Multiparametric Oled-Based Biosensor for Rapid Dengue Serotype Recognition With a New Point-Of-Care Serological Test

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Abstract: With more than 40% of the world population potentially affected, the Dengue disease is currently one of the most spread diseases, worldwide. The Dengue disease is caused by a flavivirus that has four different serotypes. The most dangerous complications (Dengue Hemorrhagic Fever or Dengue Shock Syndrome) may arise in secondary infections, when a patient is infected by two or more different virus serotypes.

In this paper, we present a new multi parametric immuno-fluorescence based test able to discriminate the Dengue serotypes using a serological test. The serological test is based on a disposable multi parametric biosensor able to detect the IgM or the IgG antibodies with good serotype specificity.

The test results are obtained in 30 minutes in a newly developed portable reader where the fluorescent signal of 3 μl of serum sample is detected. This new device is an OLED-based hand-held reader and therefore enabled Point-of-Care operation. Image processing software, integrated with the reader, allows to discriminate the virus serotypes also in presence of serotype cross-reactivity, which is currently one of the most important issues in flavivirus serological test. Due to the high test sensitivity, a very early Dengue diagnostic has also been demonstrated.

Keywords: OLED-based biosensor; Point-of-Care diagnostics; serological test; Dengue disease
1 Introduction

Currently used Dengue diagnostics, as well as diagnostics of other flaviviruses, are based on various techniques both serological and molecular [1,2]. However, two major problems affect the molecular and serological diagnostics of flaviviruses:

1) molecular diagnostics effectiveness is limited to a specific time window as virus in patient sera or urine is present till the fifth/sixth day from the infection. As typically the disease symptoms raise after the second/third day from the infection and the patient may go to the doctor few days later, it is well possible that virus is no more present in the biological samples, thus not allowing to diagnose with this method. Nevertheless, it is worth to mention that this is the most accurate method for early disease diagnostic and Dengue serotype recognition.

2) serological diagnostics focus on the detection of antibodies produced by the immunological system to face the infection. Two different kinds of antibodies are normally detected for diagnostic purposes: immuno-globuline M, IgM (present in the sera starting from the fifth/seventh day from the infection and lasting normally about three-six months) and immuno-globuline G, IgG (start to be present in the sera after eight/ fourteen days from the infection but can be detected for years). It appears evident that this diagnostics are not ideally suited for an early diagnosis. Furthermore, in flaviviruses infection there is another important issue to be considered: the cross-reactivity of antibodies raised by the immunological system against viruses of the same flavivirus family. This issue may seriously affects the accuracy of the diagnosis, in particular in that regions where several kinds of flaviviruses are present (as it is for Dengue and Zika in Latin America).

To mitigate these issues the scientific community has found a complementary serological approach by looking at the presence of the non structural protein NS1 (glycoprotein) in the patient sera [3,4]. This protein is expressed by the cells at the very first stage of the virus infection (first day) and while its role is still not completely understood, its presence seems to be related with the virus replication [5]. The detection of the NS1 protein allow an early diagnostic with a better accuracy than the methods previously described, where its expression is specifically related to the virus that has generated it. It is currently detected using either sandwich ELISA or lateral flow based diagnostics.

There is also another possible early stage and specific diagnostic, currently not extensively investigated, i.e. the detection of antibodies (IgM and IgG) against the specific NS1 protein [6]. In particular, researchers focused their attention on the Dengue serotype specificity of IgM against the serotype-specific NS1 protein [7,8]. As described in there, while a certain degree of cross-reactivity between the different NS1 serotype specific antibodies still persists, it is possible to observe that the amount of IgM against the specific serotype is higher for the right serotype as compared to the others. It is also important to note that, as described in ref. [9], the anti-NS1 IgM are raised in a very early stage of the patient infection. In the same reference it is also reported that the anti-NS1 IgM may be observed almost simultaneously with NS1 proteins in serological tests.

Therefore, following this idea, we have realized a multi-parametric, disposable cartridge by depositing a 1 μl drops of NS1 protein on the cartridge transparent substrate for each of four Dengue serotypes to detect the presence of specific anti-NS1 IgM and IgG. The sera of the Dengue patients, kindly provided by local hospitals, has been diagnosed. The tested sera have been previously characterized by other techniques (Enzyme-Linked Immunosorbent Assay (ELISA) for anti-virus IgM, IgG and protein NS1, and Reverse Transcription Polymerase Chain Reaction (RT-PCR)). Using a secondary labeled (ALEXAFLUOR 430) antibody in an inverse immuno fluorescence method, it was possible to detect the presence of Dengue anti-NS1 antibodies, identifying their serotype. The fluorescence detection has been performed using the hand-held reader developed at OR-EL d.o.o. and based on an Organic Light Emitting Diode (O-LED), which allows to optimally excite the fluorophore emission [10,11,12]. The fluorescence signal was detected using both a high sensitivity scientific CCD camera and a new CMOS sensor fitted to the reader. Specifically developed image processing software has been used to acquire the images, analyze them and perform a quantification of the fluorescence signal.

To present our work the following paper architecture has been adopted: a short description of the methods used in our experiments is given in section 2, the results obtained using the previously characterized patients sera are presented in section 3 and finally, the discussion on the obtained results is presented in the section 4.

2 Methods

2.1 Disposable cartridge preparation

Our diagnostic point-of-care system adopts a low cost disposable cartridge, developed at OR-EL d.o.o. The car-
tridge is realized using black plastic and a central hole (Fig. 1, top). A highly transparent polystyrene substrate (T > 90%, thickness 180 μm), chemically functionalized to present a hydrophilic surface, is then attached to the bottom of the hole to obtain a 150 μl reaction chamber. Two different cartridge types have been realized: the first one with a single reaction chamber and a second one with four channels to implement the fluidic circuit to feed the reaction chamber. Four 1μl spots of the four different NS1 Dengue serotype specific glycoprotein solution have been then deposited manually on the transparent substrate by the pipette.

The NS1 protein solutions have been obtained diluting a 0.5 mg/ml NS1 solution in carbonate buffer 1mM at a dilution ratio of 1:50. The four spots have then been incubated overnight at room temperature. The positions of the different Dengue NS1 spots on the cartridge transparent substrate is shown in Fig.1, bottom image.

![Figure 1: Top image: the plastic cartridge with fluidic circuit. Bottom image: geometry of the different serotype specific antigens deposition on the transparent substrate of the reaction chamber.](image)

2.2 Diagnostics test procedure

In order to detect the presence of IgG and IgM anti-NS1 in the patient’s sera the following procedure has been adopted.

A volume of 150 μl of diluted serum has been put in the reaction chamber in contact with the NS1 protein spots (serum dilution 1:50 in PBS-T, phosphate saline buffer with 0.05% of Tween 20, total amount of sera 3μl) and incubated at T > 37 °C for 15 minutes. In the case of IgM test a preliminary step for IgG removal from the patient serum has been performed.

After washing with 150 μl of PBS-T, we performed a second incubation by using a 120 μl solution of secondary antibodies (anti-IgG or anti-IgM), conjugated with AlexaFluor 430 in a dilution of 1:40, starting from a concentration of 2 mg/ml. Also this second incubation was performed at T > 37 ºC for 15 minutes. After the final wash with 150 μl of PBS-T, the dried slide has been measured to detect the fluorescence of the spots and to identify the Dengue anti-NS1 antibodies presence as well as their reactivity with the serotype specific antigens.

2.3 Fluorescence measurement

The detection of the fluorescence spot has been obtained with two setup approaches. The first one was by using a high sensitivity scientific CCD camera (Hamamatsu C8484-G03) and the second one was by using the prototype reader equipped with the CMOS sensor and the specifically developed software to perform the image processing and automated spot recognition. The results obtained with the two presented setup approaches produced comparable results (article in preparation). In this paper we discuss only the results obtained with the CCD camera. The spot fluorescence was excited with a deep blue OLED (λ_{peak} = 436 nm, FWHM= 45 nm), powered with a voltage of 7.0 V and emitting an optical power density of 85 mW/cm². For the complete OLED description see ref. [10,11,12]. The emitted radiation has been filtered with a high-pass filter with cut-off frequency at 500 nm and a transmission of T >90% in the transmission region and T< 10⁻⁵ in the blocking region. The fluorescence signal has been observed using a band-pass filter centered at 540 nm with FWHM=40 nm and with a transmission of T >90% in the transmission region and T< 10⁻⁵ in the blocking region. All images have been acquired with an Integration Time of 30 sec, with the CCD gain set at maximum. To quantify the fluorescence signal the images acquired have been despeckled after the background subtraction. A 12-bit digitizer provided intensity values from 0 to 4095. All acquired images have been processed using the open source software Image J (W.S. Rasband, U.S. National Institute of Health, Bethesda, Maryland, USA.), while the images acquired with the prototype reader, always with 12 bits digitizer, have been processed using the specific software developed at University of Bologna. The fluorescence intensity value has been obtained as the average value of the pixels gray level in the emitting spot area.
Figure 2: Fluorescence image acquired with the CCD camera and the same image with threshold applied, to outline the brightest spots: a) anti-NS1 IgM detection with NS1 spots deposited as shown in Fig. 1, b) anti-NS1 IgG detection with NS1 spots deposited as shown in Fig. 1.

3 Results

The fluorescence images acquired after sera sample processing in the disposable cartridge exhibited the presence of multiple spots due to the intrinsic cross-reactivity of the anti-NS1 antibodies. However, for most samples, one spot was clearly brighter than the others allowing the serotype recognition, as reported in ref. [9] and shown in Fig. 2.

As reported also in literature [8,9], the better serotype specificity for the current infection is achieved by considering the results of the anti-NS1 IgM test. Moreover, considering the anti-NS1 IgG test it is possible to observe the presence of previous Dengue infections due to a different Dengue serotype. This allows to recognize secondary infections and to understand the kind of serotype present in the first Dengue episode. In the case depicted in Fig. 2.b it is possible to observe the traces of a previous infection with Dengue serotype 1.

In our experiment, a 32 Dengue patient sera samples, kindly provided by Institute for Tropical Diseases, Hospital Sacro Cuore – Don Calabria, Negrar (VR), Italy, have been analyzed. All sera have been already characterized by the Hospital for the presence of IgG, IgM (against the virus) and NS1 (by different ELISA tests) and with a PCR test to identify the Dengue serotype. It has to be mentioned that there was no indication though on what day from the symptoms onset the samples were collected. Consequently, as explained in the introduction, only 19 from 32 samples have been serotype identified by PCR. The other 13 samples were virus-less, which may be due to the late blood collection (after the seventh/eighth day from infection).

Among the 32 samples, 21 have been found positive to the presence of NS1 (test BioRad and SD), 12 were positive to anti-virus IgM (using two tests, FGM and SD), 8 were with doubt (one test positive while the second negative) and 12 were negative.

The anti-virus IgG tests showed the presence of 7 positive samples, 14 negative samples and 11 with doubt. The OLED anti-NS1 test gave the following results: 28 IgM positive results and 4 IgM negative results, 17 IgG positive results, 12 IgG negative results and 3 IgG with doubt. From these results it was possible to observe that the OLED anti-NS1 test was more sensitive than the standard IgM anti-virus serological test (ELISA), being able to recognize the positive samples also at a stage where the standard ELISA test was not able to recognize them. However for these early stage sera samples, the serotype recognition was not found very precise. Only 50% of the PCR measured samples were correctly identified, due to a weak fluorescence signal. Generally, in the case of incorrectly identified samples, the first fluorescence signal was concentrated in the spot position corresponding to the Dengue serotype 3 and 4 (see Fig.3a). On the other hand, 10 out of 13 samples not identified by PCR (as presumably collected after the seventh day from the symptom onset) presented a very strong and well defined serotype definition, as shown in Fig.3c and Fig.3d. A first statistical characterization of this new test has also been carried out. After the manual production of 20 cartridges and by depositing four spots of the same antigen NS1-DEN3, the serum of the same patient was used on each cartridge. All these new tests have then been re-measured. Using this procedure we calculated the test inter and intra-assay values. Using always the same manual protocol for all the serological OLED tests, we obtained an intra-assay value of 9.4% and an inter-assay value of 12.7%.

In the further work we expect better values by using completely automated procedure, from spot deposition to fluidic sample manipulation.

4 Discussion

A serological diagnostic analysis for the simultaneous detection of different Dengue serotypes has been performed on different human sera samples. Herein,
an inverse immuno fluorescence procedure in a multi parametric disposable cartridge has been used. For both IgM and IgG anti-NS1 fluorescence detection, a cross-reactivity between different serotype specific antigens has been observed, with a strong variability for different samples. However, the serotype recognition is clearly indicated by looking at the position of the brightest fluorescence signal recorded with a CCD camera, as shown in Fig.2 and 3. The comparison with standard ELISA and PCR tests performed on the same samples lead us to 3 significant observations:

1) The use of the OLED immuno fluorescence test allows an early detection of Dengue infection, comparable with a standard NS1 ELISA test;
2) The serotype recognition seems to be not very precise using early stage disease sera samples.

Much more evident serotype recognition is observed in sample collected in a convalescent disease stage. In this case up to 85% of the PCR non-identified samples can be clearly attributed to a defined serotype in a IgM OLED based immuno fluorescence test (see Fig. 3c and d);
3) In the early stage samples, with the evidence of IgM anti-NS1 signal (while weak, around 80 counts per fluorescent spot), the serotype was not correctly identified by the spot position. In this case, the most frequently illuminated spots correspond to the positions of DEN 3 and 4 (see Fig.3a). Further investigations are planned to understand this point.

In order to better understand the relation of the immuno fluorescence Dengue serotype identification with the day of sera collection, we have obtained from another hospital the human sera samples of the same patient, but collected in specific different disease periods.

These new experiments will be presented in a forthcoming paper, which is in preparation. The already obtained results confirm our observation of a very good serotype identification (in IgM anti-NS1 test) in sera samples collected after the seventh/eighth day from the disease symptom onset.

In the case of IgG test, combined with the IgM test, it is also possible to obtain a supplementary information on the current infection. In particular when comparing the IgM and IgG test it is possible to recognize a secondary infection and understand the different serotypes involved in the primary and secondary infection. As shown in Fig. 2, it is clear that the secondary infection is due to the Dengue serotype 2 (result of IgM test). While looking at the result of the IgG test it is possible to understand that probably the primary infection was due to Dengue serotype 1. So this method can become useful also for epidemiological studies in geographical regions where more serotypes are co-circulating and where it is not easy to collect blood sera in the early stage of the disease. To apply this new multi parametric test to Point-of-Care, a portable reader, using the same cartridge, has been developed. This new reader is based on a CMOS sensor and it was tested by using the same samples described in this paper. A picture of the hand-held reader is shown in Fig. 4. The reader is operated from a laptop or a tablet via USB and the acquired image can be directly processed in the laptop using the custom developed software.

Currently we are working on an automatic fluidic circuit to enable a complete serological analysis in 30 minutes, directly at the patient site. We are confident that the...
automated system will also improve the inter and intra-assay reproducibility.

Figure 4: Hand held reader device, here shown with the case dismounted. A credit card in the forefront, is used for dimensional comparison purpose.

5 Conclusions

The OLED-based inverse immuno fluorescence multi-parametric analysis of the four Dengue serotypes has confirmed a very good sensitivity for early detection of Dengue disease (comparable with an ELISA detection of NS1 protein in sera). While the specific serotype recognition is not very accurate in the very early stage of the infection, much more accurate serotype recognition by detection of anti-NS1 IgM has been observed using blood sample collected after the seventh day from the symptoms onset. A second set of measurements (not presented here, paper in preparation) has confirmed a very good serotype specificity using these kind of sera. A Point-of-Care reader has also been developed, allowing to perform these analysis in a very short time and with high sensitivity and specificity due to the software image processing.

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7 References


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