NON INVASIVE DETECTION OF DENGUE VIRUSES FROM SALIVA: IN VITRO STUDY

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Abstract

In the previous paper, we had succeeded in developing an early detection system of dengue viruses using Sugar liganded Gold Nano Particle (SGNP) only from 6 µL serum. It has been reported that dengue virus is also detected in the saliva and urine of the patient. The evidences lead to the possibility of developing non-invasive methods of dengue virus detection. In this in vitro study, we evaluated the utility of SGNP to capture and concentrate dengue virion in 10% saliva solution. The results showed that dengue virion was successfully detected in 10% of saliva solution. Analysis of virion stability during storage showed that virions in salivary samples were stable up to 3 days at temperature wherease the RNA has significantly degraded. Although still a preliminary study, the data obtained show the prospect of SGNP as a non-invasive dengue virus detection method, as well as the development of POC (Point of Care) method. Clinical trials using saliva from dengue viruses infected patients need to be done to prove the effectiveness of the SGNP method.

Keywords: Glycoscience, real time RT-PCR, Nanotechnology, non-invasive detection

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Introduction

Dengue hemorrhagic fever, DHF, is caused by dengue virus infection, which becomes infectious diseases with the largest number of casualties in Indonesia. The dengue virus consists of 4 serotypes propagated through the aedes aigepty mosquito (Guzman *et al*., 2010). According to recent reports, DHF patients in various provinces in Indonesia are increasing (www.infodunia.net/2016/02/ penderita-dbd-di-berbagai-propinsi.html). In 2009, WHO classified DHF patients into 3, dengue without warning signs, dengue with warning sign, or severe dengue (WHO, 2009). From the accumulated data, severe dengue, often occurs in cases of secondary infection of different serotypes. Severe dengue can occur in both children and adults through ADE (antibody-dependent-enhancement) (Raphael *et al*., 2014; Balsitis *et al*., 2010). Unfortunately, until now, there is no drug or vaccine ready to be marketed. Under these conditions, the dengue virus detection with high accuracy and sensitivity is essential in setting up appropriate treatment and anticipatory programs.

Almost of the currently available dengue virus detection methods (either based, IgG / IgM, NS1, or RNA) use a patient's blood sample as a test sample. This certainly requires special expertise, to deal with dengue patients who are still infants or children. In this point of view, the use of saliva or urine as a substitute for blood, will be easier to be applied for to children or infants. Several studies related to the use of saliva and urine for the detection of dengue virus has been published (Grape *et al.,* 2011; Poloni *et al.,* 2010; Cuzzubbo *et al,* 1998; Vazquez *et al.,* 2007; Chakravarti *et al.,* 20017).

Real time RT-PCR method has many advantages in terms of sensitivity and serotyping. The problem is addressed on the low number of the virion exist in both saliva and urine that may difficult to be detected, and therefore the virion needs to be concentrated prior extraction (Poloni *et al*., 2010). In a previous study, we developed an innovative method for detecting dengue virus from only 6

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µL serum (Saksono *et al*., 2015). The basic principle of technology that we develop was the manufacture of nano-particles to capture dengue virions. The captured virus is then precipitated and dissolved in a small amount of SDS solution. By the technique, samples that were initially difficult to be detected (due to the low concentration of the RNA) then were able to be detected. In this study, we evaluated the potential use of SGNP in developing non-invasive dengue virus detection method in vitro.

Materials and Methods

Materials and Equipments

Real Time PCR Detection System type of iQ5 (BioRad). The synthesis of sugar gold nano particle (SGNP) was performed according to the previous protocol in Japan (Saksono *et al*., 2015). One-step SYBR Prime Script RT-PCR kit II (Takara Bio Inc, Japan).

Culture vero cells and DENV-2 Infection

Vero cells were cultured on MEM media with addition of 10% FBS and 1% APS. After 3 days of cultured, the supernatant was discarded and replaced with fresh MEM media containing 2% FBS and 1% APS. The vero cell was then infected with DENV 2 virus solution and cultured for 4 days at 37 ° C, 5% CO_2. The infected culture then was moved into 15 ml Falcon, and centrifuged. The obtained supernatant was then filtered using a 0.45 µM filtration. The filtrate was centrifuged at 42,000 rpm for 20 min at 4 ° C. The precipitate is then suspended with 1 mL PBS buffer pH 8.0. The centrifugation was repeated up to 2 times, and the precipitate obtained was then suspended at 500 µL PBS buffer pH 8.0 and stored at -80 ° C until use.

Preparation of dengue virus in saliva solution

Saliva is obtained from 3 healthy volunteers. Dengue virion solution was diluted 10^2 to 10^6 times, with 10% saliva in PBS (pH 8). As a control, the same virion solution was diluted with PBS.

Dengue virion capture with SGNP

Five hundred µL virion / saliva / PBS was taken from virion solution and the remained was used as non-fraction. Ten µL of the SGNP suspension was added into 500 µL virion / saliva / PBS, followed by mixing using vortex. The mixture was incubated at room temperature for 30 minutes with shaking. The SGNP-virus complex particles were then collected by centrifugation at 10,000 x g for 10 minutes. The supernatant obtained was transferred into a new eppendorf tube (as the supernatant fraction). The precipitate obtained was rinsed with 1 mL of PBS buffer and centrifuged. The precipitate was added with 20 µL 1% SDS solution and vortex to break the virion and eluate the RNA (as the ppt fraction). Non-, sup- and ppt-fractions are used as samples in real time reaction of PCR (Fig 1).

Real time RT - PCR

The PCR reactions were performed as previously reported protocol (Saksono *et al*., 2015). Two µL of RNA solution wass added into 23 µL PCR reaction. The PCR amplification was runned according to the method previously described using general primers specific for dengue viruses (Saksono *et al*., 2015). Dengue virus identification is indicated by a Tm value between 83 ºC - 84 º C (Setiati *et al*., 2006). The efficiency of capturing is calculated by the following formula:

Efficiency = [Ct (CP) Non-fraction - Ct (CP) ppt-fraction

RNA Stability

Non-, sup- and ppt-fraction were placed at room temperature for up to 3 days. Real time RT-PCR amplification is run every 24 hours to check RNA condition. The stability of RNA was evaluated by comparing the value of Ct (CP) of ppt- and non-fraction that were obtained from Real Time RT-PCR amplification. RNA stability is indicated by CT values; if it does not change means stable, and if it changes enlarged, it means degradation.

Results

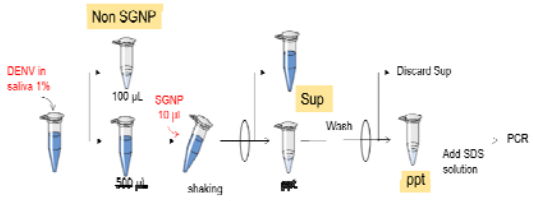

Figure 1. Strategy to capture the virion from saliva solution

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Efficiency of virion capture

Table 1. Effectiveness of SGNP to Capture virion consisted in PBS solution..

Dillution factor	SGNP				Non		Effectiveness Δ(Non - ppt)
	ppt		Sup				
	Ct (CP)	Tm#1	Ct (CP)	Tm#1	Ct (CP)	Tm#1	
$1x10^{-2}$	20.69	83.76	21.9	84.15	20.93	83.89	0.24
$1x10^{-3}$	22.57	83.77	25.76	84.19	24.96	84.08	2.39
$1x10^{-4}$	24.87	83.84	29.68	83.82	28.63	84.19	3.76
$1x10^{-5}$	27.8	83.8	31.38	83.98	32.11	84.23	4.31
$1x10^{-6}$	26.63	84.32	31.85	84.02	34.47	84.13	7.84
PC					17.39	84.67	
NC					35.04	79	

The effectiveness of virion capture in PBS samples with gradually diluted virion levels is shown in Table 1. The efficiency calculation was calculated according to the formula [1], where the smaller Ct values means the higher RNA concentration, and vice versa. As shown in Table 1, increasing dilution factor resulted in the increasing effectiveness and the highest effectiveness (of 7.84) was obtained in the sample with $1x10^{-6}$ dilution.

Saliva effect on virion capture process

Table 2. Effectiveness of SGNP to Capture virion consisted in saliva solution.

Sample (SALIVA)	SGNP	SGNP				Non		Effectiveness Δ(Non - ppt)
		ppt		Sup				
		Ct (CP)	Tm#1	Ct (CP)	Tm#1	Ct (CP)	Tm#1	
Control (PBS)	BD-03	23.32	83.14	30.51	83.06	28.4	83.2	5.08
	BD-04	23.39	83.16	30.51	83.05			5.01
A	BD-03	24.42	83.19	30.59	83.04	29.02	83.22	4.6
	BD-04	24.45	83.2	30.09	83.09			4.57
B	BD-03	25.34	83.21	29.11	83.1	30.08	82.89	4.74
	BD-04	25.29	83.25	29.92	83.13			4.79
C	BD-03	26.03	83.27	30.1	83.17	30.75	83.15	4.72
	BD-04	26.2	83.29	30.26	83.17			4.55
PC						25.93	82.83	
NC						36.06	79	

The saliva effect on the virion captured by SGNP was evaluated. The effectiveness results are summarized in table 2. The effectiveness value of control (PBS) was 5,0 whereas the effectiveness of samples (A, B and C) were vary ranged between 4.55 to 4.89. Compared to PBS (sample without saliva), the effectiveness of virion capture on saliva samples (A, B, and C) slightly decreased, ranging from 10%.

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Stability of virions and isolated RNA

Table 3. RNA stability during storage at room temperature.

Sample (SALIVA)	Day	SGNP				Non		Effectiveness Δ(Non - ppt)
		ppt		Sup				
		Ct (CP)	Tm#1	Ct (CP)	Tm#1	Ct (CP)	Tm#1	
Control (PBS)	1st	23.61	83.34	29.73	83.1	27.88	83.24	4.27
	2nd	23.81	83.25	32.11	83.09	29	83.14	4.07
	3rd	23.37	83.23	32.16	83.78	30.08	83.07	6.71
Saliva	1st	26.18	83	30.47	83.22	29.75	83.08	3.9
	2nd	34.74	83.23	31.76	82.92	30.42	82.98	-4.32
	3rd	36.13	83.28	33.58	82.85	32.29	82.85	-5.71
PC						24.42	83.67	
NC						-	61	

The stability of isolated RNA in ppt fraction has been tested for 3 days. As shown in table 3, the isolated RNA from control (PBS sample), did not have any degradation until 3 days (the Ct (CP) values were not changed), whereas isolated RNA in ppt fraction from saliva sample showed RNA degradation from day to day, as indicated by the change of Ct (CP) value.

Discussion

Indonesia is a country with the largest dengue endemic in the world. More than 100.000 cases of DF and DHF are reported annually (Hotta et al., 1970). DHF cases were first reported in 1870 (Karyanti *et al*., 2014) and since then, dengue cases have continued to rise, and outbreaked in 1973, 1988, 2007, and 2010 (Sueki *et al*., 2016). Although dengue fever case is a big problem in Indonesia, but until now, there is no specific dengue virus or drug or vaccine available yet. Therefore, early dengue virus diagnosis is important in order to reduce dengue cases. Various methods have been developed and commercialized. However, all the available dengue virus diagnostic methods are based on the patient's blood utilization. Many technical problems were faced when serum was used as a test sample such as the number puskesmas with laboratory test facilities, the population who spread to remote areas and far from the puskesmas, etc. Those facts had greatly affected the efforts of DHF management proclaimed by the government, including the type of diagnostic method. In this point, the development of non-invasive detection methods, without losing sensitivity is necessary.

Saliva contains various secreted compounds such as IgA, virions etc, that may able to be used for the detection of some acute diseases as well as infectious diseases (Sueki *et al*., 2016; Takayama *et al*., 2016; Javaid *et al*., 2016). The use of dengue-specific IgA, NS1 secreted into saliva and urine had been performed (Grape *et al*., 2011; Cuzzubbo *et al*., 1998; Vazquez *et al*., 2007; Chakravarti *et al*., 2007; Saksono *et al*., 2015). The commercialization of the IgA kit is also being tested. However, the sensitivity will vary depend on the type of dengue infected. (Saksono *et al*., 2015). Korhonen et al (2014) reported that the PCR real time method could detect dengue virus present in saliva from day 2 to day 14, although with sensitivity ranging from 60 to 70% (Essi *et al*., 2014). Another study showed that the detection of dengue virus from saliva with real time RT PCR is possible (7, Essi *et al*., 2014; Mizuno *et al*., 2007; Hirayama *et al*., 2012). However, the number of virions present in saliva is much less rather than those in serum (Poloni *et al*., 2010, Mizuno *et al*., 2007). Therefore, an effort such as raising the concentration of virion is required to increase the sensitivity of the method. We had reported our successfully developed a new highly sensitivity of dengue detection technique with only 6 µL serum (Saksono *et al*., 2015). Therefore, we set an *in*

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vitro study to evaluate the potential uses of the method to detect the virion in saliva, including the effectiveness of SGNP in capturing the virion, and the stability of RNA during storage. The strategy for capturing the virion from saliva solution was shown in figure 1.

The effectiveness of SGNP in capturing virion was evaluated using gradually diluted virion in PBS. As shown at table 1. The capturing efficiency is increasing as well as the increasing dilution factor. This is due to the number of virion is decreasing gradually. Interestingly, even the Ct(CP) value of Non-fraction with $1x10^{-6}$ dillution was 34.47, additional SGNP had increased the Ct(CP) value (of 26.63). The data suggested that SGNP can efficiently captured the virion and improved the sensitivity of dengue detection using PCR amplification method.

Next, we evaluate the effect of saliva on the capturing virions. Saliva were collected from 3 volunteers and then were used to prepare the sample virion (with dilution factor of $1x10^{4}$) in 1% saliva/PBS. We used PBS solution as control. As shown at table 2, the effectiveness of SGNP only reduced up to 10% when samples contain 1% of saliva. This decrease in effectiveness, was due to inhibition by the compounds contained in the saliva and or the viscosity of the samples. However, if we compared the ability of SGNP to increase the Ct(CP) value from 34.47 to 26.63 in table 1(samples with dilution factor $1x10^{-6}$), the decrease value of 0.5 ~1 will not affect significantly into effectiveness of SGNP in capturing the virion. Then, we evaluated effect of saliva on the stability of RNA during storage at room temperature. As we know, that saliva contains many biological active compounds that may degrade the RNA during storage. Our observation for 3 days storage showed that the Ct(CP) value in ppt fraction of samples decreased gradually, whereas those of control remained stable. This suggested that saliva contains active compounds that may cause RNA degradation. On the other hands, Ct(CP) value of non-fraction also slightly decrease during storage. However, data showed that the virion form is more stable rather than ppt form. The data also suggested the easiness in handling samples when its should be send from far away. We can just collect the saliva from patients suspected of dengue hemorrhagic fever from the village and send to the laboratory in city.

Overall, in vitro assay results of the SGNP method for detecting dengue virus from saliva have a great chance to be applied. Many benefits such as easiness in handling the samples from the place that may far away from laboratory, the high stability of virion, and highly effectiveness of SGNP in capturing the virion in the sample containing 1% saliva, greatly contributes in the utility of saliva in dengue detection system. However, Clinical trials using saliva from DHF patients as well as suspected DF will be done, in collaboration with UI Medical Faculty.

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