

NANO-GOLD PARTICLES MEDIATED DETECTION OF NS1; AN EARLY DIAGNOSTIC MARKER OF DENGUE VIRUS INFECTION

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ABSTRACT

In this study, an immunoassay is developed for qualitative determination of NS1 antigen of dengue virus in serum using nano-gold particles. This diagnostic device comprises of four components such as sample pad, conjugation pad, nitrocellulose membrane and absorbent pad. Anti-NS1 antibodies and rabbit anti-mouse IgG antibodies were coated on nitrocellulose membrane as a test and control line respectively. Assay device was evaluated by purified NS1 and prior ELISA confirmed NS1 positive and negative samples. The diagnostic device showed promising results to detect NS1.

Key words: Dengue Virus, Diagnostic Device, Colloidal Gold Particles, Rapid Diagnosis, NS1 antigen, ELISA.

INTRODUCTION

Dengue virus is one of the major public health problems especially in the tropical countries. Dengue virus belongs to the Genus *Flavivirus* of the Family *Flaviviridae*, and this virus includes four types of strains (DNV-1, DNV-2, DNV-3 and DNV-4) identified to be evolved in non-human primates (Wang *et al.*, 2000). Dengue virus is a membrane-enclosed single stranded RNA virus having positive sense genome comprising of three structural and seven nonstructural proteins (Henchal and Putnak, 1990). Pakistan had first case of dengue fever in 1994 (Khan *et al.*, 2010) and since then it has been circulating throughout the years, but the current epidemic of dengue fever (DF) was worst that happened because of floods in Monsoon. More than 31,000 cases have been reported mainly in the Punjab province of Pakistan.

The non-structural 1 (NS1) protein is believed to be responsible for the pathogenicity of dengue virus (Tajima *et al.*, 2008). NS1 is a highly conserved glycoprotein having molecular weight of 46 kDa and this protein may be present in intracellular, cell surface, as well as in secreted forms. This protein has 12 invariant cysteine residues that form six intermolecular disulfide bonds and two N-glycosylation sites. The NS1 protein is transported through the Golgi pathway to the plasma membrane and then secreted out of the mammalian cells (Crooks *et al.*, 1994). The NS1 appears in higher concentrations in the sera of dengue hemorrhagic fever (DHF) patients as compared to those suffering from DF, which indicates its association with the disease severity (Avirutnan *et al.*, 2006). On account of its high concentration in the serum, it could be used for the early diagnosis of DF or DHF by using immunochromatographic device or ELISA (Peeling *et al.*, 2010).

Different techniques are used for the diagnosis of Dengue Virus infection like Virus isolation, RNA detection, Immuno-histo chemistry, IgM, IgG detection and NS1 detection by ELISA but these techniques are highly expensive, time consuming, require well equipped labs and expertise. Most rapid assays are the immunochromatographic (lateral-flow) strips that are available in the kit form. These assays require no other reagent and specialized equipment, and test results can be obtained in less than 15 minutes. Immunochromatographic strips incorporate both antigen and signal reagent into a nitrocellulose strip. The specimen is applied to an absorbent pad, the antigen combines with signal reagent, and migrates through the strip. A positive reaction results in a visual line on the membrane where viral antigen has been applied (Branson, 2000). Immuno chromatographic strips may be used for diagnostic purposes of various disease and biological samples in the form of whole blood, serum, or plasma may be applied on the strips. However, some IC assays can be performed with finger-stick specimens, saliva or oral fluids which are very cost effective and makes this diagnostic method ideal for screening of the viral infections (Sia *et al.*, 2004).

Pakistan is facing outbreaks of dengue virus, where the diagnosis and management of the dengue viral infection is the main issue due to complicated, time consuming and expensive procedures to diagnose infection at early stage. Aim of the present study is to develop immunochromatographic lateral flow device locally, using nano-gold labelled anti-NS1 antibodies to detect NS1 Ag in the serum which could ensure rapid, cost effective and early diagnosis of the dengue virus infection.

MATERIALS AND METHODS

Preparation of colloidal gold Particles: Colloidal gold particles of 20 nm size were prepared by the reduction of chlorauric acid (Brust *et al.*, 1994). 50 mg of chlorauric acid (Sigma) was dissolved in 500 ml of deionized water and boiled with continuous stirring to reduce chlorauric acid. 12.5 ml of 1% Sodium citrate was added, by addition of trisodium citrate color changed from straw yellow to dark blue and finally to reddish color. The colloidal gold particles solution was cooled at room temperature and pH was adjusted at 7.4 by 1 M K₂CO₃. The solution was stored at 4 °C.

ELISA to confirm the interaction of recombinant NS1 Ag and anti-NS1 antibody: ELISA was performed in order to confirm antigen antibody interaction (Samra *et al.*, 2006). 2 µg of recombinant NS1 antigen was coated on microtiter plate, Anti NS1 antibody as primary antibody and anti-mouse IgG horseradish peroxidase (HRP) labeled as secondary antibody used in this assay, while mouse IgG was used as an isotype control.

Conjugation of Anti-NS1 antibody with colloidal gold particles: Anti-NS1 antibody was conjugated with gold particles (Faulk and Taylor, 1971; Romano *et al.*, 1974). 25 µg of anti-NS1 antibody was added to 1 ml of gold particles solution that was gently inverted for 30 min at room temperature and centrifuged at 12000 rpm at 4 °C for 1 hour. The pellet was resuspended in 0.5 ml stabilizing buffer (0.15 M sodium chloride, 50 mM Tris-Cl, pH 7.4, 0.5 mg/ml polyethyleneglycol-4000 and 0.001% sodium azide). Antibody-gold conjugation was confirmed by immunodot blot (Brust *et al.*, 1994).

Immunodot blot analysis: This assay was performed to evaluate interaction of anti-NS1 gold complex, and the presence of NS1 antigen (Brust *et al.*, 1994; Hsu, 1984) with little modifications. Nitrocellulose membrane was cut into a 3 x 5cm piece, and then 5µl of purified NS1 antigen was spotted on the membrane and then it was allowed to dry. To avoid non-specific binding, membrane was dipped in the blocking buffer (3 % BSA in TBS) for 45 minutes at 37°C. Membrane was washed by 1X TBS and allowed to dry, then membrane was dipped in anti-NS1 gold complex for 45 min with constant shaking and results were noted.

Preparation of Strip for Lateral flow assay: Nitrocellulose membrane was cut into 3.0 cm x 0.3 cm strip. 2 µl of purified anti-NS1 antibody (0.1 µg / ml) was spotted on the membrane 1.0 cm above the bottom of the paper as the test line. Whereas, 2 µl of goat anti-mouse IgG (0.1 µg / ml) was spotted as a control line 1.0 cm above the test line. The membrane was allowed to air dry for 45 minutes at 4°C and then blocked by using 3% bovine serum albumin. The conjugate pad was a strip of 0.8 cm x 0.3 cm of polyester sheet. 100 µl of gold labeled

anti-NS1 antibody was loaded on to the conjugate pad and dried at 4 °C. The conjugate pad was attached at the bottom of nitrocellulose membrane. A sample application pad of 0.5cm x 0.3 cm strip of polyester sheet was attached onto the conjugate pad. A strip of 0.5cm x 0.3cm of 3mm Whatman paper was attached as absorbent pad to the end of membrane opposite to the conjugate pad. This strip was laminated on a plastic strip (3.0 cm x 0.3 cm) and stored at 4°C. The overall structure of the device is shown in Fig 1. This strip was evaluated by purified NS1 antigen purchased from Abcam (UK) as well as prescreened NS1 positive serum samples.

RESULTS AND DISCUSSION

The strip development is a continuation of the efforts that have been made for years to apply modifications of ELISA in such a way that they can be used for the field diagnostic approaches. Many other research studies have also focused on using a nitrocellulose membrane as test template for binding and detecting antigens by the principle of immunodot blot assay and this aspect has also been practiced in some research applications (Pappas *et al.*, 1983; Stott, 1989).

Integrity of NS1 antigen and anti-NS1 antibody purchased from Abcam (UK) was determined by SDS-PAGE. The analysis of SDS-PAGE revealed that both antigen and antibody were in their integrated forms signified by their respective molecular weights. Moreover, the affinity of NS1 antigen against anti-NS1 Ab was determined by using ELISA (Samra *et al.*, 2006) while isotype mouse IgG was used as negative control. Change in the color of test wells (having anti-NS1 antibody) after the addition of substrate and OD values measured at 450 nm revealed that NS1 antigen exhibited highest affinity against anti-NS1 antibody, while NS1 Ag did not show any affinity against isotype control which confirmed the specificity of anti-NS1 antibody as shown in Figure 2. The antigens and antibodies may therefore be used effectively for the specific antigen-antibody interactions in the detection assays.

Anti-NS1 antibody was labeled with gold nanoparticles for dip-strip preparation. Colloidal gold nanoparticles exhibit the significant electronic and optical properties and they are very small in size so they tend to be preferentially used in the diagnostic applications as probe for labeling antibodies rather than using enzyme-labels (Zharov *et al.*, 2003). Citrate reduction is known as the most efficient and simple method for the preparation of colloidal gold nanoparticles (Turkevich *et al.*, 1951). Initially, sodium citrate helps in reducing the chlorauric acid for preparing gold nanoparticles but after that charged citrate ions surround the outer shell of gold particles thus making them charged so that they do not attract each other and avert self-aggregation (Freus, 1973). In the present study, colloidal gold particles of

approximately 20nm size were prepared by the citrate reduction method. Chlorauric acid was reduced deep blue color upon addition of 1% tri-sodium citrate in chlorauric acid (Fig 3, B). The reduction reaction was completed by further heating and stirring that result in the appearance of brick red color (Fig 3, C) and final pH was adjusted to 7.4 by adding K_2CO_3 that gave pink color of gold suspension (Fig 3, D). Final pH of colloidal gold was adjusted to 7.4 because this is the optimal pH for conjugation of gold nano-particles to anti-NS1 antibody and K_2CO_3 was used as pH stabilizer. If the pH of suspension is not according to the isoelectric point of the particular protein to be conjugated then conjugation fails to take place. Therefore, the final pH of colloidal gold was adjusted necessarily to 7.4 (Sharma *et al.*, 2006).

Anti-NS1 antibody was conjugated with gold nano-particles under constant agitation at room temperature. Then the resultant gold-anti-NS1 pellet was stored at 4 °C in the stabilizing buffer. The formation of gold-anti-NS1 complex was evaluated by immune-dot blot assay while mouse IgG was used as negative control. In order to confirm the successful formation of gold-anti-NS1 complex, nitrocellulose membrane was first spotted with NS1 antigen and then allowed to react with the previously formed Anti NS1-Gold and mouse anti IgG-Gold complex. Appearance of reddish pink blot on the nitrocellulose membrane indicated that Anti NS1-Gold complex was formed successfully.

The reddish pink spot indicated the interaction of antigen (NS1) and anti-NS1 antibody labeled with gold nano-particles as shown in Fig 4. Therefore, nano gold labeled anti-NS1 antibody indicates potential to be used for the dip-strip preparation. Dip-strip based on the immune-chromatographic techniques and lateral flow assay was prepared in the present study. For the labeling of antibodies, commonly nanoparticles are used because they are very specific in binding and small in size so they do not create any hurdle in the sample flow through the strip (Leuvering *et al.*, 1980). Most recently, gold nanoparticles are being employed for this purpose (Kusano *et al.*, 2007). Dipsticks are typically made up of nitrocellulose like fragile membrane that is supported by plastic covering just leaving the sample application pad open. Application and conjugation pads are typically made of cross-linked silica that facilitates the flow of sample during chromatographic assay. Test line contains the specific antibody against which analyte (antigen) is to be targeted so color development at the test line gives the result whereas control line is used to assess the complete flow of sample throughout the strip (Kolossova *et al.*, 2007). Sample flows due to the capillary force imposed by the nitrocellulose membrane of the strip and this flow is maintained by the absorbent pad. Absorbent pad is attached at the end of strip so that it will sustain the sample flow by wicking it to the control line (Posthuma-Trumple *et al.*, 2009).

Anti-NS1 antibody was applied at test line on the strip, made up of nitrocellulose membrane to detect the presence of NS1 antigen in the sample while mouse Ig G was applied at control line to indicate the complete flow of the sample. Sample application pad constituted polyester sheet that facilitated the smooth flow of sample across the strip due to its capillary force and this force was maintained by the absorbent pad of glass fiber and whatman paper. The absorbent pad sustained the sample flow across the strip by wicking it to the control line showing complete flow of the sample. Conjugate pad of polyester sheet was affixed with gold-labeled anti-NS1 Ab so that the sample containing NS1 antigen can form complex with the gold-labeled antibody and gives colored reaction at the test line.

Our locally prepared dip strip was evaluated by recombinant NS1 solution and also by dengue serum samples containing NS1 pre-screened by NS1 ELISA. Purified NS1 antigen in the sample reacts with Anti NS1-gold complex on the conjugation pad and moves towards the nitrocellulose membrane where it combines with Anti NS1 antibody jetted on test line and forms a red line. Purified NS1 and NS1 ELISA confirmed serum samples were run on the strip and pink line appeared on both test and control area, while no pink test line was appeared on samples that were collected from healthy individual when applied on the strip (Fig 5).

The positive and negative samples were validated by ELISA. These results of ELISA were in accordance with those of dip-strip immunoassay indicating the scope of this dip strip as a successful and cost-effective diagnostic tool for dengue virus infection.

96 well maxisorp plate was coated with 2μ recombinant NS1 Ag and its interaction with anti-NS1 Ab and isotype control was analysed by using HRP labeled anti-mouse Ab and TMB substrate. The interaction was assessed by the change in color of the substrate which was measured at 450 nm. The results confirmed the interaction between anti-NS1 Ab and recombinant NS1.

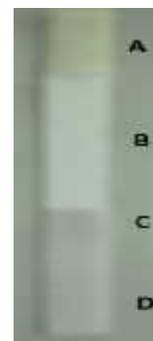


Fig 1: Locally Prepared Dip Strip: (A) Absorbent pad that is made of glass fiber and watman filter paper (B) Nitrocellulose membrane (C) Conjugation Pad containing the glass fiber to ensure uniform and smooth flow of sample (D) Sample Pad.

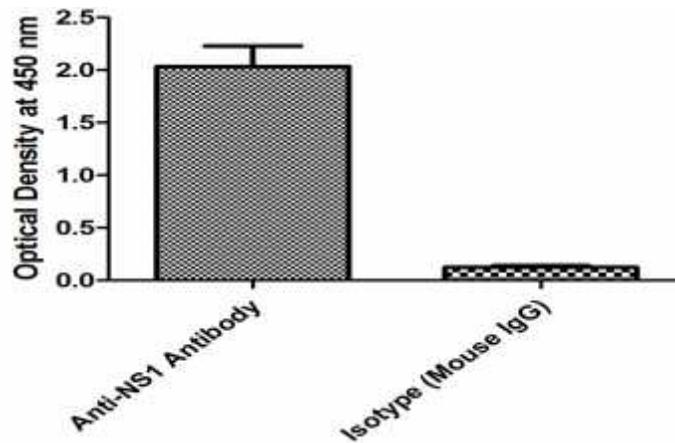


Fig 2: ELISA to assess the interaction between anti-NS1 Ab and recombinant NS1.

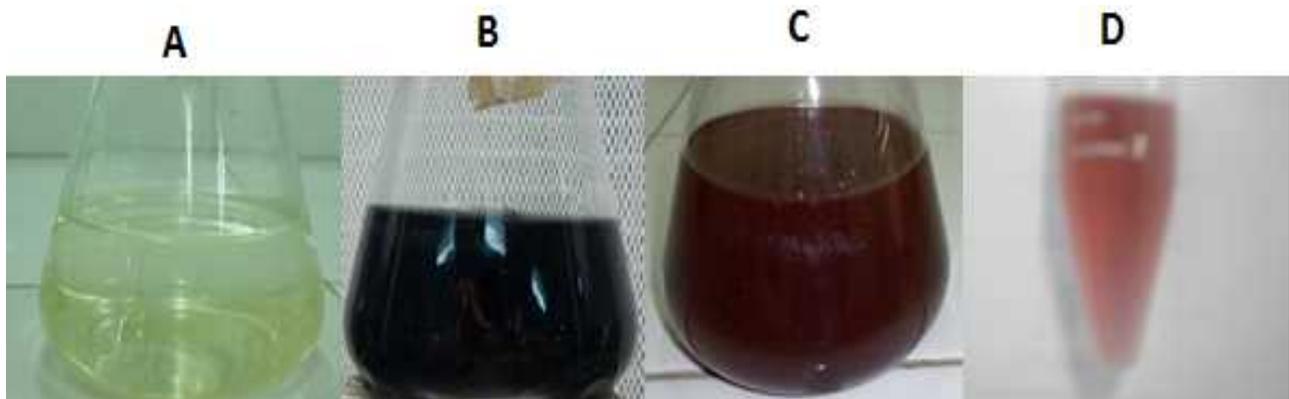


Fig 3: Preparation of colloidal gold particles: Colloidal gold particles prepared by the reduction of chlorauric acid with the help of 1% trisodium citrate under heating and vigorous stirring. During heating the solution showed different colored phases as shown in this figure (A) Slight yellow color produced by the addition of chlorauric acid in deionized water. (B) Deep blue color developed when 1% trisodium citrate was added showing the reduction of chlorauric acid. (C) Upon further heating and stirring the reaction completed and final brick red color developed (D) pH adjusted by K_2CO_3 at 7.4 and gave pinkish red appearance.



Fig 4: Immunodot Blot: (A) Positive Control: 5 μ g of purified NS-1 antigen was coated on nitrocellulose membrane and blocked with 3% BSA. 50 μ l of the gold conjugated anti-NS1 antibody was added on the membrane to assess its interaction with NS1 antigen. A reddish pink spot was appeared on the membrane after washing with PBS to confirm interaction of NS1 Ag and anti-NS1 Ab. (B) Negative control with IgG Isotype: no colored spot t indicating no binding of NS1 antigen with mouse IgG (isotype).

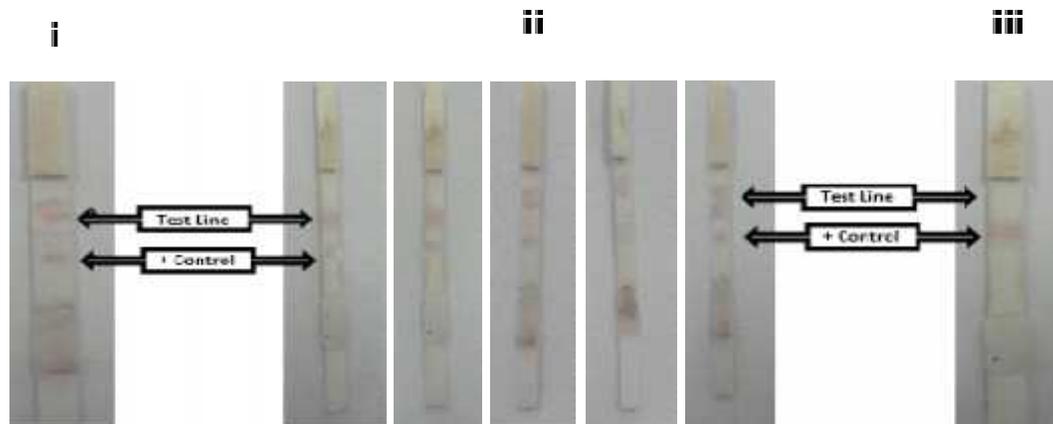


Fig 5: Evaluation of the Dip Strip: (i) Purified NS1 antigen (1.5µg/µl). (ii) Five pre-screened ELISA confirmed NS1 serum samples. (iii) Control serum of healthy individual. Samples were added on sample application pad and allowed to flow on strip for 10 minutes and noted the results.

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