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DETECTION OF CHIKUNGUNYA VIRUS FROM SERA OF DENGUE-SUSPECTED PATIENTS IN THE PHILIPPINES

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ABSTRACT

Due to the similar symptoms with dengue fever (DF) and dengue hemorrhagic fever (DHF), Chikungunya (CHIK) virus infection has been diagnosed as DF or DHF. In this study, we examined more than 300 serum samples collected from dengue suspected patients in San Lazaro Hospital and St. Luke's Medical Center. Reverse transcriptase-polymerase chain reaction (RT-PCR) and antigen sandwich enzyme-linked immunosorbent assay (ELISA) were applied for CHIK virus detection. IgM-indirect immunofluorescence antibody test (IgM-IFA), IgM capture ELISA and IgG indirect ELISA were applied for detection of serological evidence of CHIK virus infection. Although we could not detect any Chikungunya virus by RT-PCR, 4.1% of the examined samples showed high titer for Chikungunya virus by antigen sandwich ELISA. By

IgM-FA, 15.3% was positive for CHIK virus. Thirteen point seven percentages showed positive for CHIK virus by IgM-capture ELISA. Eighteen point five percentages showed positive for CHIK virus by IgG indirect ELISA.

Key words: Chikungunya (CHIK) virus, dengue virus, dengue fever (DF), dengue hemorrhagic fever (DHF), RT-PCR, antigen sandwich ELISA, IgM-IFA, IgM capture ELISA, IgG indirect ELISA, Philippines

INTRODUCTION

Chikungunya (CHIK) virus belongs to genus *alphavirus* in the family *Togaviridae*. CHIK virus infection is one of the mosquito-borne diseases. It is transmitted by mosquito, *Aedes aegypti* and *Aedes furcifer-taylori* in Africa, India and southeast Asia including the Philippines (Hayes *et al.*, 1986). Due to the similar symptoms with dengue fever (DF) and dengue hemorrhagic fever (DHF), CHIK virus infection has been diagnosed as DF or DHF (WHO, 1997). CHIK virus infection sometimes caused outbreak (Carey *et al.*, 1969; Thaikruea *et al.*, 1997) and also disappeared 7 to 8 years to several decades (Burke *et al.*, 1985; Pavri, 1986).

Previously, Basaca-Savilla reported that 6.94% of 55 clinically diagnosed influenza cases had HI antibody for CHIK virus and 1.5% of 67 children aged 0 to14 years had HI antibody for CHIK virus in the Philippines (1966). Three cases of CHIK virus infection was diagnosed by IgM capture ELISA; the CHIK virus was also isolated from one of the patients in June and November 1985 and in January 1986 in the Philippines (Hayes *et al.*, 1986).

The aim of our study was to clarify the impact of CHIK virus infection among suspected DF/DHF patients in the Philippines. We examined more than 300 serum samples that were collected from dengue suspected patients in San Lazaro Hospital and St. Luke's Medical Center. Reverse transcriptase-polymerase chain reaction (RT-PCR) and antigen sandwich enzyme-linked immunosorbent assay (ELISA) were used for CHIK virus detection. IgM-indirect immunofluorescence antibody test (IgM-IFA), IgM capture ELISA and IgG indirect ELISA was applied for detection of serological evidence.

PATIENTS AND METHODS

Patients

All patients had been admitted to the San Lazaro Hospital or St. Luke's Medical Center as suspected DF or DHF patients. The clinical condition was graded by medical staff according to World Health Organization guidelines (WHO, 1986).

Virus and cells

C6/36 Aedes albopictus cells (Igarashi, 1978) were infected with CHIK virus (Kenyan Strain). CHIK virus was plaque purified three times using baby hamster kidney (BHK-21) cells.

RT-PCR

Serum separated from blood sample was inoculated to C6/36 cells then incubated 7days for dengue virus and CHIK virus detection. Viral RNA was extracted from 100 µl of infected culture fluid (ICF) of C6/36 cells by 300 µl of Trizol-LS (Gibco BRL, Grand Island, N.Y.) and 80 µl of chloroform. The mixture was centrifuged for 10 min at 14,000 x g, the aqueous phase was mixed with equal volume of 2-propanol, centrifuged for 15 min at 14,000 x g for 15 min for RNA precipitation. The RNA precipitate was washed once with 75% ethanol, and re-suspended in 20 µl of RNase-free water. The complementary DNA (cDNA) synthesis was performed for 30 min at 37 °C in 1.5ml tube containing 20 µl of RNA template, 200 units of reverse transcriptase (SuperScript TM II RT, Gibco-BRL), 50 µM of Random hexamer (Takara, Kyoto, Japan), RNase inhibitor (40unit, 5 Prime→3 Prime, Inc. Boulder, CO), 1 x conc. reaction buffer and 0.2mM of dNTP (Takara) in a 40 µl reaction volume. Oligonucleotide primers (CHIK/NS1/S and chik/ns1/c) for PCR were chosen from aligned nucleotide sequences of the nonstructural protein 1 (NS1) gene of CHIK and O'Nyong-nyong (ONN) viruses (Hasebe, personal communication). The PCR was performed in 0.2 ml thin-walled tube containing 5 µl of cDNA template, 1.25 units of Taq DNA polymerase (Amersham Pharmacia biotech, Arlington Heights, IL), 1 x conc. reaction buffer and 0.2mM of dNTP (Takara), 1 µM of sense primer and 1 µM of antisense primer in a 50 µl reaction volume. The condition for PCR was at 94 °C for 3min followed by incubation at 94 °C for 1 min, 54 °C for 1 min 30sec, 72 °C 2 min for 25 cycles in a model 2400 thermocycler (Perkin-Elmer Corp., Norwalk, CT). The last elongation step at 72 °C was increased 5 min to ensure complete extension of the PCR products.

Antigen sandwich ELISA

The antigen sandwich ELISA procedure selected was similar to that reported by Thant et al. (1995) with the following modifications. The 96-well flat bottom micro plate (Nalge Nunc International, Roskilde, Denmark) was coated overnight at 4 °C with 100 µl of capture antibody (256 ELISA units). The capture antibody was prepared from CHIK virus hyperimmunized rabbit. Each well was inactivated with 100 µl of Blockace (Yukijirushi, Japan) for 60 min at 37 °C, washed 3 times with PBS-0.05% Tween 20 for 3 min each and drained. After emptying and washing the plate, 100 µl of sample ICF and positive standard antigen were added in each of duplicate wells and incubated 60 min at 37 °C. Positive standard antigen (80 ELISA units) was prepared by inoculation of CHIK virus to BHK-21

cells in 5 days incubation at 37 °C. After the plate was washed, 100 µl of detection antibody (2000 ELISA units) was added in each well, then incubated for 60min at 37 °C. The detection antibody was selected from anti-CHIK virus IgG high titer human sera by CHIK virus IgG indirect ELISA. The plate was washed, and then reacted with 100 µl of horseradish peroxidase (HRPO)-conjugated anti-Human IgG goat serum (1:3000 dilution, Zymed Laboratories. Inc., So. San Francisco, CA). After 60 min incubation at 37 °C, the plate was washed, and 100 µl of substrate solution containing o-phenylenediamine dihydrochloride (OPD) and 0.02% hydrogen peroxide added. After 60 min incubation at room temperature in the dark, the reaction was stopped by adding 100 µl of 1N hydrochloric acid to each well. The optical density (OD) of the sample ICF of antigen sandwich ELISA were measured at 492 nm by an ELISA reader (SpectraMAX190, Molecular Devices Co., Sunnyvale, CA) exceeded positive standard antigen of that were considered as high titer.

IgM-IFA

The IgM-IFA procedure selected was similar to that reported by Henchal et al. (1982) with following modifications. Uninfected and infected BHK-21 cells mixed were at 2:3 ratio, then spotted on 8 well fluorescent antibody slides (ICN Biomedicals, Inc., Aurora, OH) 10µl of cell suspension in each well. Before staining, each slide was inactivated with Blockace (Yukijirushi, Sapporo, Japan) for 6o min at 37 °C, washed 3 time with PBS for 5min each and drained. Ten µl of the test and control sera diluted 1:10 with PBS-2% normal goat serum (Dako A/S, Denmark) were added to each well and incubated for 60 min at 37 °C in a humid chamber. After washing the slides as described above, the cells were reacted with 10 µl of FITC-conjugated anti-human IgM (µ chain specific) goat serum (MBL, Nagoya, Japan) diluted 1:800 with PBS-2%normal goat serum and incubated for 60min at 37 °C in humid chamber. After washing the slides as described above, mounted with FluoroGuard ™ Antifade Reagent (BioRad, Hercules, CA) and sealed with nail polish. The slides were viewed with 20 x or 40 x objectives using a fluorescent microscope Axiolab (Carl Zeiss, Germany).

IgM capture ELISA

The IgM capture ELISA procedure used was similar to that reported by Bundo and Igarashi (1985) with following modifications. The 96-well flat bottom micro plate (Nalge Nunc International) was coated with 100 µl of goat anti-human IgM (µ-chain specific, 5 FC units, Cappel/ICN Pharmaceuticals, Inc. Aurora, OH) overnight at 4 °C. Each well was inactivated with 100 µl of Blockace (Yukijirushi) for 60 min at 37 °C, washed 3 times with PBS-0.05% Tween for 3 min each and drained. After emptying and washing the plate, 100 µl of the test and control sera diluted 1: 100 with Blockace containing 10% serum bovine CADET (ICN Biomedicals, Inc.) were added into each duplicate wells and incubated for 60 min

at 37 °C. The wells on the plate were washed and 100 µl of CHIK virus antigen (64 ELISA units, prepared in C6/36 cells) was added and incubated for 60 min at 37 °C. After washing, the wells were reacted with 100 µl of CHIK virus hyperimmunized rabbit serum (262 ELISA units). The plate was incubated for 60 min at 37 °C, washed, and then reacted with 100 µl of HRPO-conjugated anti-rabbit IgG rat serum (1:1000 dilution, Zymed Laboratories, Inc.). After 60 min incubation at 37 °C, the plate was washed, and added 100 µl of substrate solution containing OPD and 0.02% hydrogen peroxide. After 60 min incubation at room temperature in the dark, the reaction was stopped by adding 100 µl of 1N hydrochloric acid. The OD at 492 nm was measured by an ELISA reader. A positive : negative (P:N) ratio was obtained by dividing the OD492 of the test specimen by OD492 of the negative standard. The specimen showing P:N ratio greater than or equal to 2.0 was considered positive, provided the standard positive specimen definitely showed positive result. Negative standard serum was selected from CHIK IgM IFA and CHIK IgG indirect ELISA double negative samples.

IgG indirect ELISA

The IgG indirect ELISA procedure selected was similar to that reported by Bundo et al. (1981) with following modifications. The 96-well flat bottom micro plate (Nalge Nunc International) was coated with 100 µl of CHIK virus antigen (20µg/ml) overnight at 4 °C. CHIK virus antigen was prepared by ultracentrifugation using Sucrose gradient. Each well was inactivated with 100 µl of Blockace (Yukijirushi) for 60 min at 37 °C, washed 3 times with PBS-0.05% Tween 20 for 3 min each and drained. After emptying and washing the plate, 100 µl of the test sera diluted 1: 100 with Blockace containing 10% serum Bovine CADET (ICN Biomedicals, Inc.) and two fold serially diluted positive standard serum were added into each duplicate wells and incubated for 60 min at 37 °C. The plate was washed, and then reacted with 100 µl of HRPO-conjugated anti-Human IgG goat serum (1:3000 dilution, Zymed Laboratories, Inc.). After 60 min incubation at 37 °C, the plate was washed, and added 100 µl of substrate solution containing OPD and 0.02% hydrogen peroxide. After 60 min incubation at room temperature in the dark, the reaction was stopped by adding 100 µl of 1N hydrochloric acid. The OD at 492 nm was measured by an ELISA reader. The titers of the samples were determined by comparison with serially diluted positive standard. The titer greater than or equal to 100 was considered as positive.

RESULTS AND DISCUSSION

RT-PCR

No positive sample was found in 315 samples examined by CHIK RT-PCR using CHIK NS1 primers. We also examined 44 of cDNA samples for PCR using Toga-alphavirus consensus primers (M2W and cM3W) which can widely amplify

alphaviruses (Pfeffer et al., 1997), also could not find any positive sample. The isolation of arboviruses requires several times of blind passages in suckling mice or in cell lines (Pfeffer et al., 1997). Therefore, in case of low virus titer due to slow virus growth or low adaptation to C6/36 cells, it might be difficult to detect the presence of the virus by RT-PCR.

Antigen sandwich ELISA

Six hundred forty ICF samples were examined by CHIK antigen ELISA to detect the existences of CHIK virus protein. The OD of 26 ICF samples showed greater than or equal to that of positive standard ICF. As a threshold value of antigen sandwich ELISA, other researchers used much lower criteria such as a mean absorbance of 50 wells for supernatant from uninfected C6/36 mosquito cells or BHK-21 cells (Greiser-Wilke et al., 1991) or more than a two fold ELISA-OD above that of the negative control (Pandey et al., 1999). Table 1 shows the increase of high titer samples in rainy season in the Philippines, 1999. Therefore, there is a possibility that CHIK virus infection occurred from August to December in 1999.

IgM-IFA

Five hundred ninety serum samples were examined by IgM-IFA to detect the existence of IgM against CHIK virus. Ninety (15.3%) were positive (Table 2). Using the same samples, 302 (51.2%) were only positive for dengue, 44 (7.5%) were double positive for dengue and CHIK by either RT-PCR or IgM capture ELISA (Matias et al., 2000).

IgM capture ELISA

Three hundred forty three serum samples were examined with IgM –IFA. Forty seven (13.7%) were found to be positive (Table 2). Using the same samples, 157 (45.8%) were only positive for dengue, 19 (5.5%) were double positive for dengue and CHIK by either RT-PCR or IgM capture ELISA (Matias et al., 2000). To determine P:N ratio, we arbitrarily chose negative standard serum among CHIK IgM-IFA and CHIK IgG indirect ELISA double negative samples. Therefore, there is a possibility of existence of false positives and false negatives in the above described results. Further experiments for selection of negative standard such as hemagglutination inhibition test or some other serological test will be required. Although the results of IgM-IFA and IgM capture ELISA were similar (Table 2), the association between these two tests was not statistically significant (Chi-square (X^2) value: 0.47, degrees of freedom (df): 1, p value: $0.40<p<0.50$) due to unknown reason.

Table 1. Monthly trend of CHIK virus appearance in 1999 by CHIK antigen sandwich ELISA.

Month	Total	<0.2*	0.2~0.5*	0.6~0.9*	1.0~1.3*	1.3<*
January	59	59	0	0	0	0
February	32	32	0	0	0	0
March	17	16	1	0	0	0
April	33	33	0	0	0	0
May	44	43	1	0	0	0
June	70	68	2	0	0	0
July	62	58	4	0	0	0
August	127	40	64	13	10	0
September	92	53	18	12	9	0
October	37	25	8	3	0	1
November	50	31	11	3	2	3
December	17	11	5	0	0	1
Total	640	469	114	31	21	5

*---Titer of sample OD492/ positive standard OD492 was indicated.

Table 2. The results of serological examinations for CHIK virus infection.

Type of examination	Positive	Negative	Total
IgM-IFA*	90 (15.3%)	00 (84.7%)	590 (100%)
IgM capture ELISA*	47 (13.7%)	296 (86.3%)	343 (100%)
IgG indirect ELISA	91 (18.5%)	401 (81.5%)	492 (100%)

*---The association between these two tests was not statistically significant.
Chi-square (X^2)value: 0.47, degrees of freedom (df): 1, p value: $0.40<p<0.50$.

IgG indirect ELISA

To detect the existence of IgG against CHIK virus, 492 serum samples were examined by IgG indirect ELISA. Ninety one (18.5 %) were positive (Table 2). Fig. 1 shows that IgG positive ratio was constantly around 20% except for 0 to 4 and 25 to 29 year old groups. Aside from antigen sandwich ELISA, examinations for antigen detection such as RT-PCR, plaque assay, one-day old mice inoculation did not show any clear positive result (data not shown). As a another approach, blind passages in C6/36 or other cell lines (BHK-21, Vero), hemagglutination test of ICF and IFA for antigen detection using serum sample inoculated cell lines might be worthwhile to perform.

The results of IgM-IFA and IgM capture ELISA indicated that at least 7 to 8 % of DF/DHF suspected patients was only positive for CHIK virus. The results of antigen ELISA and IgG indirect ELISA also support the existence of CHIK virus infection in the Philippines. Therefore, careful and continuous monitoring of appearance of CHIK virus infection throughout the year and every year will be important to determine whether it only appeared during the rainy season of 1999 or not. If CHIK virus infection constantly appears every year in the Philippines, the introduction of vaccination for CHIK virus might be one of the effective solutions. In the U.S.A., researchers have already developed a live attenuated vaccine strain (CHIK 181/clone 25) for CHIK virus infection (Levitt et al., 1986). As part of the safety tests of the CHIK virus vaccine, CHIK 181/clone 25, Turell and Malinoski examined the transmissibility of the vaccine virus from vaccinated monkeys to a second vertebrate host by mosquito (1992). They also examined the possibility of its reversal to a more virulent form, not only in the original immunized vertebrate host, but also in an arthropod vector (1992). Fortunately unlike DF. DHF, immune enhancement phenomenon has never been reported in CHIK virus infection. Therefore the introduction of CHIK virus vaccine might be much more acceptable to the society where CHIK virus infection and DF/DHF coexist, than that of the vaccine for DF/DHF at this time.

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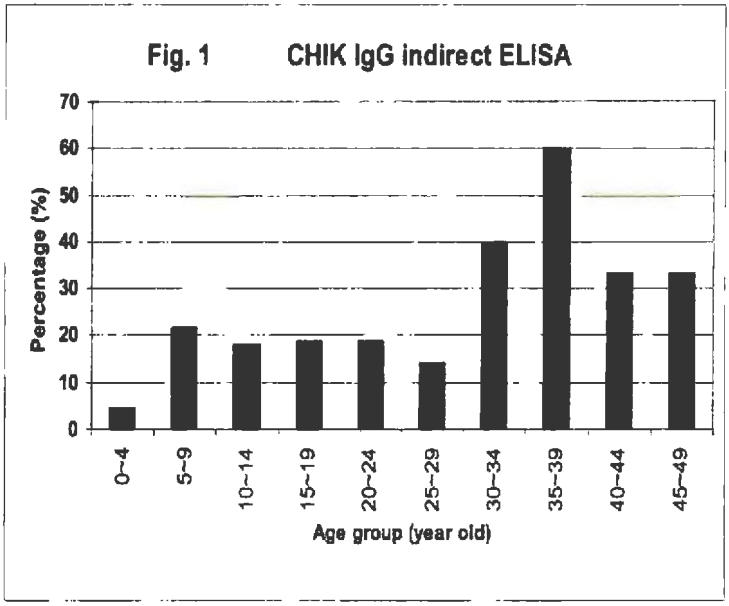

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