International Journal of Engineering Science Invention (IJESI)
ISSN (Online): 2319 – 6734, ISSN (Print): 2319 – 6726
www.ijesi.org ||Volume 8 Issue 03 Series. I || March 2019 || PP 78-84

Study on Virological Analysis of Dengue Viral Infection in Kazaure Local Government, Jigawa State

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Abstract:*The study focuses on Virological analysis of Dengue fever among the patients attending Kazaure General Hospital. A total of 233 serum samples were collected from patients with suspected cases of Dengue Fever (DF) from 2016 to 2018. Dengue virus was successfully isolated from 23 samples by culture on C6/36 and LLC-MK2 cells; it was then detected by indirect immunofluorescence assay (IFA). The cytopathic effect (CPE) of dengue virus on C6/36 appeared in most of the samples within 1-4 days post-inoculation comparing to 7-12 days on LLC-MK2 cells, and this was characterized by the ability to induce syncytia and multinucleated giant cells. On the other hand, by using RT-PCR technique, 23 (5.3%) samples were positive. All samples with positive cell culture results were detectable by RT-PCR. Dengue virus type 1 (DENV-1) was the dominant serotype followed by DENV- 3 and DENV-2, while DENV-4 was not detected in tested samples. These results indicate that DENV-RNA detection by RT-PCR is more sensitive than virus isolation. We suggest that the high sensitivity coupled with the turnaround time, have made the RT-PCR a better choice as a routine test for DENV diagnosis.*

Date of Submission: 17-02-2019 Date of acceptance: 03-03-2019

I. Introduction

Dengue virus (DENV) is the cause of dengue fever in Asia, and African countries including Nigeria. It is a mosquito-borne single positive-stranded RNAvirus of the family Flaviviridae; genus Flavivirus. The earliest change detectable on laboratory investigations is a low white blood cell count, which may then be followed by low platelets and metabolic acidosis. Isolation and diagnosis of Dengue virus rely on virological and serological investigations.

The number of reported dengue cases has increased since the 1980s due to factors such as unplanned urbanization, lack of surveillance and vector control, poor public health, international travel and virus and vector evolution (Guzman and Kouri 2002, Gubler 2011b).

Understanding risk factors to infection is important for public health control programs. The evaluation of male-female difference in infection rates for instance has been difficult to discern. Three independent studies from dengue epidemics in Singapore and India found that the risk of infection in males was two times higher in females (Goh et al. 1987, Agarwal et al. 1999, Wali et al. 1999). A few studies in South America including out recent study in Nigeria reveal that both sexes are equally affected (Vasconcelos et al. 1993, Rigau-Perez et al. 2001, Oyero and Ayukekbong 2014). Taken together, a comprehensive evaluation of sex difference in infection rate requires well-designed studies that would take into consideration both biological and social factors that drive dengue transmission in the population.

The contribution of climate change to DENV transmission has been investigated previously and the incidence and, in particular epidemics of dengue has been common during the rainy season (Hales et al. 1996, Keating 2001). The availability of favorable breeding grounds for the mosquito vector enhances the spread of DENVs. Due to water requirements for breeding, mosquito densities speak during the wet season, resulting in an increase in the number of dengue cases during this period (Hales et al. 2002). The poor drainage system and inadequate waste disposal in most Nigeria cities results in the presence of stagnant water bodies and water collected in waste metal containers and vehicle tires. These media serve as breeding sites for the mosquito vectors which are the agents of DENV transmission (Baba and Talle 2011). The increase in the number of susceptible individuals in these areas also enhances the risk of human to mosquito transmission and vice versa. Therefore, due to the nature of the route of infection, those at greatest risk of infection are those in regular exposure to the mosquito vector. A high IgGseroprevalence has be reported among adults >40 years of age compared to those younger than 40 years of age which is consistent with increased in vector exposure with age (Oyero and Ayukekbong 2014).

Dengue is the most rapidly spreading mosquito-borne viral disease with an estimated incidence of 390 million cases per years (Simmons et al. 2012, Bhatt et al. 2013). It is regarded as the most important arboviral

disease worldwide (Gubler 2011a) and it is estimated that every year between 2.5-3.6 billion people in over 125 endemic countries are at risk including 120 million travelers to these regions (Gubler 2002a, Guzman and Kouri 2002). About 2 million cases evolve to dengue hemorrhagic fever and about 20,000 may culminate to death (Gubler 2002a, Shepard et al. 2011). The first isolated case of dengue in Nigeria was in the 1960s (Carey et al. 1971, Amarasinghe et al. 2011), but dengue is not a reportable disease in this country with most cases often undiagnosed, misdiagnosed as malaria or referred to as fever of unknown cause. Dengue IgMseroprevalence of 30.8% was reported in Nigeria among febrile children (Faneyeet al., 2013), while another study in the north of the same country among healthy children revealed a seroprevalence of 17.2% (Oladipo et al., 2014). The finding from the later study needs to be interpreted with caution as it's not clear from the study when samples were collected considering it is well established that dengue IgM antibody production may last for a couple of weeks after infection (Schwartz et al. 2000). Our recent survey of dengue IgG antibodies in Ibadan, Nigeria showed a seroprevalence of 73% among febrile patients age 4 – 82 years. A further investigation of samples for active dengue infection by non-structural 1 (NS1) antigen analysis revealed an NS1 seroprevalence of 35% (Oyero and Ayukekbong 2014). These data are consistent with the fact that dengue is an endemic and emerging cause of fever in Nigeria. However, the disease is neglected, under recognized and under reported in Nigeria due to lack of awareness by health care providers and lack of prioritization by the public health authorities.

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Dengue virus (DENV) causes epidemics throughout the subtropical and tropical regions (Block et al., 1988; Guzman and Kouri, 2003). The disease is caused by a single stranded RNA virus. This virus belongs to the family Flavividae and has four closely related but serologically distinct types: DENV-1, DENV-2, DENV-3, and DENV-4 (Clarke, 2002). All four types are implicated in causing classical dengue fever (DF) or life–threatening syndrome, dengue hemorrhagic fever (DHF), which is characterized by abrupt onset of vascular leakage and the dengue shock syndrome (DSS) in DHF with evidence of poor perfusion, e.g. shock (Gubler, 1997, 1998). The virus is transmitted to human by bites of infected Aedes aegypti and Aedes albopictus mosquitoes (Chen and Wilson, 2004). The clinical picture ranges from rela- tively mild dengue fever, accompanied with characteristic symptoms such as fever, headache, retro- orbital pain, arthralgia, rash and myalgia, to severe DHF and DSS. The primary infection with one of the four Dengue serotype provides lifetime homologous immunity, but only weak cross protection against other serotypes. Secondary infection with a heterologous serotype is implicated in the increased association with DHF/DSS as the result of antibody-dependent enhancement (Guzman and Kouri 1996). The routine laboratory diagnosis of dengue virus infection is primarily achieved by RT-PCR (Drosten et al., 2002; Poersch et al., 2005; Gomes et al., 2007; Saxena et al., 2008; Yamada et al., 2002) and recently by NS1 dengue antigen Elisa. Serological diagnosis is carried out by detection of IgM and IgG antibodies by ELISA. Isolation of dengue virus in cell culture also plays an important role in dengue detection and serotyping. The first Isolation of DENV from a fatal case of DHF in adults in Dutse jigawa was reported after the first dengue outbreak in 1994 by Fakeeh and Zaki (2001). This paper report the results of dengue surveillance of serum samples tested from 2016 to 2018 in Arbovirus Unit of Pasteur Institute, Dakar, Senegal using two cell lines C6/36 and LLC-MK2 followed by virus detection using indirect immunfluroscence assay (IFA). In parallel, dengue virus RNA was directly detected in the same serum samples by RT-PCR. DENV typing was carried out by IFA using specific monoclonal antibodies against dengue 1 to 4 and by RT-PCR using serotype-specific primers.

II. Materials And Methods

Cell cultures

A. albopictus mosquito cells line (C6/36) is the most commonly used cell culture system as it provides a relatively rapid, sensitive, and economic means of dengue virus isolation (CRL-1660, ATCC). This cell line was grown in Eagle minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), and 2 mM glutamine under 5% CO2 at 28°C (Tesh, 1979; Kuno et al., 1985; Diamond et al., 2000; Ter Meulen et al. 2000). The mammalian rhesus monkey kidney cells, LLC-MK2 were cultured in Dulbecco modified Eagle medium (DMEM) supple- mented with 10% FBS, Penicillin (5 U/ml) and streptomycin (5 g/ml). The cells were incubated at 37°C in 5% CO2 atmosphere (Guzman et al., 1984).

Patient samples

A total of 233 serum samples were collected from patients hospitalized at Kazaure General Hospital between January 2016 and February 2018. All samples were taken from patients 1 to 8 days after onset of suspected dengue symptoms (fever, retro-orbital pains, muscle pains…etc). Serum samples were tested immediately or stored at - 80°C until use. A convalescent sample for viral diagnostic serology could not be collected two weeks after fever onset because the majority of patients did not return for follow-up investigations.

Dengue virus isolation on cell culture

These two cell lines C6/36 and LLC-MK2 have been routinely used in our laboratory for dengue virus isolation. Patient serum was diluted 1:10 with medium and passed through a 0.22 µm filter, 100 µl of the filtered diluted serum was added to 80% confluent monolayer of C6/36 or LLC-MK2 cells in cell culture tubes and incubated for 1h at 28°C for C6/36 cells and at 37°C for LLC-MK2 cells. After adding 1 ml of medium, cultures were incubated and examined daily for appearance of cytopathological effect (CPE). The CPE of dengue virus in C6/36 appeared between 1 to 4 days post inoculation while in LLC-MK2 around 7 to 12 days. Infected cells with CPE were harvested and tested by IFA using a polyclonal antibody pool. If CPE did not appear after 7 days on C6/36 or after 12 days on LLC-MK2, the infected cells were still harvested and tested by immunofluorescence for identification of DENV. For serotyping dengue virus specific monoclonal antibodies (Robert Koch-Institute in Berlin /Germany) against serotypes 1 to 4 were used with dilution of 1:50 in PBS (Henchal et al., 1983).

Polyclonal antibody pool

Human sera with high antibodies Immunoglobulin G (IgG) titers in capture ELISA (PanBio, Australia), were pooled and filtered with 0.45 µm Nunc Filter. Pooled sera were tested with ELISA and IFA and the optimal dilution for IFA primary antibody was determined (data not shown). This pooled serum was used as internal laboratory reference serum.

Indirect immunfluorescence assay (IFA)

DENV infected C6/36 or LLC-MK2 cells were mixed with non- infected cells and deposited on Teflon coated 8-well slides. The slides were air dried inside a bio-safety cabinet and fixed in chilled acetone/methanol (1:1) for 20 min., the wells were overlaid with 30 µl of specific polyclonal (Serum pool 1:1000 dilution) or monoclonal antibodies against DENV for serotyping. The slides were incubated in a moist chamber at 37°C for 60 min before they were washed three times in PBS. The bound antibody was detected with fluorescein-isothiocyanate (FITC)-conjugated goat anti-human IgG (Sigma, Chemicals Co.) diluted 1:1000 PBS if the first antibody is human serum or goat anti-mouse IgG (Sigma, Chemicals Co.) diluted 1:500 in PBS and 0.2% Evans blue (Sigma Chemicals Co.) if the first antibody is mouse monoclonal antibodies. The slides were washed, mounted with Fluoprep (BioMerieux) and finally, examined under a Leitz fluorescence microscope.

Detection and typing of dengue virus by real time RT-PCR

RT-PCR was performed on the 233 patients' serum samples. The light cycler (Roche v 2.0) was used. Viral genome was amplified using Oligonucleotide primers as described by (Drosten et al., 2002) and PCR products were detected using FAM-labeled hybridization probes, 20 pmol/µl of each primer (TIB-MOLBIOL, DenS2: 5'-GGATAGACCAGAGATCCTGTGT, DenAs1-3: 5'- CATTCCATTTTCTGGCGTTC, DenAs4: 5'-CAATCCATCCATCTT- GCGG-CGCTC) and 10 µM of DENV probe (TIB-MOLBIOL, DenP: 5'-6FAM-CAGCATCATTC CAGGCACAGTXT--PH), Specific Oligonucleotide with 5' FAM-labeled probe is designed for each type of DENV, preparing 4 tubes for each sample using Qiagen QuantiTect Probe RT-PCR kit.

Table 1. characterization of Dengue virus stains isolated fron dengue patients in kazaure local govt.

No. of sera tested	Dengue virus isolation in cell culture					Dengue virus Detection by RT-PCR				
	Positive virus isolation (%)	DENV serotypes identified by monoclonal antibodies (%)				RT-PCR positive (%)	DENV serotypes typing by RT-PCR (%)			
		1	2	3	4		1	2	3	4
199	64	61	1	2	0	78	75	1	2	0
25	4	2	0	2	0	6	5	0	1	0
9	2	2	0	0	0	3	2	0	1	0
233	70 (30.04)	65 (92.8)	1 (1.4)	4 (5.7)	0 (0)	87 (37.3)	82 (94.3)	1 (1.1)	4 (4.6)	0 (0)

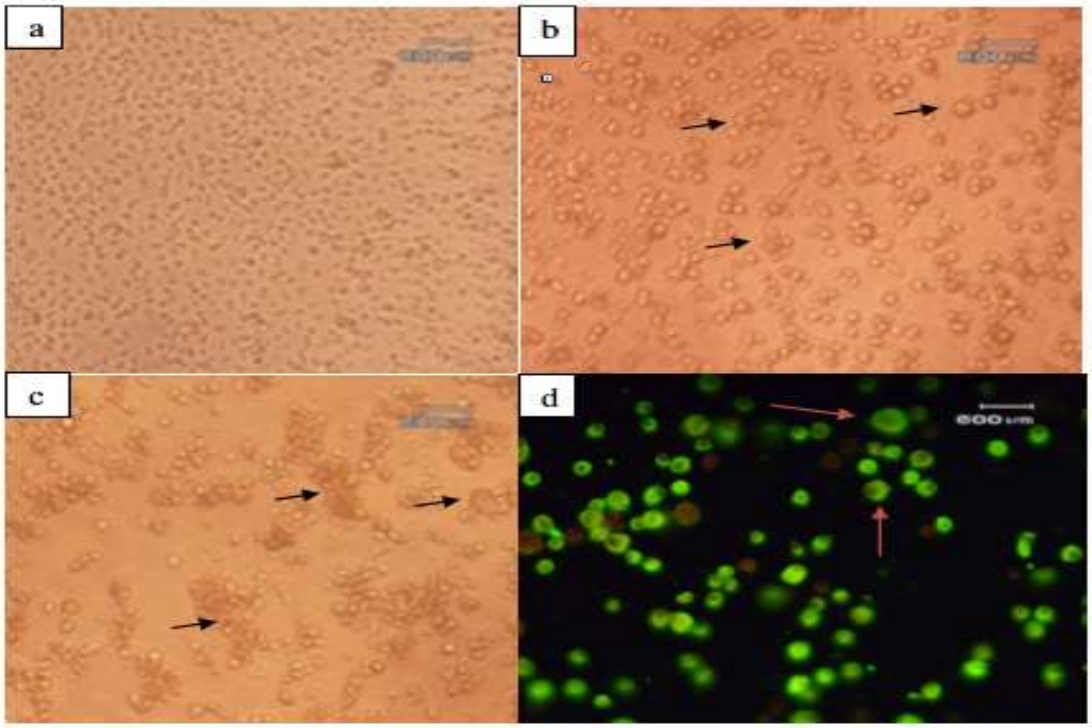

Figure 1. Dengue infected C6/36 cells. a) Monolayer of uninfected C6/36 cells. b) CPE of dengue virus, 1 day after inoculation of C6/36 cells with patient serum. The infected cells became round with light swelling and few aggregations. c) On the second day after virus inoculation, increased cell aggregation and formation of multinucleated giant cells as a result of cell-cell fusion. (arrows). d) Dengue infected C6/36 cells, 5 days after infection stained with specific monoclonal antibodies against serotype 1.

III. Results

Dengue virus isolation and CPE characterization

Dengue virus infection was confirmed in 23 out of 233 cases by inoculation in C6/36 cells and in LLC-MK2 cells (Table-I). The CPE of dengue virus in C6/36 appeared between 1 to 4 days post inoculation, but in LLC-MK2 after 7-14 days. CPE in C6/36 cells was characterized as follow: first, the cells became round and swollen, then small aggregates would appear, as shown in (Figure 1b and 1c). Finally, after several days multinucleated giant cells, syncytia, and many degenerated cells and cell debris were observed. Some cells showed necrosis and become detached from the tubes at later stages of infection. The CPE of DENV in LLC-MK2 cells was characterized by the ability of these cells to form multinucleated giant cells and syncytia after 7 to 14 days post inoculation with patient's serum. The rounding and swelling of the giant LLC-MK2 cells were well marked as shown in (Figure 2b and 2c). Infected cells with CPE appearance were havested and tested by IFA using the polyclonal antibody pool (1:1000). Positive cultures exhibit clear positive cytoplasmic fluorescence (Figure 2d).

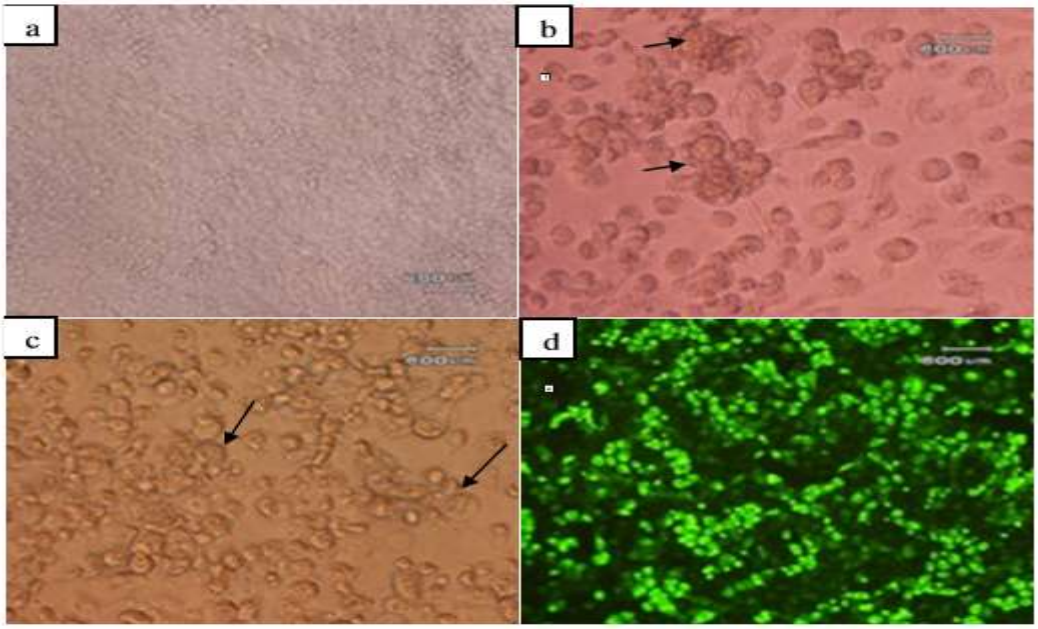

Figure 2. Dengue infected LLC-MK2 cells. a) Confluent monolayer of LLC-MK2 non-infected monkey kidney cells. b) The infected cells became round with light swelling and few aggregations. c) Seven days after inoculation of LLCK2 cells with serum from infected patient with DF, diffuse type of CPE typical of DENV all over the monolayer. Infected cells became rounded, swollen, syncytia and multinucleated cells can be seen. Also some degenerated cells (arrows) Giant cell formation and aggregation characterize the LLC-MK2 cells. d) Indirect Immunoflourescent antibody testing with polyclonal antibodies identifying dengue virus in tissue culture of LLC-MK2 cells. Dengue infected LLC-MK2 cell, 10 days after infection using polyclonal antibodies (1:1000).

Dengue virus typing by IFA using monoclonal antibodies

Figure (1d) shows positive immunofluorescence tested in the cytoplasm with the nucleus laying at the edge of the cell (eccentric). This is typical for the typing results for the three dengue forms in our studies. Typing of 23 isolated dengue virus strains showed that three dengue serotypes were detected simultaneously in the three year period from 2016 to 2018. Serotype DENV-1 was the main isolated serotype (19 of 23 positive samples), followed by dengue type 3 (3 of 23 positive samples) and dengue type 2 (1 of 23 positive samples) DENV-4 was not found.

IV. Discussion

Dengue virus isolation on cell culture from patient's serum and mosquitoes remains the "gold standard," but this method is still difficult due to several reasons:

- The lower sensitivity of cells in comparison with RT-PCR.
- A longer time needed (1 to 2 weeks) for virus growth, detection by IFA and to identify the isolated virus with serotype-specific monoclonal antibodies, as DENV sometimes requires multiple passages before inducing CPE in the infected cells.
- The isolation of dengue virus from clinical specimens is frequently possible only during the viraemic-phase (acute dengue fever) which occurs in the first 5 days after onset of the symptoms.
- The viability of virus isolation depends on the proper handling and prompt delivery of the specimen to the laboratory.
- The CPE produced in mosquito cell culture by many dengue viruses is difficult to detect, and it can be morphologically variable.
- The work in a Bio-safety Laboratory Level three (BSL-3) is required for culturing of dengue virus in many countries including Nigeria

Therefore many laboratories in the world do not perform dengue virus isolation in cell culture routinely. In recent years, RT-PCR has proved to be very useful in dengue diagnosis and has been able to detect dengue viruses up to the 8th day after the onset of the symptoms (Yong Yean Kong et al., 2006). Most important, it is rapid and can be done in few hours after receiving the sample; consequently, a proper treatment can be started soon enough (within 4 h) to avoid complications. Finally RT- PCR has demonstrated high sensitivity and specificity (Dorsten et al., 2002). However, the virus isolation in cell culture cannot be replaced completely by RT-PCR, as the isolation of live DENV on cell culture will still remain a valuable tool for further dengue research such as; genome sequencing, preparation of vaccine, immunizing of animals, performing of

animal experiments to study the pathogenesis of the disease, and producing of monoclonal and polyclonal antibodies for diagnostic proposes.

In this study the molecular method based on RT-PCR has been compared with the cell culture method, The results have shown that the application of RT-PCR on 23 culture-positive blood samples gave a specificity and sensitivity of 100% and allowed a rapid diagnosis. In contrast, virus isolation was not successful on all RT-PCR positive samples; only 20 virus isolates were detected from 23 RT-PCR positive serum samples. These results indicate that RT-PCR technique is more sensitive than virus isolation from clinical samples. It must be emphasized, however, that RT-PCR should not be used as a substitute for virus isolation. The availability of virus isolates is important for characterizing virus strain differences, since this information is critical for viral surveillance and pathogenesis studies. Dengue virus serotype analysis is important in epidemiological and pathological studies. Three dengue serotypes were detected over the three-year period from 2016 to 2018. Typing of the disease in this study was done using specific monoclonal antibodies and by RT- PCR. We have found no discrepancies between serotypes detected in RT-PCR and IFA. Our dengue typing results indicate that dengue virus types 1, 2 and 3 still circulating in Nigeria, dengue serotype 1 being responsible for most of our severe DF and DHF during this period). During 1994 to 1997 the study of Fakeeh and Zaki. (2001) had shown that DENV-2 was the most frequent serotype in primary dengue constituting (66.7%) of the positive samples, followed by DENV-1 and DENV-3. Our present paper demonstrates that DENV-1 was the dominant isolate followed by DENV-3 and DENV-2 during study period from 2016-2018. The long-term persistence of dengue virus and continuously circulation of three serotypes 1, 2, 3 since 1994 in Nigeria, with one serotype emerging as the cause of each periodic epidemic probably indicates that the circulating serotype changes over time as has been demonstrated in other endemic situations for dengue viruses (De Simone et al., 2004; Nisalak et al., 2003, Cummings et al., 2004; Timothy et al., 2002). Multiple factors influence dengue transmission and serotype circulation in Nigeria like the cycle of epidemics, the emergence of new dominant serotypes, local environment, vector breeding, the pre-existing dengue serotype specific antibodies in population over time and their capacity to induce epidemics or severe dengue diseases. All these factors required extensive study by concerned Jigawa authorities to prevent and minimize future outbreaks among local residents.

References

[1]. Ayyub M, Khazindar AM, Lubbad EH, Barlas S, Alfi AY, Al-Ukayli S (2006). Characteristics of dengue fever in a large public hospital, Nigeria.

[2]. J. Ayub. Med. Coll. Abbottabad, 18: 9-13. Block J, Kay BH, Hall RA, Gorman BM (1988). Isolation and characterization of dengue viruses serotype 1 from an epidemic in northern Queensland, Australia. Arch. Virol., 100: 3-4, 213-220.

[3]. Chen LH, Wilson ME (2004). Transmission of dengue virus without a mosquito vector: nosocomial mucocutaneous transmission and other routes of transmission. Clin. Infect. Dis., 39 (6): e56-e60.

[4]. Cummings DA, Irizarry RA, Huang NE, Endy TP, Nisalak A, Ungchusak K, Burke DS, Yamada K, Takasaki T, Nawa M, Kurane I (2004). Travelling waves in the occurrence of dengue hemorrhagic fever in Thailand. Nature, Jan 22; 427(6972): 344-447.

[5]. Clarke T (2002). Dengue virus: break-bone fever. Nature, 416 (6882): 672-674.

[6]. De Simone TS, Nogueira RMR, Araújo ESM, Guimarães FR, Santos FB, Schatzmayr HG, Souza RV, Teixeira Filho G, Miagostovich MP (2004). Dengue virus surveillance: the co-circulation of DENV-1, DENV-2 and DENV-3 in the State of Rio de Janeiro, Brazil. Trans R Soc. Trop. Med. Hyg., 98(9): 553-562.

[7]. Diamond MS, Edgil D, Roberts TG, Lu B, Harris E (2000). Infection of human cells dengue virus is modulated by different cell types and viral strains. J. Virol., 74(17): 7814-7823.

[8]. Drosten S, Gottig S, Schilling S, Asper M, Panning M, Schmitz H, Guenther S (2002). Rapid detection and quantification of RNA of Ebola and Marburg viruses, Lassa virus, Crimean-Congo hemorrhagic fever virus, Rift Valley fever virus, dengue virus, and yellow fever virus by real-time reverse transcription-PCR. J. Clin. Microbiol., 40(7): 2323-2330.

[9]. Fakeeh M, Zaki AM (2001). Virologic and serologic surveillance for dengue fever in Jeddah, Saudi Arabia, 1994-1999. Am. J. Med. Hyg., 65(6): 764-767.

[10]. Gomes AL, Silva AM, Cordeiro MT, Guimaraes GF, Marques ETJr, Anath FG (2007). Single-tube nested PCR using immobilized internal primers for the identification of dengue virus serotypes. J. Virol. Methods, 145(1): 76-79.

[11]. Guzman G, Kouri G (2003). Dengue and dengue hemorrhagic fever in the Americas: lessons and challenges. J. Clin. Virol., 27(1) 1-13.

[12]. Guzman G, Kouri FG, Soler NM, Morier DL, Vazquez RS (1984). Isolation of dengue virus type 2 from the serum of patients using the suckling mouse and cultured LLC-MK2 cells]. Rev. Cubana Med. Trop., 36(1): 4-10.

[13]. Gubler DJ (1997). Dengue and dengue hemorrhagic fever: its history and resurgence as a global public health problem. In: Gubler DJ, Kuno G., editor. Dengue and dengue hemorrhagic fever. London: CAB International.; pp. 1–22.

[14]. Gubler DJ (1998). Dengue and dengue hemorrhagic fever. Clin. Microbiol. Rev., 11(3): 480-496.

[15]. Guzman MG, Kouri G (1996). Advances in dengue diagnosis. Clin. Diagn. Lab. Immnunol., 3: 621-7.

[16]. Henchal EA, McCown JM, Seguin MC, Gentry MK, Brandt WE (1983). Rapid identification of dengue virus isolates by using monoclonal antibodies in an indirect immunofluorescent assay. Am. J. Trop. Med. Hyg., 32: 164–169

[17]. Khan NA, Azhar EI, El-Fiky S, Madani HH, Abuljadial MA, Ashshi AM, Turkisatni AM, Hamouh EA (2008). Clinical profile and outcome of hospitalized patients during first outbreak of dengue in Makkah, Saudi Arabia Acta Tropica, 105(1): 39-44.

[18]. Kong YY, Thay CH, Tin TC, Devi S (2006). Rapid detection, serotyping and quantitation of dengue viruses by TaqMan real-time one-step RT-PCR. J. Virol. Methods, 138(1-2): 123-30.

[19]. Kuno G, Gubler DJ, Velez M, Oliver A (1985). Comparative sensitivity of three mosquito cell lines for isolation of dengue viruses. Bull. World Health Organ, 63(2): 279-286.

[20]. Nisalak A, Endy TP, Nimmannitya S, Kalayanarooj S, Thisayakorn U, Scott RM, Burke DS, Hoke CH, Innis BL, Vaughn DW (2003). Serotype specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. Am. J. Trop. Med. Hyg., 68(2): 191–202.
[21]. Poersch CDO, Pavoni DP, Queiroz MH, Borba DL, Goldenberg S, Santos CND, Krieger MA (2005). Dengue virus infections: comparison of methods for diagnosing the acute disease. J. Clin. Virol., 32(4): 272-277.
[22]. Saxena P, Dash K, Santhosh SR, Shrivastava A, Parida M, Rao Pl (2008). Development and evaluation of one step single tube multiplex RT-PCR for rapid detection and typing of dengue viruses. Virol., J. 5(1): 20.
[23]. Tesh RB (1979). A method for the isolation and identification of dengue viruses, using mosquito cell cultures. Am. J. Trop. Med. Hyg., 28(6): 1053-1059.
[24]. Ter Meulen J, Grau M, Lenz O, Emmerich P, Schmitz H, Oh F, Jaspert R, Niedrig M (2000). Isolation and partial characterization of dengue virus type 2 and 4 strains from dengue fever and dengue haemorrhagic fever patients from Mindanao, Republic of the Philippines. Trop. Med. Int. Health, 5(5): 325-329.
[25]. Timothy PE, Nisalak A, Chunsuttiwat S, Libraty DH, Green S, Rothman AL, Vaughn DW, Ennis FA (2002). Spatial and Temporal Circulation of Dengue Virus Serotypes: A Prospective Study of Primary School Children in Kamphaeng Phet, Thailand. Am. J. Epidemiol., 156: 52- 59.
[26]. Yamada K, Takasaki T, Nawa M, Kurane I (2002). Virus isolation as one of the diagnostic methods for dengue virus infection. J. Clin. Virol., 24(3): 203-209.

Mustapha Bashir Kazaure" Study on Virological Analysis of Dengue Viral Infection in Kazaure Local Government, Jigawa State" International Journal Of Engineering Science Invention (Ijesi), Vol. 08, No. 03, 2019, Pp 78-84