but the binding of antigen and antibody in solid phase which requires optimum of their concentration. The design of rapid assay requires kinetics at a unequilibrium state. In this application, the primary parameter that controls the final result is the capillary flow rate. The slower the membrane flow rate, the higher sensitivity. The membrane pore size of nitrocellulose membrane also affects the result of this assay. Ultimately, it becomes necessary to adjust the proper concentration of the VP7 protein and protein A at the test and control line respectively, which contributes to the overall performance of this assay. It is straightforward to vary capture reagent concentration, detector particle concentration and the volume of sample being analyzed. Overall, this assay is mainly dependent upon all the above parameters to get a satisfactory result. Further assessment of these factors and modifying the parameters was then executed to accommodate the required objectives.

4.1. Gold Synthesis and characterization

Due to have high negative zeta potential, the gold nanoparticle solution with range of 15 to 40 nm particle size are stable for long duration in absence of any special stabilizing agent (Irina *et al*., 2010) because this high negative zeta potential prevents the aggregation of the particles. The gold nanoparticles were synthesized using different concentration of auric chloride solution which was reduced to auric ion of nanometer size by sodium citrate (Turkevich *et al* 1951). These particles were characterized for their size, potential and their λ_{max} to get the optimum properties required in the study. Fig.7a, 7b and 7c show the TEM images of gold nanoparticles of 10mM, 15mM and 20mM respectively. The size of gold nanoparticle has

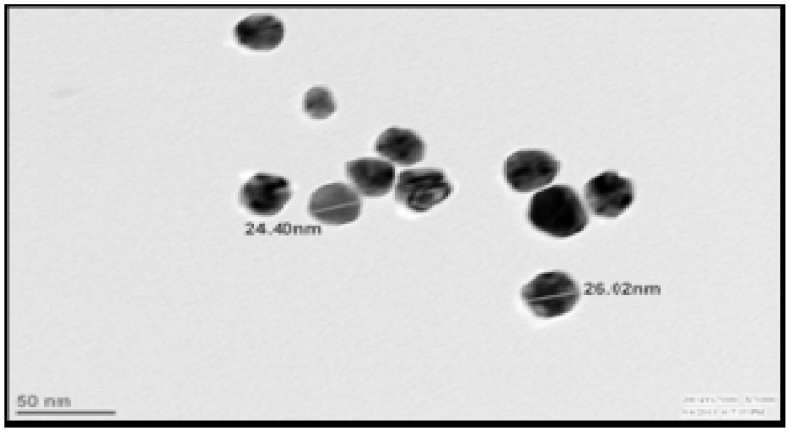


Fig. 7a : Characterization of 10 mM gold nanoparticle under TEM

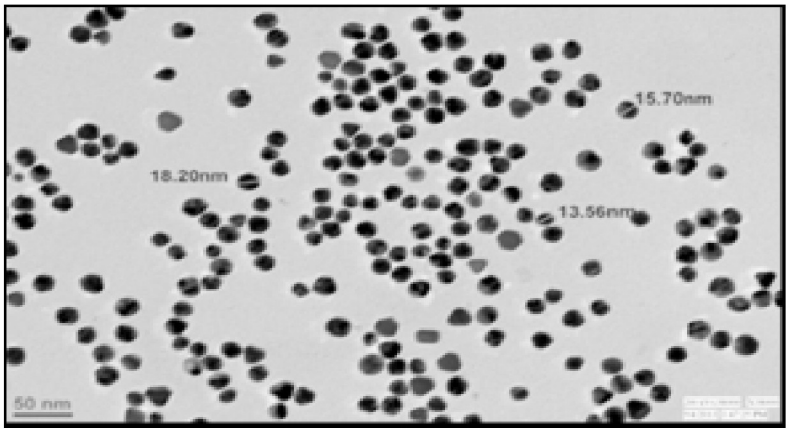


Fig. 7b : Characterization of 15 mM gold nanoparticle under TEM

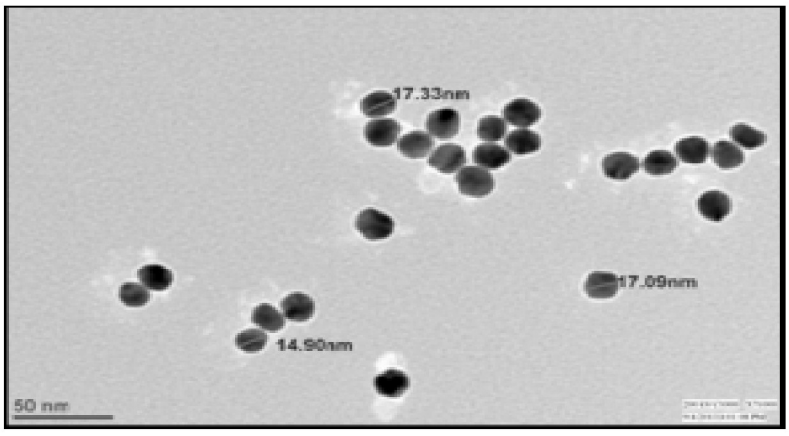


Fig. 7c : Characterization of 20 mM gold nanoparticle under TEM

been determined by measuring the diameter of whole particles on TEM. The average diameter of gold nanoparticle solution was found in the range of 10 to 30 nm for all three concentrations with some particle of higher and some particles of lower size distribution. Here, it is apparent that the size of gold nanoparticle decreases with increase in concentration chloroauric acid but it needs to be validated and confirmed. The TEM images show that gold colloid is in mono dispersion state, this is because of negatively charged layer of citrate ions, which repel each other. Moreover, the TEM images show that the most of the gold nanoparticle are round or spherical in shape which is required for conjugation of antibody. In the present study, the size of 10mM, 15mM and 20mM of gold nanoparticle was found around 25nm, 18nm, and 15nm respectively against the average size of 18nm (Verma *et al*., 2014). The effect of conjugation to the size of the gold nanoparticle was also seen in this study. The size of bare gold nanoparticle and the conjugated gold particle under TEM are shown in the Fig. 8a and Fig. 8b respectively. In this study, it was found that the size of the bare gold nanoparticle and the conjugated gold nanoparticle are around 27nm and 45 nm respectively.

Similarly the dynamic light scattering study was conducted to determine the mean zeta potential, their mobility and their conductivity of the bare gold nanoparticles. Table 1 shows the zeta potential, mobility, conductivity, effective voltage and wall zeta potential of gold nanoparticle solution. For zeta potential distribution study, peak number and peak area gives important explanation. For each sample, three cycles of different counts were run and average of the counts was taken. Beside size distribution, the zeta potential measurement is also important for characterization. The negative charge on nanoparticle due to citrate ions is another important indicator for particle size (Amir *et al.,* 2009). In present study, zeta potential of the synthesized nanoparticles for all samples were highly negative with mean potential -11.5mv, -30.5mv and -55mv of 10mM, 15mM and 20mM gold solution respectively. Fig 9a and Fig 9b show the dynamic light scatering for gold nanoparticle of 10mM and 20mM respectively. The graphs of dynamic light scattering of the gold nanoparticle are shown here, which is nothing but the graph between the total counts vs apparent zeta potential of individual gold nanoparticle. Here it is seen that the zeta potential of gold nanoparticle fall towards negative side of the X axis. Fig. 10a, Fig. 10b and Fig. 10c shows the zeta potential of 10 mM, 15 mM and 20 mM gold nanoparticle respectively.

Table 1: Size and zeta potential distribution of gold nanoparticle

Record	Type	Sample Name	Temperature (°C)	Zeta potential (mV)	Mobility (µmcm/Vs)	Conductivity (mS/cm)	Effective Voltage (V)	Wall Zeta Potential (mV)
1	Zeta	GNP 10MM 1	25	-11.3	-0.8843	0.996	151	-1.58
2	Zeta	GNP 10MM 2	25	-11.1	-0.8732	1.04	151	-2.97
3	Zeta	GNP 10MM 3	25	-19.3	-1.514	1.07	151	-4.44
4	Zeta	GNP 15MM 1	25	-30.40	0.02382	1.19	151	-10.38
5	Zeta	GNP 15MM 2	25	-31.87	-0.01465	1.32	151	-12.196
6	Zeta	GNP 15MM 3	25	-29.27	-0.02157	1.31	150	-13.0736
7	Zeta	GNP 20MM 1	25	-57	-4.471	1.15	151	-35.9
8	Zeta	GNP 20MM 2	25	-59.2	-4.643	1.22	151	-35.7
9	Zeta	GNP 20MM 3	25	-57.1	-4.478	1.21	151	-44.3
Mean(1-9)			25	-23.9	-1.875	1.17	151	-13.5
Std Dev			0	26.2	2.055	0.114	0.333	19.1

Colloidal gold conjugates have found widespread use due to their high stability and the unique optical properties of gold nanoparticles. For conjugation, antibody was directly adsorbed on the colloidal gold particle surface. Zeta potential can be very good tool for characterization of antibody gold conjugate. Gold nanoparticles synthesized using citrate reduction method is negatively charged due to citrate capping (Brewer *et a*l., 2005; Sugunan and Dutta *et al*., 2005). The property of citrate capped gold nanospheres can be exploited for the electrostatic interaction with some positively charged biomolecule. Antibody was directly adsorbed on the colloidal gold particle surfaces, mediated mainly by London-Vander Waals force and hydrophobic interaction (Hermanson *et al*., 1992) or through chemisorptions of native thiol groups present in their chemical structure and the amino groups on the antibody are positively charged and attracted towards the negative gold surfaces and hydrophobic pockets of antibodies will adsorb physically on the bare particle surfaces. In general, bioconjugation is pH dependant and the pH of the antibody and gold solution must be maintained at or above the isoelectric point of antibody (Hermanson *et al*., 1996) This adsorption will cause reduction in potential indicating formation of conjugate.

Spectrophotometry is another important aspect for characterization of gold nanoparticles by which the absorption spectrum of each concentration gold nanoparticle was derived. With increase in size of particle, the absorption peak shifts to longer wavelength and

Effect of conjugation on size of gold nanoparticle

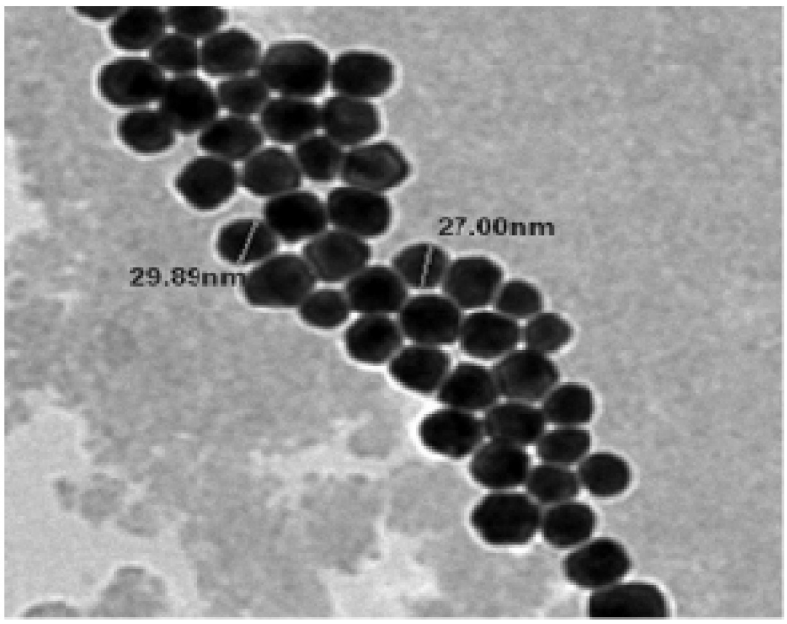


Fig. 8a : Size of bare gold nanoparticle under TEM

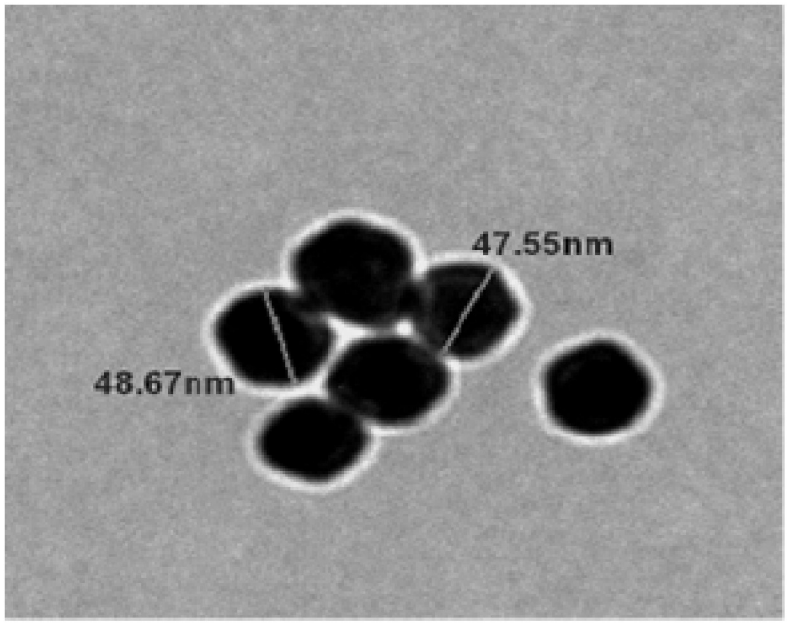


Fig. 8b : Size of antisheep IgG conjugated gold nanoparticle under TEM

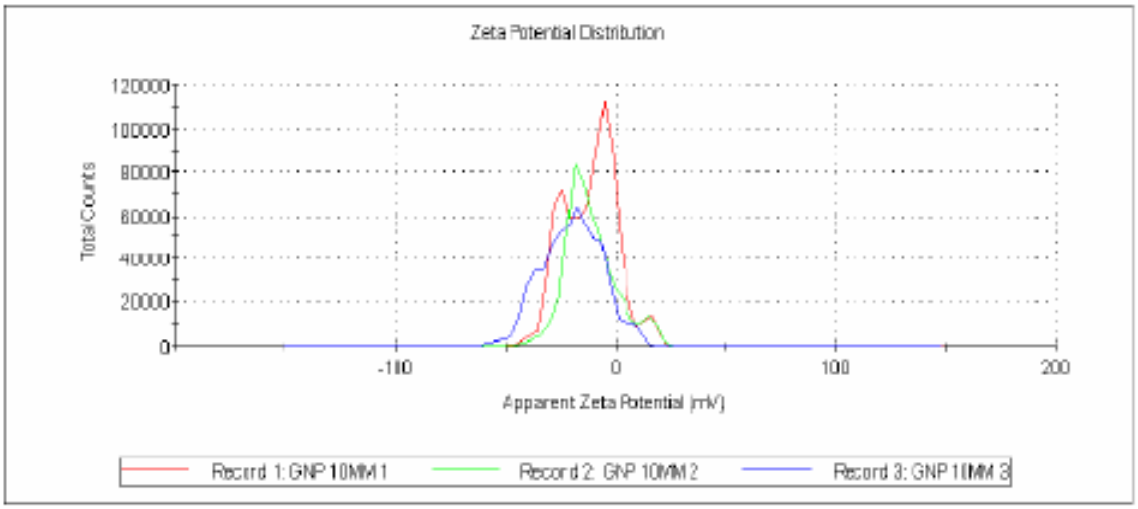


Fig. 9a : Characterization of 10 mM Gold Nanoparticle by dynamic light scattering

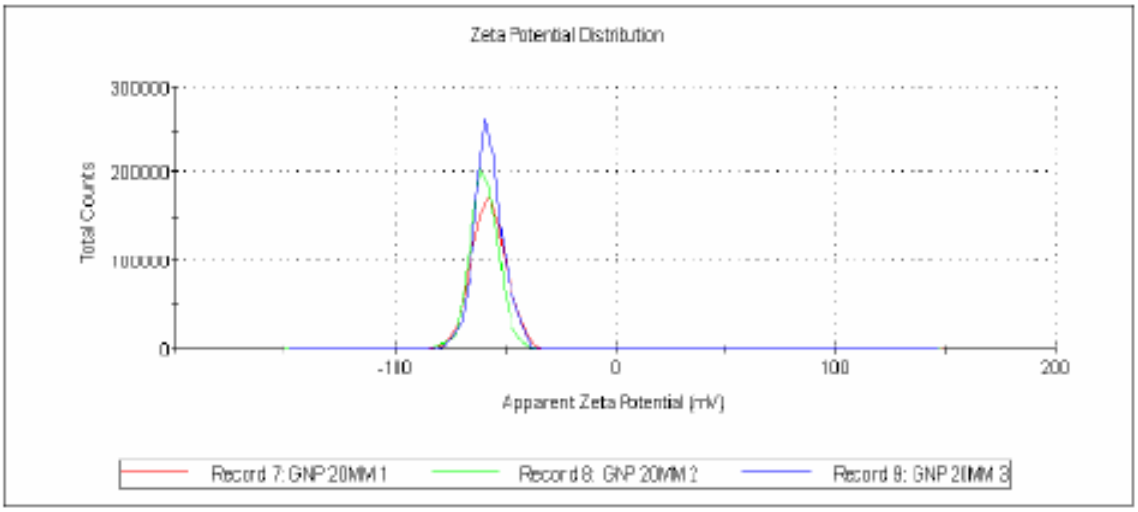


Fig. 9b : Characterization of 20 mM Gold Nanoparticle by dynamic light scattering

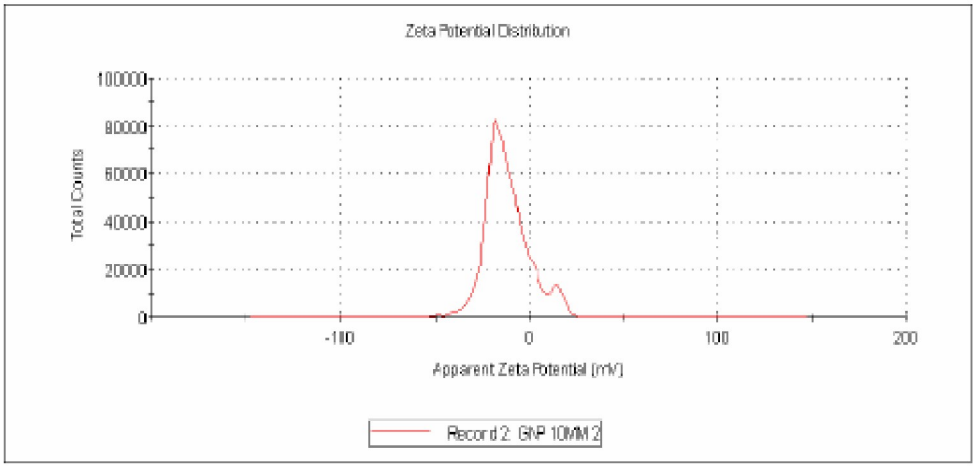


Fig. 10a : Zeta potential of 10 mM Gold Nanoparticle

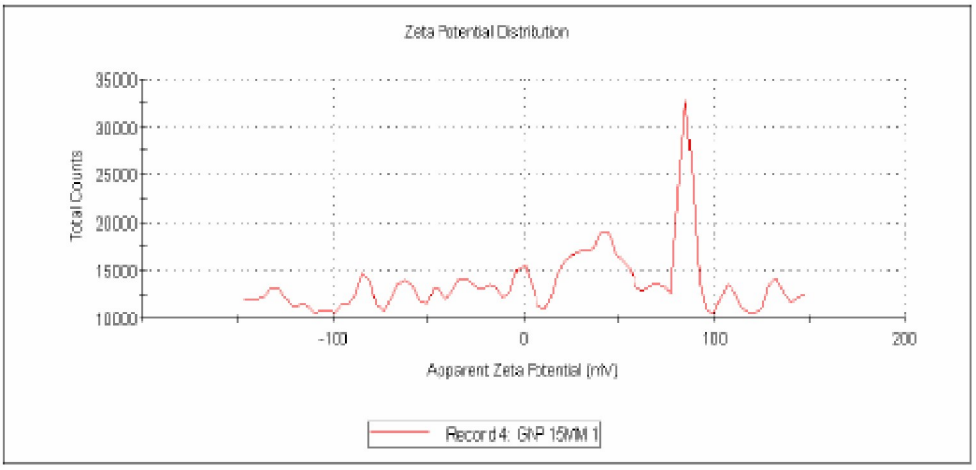


Fig. 10b : Zeta potential of 15 mM Gold Nanoparticle

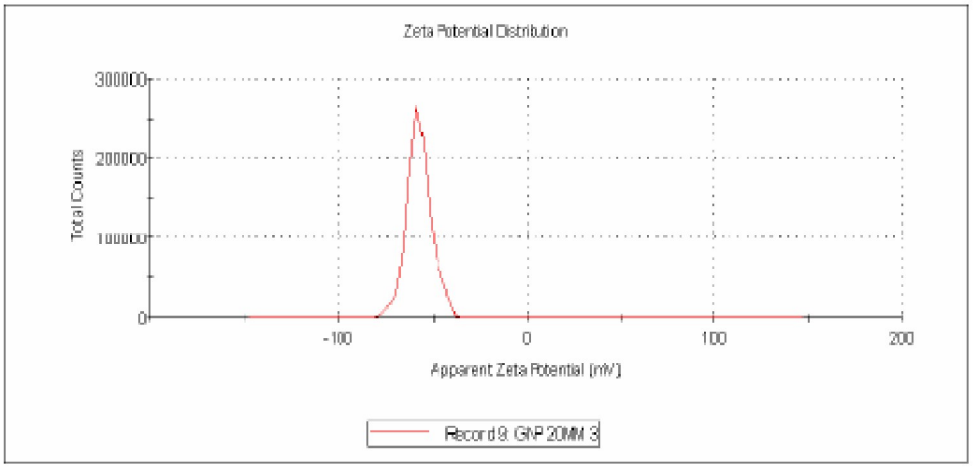


Fig. 10c : Zeta potential of 20 mM Gold Nanoparticle

the width of absorption spectra is related to the size distribution range. Generally, gold nanoparticles display a high single absorption peak in the visible range between 520 -530 nm, due to its high surface Plasmon resonance properties. This gives brilliant red color to gold nanoparticle which varies according to their size. In present study the absorption of gold nanoparticle was measured in single beam spectrophotometer and absorption maxima was noted in range of wavelength (360-590nm). The readings are shown in the Fig. 11a, 11b and 11c for 10 mM, 15 mM and 20 mM GNPs respectively. It is seen that the absorbance of gold nanoparticle is maximum at 520-530 nm. Due to the absorption spectrum falls in the visible region, we can easily detect what is the change occurring on the surface of gold particle, that's why the gold nanoparicle is now extensively used in lateral flow assay and in SPR based biosensor.

4.2 Purification and quantification of IgG from hyperimmunosera

The purified IgG was prepared from hyperimmunosera by using Himedia IgA Purification kit. The quantification of IgG was measured by Bradford method and with Bicinchoninic acid assay.

Table 2: Results of Bradford method of Protein Assay

Quantity of BSA	0.15 N NaCl	Bradford regent	Absorbance A_{595}
0 µg (0 µl)	100 µl	1 ml	0.00
50 µg (10 µl)	90 µl	1 ml	0.094
100 µg (20 µl)	80 µl	1 ml	0.197
200 µg (40 µl)	60 µl	1 ml	0.380
300 µg (60 µl)	40 µl	1 ml	0.580
400 µg (80 µl)	20 µl	1 ml	0.781

Concentration of purified IgG

Quantity of sample	0.15 N NaCl	Bradford regent	Absorbance A_{595}
5 µl	95 µl	1 ml	0.423
10 µl	90 µl	1 ml	0.396

The graph of Bradford assay is shown here in Fig.12. By graphical analysis and calculation of above data, the concentration of purified IgG was found to be 240ug/ml.

Table 3: Results of Bicinchoninic Acid (BCA) Protein Assay

Tube	Bovine Serum Albumin	Diluent (µl)	Final Concentration(µg/ml)	Optical density
A	300µl from Stock	0	2000	1.7
B	150µl from Tube A	150	1000	0.857
C	150µl from Tube B	150	500	0.423
D	150µl from Tube C	150	250	0.215
E	150µl from Tube D	150	125	0.107
F	150µl from Tube E	150	62.5	0.050
G	150µl from Tube F	150	31.25	0.023
H	150µl from Tube G	150	15.625	0.011
I	150ul from Tube H	0	0	0
Sample($E_{1)}$)				0.210
Sample(E_2)				0.240
Sample (E_3)				0.200

The calibration graph of Bicinchoninic Acid (BCA) Protein Assay is shown in Fig. 13. By graphical analysis and calculation of above data the concentration of purified IgG was found to be 240ug/mg.

4.3 Construction of immunochromatographic test system

Now a day's chromatographic system is principle immune detection method for onsite detection. This method is qualitative and semi quantitative. In present study, the chromatographic test strip was developed by using antibody labeled gold nanoprobe to bluetongue sera. A schematic diagram of immunochromatographic test strip is shown in Fig. 1. The sample pad and colloidal gold Ab conjugate release pad were placed at the top LFA lamellate of the system and the absorbent pad was placed at the bottom of the nitrocellulose membrane. The purified VP7 protein on test line and protein A /G on control line were immobilized as shown in Fig. 4b.

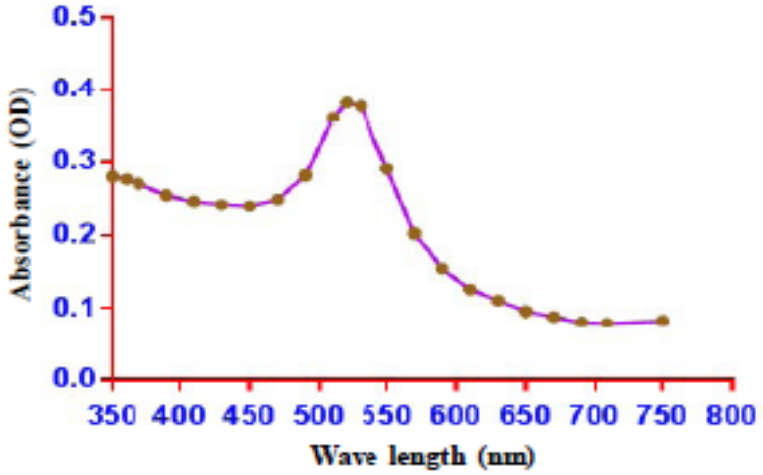


Fig. 11a : Characterization 10 mM gold nanoparticle by uv-vis spectrophotometry

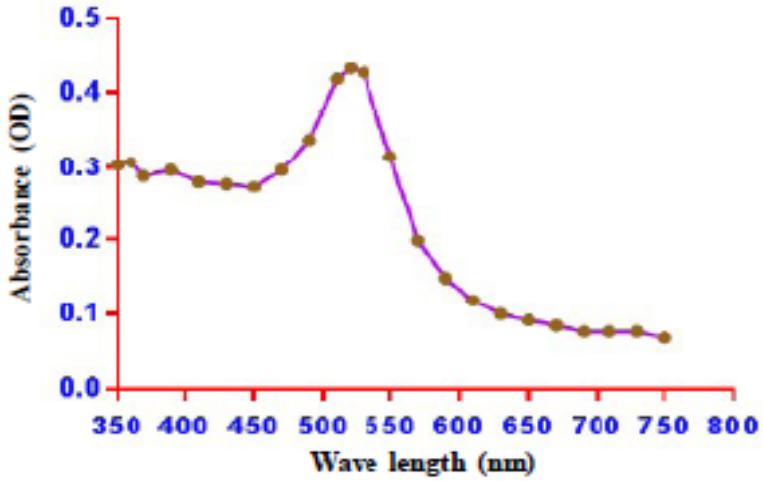


Fig. 11b : Characterization 15 mM gold nanoparticle by uv-vis spectrophotometry

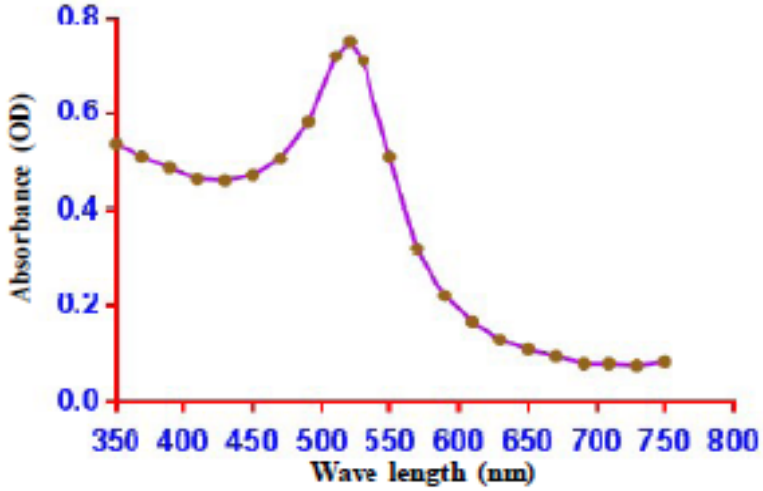


Fig. 11c : Characterization 20 mM gold nanoparticle by uv-vis spectrophotometry

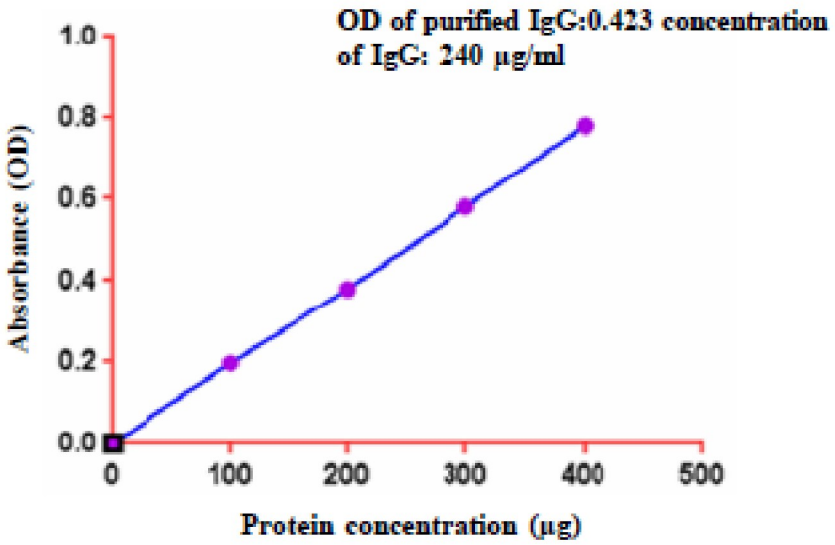
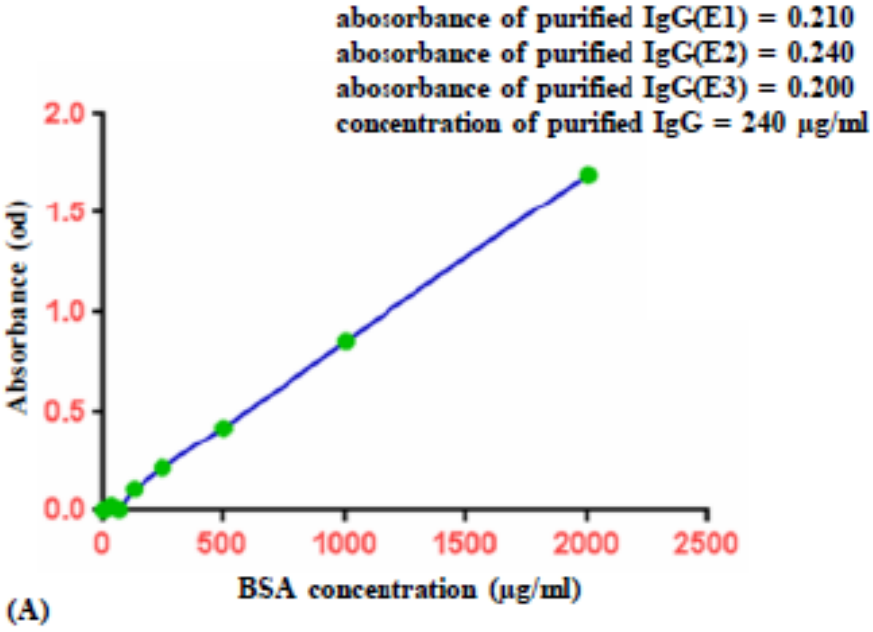
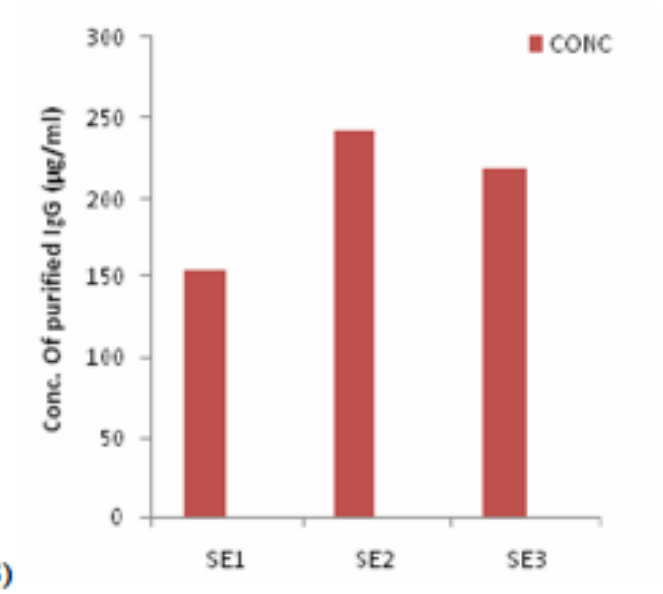


Fig. 12 : Calibration graph of Bradford assay (absorbance A595 vs concentration of BSA in microgram)



(A)



(B)

Fig. 13 : Calibration graph of bicinoconic acid assay (absorbance A595 vs concentration of BSA in microgram)

In the assay, the gold antibody probe was applied to release pad containing a fixed quantity of analyte to be detected which reacts with secondary antibody. Then aqueous buffer was applied to the sample pad, which flows downward through the capillary action and carrying the whole complex. As the reaction mixture reached to the test line of purified VP7, there was a binding of primary antibody and conjugated secondary antibody to the VP7. So the line was appeared in case of positive sample at test as well as the control line was appeared as the protein G bind to the all mammalian IgG. But in case of the negative sample, the anti sheep Ab conjugated gold cannot bind to the VP7 as there is absence of serum IgG in sample. So there was no formation of line at test, but the control line was appeared as protein G binds to the gold conjugated secondary IgG. The intensity of color band can also be visualized as a way for estimation of bluetongue serum IgG not only qualitatively but also semi quantitatively.

4.3.1 Impact of capillary flow rate on strip sensitivity

The capillary flow rate is the speed at which a sample front moves along a membrane strip when liquid is introduced at one end. This value is very difficult to measure accurately since the rate decays exponentially as the liquid moves forward along the membrane. An easier parameter to measure is the capillary flow time, which is nothing but the time required for liquid to move along the strip of a defined length. This value is typically expressed as sec/cm and inversely related to flow rate. The flow rate of a membrane depends on the aggregate properties of the porous structure. A discussion of these parameters is helpful in understanding the capillary flow.

4.3.2 Impact of width of the test and control lines

Since bed volume increases with the thickness, the width of the test and control lines can be affected. In most instances the capture reagents are applied to the membrane at a constant volume per unit distance. The liquid is absorbed into the membrane, displacing the air from a comparable volume within the pores. The liquid usually penetrates down into the membrane and then moves laterally. Spreading of the reagent on the relatively thicker membranes because there is less depth to allow downward penetration of the liquid. If the same mass of capture reagent is spread out over a wide area, the detector reagent will be similarly diffused, the net result being less color intensity and lower sensitivity.

4.4. Performance testing for chromatographic strip and standardization of the rapid assay

A sensitive colloidal gold immunochromatographic assay using a VP7 protein was devolved for the rapid detection of BTV antibodies in the clinical field samples. The VP7 protein should be optimized to get a proper line on the test. Some scientist found the optimal concentrations of purified recombinant VP7 protein was 2.0 mg/ml for better sensitivity of the strip. (Yang *et al*., 2010), but here we used 480 µg/ml of VP7 protein on the test line. Various parameters that influenced the assay performance were investigated and optimized

(1) Type of Membranes: It should be made from nitrocellulose due to its hydrophobicity, so the type and amount of detergent or surfactants used are normally fixed for compatibility with casting process

(2) Pore size: It is a measure of the diameter of the largest pore and pore size distribution is a measure of the range of pore sizes. We selected three different strips of 8µ, 10 µ and 15 µ. Out of these, 10 micron was found more effective than other.

(3) Size of gold nanoparticle: In our research we use 20nm, 30nm and 40nm size sigma gold solution. Based on sensitivity point of view, 30nm was found to be most suitable one. The suitable size of the gold was found 20 to 30 nm, if the size of the gold particle is less than 20 nm, then there will be formation of diffuse line on the both test and control line and if the size of the gold nanoparticle is more than 30 nm, then the gold nanoparticle cannot flow in the nitrocellulose membrane, so there is also formation of bands other than the test and control line.

(4) Detection limit: In this study, we have determined the detection limit of this assay. For this we analysed the purified hyperimmunosera, serial two fold dilution (0, 2, 4, 8, 16, 32, 64, and 128) was done from purified IgG which concentration was measured to be 240 µg/ml at 0 fold dilutions by Bradford and bicinchoninic method i.e means 240ug, 120 µg, 60 µg, 30 µg, 15 µg, 7.5 µg, 3.75 µg and 1.875 µg of purified IgG were applied to sample pad. The gold conjugate was made by physical adsorption method by taking antisheep IgG with gold nanoparticles of different size. Then running

buffer (PBS-T, pH of 7.5) was poured on sample pad drop by drop to carry the whole complex of gold-antibody conjugate and analyte through nitrocellulose membrane. The complex was allowed to run up to the top of nitrocellulose membrane and strips were kept for drying. The image containing color signal was produced on strips immediately. Before this analysis, the gold-Abs conjugate was tested for development of control without addition hyper immune sera to check whether the conjugate is working properly or not. The analysis results for BTV purified hyperimmunesera with 10 and 15 micron immunochromatographic strip by visual observation are shown and presented in Fig 14a and 14b respectively. Here it was seen that, up to 128 dilution both test and control line were appeared after that the test line was not seen only control line was appeared. In absence of BTV antibody, the binding of immune gold labeled secondary antibody to the VP7 protein at test line does not occur. But when there is presence of BTV Ab in sample, can bind to VP7 protein and there is appearance of the line on test. The minimum concentration of BTV Ab, which made the color of test line to appear but with a strong red band at control line, was determined to be the detection limit of visual immunochromatographic strip test. The results of repeated experiments under optimized condition for analysis of purified IgG was verified and the detection limit of this assay was found to be 1.875 ug/ml.

(5) Analysis time: The analysis process was very simple and easy to use and can be completed within 2-3 minutes.

(6) Ratio of gold conjugate and purified IgG : The device was also tested with taking different combination of purified IgG and conjugate i.e. 3:4, 4:8, 5:10, 6:12, 3:3 ratio and the result is shown here in the Fig. 15. It was found that the effective ratio of conjugate and purified IgG was 6:12.

Sensitivity and specificity

Regarding clinical sample, c-ELISA has been extensively used as it has good specificity and sensitivity (Battenet *et al*., 2009), but in this study we used indirect ELISA as the reference

test (De *et al.,* 2009) Here the device was also checked successfully for testing BTV IgG from clinically affected field sheep sample and is shown in Fig. 16a, which were parallel tested with indirect ELISA and results were found almost similar. About 156 samples were tested with indirect ELISA, it was found that 29 samples were found to be positive and 127 samples were found to be negative and by lateral flow assay it was found that 24 were found to be positives and 132 samples were found to be negative.

Table 4: Showing the results of comparison between Indirect ELISA and lateral flow assay

		Indirect ELISA		
		Positive	**Negative**	**Total**
Lateral flow assay	**Positive**	24	1	25
	Negative	5	126	131
	Total	29	127	156

From table, it was seen that,

True positive (a) 24

False negative(c) 5,

False positive (b) 1,

True negative (d) 126 were found.

Relative to indirect ELISA,

Sensitivity: a/a+c ×100 = 82.76%

Specificity: d/b+d×100 = 99.21%

So it was found that the sensitivity and specificity of this developed lateral flow assay were high i.e 82.76% and 99.21 % respectively as compared to specificity (97.6%) and sensitivity (100%) (Yang *et al.,* 2010), using the c-ELISA as a reference test. Here, the kappa factor we found to 0.870 as compared to the results obtained by c-ELISA and ICS (kappa = 0.930) (Yang *et al*., 2010). Fig. 16b shows the developed kit which was tested with PBS, HIS and field sera.

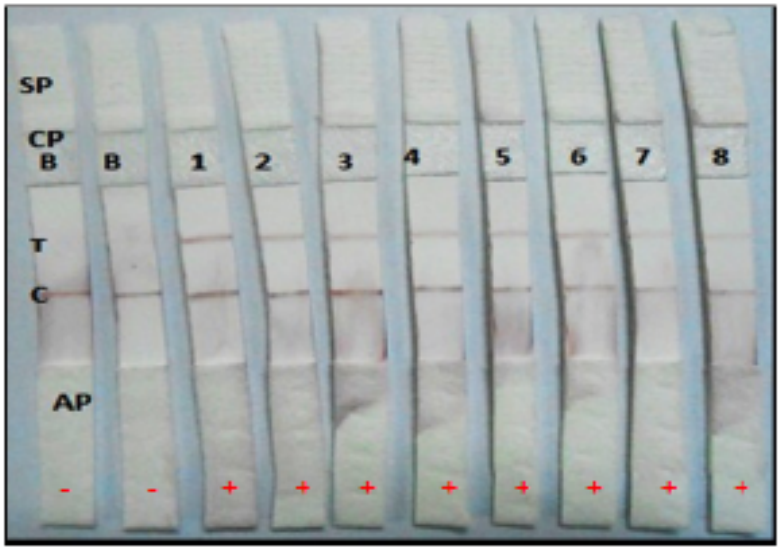


Fig. 14a :Showing the 10 micron strip after testing with two fold dilution of purified hyper immunosera (left) i.e First two as blank (Tested with PBS) and 1 to 8 as 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 dilutions, respectively i.e (240ug, 120ug, 60ug, 30ug, 15ug, 7.5ug, 3.75ug, 1.875ug/ml of IgG)

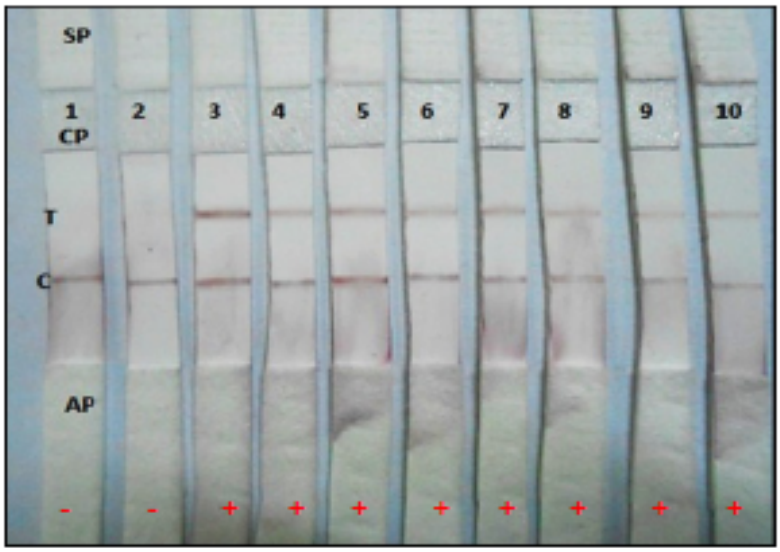


Fig. 14b :Results of 15 micron strip after testing with two fold dilution of hyperimmunosera (left) i.e 1, 2 as blank and 3 to 10 as 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 dilutions, respectively

Effect of the ratio of the purified IgG of HIS and the conjugate on the appearance of the test line and control line on the lateral flow device

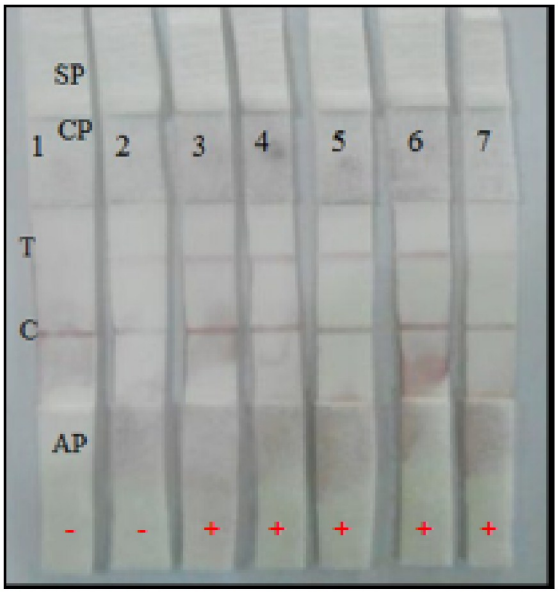


Fig. 15 : Results of lateral flow strip after testing with HIS and conjugate in different ratio strip 1 to 7 (l) showing as blank, 3:4, 3:6, 4:8, 5:10, 6:12 and 3:3, respectively

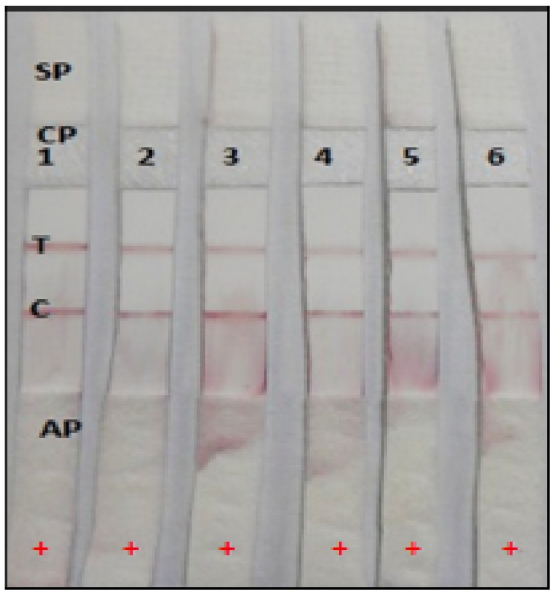


Fig. 16a :Showing the results of lateral flow assay after testing with unknown field sheep sera sample

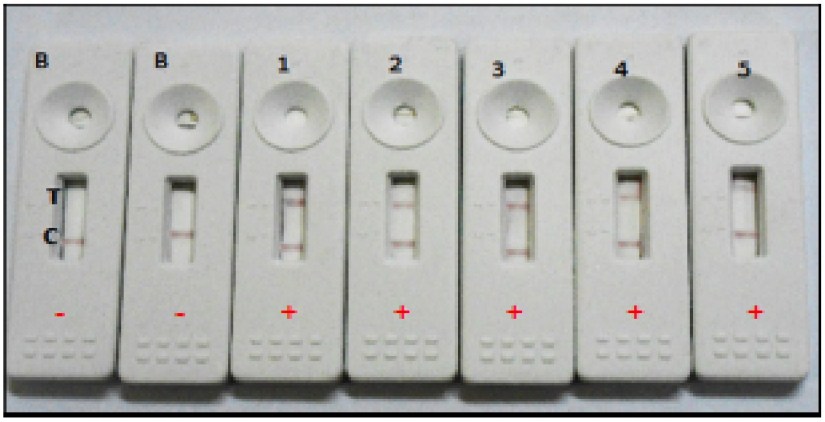
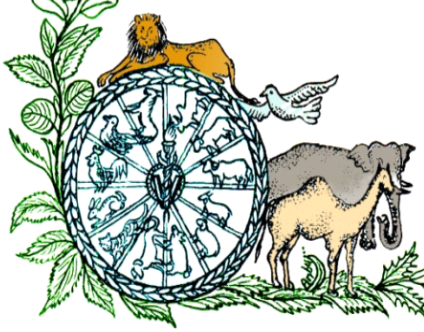


Fig. 16b :Showing first two as blank and 1 to 5 showing the results of BTV lateral device with HIS and field sera, first two as blank (tested with PBS) and 1, 2, 3 with HIS, and 4, 5 with field sera

Due to its high sensitivity and specificity, this assay may provide new platform for diagnosis of bluetongue in small ruminants at field level. This device can be commercialized and patented for further application after validation with a lot of sample.

Summary and Conclusions



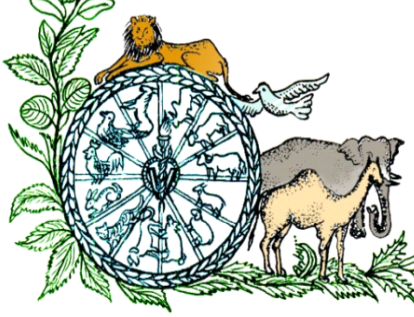
5 SUMMARY AND CONCLUSIONS

Gold nanoparticles are the most biocompatible, highly stable and of high optical surface Plasmon properties. In present study, synthesis of gold nanoparticles, their characterization and application in development of lateral flow assay for bluetongue antibody were studied. Gold nanoparticle was synthesized by sodium citrate reduction method. For this, 50 ml of auric chloride solution of 10 mM, 15 mM, 20 mM were heated up to boiling and trisodium citrate was added with simultaneous stirring. This solution was boiled till development of brilliant red colored colloidal gold suspension. This colloidal gold suspension was kept at 4°C. The gold nanoparticles were characterized for morphology, shape and size by TEM, its zeta potential by dynamic light scattering and absorption spectrum by UV-VIS spectroscopy. Most of the gold nanoparticles were ranging from 10 to 30 nm. Bluetongue is an acute febrile viral disease of small ruminants. The morbidity and mortality rates due to bluetongue can be raised up to 100% and 30% respectively in severe outbreaks. Due to its high economic loss, it is necessary to diagnose the disease properly with in short period. So a rapid sensitive diagnostic assay should be developed, which will be applicable under field condition. So the lateral flow assay may be substitute for this against the avaible conventional diagnostic methods. The chromatographic strip test is a very simple diagnostic assay which can be used by unskilled personnel without any need of sophisticated machines. This assay can be used for qualitative as well as for semi quantitative evaluation of analytes with easy visual antigen and antibody interaction on real time. The lateral flow device was prepared by assembling the nitrocellulose membrane, conjugate pad, sample pad and absorption pad with support on solid plastic laminate. The antirabbit and antisheep antibodies were conjugated with gold nanoparticle with simple

hysical adsorption method for testing with hyper immune sera and field suspected sera for bluetongue respectively. Purified VP7 protein (480 µg/ml) and protein G (1mg/ml) were spotted on test and control line respectively by easy printer. The VP7 protein was expressed and the hyperimmunosera was raised against VP7 protein in rabbit. The hyperimmunosera was purified by protein A based affinity chromatography and concentrated with small centrifugal device having 10 KDa MW cut off. Quantification of antibody was done by Bradford and Bicinchoninic acid method. The concentration of purified IgG was found to be 240 µg/ml by extra plotting the graph. The conjugate of gold colloid and secondary antibody was formed by using KH_2PO_4 (pH 7.5). The secondary IgG at 50 µg/ml was prepared by diluting it with 5Mm KH_2PO_4 (pH 7.5) buffer to final volume of 100 µl and mixed with 900 µl of gold colloidal solution. The above solution was centrifuged at 13000 RPM for 20 mins at 4p c .The pellet again was suspended with preservative solution, again centrifuged and the pellet was sonicated for 30 sec and was stored in preserving buffer at $4^{o}C$ for further use. This colloidal gold-antibody conjugate forms the basis for development of immune chromatographic assay for bluetongue. The optimization of device parameters such as wicking rate, pore size, assay time, gold nanoparticle size etc was done. The purified IgG which concentration was 240ug/ml, was tested with this strip by taking 2 fold dilutions (0, 2, 4, 8, 16, 32, 64, 128, 256 fold of dilution) i.e 240 µg, 120 µg, 60 µg, 30 µg, 15 µg, 7.5 µg, 3.75 µg, 1.875 µg of BTV Ab were tested with the strip. From each diluted sera 8 µl was mixed with 4ul of antibody gold conjugate. The above mixed solution was applied over the conjugate pad of the strip. Then running buffer was poured on the sample pad drop by drop to carry the whole complex of gold Ab and BTV IgG through nitrocellulose membrane. The complex was allowed to run up to the top of nitrocellulose membrane. The color signals were produced on test and control line. The minimum limit of detection of BTV IgG was found to be 1.875µg/ml that means up to 1.875 µg/ml of minimum concentration of bluetongue Ab can be detected by this assay. Gold nanoparticles are the most versatile material for visual sensing to this device and the compatibility of gold nanoparticle with biological molecules like antibody is excellent. The results of this qualitative one step test take only 2-3 minutes. Visual evaluation, rapidity and simplicity of results have practical advantages over existing conventional immunoassay formats. 156 field samples were tested by indirect ELISA and also parallel tested with lateral flow strip. Out of 156, 29 samples

showed positive and 127 samples showed negative by indirect ELISA. But by lateral flow device, 25 and 131 samples showed positive and negative respectively. The sensitivity and specificity of the lateral flow assay were found to be 82.76% and 99.21% respectively. From these results it is concluded that, the bluetongue disease of affected animal can be detected with this rapid assay and giving a better platform for reducing the economic loss of the country.

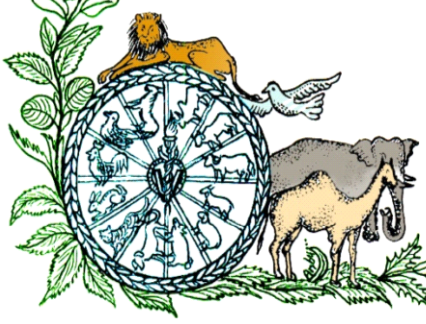
Mini Abstract



6 MINI ABSTRACT

Rapid assay is a very convenient and reliable real time screening test for detection of various analytes. Bluetongue is a non contagious, cullicoides borne viral disease of small ruminants caused by *orbivirus* of family *reoviridae*. In this study, lateral flow device was developed against bluetongue antibody by using VP7 protein for rapid diagnosis of this disease. VP7 protein was expressed in house and hyperimmunesera was purified to use as a positive sample. The purified IgG was concentrated and characterised with Bradford and Bicinchoninic acid assay. Gold nanoparticle was synthesized and characterized by U/V spectroscopy, transmission electron microscopy and with dynamic light scattering. The size of the gold nanoparticle was found around 18±5 nm with different shapes and its zeta potential was found around -55.4±3mv. The antirabbit and antisheep antibodies were conjugated with gold nanoparticle with physical adsorption method. The device was assembled with the nitrocellulose membrane which contain test and control line stripped with VP7 and protein G respectively. The minimum concentration of VP7 and protein G was found to be 480ug and 1mg per ml respectively. This device was also tested with the field sera and compared with indirect ELISA parallelly and produced satisfactory result. The lowest concentration of the BTV Ab that made the test line to appear, was determined to be the limit detection for this assay. In this present report, the limit detection for bluetongue assay was found to be 1.875 µg/ml. The sensitivity and the specificity of this assay was found to be 82.76% and 99.21 % respectively The overall assay time was determined to be 2-3 mins.This device may provide a new platform at field level rapid diagnosis of bluetongue with low cost and with high sensitivity.

लघु सारांश



7 लघु सारांश

तवरित जाँच विभिन्न विश्लेषण पदार्थ का पता लगाने के लिए एक बहुत ही सुविधाजनक और विश्वसनीय एवम वास्तविक समय में जांचने की परीक्षा है। कुलीकोइड्स जनित वायरल रोग 'ब्लूटंग', जो कि एक गैर संक्रामक बीमारी है छोटे जुगाली करने वाले जानवरों में पाई जाति है जो रिओविरिडै परिवार के ओर्बिवायरस की वजह से होती है। इस अध्ययन में तुरन्त जांच के लिए वीपी7 प्रोटीन का उपयोग करके ब्लूटंग बीमारी के एंटीबॉडी के खिलाफ तवरित जांच अन्तर विकसित किया गया है। वीपी7 प्रोटीन कोप्रयोगशाला में अभिव्यक्त किया गया एवम हाइपर प्ररतिरक्षा सीरम एक सकारात्मक नमूने के रूप में उपयोग करने के लिए शुद्ध किया गया। पहले शुद्ध आईजीजी को संकेन्द्रित किया गया, तत्पश्चात ब्रैडफोरड और बिसिनोकनिक एसिड परख जांच के द्वारा इसकी मात्रा का पता किया गया। पहले सोने के नैनोपार्टिकल को संश्लेषित किया गया। तत्पश्चात यू/वी स्पेक्ट्रोस्कोपी, ट्रांसमिशन इलेक्ट्रॉन माइक्रोस्कोपी और डायनामिक लाइट स्कैटरिंग की मदद के द्वारा इनका लक्षण वर्णन किया गया। सोने के नैनोपार्टिकल का आकार 18±5 एनएम पाया गया जो विभिन्न आकृतियोंके हैं और इनका जीटा पोटेंशियल –55.4±3 एमवी के आसपास पाया गया। भौतिक सोखनाविधि के द्वारा एंटी–रैब्टि एवं एंटी–शीप प्रतिरक्षीयों को सोनके नैनोपार्टिकल के साथ संयुग्मित किया गया। इस संयुग्मित को नाइट्रोसेलूलोज़ झिल्ली के साथ संकलित किया गया जिस पर क्रमशः वीपी7 प्रोटीन कि परीक्षण रेखा एवं प्रोटीन–जी की नियंत्रण रेखा शामिल है। वीपी7 और प्रोटीन–जी की न्यूनतम संकेद्रण संख्या क्रमशः 480 माइक्रोग्राम एवं 1 मिलीग्राम प्रति मिलीलीटर रखी गई। 156 क्षेत्र नमूनों की तुलना हमने अपने बनाए हुए यंत्र के द्वारा परिक्षण किया और जब हमने दोनों कि तुलना की तो परिणाम संतोषजनक मिला। इस जांच की परिक्षण रेखा की निचली सीमा के निर्धारण हेतुवीटीवी ऐलिसाड़ी की जो निचली संकेन्द्रण संख्या पाई गई वही नमूनों की निचली संकेन्द्रण संख्या पता लगाने की सीमा है। इस वर्तमान विवरण में ब्लूटंग कि जांच की सीमा 1.875 माइक्रोग्राम/मिलीलीटर पाई गई। जांच की सीमा के लिए क्रमशः संवेदनशीलता और इस जांच की विशिष्टता 82.76% एवं 99.21% पाईगई। समग्र जांच की सीमा का समय 2–3 मिनट पाया गया। उच्च संवेदनशीलता एवं कम लागत के साथ ब्लूटंग बीमारी की तुरन्त जांच हेतु यह यंत्र एक नयी दिशा प्रदान कर सकता है।

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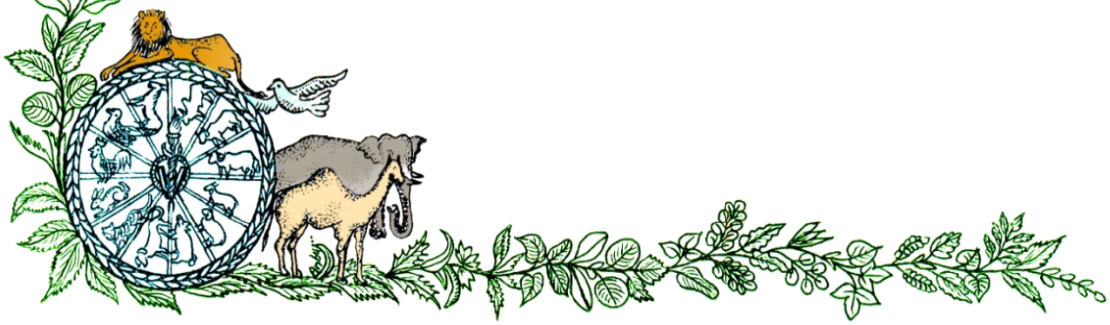

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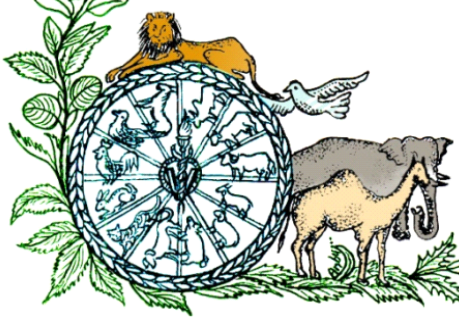
Appendix



APPENDIX

SOLUTIONS PREPARED

1.	**Auric Chloride (1%)**	
	Auric chloride (49%)	1 gm
	Milli Q water	49 ml
2.	**Trisodium citrate (1.5%)**	
	Sodium citrate tribasic	0.03 gm
	MilliQ water	2 ml
3.	**Bovine serum Albumin (10%)**	
	BSA	100 mg
	KH_2PO_4 (50 mM, pH 9.0)	1 ml
4.	**Polyethylene glycol (1%)**	
	PEG (200000)	10 mg
	KH_2PO_4 (50 mM, pH 7.5)	1ml
5.	**KH_2PO_4 Buffer (50mM)**	
	KH_2PO_4	136.09 mg
	Milli Q water	20 ml
6.	**Preservative solution (pH 8.2)**	
	A). BSA (1%)	200 mg
	B). PEG-20000 (0.05% W/V)	10 mg
	C). NaN_3 (1% W/V)	20 mg
	D). NaCl (150 mM)	175.5 mg
	E). Tris-HCl(20 mM)	20 ml
7.	**Phosphate buffered saline (PBS)**	
	NaCl (137mM, MW: 58.44)	8.00 gm
	KCl (2.7mM, MW: 74.55)	0.20 gm
	Na_2HPO_4 (4.3mM, MW: 141.96)	0.61 gm
	KH_2PO_4 (1.4mM, MW: 136.09	0.19 gm
	Distilled water	1000 ml

The pH of prepared solution should be 7.2 .

8. **Protein A Based IgG Purification kit buffer formulation**

Use the following recipes to prepare the buffers supplied with the IgG purification kit with protein A Based column.

Binding Buffer: 1.5M glycine/NaOH buffer, 3M NaCl (pH 9),Add 112.6 g glycine (free base; MW 75.07), 175.3 g NaCl (MW 58.44),1.0 gm sodium azide to 800 ml distilled water. Tritate with 5M NaOH to pH 9.0.Make up final volume to 1L with distlled water.

Elution Buffer. 1M sodium citrate buffer pH 5.5, add 23.44 g citric acid (trisodium salt,dehydrate; MW 294.1), 3.872 g citric acid (anhydrous; MW 192.1), 1.0 gm sodium azide to 900 ml distilled water. Make the volume to 1L with distilled water.

Neutralization buffer: (1M Tris/ HCl buffer pH 9.0). Add 103.72 g Tris base MW 121.1), 22.72 g Tris hydrochloride (MW 121.1), 22.72 g Tris hydrochloride (MW 157.6), 1.0 g sodium azide to 800 ml distilled water. Make up the final volume to 1Lwith distilled water.

VITAE

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M.V.Sc	2014	I.V.R.I, Izatnagar	84.70%	1st class

Membership in Proffesional bodies

1. Odisha veterinary council
2. Veterinary Council of India

Award & Fellowship

1. ICAR junior research fellowship during M.V .Sc
2. Received 2nd prize for poster presentation of conference "ICAMA-2014" held at university Allahabad.
3. Received merit scholarship in all years during B.V.Sc and A.H.