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Development of a Colloidal Gold- based lateral flow immunoassay for the rapid detection of glycosylated hemoglobin (HbA1c) in whole blood

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ABSTRACT

Globally, diabetes mellitus (DM) is one of the major health problems. DM prevalence is increasing rapidly and causing disability and death. So, early and accurate diagnosis of DM is very important to prevent the further complications. In this study, a lateral flow immunoassay (LFA) was developed to detect glycosylated hemoglobin (HbA1c). Colloidal gold particle labeled monoclonal anti-glycosylated hemoglobin (clone 16) was used as the detector reagent. Monoclonal antibodies for anti-hemoglobin (clone 6) and anti-mouse IgG were immobilized in test and control lines, respectively, of a nitrocellulose membrane, acting as the capture reagents. The LFA was used to detect HbA1c in 500 sera from clinically proven DM patients. The intensity and number of the test lines on the LFA strips differentiate normal, pre-diabetic (under control) and diabetic (elevated levels of HbA1c). The results from our LFA were validated and compared with Alere Afinion AS 100 HbA1c analyzer and Bio-Rad variant II- HPLC obtained good data. Moreover, the test strip was stable at 4-30°C temperature for up to 6 months. The HbA1c LFA was highly selective for HbA1c and showed no cross reactivity against HbA0, glycated HbA0, HbA2. Thus, the developed colloidal gold based LFA proved to be simple, rapid, inexpensive, point-of-care method with good specificity and sensitivity.

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INTRODUCTION

Worldwide, diabetes is one of the major health problems and is one of the leading causes of disability and death (WHO 1999, Chen, L., 2012). Diabetes is defined as a group of metabolic disorder characterized by persistently elevated levels of blood glucose. Poor management of blood glucose levels usually results in long term complications including damage, dysfunction to various organs, especially kidneys, heart, blood vessels, eyes, and nerves (NDDG 1972, Nagababu Pyadala *et al.,* 2016). Therefore continuous monitoring of blood glucose is recommended for

the diagnosis and treatment of diabetes. For blood glucose determination, we require three to four tests a day due to a large fluctuation of blood glucose concentrations (Heller, A *et al.,* 2008). In 2010 American Diabetes Association standards of medical care, included the screening of glycosylated hemoglobin (HbA1c ≥ 6.5 %) as a criterion for the diagnosis and management of diabetes mellitus (Zinman, B. *et al.,* 2010). Globally HbA1c is used as a biomarker for long-term glycemic control and also in the prevention and delay of the development of hyperglycemic complications. More recently the screening of HbA1c is used not only for monitoring and management of the disease but also, as a screening test for the identification of patients at risk of developing the complications (Genuth S *et al.,* 2003,8 Sato KK *et al.,* 2009, Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 33, Edelman D *et al.,* 2004).

Glycosylated hemoglobin (HbA1c) is hemoglobin bound to glucose in red blood cell. HbA1c is formed by a non-enzymatic reaction between plasma glucose and N-terminal valine of the β chain of hemoglobin, which is two step processes. The initial step takes minutes to hours to form aldimine complex (reversible reaction), followed by Amadori rearrangement (irreversible reaction) to form the stable ketoamine HbA1c. This glycation reaction will occur slowly and continuously over the lifespan of a red blood cell (120 days). The HbA1c percentage will provide glycemic history and also reflects the average blood glucose concentration over the entire life span of erythrocytes (Bunn HF *et al.,* 1978). A wide range of analytical methods exists for the analysis of HbA1c percentage, including ion-exchange chromatography (Eckerbom, S *et al.,* 1994), electrochemical methods (Park, J. Y *et al.,* 2008, Liu, S. Q *et al.,* 2006, Chang, B. Y *et al.,* 2012, Son, S. U*et al.,* 2006, and Qu, L. *Et al.,* 2009), boronate affinity chromatography (Frantzen, F *et al.,* 1997, Psotova, J. *Et al.,* 1995), electrophoresis (Jenkins, M *et al.,* 2003), and immunoassays (Ikeda, K. *Et al.,* 1998, Metus, P. *Et al.,* 2004). To perform the above methodologies the well-trained technician, a large number of samples and expensive instruments with long turnaround time are required. Hence analytical methodologies are not suitable to screen diabetic patients in the resource-limited settings, like lack of laboratory infrastructure and sophisticated tools for detection.

Therefore, the present study aims to develop rapid inexpensive, user-friendly, semi-quantitative and point of care diagnostic assay for detection of glycosylated hemoglobin in whole blood samples of diabetic patients.

METHODS AND MATERIALS

The majority of this study was performed at Genomix Molecular Diagnostics Pvt. Ltd. (Hyderabad, Telangana, India) and MNR Medical College & Hospital, Sangareddy, Telangana. Monoclonal antibodies for anti-hemoglobin (Capture antibody, Arista Biologicals Inc, Allentown, PA 18101, USA), anti-glycosylated hemoglobin (HbA1c-detection antibody, Arista Biologicals Inc, Allentown, PA 18101, USA), and Gold conjugates (Arista Biologicals Inc, Allentown, PA 18101, USA) were used for assay development. The laminated nitrocellulose membrane, Sample pad, and absorbent pad were purchased from MDI-membrane technologies, Ambala Cantt, India. The mouse IgG and goat anti-mouse IgG was obtained from Arista Biologicals Inc (Allentown, PA 18101, USA). The other entire chemical like sodium dodecyl sulphate (SDS), Phosphate buffer (PBS), Tris buffer (hydroxyl methyl amino methane), and Bovine Serum Albumin (BSA) were purchased from Thermo Fisher Scientific, Waltham, MA, USA.

Apparatus

Bio-Dot Quanti-2000 Bio-jet apparatus and SS Programmable Strip Cutter (Model M-70) from advanced sensor systems Pvt. Ltd. (Ambala Cantt-133006, India) were used for the coating of antibodies and cutting the test strips. Finally, the signal from the lateral flow assay was measured with an ESE quant Lateral Flow Reader (QIAGEN Lake Constance GmbH, Germany).

Sample Collections

A total of 500 whole blood samples were collected in vacutainer with EDTA from diabetic patients attending the diabetic clinic during the period from January 2013 to December 2016. 3 ml of whole blood was collected from anti- cubital vein from each diabetic patient.

Development of lateral flow assay for estimation of HbA1c

The lateral flow assay (LFA), a rapid and simplified version of ELISA was developed to test the presence of glycosylated hemoglobin in the test human blood sample as seen in the picture (figure 1). The assay or the kit is a compact device housed in a plastic casing. It consists of a nitrocellulose membrane detection strip flanked at one end by a reagent pad and at the other end by an absorption pad. A sample application pad flanks the reagent pad and these components were laminated on a plastic backing which holds them in a systematic way to optimize the liquid flow.

The detection strip contains Hb1Ac specific monoclonal antibody (clone 16 from Arista Biologicals Inc.) and at test line anti haemoglobin

(clone 6 from Arista Biologicals Inc.) as well as a reagent control (control line) (anti-mouse IgG) immobilized as distinct lines. The conjugation pad or reagent pad contains dried and stabilized detection reagent consisting of a colloidal gold-conjugated with anti- specific monoclonal antibody as detection reagent in the antigen detection flow assay from blood samples. The starting material was at a concentration of 1mg/ml in phosphate buffered Saline (PBS). The gold particles were prepared at 20 to 60 nanometer size and were used to conjugate Hb1Ac specific monoclonal antibody and the resultant complex (50 ODs) was blocked, stabilized and dispersed on to the conjugation pad at 1.5 µl per centimetre and air dried.

The Monoclonal capture antibody was applied to the detection strip as a one-mm-wide line by using a Bio Dot Quanti-2000 Bio jet apparatus. Anti-Mouse IgG was applied in a second line to function as a reagent control line. The detection antibodies were applied to the conjugate pad by using a Bio Dot Air jet Quanti 2000 apparatus. Strip tests are assembled/ laminated on a plastic support by mounting the sample application pad, conjugation pad or reagent pad, detection strip, and absorption pad onto a rigid support and placing the strips in a plastic assay device with a round sample well positioned above the sample pad and a square detection and control window above the detection strip. Finally, whole device was sealed in a moisture-resistant protective three layer aluminium foil containing a silica gel bag as desiccant. The antigen preparation method, the amount and concentration of capture antibody, and the amount of detection reagent was optimized in a step-by-step procedure using a panel of well-defined positive and negative control blood samples from known diabetic or normal people. The sample buffer consisted of phosphate-buffered saline, pH 9.0, containing stabilizers at desired concentrations. The flow assay is performed by the addition of 5 µl of fresh blood directly onto the sample application pad in the sample well of the plastic assay device. Following the addition of 120 µl of sample buffer, the result is read 10 to 15 min later by visual inspection for staining of the antigen and control lines in the test window of the device. The control line should stain in all cases. The assay is scored negative when no staining of the test line is observed and positive when a distinct staining (Red to maroon) of the test line is observed indicating that the person has glycosylated hemoglobin. The amount of glycosylated is semi quantified using the specific ESE lateral flow scanner.ds as described by Raaman, 2006.

Test procedure

5µl of human whole blood was added to the sample well with 120 µl of buffer and incubated for 5-10 minutes for the development of colour bands.

Stability test

390 aluminium foils packets containing silica gel were prepared, each with one HbA1c lateral flow immune assay kit. Later these 390 kits were divided in to three groups, each group having 130 kits individual groups were stored at different temperatures like, 4°C, 25-30°C, and 45°C for 180 days (6 months) as per WHO guidelines (23,24,25). For every 15 days up to 6months, the kits were retrieved from the accelerated conditions were tested with HbA1c samples.

Detection Limit

4.8% (30 mmol mol−1) HbA1c calibrator was diluted two folds 7 times (1:0, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) with haemolysis reagent (distilled water with blood stabilizers), eight hemoglobin A1c immunoassay test kits were tested (including one negative control strip, tested with in-house buffer). Calibrator (5 µl) at different concentrations was dispensed directly onto the NC membrane. For the negative control strip, in-house buffer was dispensed directly onto the NC membrane. All strips were washed with phosphate buffer containing Tween-20. The detection limit was determined by the degree of visibility of test lines on the strips.

RESULTS

A total of 500 whole blood samples were collected from MNR medical college and Suraksha poly clinic in that 50 blood were chosen selectively collected samples conformed by the HPLC with known values (4% - 14%) were used to validate the performance of our lateral flow assay for the quantification of HbA1c levels. All the above 50 samples were subjected to testing with a Alere Afinion AS 100 HbA1c analyser, Bio-Rad variant II-HPLC and our in-house hemoglobin A1c lateral flow assay, respectively.

Calibration curve for quantitative estimation of HbA1c

HbA1c levels were quantitatively estimated in blood samples by plotting a calibration curve between HbA1c levels versus signal intensity measured by the ESE Quant lateral flow reader as shown in the figure 2. ESE Quant lateral flow reader was used to measure the signal intensity. This was designed specifically to detect colloidal gold reflectance on a lateral flow platform.

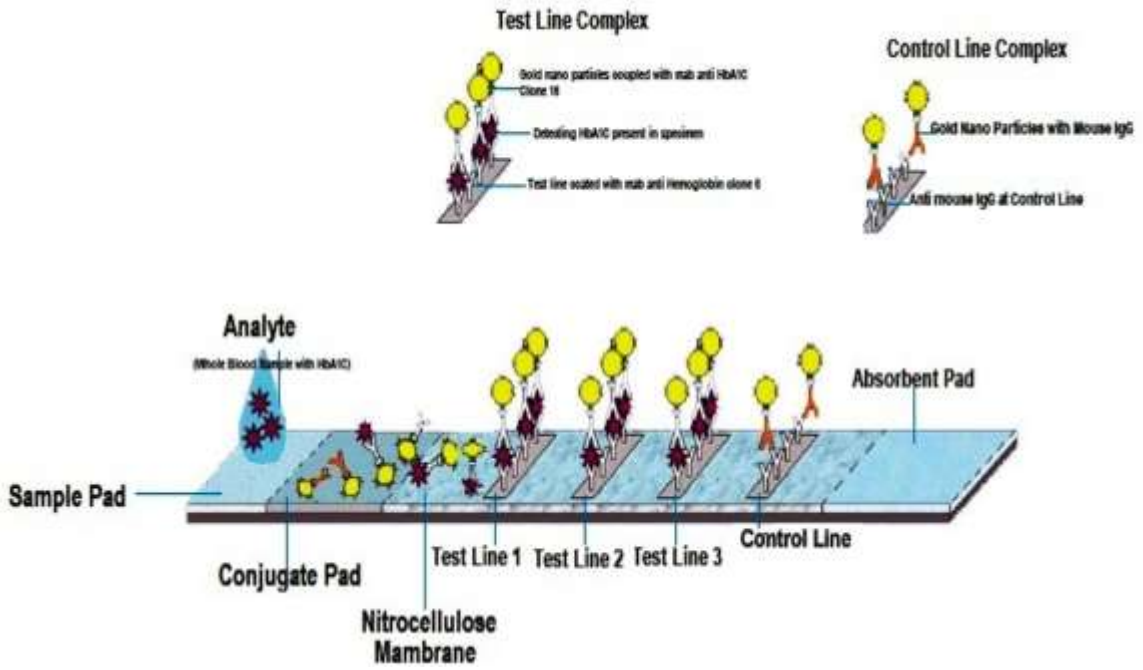

Figure 1: Schematic representation for detection of HbA1c by using sandwich immunoassay on lateral flow strips

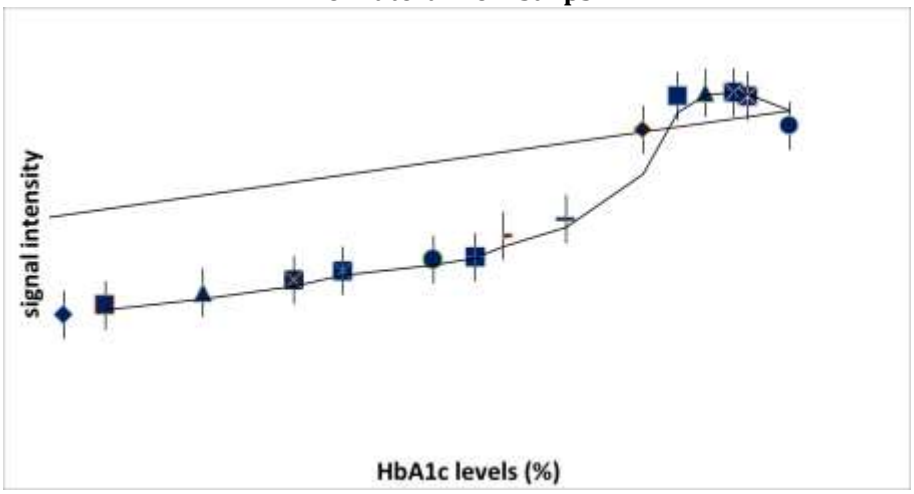

Figure 2: Signal intensity of test lines versus concentration of HbA1c (%)

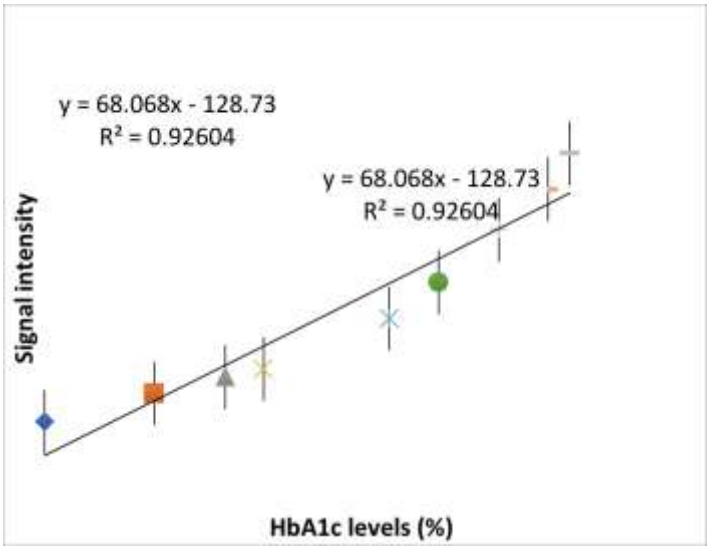

Figure 3: The dynamic range of HbA1c detection falls in between 4%-14 % with R2 = 0.926

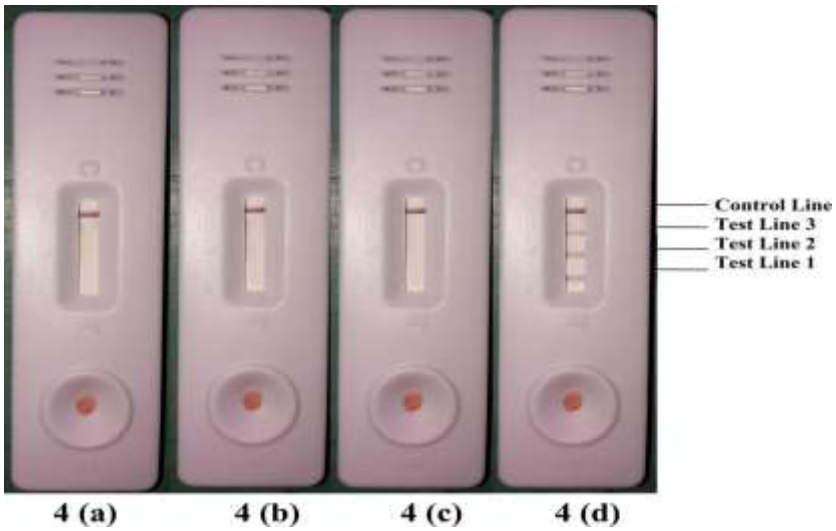

Figure 4: Purified synthetic reagents were used to test the cross reactivity or selectivity of the lateral flow immune assay.

(4a) HbA1c kit was tested with purified HbA0 (non-glycated fraction of HbA). (4b) HbA1c kit was tested with purified glycated HbA0. (4c) HbA1c kit was tested with purified HbA2. (4d) HbA1c kit was tested with purified HbA1c. No cross reactivity was observed.

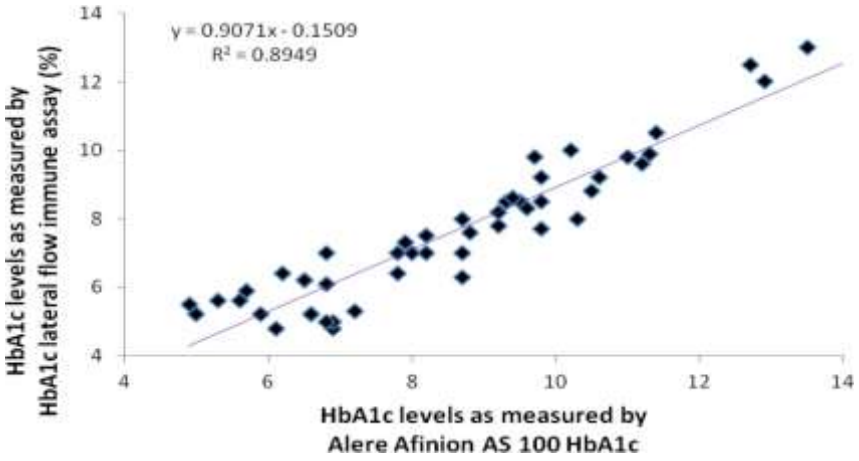

Figure 5a: Regression plot. HbA1c lateral flow immune assay (LFA) versus Alere Afinion AS 100 HbA1c, 50 blood samples

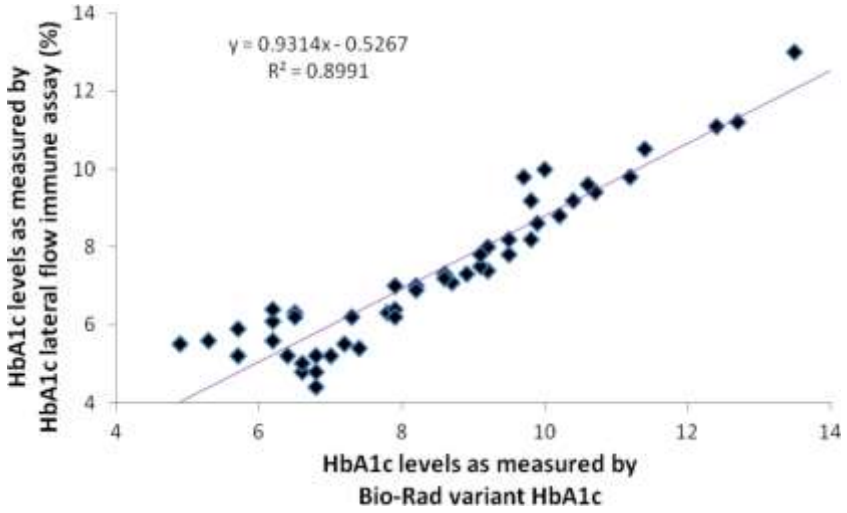

Figure 5b: Regression plot. HbA1c lateral flow immune assay (LFA) versus Bio-Rad variant HbA1c, 50 blood samples

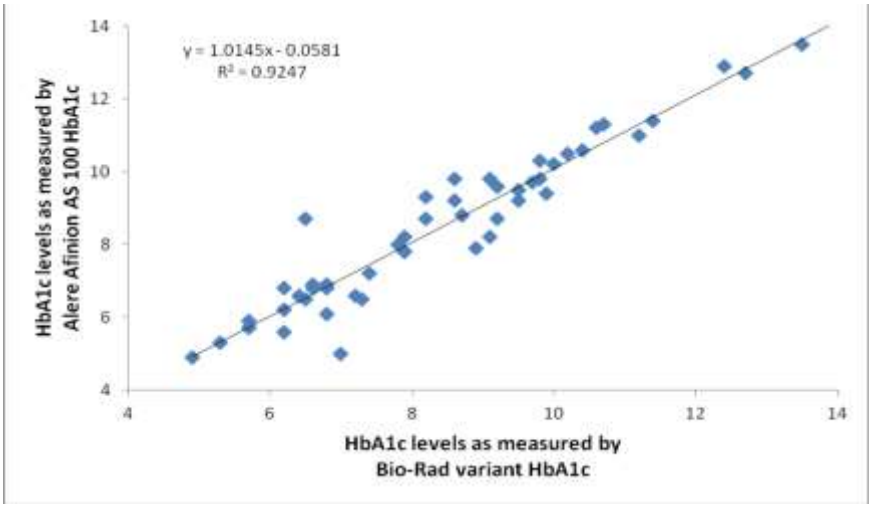

Figure 5c: Regression plot. Bio-Rad variant HbA1c versus Alere Afinion AS 100 HbA1c. 50 blood samples

Table 1: Three distinct groups of blood samples

Distinct Groups	HbA1c % (old units)	HbA1c mmol/mol (new units)
Group 1	< 6.5 %	48 mmol/mol
Group 2	6.5-7.0%	48-53 mmol/mol
Group 3	>7.0 %	53 mmol/mol

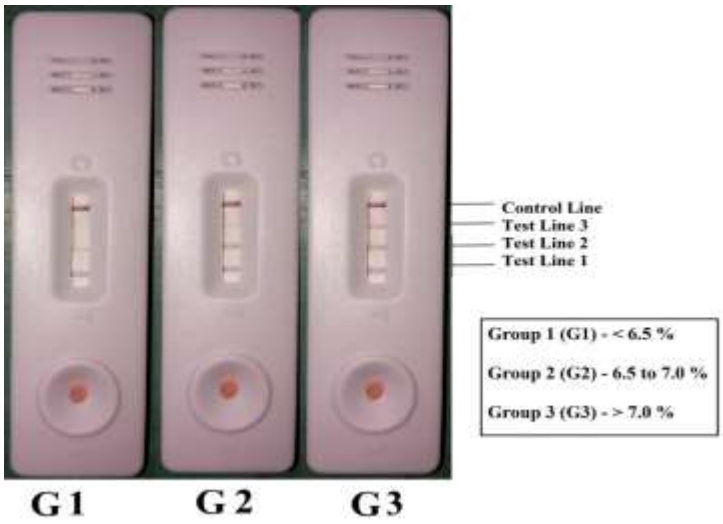

Figure 6: Three distinct groups of blood samples, group I (<6.5%, 48 mmol mol−1), group II (6.5%–7.0%, 48–53 mmol mol−1), and group III (>7.0%, 53 mmol mol−1) were tested with HbA1c LFA. The signal intensity of test lines increased with the increase in HbA1c lev

The reflectance signal intensity generated in test lines T1, T2, T3 was summed and plotted against the HbA1c levels (%).

Selectivity test/ cross reactivity test

Cross reactivity test was performed on our lateral flow assay by using purified HbA0 (non- Glycated fraction of HbA), HbA0 (Glycated species of HbA), HbA2 (which contains 2 α and 2δ chains), and HbA1c (in which glucose bounds to β chain). Then 5 µl of purified cross reactants was added to the sample well with 120 µl of buffer and incubated for 5 minutes for the development of colour bands. The test strips were then read for signal intensity measured by the ESE Quant lateral flow reader as shown in the figure 4.

Performance of lateral flow assay for estimation of HbA1c

The fifty samples with known values were used to test the in-house hemoglobin A1c lateral flow assay (HbA1c LFA). Based up on the signal intensity of the test lines measured by ESE lateral flow reader the HbA1c levels are estimated. we compared the results obtained by our lateral flow assay to the results obtained from Alere Afinion AS 100 HbA1c and Bio-Rad variant II- HPLC by regression analysis as shown in the figure 5 (5a,5b,5c).

DISCUSSION

HbA1c levels were quantitatively measured in the blood samples by plotting a calibration curve between HbA1c levels versus signal intensity measured by the ESE Quant lateral flow reader as shown in the figure 2. Sigmoid shape curve was observed as shown in the figure 2. The signal intensity increases with HbA1c levels, and gets saturated when at 14% HbA1c levels. The signal intensity decreases with HbA1c levels >15%. A linear range was established between 4-14% as shown in the figure 3. Regression R2 = 0.92, indicating a good linear relationship between signal intensity and HbA1c levels. This calibration curve depicts that the lateral flow is very sensitive to HbA1c levels with small increments. A wide gap was observed in signal intensity between distinct HbA1c levels. Thus allowing the end users for semi quantitative visual interpretation of the results and preventing confusion. Along with visual interpretation, a portable ESE lateral reader can be used to perform quantitative measurement, thus, enabling to differentiate between HbA1c levels.

A cross reactivity test was performed to determine the selectivity of the lateral flow assay against HbA1c. Thus, strengthening the principle that amplification of signal occurs only when HbA1c is bound to the detection antibody (mAb anti hemoglobin A1c, clone 16) and subsequently forms a sandwich with the capturing antibody (mAb anti hemoglobin, clone 6) at test line. Cross reactivity test was performed on our lateral flow assay by using purified HbA0 (non- Glycated fraction of HbA), HbA0 (Glycated species of HbA), (which contains 2α and 2δ chains), and HbA1c. This test was performed, because anti-hemoglobin antibody at test line bound to all types of hemoglobin, it is difficult to ensure that non-HbA1c forms immune-complexes. Cross-reactivity test was performed as shown in the figure 4. The end result indicated that the sandwich was formed and signal intensity detected only when HbA1c is present in the sample. The mAb anti-hemoglobin A1c antibody was highly selective for the N- terminal glycation cite at the valine in the β-chain. The other forms of hemoglobin [as the N- terminal glycation cite at the valine in the β-chain is absent) will not bind and produce signal at test line.

Stability test

We performed a stability study for 6 months on our lateral flow immune assay. 390 lateral flow cassettes were stored in aluminium foils along with silica gel at three different temperature conditions like 4°C, 25-30°C and 45°C for 180 days (6 months). Over the duration of 180 days at 15 days interval the lateral flow strips were obtained 13 times from three different accelerated storage conditions and tested using known sample values. The stability of LFA was assessed by measuring the loss of signal over the 6 months of evaluation period at accelerated stability conditions (Hornback, L. A 2010, WHO Prequalification TGS–2 2015, and ICH Q1E Evaluation of stability data 2004). The outcome the results depends on the storage conditions of the end product. To reveal the effect of temperature storage on kit performance the LFA kits stored at different accelerated storage conditions as follows: a) 4°C in a cold chain refrigerated condition up to 6 months, b) 25-30°C temperature conditions up to 6 months c) 45°C in an incubator up to 6 months. The kits those were stored at 4°C and 25-30°C showed similar and consistent results till the assessment time. The kits those were stored at 45°C showed improper results at the end of the assessment time for a period of 4 months (120 days) showed consistent results, later on the stability of the LFA got decrease in terms of band intensity and in flow rate. This observation indicated that the developed LFA is reasonably stable 6 months when storage at 4-30°C. So we are recommending to store LFA in between 4-30°C.

Performance of lateral flow assay for estimation of HbA1c

Fifty samples with known values were used to test the in-house hemoglobin A1c lateral flow assay (HbA1c LFA). Based up on the signal intensity of the test lines measured by ESE lateral flow reader, the HbA1c levels are estimated. The results obtained from lateral flow assay were compared to the results obtained from the Alere Afinion AS 100 HbA1c and Bio-Rad variant II- HPLC by regression analysis as shown in the figure 5. Through regression analysis, the correlations between the results of HbA1c LFA and Alere HbA1c kit was found to be 0.894 (Figure 5a) and for the Bio-Rad variant II HPLC was 0.899 (Figure 5b),whereas the correlation between the Bio-Rad variant II- HPLC and Alere HbA1c kit test was 0.924 (Figure 5c). The correlation results between Alere HbA1c kit and Bio-Rad variant II- HPLC was higher, whereas the correlation between our lateral flow immunoassay and these standard methods was quite similar with minimal differences as shown in the figure (figure 5a & 5b.). The correlation between our lateral flow immune assay and standard methods are quite similar and this can be attributed to the methods like Bio Dot Air jet Quanti 2000 machine and other analytical methods used. Several analytical assays performed using buffer with range of pH (from 6 to 11) suggested that the optimal pH condition for the best flow for the LFA devices. Use of highly alkaline buffer explains the similarity of the correlation values between our LFA and Standered analytical

methods. The Alere HbA1c kit and Bio-Rad variant II- HPLC are commercially available methods with clinically proven results, whereas our LFA is newly developed prototype which requires further optimization to get good sensitivity and specificity.

To assess the performance of LFA 50 samples were compared with known HbA1c values obtained through gold Standered method HPLC. Based up on the HbA1c levels the 50 samples were categorised in to three distinct groups, as motioned in the table 1. Whole blood samples with lower HbA1c levels (<6.5or 48 mmol/mol) showed two distinct lines at T1 and T2 on the lateral flow immune assay. Whole blood samples with intermediate HbA1c levels (6.5 -7.0 % or 48 – 53 mmol/mol) showed three distinct line at T1,T2 and T3, but T3 line was faint. The lateral flow tested with the whole blood sample containing higher HbA1c levels (>7.0% or 53 mmol/mol) showed three test lines at T1, T2 and T3 as shown in the figure 6. No false positive results (>6.5% HbA1c) were observed for blood samples in the normal HbA1c levels category (<6.5% HbA1c), indicating that the lateral flow immune assay shows potential for use in both diabetes care and diagnosis. Due of the intensity gaps observed in response to increasing HbA1c levels in blood samples and the consistency of the signal pattern generated on the lateral flow immune assay for the different groups of HbA1c levels, the end users without a lateral flow reader can also interpret the results on our LFA through naked eye. When compared LFA with other existing point-of-care devices in measuring HbA1c levels, LFA shows promise as a portable device for diagnosis and diabetic care. For example, unlike zinc boric acid conjugate lateral flow strip (Biohermes device for HbA1c), this device does not require sample pre-treatment and direct detection of HbA1c levels can be tested using whole blood samples without dilution. Moreover, in this assay in- house (neutral) buffer is used only for rinsing. But does not require two types buffers which are used in zinc-boric acid conjugate lateral flow strip (R.P. McCroskey, C.E. Melton, Google Patents, 2010 and E. Sundrehagen, Google Patents, 2014).

Based up on the results obtained so far through LFA, compared to HPLC and LFA has proved to be simple, rapid, inexpensive, point-of care method with good specificity and sensitivity.

Limits of Detection

A range of dilution factor (1:0, 1:2, 1:4, 1:8, 1:16, 1:32, and 1:64) was performed using 4.8% HbA1c sample (30 mmol/mol). After performing of this test it has been concluded that 1:32 dilution was chosen as a limit of detection for LFA beyond this range the visibility of the test was not clear when the HbA1c levels were <0.937 mmol/mol.

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