International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Circulation of Dengue Serotypes in Local Population of District Lahore, Pakistan

Ammad ud din[a], Imran Riaz Malik[b*], Rabail Alam[c], Ghulam Mujtaba[d], Mehmood-ul-Hassan Qazi[e]

[a,c,e] *Institute of Molecular Biology and Biotechnology (IMBB), The University of Lahore, Pakistan*

[b] *Department of Biotechnology, University of Sargodha, Pakistan*

[d] *Institute of Nuclear Medicine and Oncology, Lahore, Pakistan*

[b] *Email: imranriaz428@gmail.com*

Abstract

Infection with dengue virus (DENV) is considered as serious public health issues internationally as amounted to 2.5 billion people are at infection risk throughout the world. Dengue is now endemic in Pakistan. Till now, no licensed vaccine is available against dengue virus infection. The main purpose of this study was to find out differences in the levels of IgM and IgG on gender basis as well as distribution of dengue serotypes in the local population of district Lahore, Pakistan. Fifteen blood samples including 3 control sample were collected from dengue infected patients and statistical results showed significantly higher mean levels of IgM (1.12 $\pm$ 0.09) and IgG (2.07 $\pm$ 0.56) antibodies in patients as compared to control groups for IgM (0.34 $\pm$ 0.05) and IgG (0.10 $\pm$ 0.05) antibodies respectively. Statistical results on Gender base showed significantly higher mean levels of IgM (1.10 $\pm$ 0.19) in males and of IgG (2.27 $\pm$ 0.74) in females. The enveloped gene of 1.5 kb was successfully amplified through polymerase chain reaction and cloned in pTZ57R/T. The cloned gene was then confirmed through restriction digestion. Out of 15 dengue samples, only 3 dengue samples were successfully amplified using polymerase chain reaction which all belongs to serotype 2 In future, it may contribute in development of treatment to dengue infection with dengue virus type 2 which is more prevalent in district Lahore.

Keywords: Dengue; Envelope Encoding Gene; IgG; IgM; Serotypes Introduction.

---

* Corresponding author. Phone: +92334483020

E-mail address: imranriaz428@gmail.com.

1. Introduction

It is estimated that about 2.5 billion people are at the risk of dengue infection worldwide. Each year about 50 – 100 million dengue infections occurred throughout the world. Out of them more than 1.5 million individuals show the clinical presentation and about 500,000 infections lead towards severe disease conditions like dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Approximately about 25000 deaths report each year as a result of dengue infection, especially in children [20].

It is believed that dengue virus came in Pakistan in the form of infected mosquito eggs placed in tyres at Karachi sea port [1, 4]. Data suggested that in last 20 years about seven epidemics had been reported from Pakistan [17] but the outbreak appeared in 2011 was the worst outbreak in the history of Pakistan. This outbreak involved 23, 252 laboratory confirmed cases and 361 deaths. A large number of cases reported from Punjab province but Sindh province was also badly affected [22].

Infection with dengue virus (DENV) is considered as major public health issue internationally [13]. Dengue viruses are belongs to family Flaviviridae [23]. *"Aedes aegypti"* a species of mosquito considered as principle vector for the transmission of dengue virus [16]. *Aedes aegypti* larvae usually finds at standing clean water reservoirs like fresh water buckets, water storage containers and discard tyres [2, 9, 14, 5].

Dengue virus has positive sense, single stranded RNA genome consist of 10,700 nucleotides [15]. Dengue virus genome encodes three structural and seven nonstructural proteins [20]. The structural proteins are named as capsid (C), membrane (M) and envelope (E) and non - structural proteins are named as NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 [15]. Dengue virus exists as four serotypes. These four serotypes are antigenically distinct from each other denoted as DENV – 1, DENV – 2, DENV – 3 and DENV – 4 [7].

As there is no licensed vaccine available for dengue virus infection till now so a safe and effective vaccine is the urgently needed [21]. Till now all vaccines (in development phase) have aim to produce immunity against all four serotypes but there are hurdles in development of such vaccines [8]. The main purpose of this study was to find out differences in the levels of IgM and IgG on gender basis as well as distribution of dengue serotypes in the local population of district Lahore, Pakistan.

2. Materials & Methods

2.1. Collection of blood samples

Blood samples (2-3 ml) were collected from dengue virus patients with the help of expert technicians and consent of patients. Blood samples were drawn from forearm in 5 ml sterile BD syringes using aseptic vein puncture technique.

2.2. Primers designing

A set of serotype specific primers were designed to amplify envelope gene from serum sample of patients infected with dengue virus. Serotype specific forward (F) and reverse (R) primers were designed from gene bank sequence (Accession NO: JN030340) by using Primer 3 software (Table 1).

Table 1: List of oligonucleotides primers used for the amplification of enveloped gene

Primers Name	**Primer Sequence (5′ - 3′)**	**Amplified Product (kb)**
EDENV-1F	F':5-GGCGAATTCATGCGATGCGTGGGAAT-3 *EcoRI*	**1.5**
EDENV-1R	R':5-TACTCGAGCGCCTGAACCATGACTC-3 *XhoI*	
EDENV-2F	F':5-GCGGCAAGCTTATGCGTTGTATTGGAATA-3 *HindIII*	**1.5**
EDENV-2R	R':5-TACTCGAGAGCCTGCACCATAACTCCCAAA-3 *XhoI*	
REDENV-2F	F': 5-GCGGCAAGCTTGCTCCTTCAATGACAATG -3 *HindIII*	**1.5**
REDENV-2R	R': 5-TACTCGAGGCTCACAACGCAACCACTATC-3 *XhoI*	
EDENV-3F	F':5-GCGGTCGACATGAGATGTGTGGGAGT-3 *SalI*	**1.5**
EDENV-3R	R':5-CACTCGAGATAGAGTGTGATGATTCC-3 *XhoI*	

2.3. Screening of samples

Samples were screened on the basis of presence of dengue virus IgM and IgG antibodies. Only dengue virus IgM or IgG positive samples were selected for analysis. Viral RNA was isolated from 100ul patient sera using Nucleospin Viral RNA isolation kit (Machery Nagel) and cDNA was synthesized using reverse transcriptase.

2.4. Amplification of envelope gene

Two rounds of amplifications were carried out through Polymerase Chain Reaction (PCR); first round PCR was involved first set of specified primers (REDENV2) and 3µl cDNA as template. Thermal cyclic condition were: Initial Denaturing at 94 °C for 3 minutes, Denaturing at 94 °C for 1 minute, Annealing at 57 °C for 1 minute, Extension at 72 °C for 1 minute, Go to (2) Cycle 35 and Final Extension at 72 °C for 10 minutes. Second round Nested PCR was involved second set of specified primers (EDENV2) and first PCR product 2µl as template. Thermal cyclic condition were: Initial Denaturing at 94 °C for 3 minutes, Denaturing at 94 °C for 1 minute, Annealing at 56 °C for 1 minute, Extension at 72 °C for 1 minute, Go to (2) Cycle 35 and Final Extension at 72 °C for 10 minutes. Dream *Taq* Green PCR Master Mix (2x) was used for Polymerase Chain Reaction (PCR) according to manufacturer's protocol. PCR was performed in 0.2 ml PCR tubes containing 25µl total reaction mixture. Nuclease free water was used as PCR water.

2.4 Construction of cloning vector

Full length PCR amplified envelope gene (1485 bp) was then ligated to the pTZ57R/T vector (2886 bp) using T4 DNA ligase. After ligation, the constructed vector containing a gene of interest was named pAIQ.EDENV-2 (Figure 1).

2.5. Restriction analysis

The "Heat Shock Method" was used for the transformation of competent cells (DH5 alpha), with pAIQ.EDENV-2.The GeneJET Plasmid Miniprep Kit was used for plasmid isolation with inserted gene and were confirmed through restriction digestion with *HindIII and XhoI*. Agarose gel of 1.5% was used to analyze amplified product, plasmid DNA and restriction digestion.

2.6. Statistical analysis

Statistical analysis was performed on data using SPSS software. The results were interpreted and finally presented in graphical format.

3. Results

3.1. Samples screening

From 15 collected samples; 3 samples were negative control while remaining 12 samples were positive on the IgM and IgG basis. These positive samples were further screened through Polymerase Chain Reaction (PCR) by using set of specified primers for envelope gene of dengue virus type 2, 3 and 4. There were significant higher mean levels (1.12 ± 0.09) of IgM were observed in dengue patients as compared to the control group levels (0.34 ± 0.05) as $p < 0.05$. Same trend was found in IgG, where higher mean levels (2.07 ± 0.56) of IgG were observed in dengue patients as compared to the control group levels (0.10 ± 0.05) as $p < 0.05$ (Table 2).

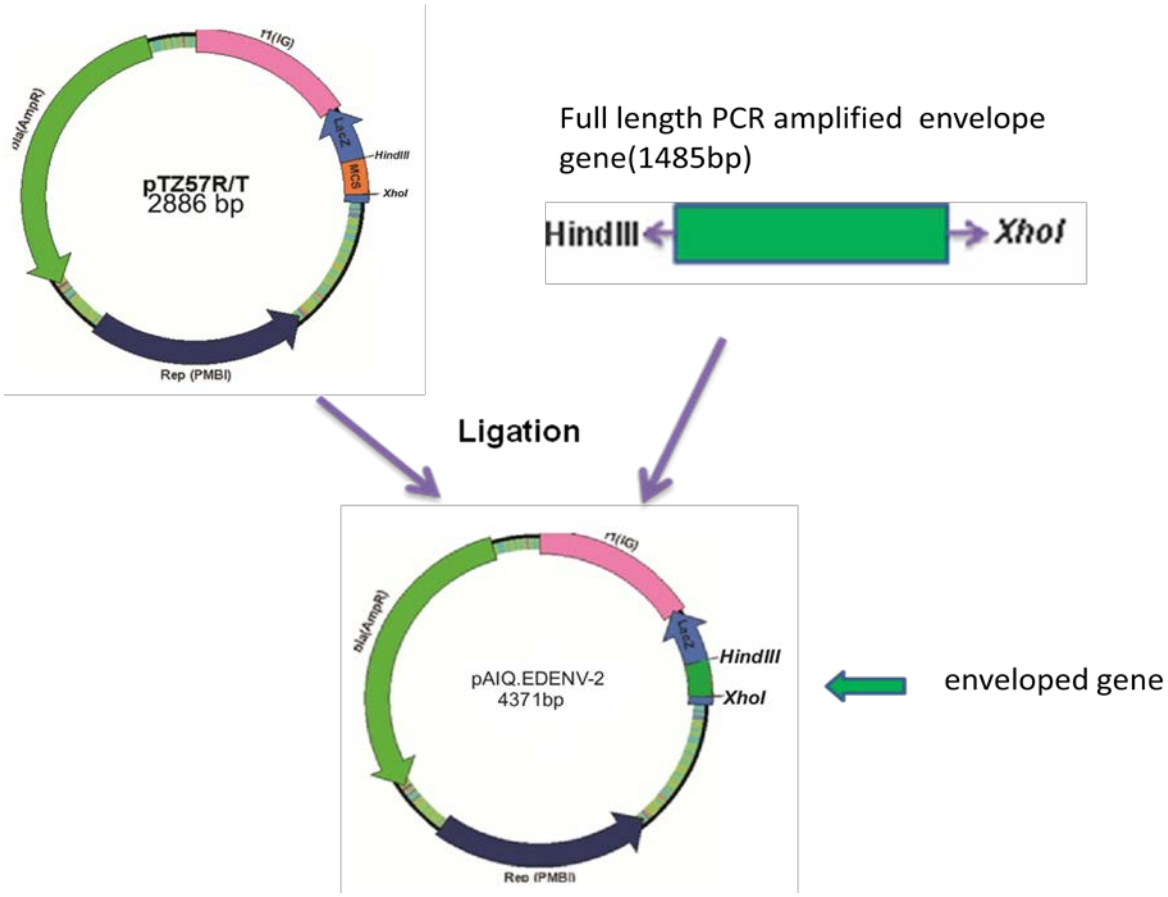

Figure 1: Construction of pAIQ.EDENV-2; Full length envelope gene was ligated to the pTZ vector. The resulted construct was designated as pAIQ.EDENV-2.

Table 2: Comparison of IgM and IgG in Dengue Patients and Control Group

	Groups	N	Mean	Std. Error Mean	p-Value
IgM	Control	3	0.34	0.05	0.002*
	Patients	12	1.12	0.09	
IgG	Control	3	0.10	0.05	0.005*
	Patients	12	2.07	0.56	

*Significant at P<0.05

Table 3 showed significant higher mean levels (1.10 $\pm$ 0.19) of IgM were observed in male as compared to the female levels (0.86 $\pm$ 0.12). But in the case of IgG opposite trends were found, where higher mean levels (2.27 $\pm$ 0.74) of IgG were observed in female as compared to the male levels (0.99 $\pm$ 0.58).

Table 3: Comparison of IgM and IgG on Gender Basis

	Gender	N	Mean	Std. Deviation	Std. Error Mean
IgM	male	7	1.10	0.52	0.19
	female	8	0.86	0.36	0.12
IgG	male	7	0.99	1.53	0.58
	female	8	2.27	2.11	0.74

3.2. Amplification of envelope gene

The envelope gene was PCR amplified by using two sets of specified primers. The amplified product were analyzed on 1.5% agarose gel, a targeted band of 1485bp was appeared (Figure 2).

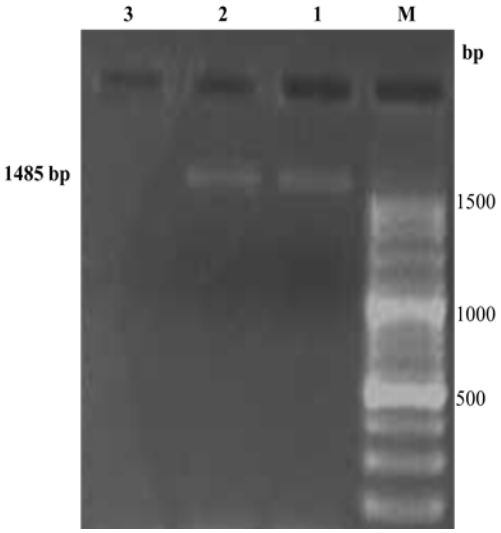

Figure 2: PCR amplified product of Envelope (E) Gene. Lane M (100 bp DNA Ladder); Lane 1 & 2 (Amplified envelope gene 1485 bp); Lane 3 (Negative control)

3.3. Restriction digestion

On digestion of recombinant plasmid (pAIQ.EDENV-2) with *HindIII and XhoI* restriction enzymes, two bands, one corresponding to pTZ57R/T vector (2886 bp) and second of envelope gene (1485 bp) were observed on 1.5% agarose gel (Figure 3) .

4. Discussion

Dengue viruses (DENVs), belongs to family Flaviviridae, are responsible for dengue infections in humans [23]. According to estimation more than 2.5 billion people lives in areas of infection risk and each year about 25000

deaths are reporting due to dengue virus infection [20]. Data shows that about seven epidemics had been reported from Pakistan during last 20 years [17] but the outbreak of 2011was the worst outbreak with 23, 252 laboratory confirmed cases and 361 deaths [22].

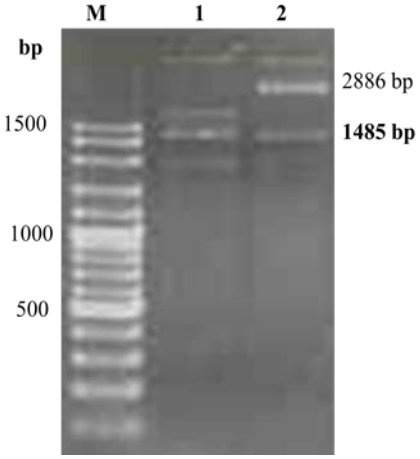

Figure 3: Restriction Digestion of pAIQ.EDENV-2. Lane M (100 bp DNA Ladder); Lane 2 (Upper band 2886 bp), (lower band1485 bp) .

The main purpose of this study was to find out differences in the levels of IgM and IgG on gender basis and distribution of dengue serotypes in the population of district Lahore, Pakistan. Fifteen blood samples were collected from dengue infected patients and statistical results showed significantly higher mean levels of IgM (1.12 $\pm$ 0.09) and IgG (2.07 $\pm$ 0.56) antibodies in patients as compared to control groups for IgM (0.34 $\pm$ 0.05) and IgG (0.10 $\pm$ 0.05) antibodies respectively. Statistical results on Gender base showed significantly higher mean levels of IgM (1.10 $\pm$ 0.19) in males and of IgG (2.27 $\pm$ 0.74) in females. Dengue IgM capture ELISA and IgG ELISA are both sensitive and specific assay for the detection of antibodies [3,11]. Assay that apply antigen from Dengue virus type 1 through 4 have a high sensitivity and specificity, range from 90 to 100% but unable to discriminate four serotypes [11, 18].

There are four antigenically distinct DENV serotypes DEN-1, DEN-2, DEN-3 and DEN-4 [6]. Among three structural proteins DENV envelope protein (~ 495 amino acids) has prime importance. As a surface protein, envelope (E) protein not only facilitates the attachment of DENV with host cell but also plays an important role in virus entry to the cell [19, 12]. Envelope (E) protein contains three structural domains, Domain I, II and III [10]. In the past, mostly domain III was selected for amplification and cloning purposes.

In this research work, full length envelope (E) gene of 1485 bp was amplified and cloned. The RNA was isolated from serum of dengue virus infected patients. PCR was performed by using set of specified primers (EDENV2) for dengue virus type 2. Gel electrophoresis was used to visualize the PCR product and then amplified product was purified. The purified product was cloned into pTZ57R/T vector to construct recombinant pAIQ.EDENV-2. The plasmid DNA from constructed clone was isolated and purified by restriction digestion with HindIII and XhoI. The results indicate the proper ligation of envelope (E) gene to pTZ vector.

It is suggested that further work can be planned for sub-cloning of envelope (E) gene in expression vector to construct fusion plasmid and transformation into competent host for bacterial expression. The antigenicity of expressed protein can also evaluate by using suitable animal model. There is also a hope that in future, the study can contribute in understanding of disease pathogenesis, development of a safe and effective vaccine, and in establishing novel diagnostic tools and therapeutics.

5. Conclusion

There were higher mean values of IgM and IgG exist for dengue virus patients as compared to the control group. At the same time IgM values were observed relatively high in males as compared to females while IgG values were observed relatively high in females as compared to male. Dengue serotype 2 is more prevalent in local population of Lahore district.

References

[1]. CG. Hayes. S. Baqar S. T. Ahmed. MA. Chowdhry. and WK. Reisen. “West Nile virus in Pakistan, Sero-epidemiological studies in Punjab Province.” *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 76, pp. 431-436, 1982.

[2]. DJ. Gubler. “Dengue and dengue haemorrhagic fever.” *Clinical Microbiology Reviews*, vol. 11, pp. 480-496, 1998.

[3]. DW. Vaughn. A. Nisalak, T. Solomon. S. Kalayanarooj. MD. Nguyen. R. Kneen. *et al*. “Rapid serologic diagnosis of dengue virus infection using a commercial capture ELISA that distinguishes primary and secondary infections.” *American Journal of Tropical Medicne and Hygien*, vol. 60, pp. 693-698, 1999.

[4]. E. Khan. R. Hasan. V. Mehraj. A. Nasir. J. Siddiqui. and R. Hewson. “Co-circulations of two genotypes of dengue virus in 2006 out-break of dengue hemorrhagic fever in Karachi, Pakistan.” *Journal of clinical virology*, vol. 43, pp.176-179, 2008.

[5]. E. Wang. H. Ni. R. Xu. ADT. Barrett. SJ. Watowich. DJ. Gubler. and SC. Weaver. “Evolutionary relationships of endemic/epidemic and sylvatic dengue viruses.” *Journal of Virology*, vol. 74, pp. 3227-3234, 2000.

[6]. EA. Henchal. and JR. Putnak. “The dengue viruses.” *Clinical Microbiology Reviews*. issue. 3, vol. 4, pp. 376-396, 1990.

[7]. EC. Holmes.and SS. Burch. “The causes and consequences of genetic variation in dengue virus.” *Trends in Microbiology*, vol. 8. Pp. 74-77, 2000.

[8]. EM. Castilow. MR. Olson. and SM. Varga. “Understanding respiratory syncytial virus (RSV) vaccine-enhanced disease.” *Immunology Research*, vol. 39, pp. 225-239, 2007.

[9]. F. Rodhain. "The role of monkeys in the biology of dengue and yellow fever." *Comparative Immunology Microbiology and Infectious Diseases*, vol. 14, pp. 9-19, 1991.

[10]. J. Abd-Jamil. C. Cheah. and S. AbuBakar. "Dengue virus type 2 envelope protein displayed as recombinant phage attachment protein reveals potential cell binding sites." *Protein Engineering Design & Selection*, Issue 21, vol. 10, pp. 605-611, 2008.

[11]. J. Groen J. P. Koraka. J. Velzing. C. Copra. AD. Osterhaus. "Evaluation of six immunoassays for detection of dengue virus-specific immunoglobulin M and G antibodies." *Clinical and Diagnostic Laboratory Immunology*, vol. 7, pp. 867-871, 2000.

[12]. JA. Mondotte. PY. Lozach. A. Amara. and AV. Gamarnik. "Essential role of dengue virus envelope protein N glycosylation at asparagine-67 during viral propagation." *Journal of Virology*, vol. 81, pp. 7136-7148, 2007.

[13]. JS. Mackenzie. DJ. Gubler. and LR. Petersen. "Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and Dengue viruses." *Nature Medicine*, vol. 10, pp. 98-109, 2004.

[14]. JSM. Peiris. WPI. Dittus. and CB. Ratnayake. "Seroepidemiology of dengue and other arboviruses in a natural population of toque macaques *(Macaca sinica)* at Polonnaruwa, Sri Lanka." *Journal of Medical. Primatology*, vol. 22, pp. 240-245, 1993.

[15]. KC. Leitmeyer. DW. Vaughn. DM. Watts. R. Salas. I. Villalobos. D. Chacon. *et al*. "Dengue virus structural differences that correlate with pathogenesis." *Journal of virology*, issue. 73, vol. 6, pp. 4738-4747, 1999.

[16]. LA. Moreira. I. Iturbe-Ormaetxe. JA. Jeffery. G. Lu. AT. Pyke. LM. Hedges. *et al*. "A *Wolbachia Symbiont* in *Aedes aegypti* limits infection with Dengue, Chikungunya, and *Plasmodium*." *Cell*, vol. 139, pp.1268-1278, 2009.

[17]. M. Idrees. W. Hussain. H. Rehman. GN. Tayyab. S. Afzal. Z. Fatima. *et al*. "Dengue virus serotype 2 (DEN-2): the causative agent of 2011-Dengue epidemic in Pakistan." *American Journal of Biomedical Sciences*, Issue. 4, vol. 4, pp. 307-315, 2012.

[18]. P, Koraka. H. Zeller. M. Niedrig. AD. Osterhaus. J. Groen. "Reactivity of serum samples from patients with a flavivirus infection measured by immunoflourescence assay and ELISA." *Microbes and Infection*, vol. 4, pp. 1209-1215, 2002.

[19]. RM. Marks. H. Lu. R. Sundaresan. T. Toida. A. Suzuki. T. Imanari. *et al*. "Probing the interaction of dengue virus envelope protein with heparin: assessment of glycosaminoglycan-derived inhibitors." *Journal of Medicinal Chemistry*, vol. 44, pp. 2178-2187, 2001.

[20]. SM. Tomlinson. RD. Malmstrom. and SJ. Watowich. “New approaches to structure - based discovery of dengue protease inhibitors.” *Infectious Disorders – Drug Targets*, issue. 9, vol.3, pp. 1-17, 2009.

[21]. WE. Brandt. “From the World Health Organization: development of dengue and Japanese encephalitis vaccines.” *Journal of Infectious Diseases*, vol. 162, pp. 577-583, 1990.

[22]. World Health Organisation(WHO).“Weekly epidemiological monitor.” Eastern Mediterranean Regional Office (EMRO), Cairo, Egypt, issue. 5, vol. 23, 2012.

[23]. Y. Lai. Y. Chung. H. Tan. H. Yap. G. Yap. EE. Ooi. and L. Ng. “Cost-effective Real-Time Reverse Transcriptase PCR (RT-PCR) to screen for dengue virus followed by Rapid Single-Tube Multiplex RT-PCR for serotyping of the virus.” *Journal of Clinical Microbiology*, issue. 45, vol. 3, pp. 935-941, 2007.