Enzyme-linked Immunosorbent Assay (ELISA) for Antibody Titers against Chikungunya Virus on Human Sera from Kenya

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Abstract: Antibody titers against Chikungunya (CHIK) virus were determined by indirect ELISA on 337 human sera obtained from Kisumu, Nakuru, Nairobi, and Nyeri areas in the Republic of Kenya. Comparison of anti-CHIK immunoglobulin (Ig-) and IgM-antibody occurrence was made on the basis of sampling areas and between adults and children. Adults had higher titers and antibody positive rates of Ig-ELISA than children in all areas sampled. On the other hand, IgM-ELISA levels were low in both children and adults. Many specimens with significant levels of Ig-ELISA did not possess hemagglutination inhibition or neutralization antibodies against CHIK. This result appears to suggest the occurrence of some alphavirus(es) in the study areas cross-reacting with CHIK, as suggested by the cross-ELISA tests using mouse immune sera.

Key words: Chikungunya virus, ELISA, Kenya.

INTRODUCTION

Serological studies continue to be very important in the diagnosis and epidemiological assessment on the prevalence of many arboviruses. Antibody titers for arboviruses have classically been measured by the complement-fixation (CF), hemagglutination-inhibition (HI) and neutralization (N) tests. The principle of enzyme-linked immunosorbent assay (ELISA) as described by Engvall and Perlman (1971) has been applied to detect etiological agents of various infectious diseases and also antibody titers against these antigens (Voller *et al.*, 1976; Sever and Madden, 1977). Although the method has been described as simple, more rapid and sensitive than conventional tests like CF, HI, and N, there have been only a few reports on its application to arboviruses (Hofmann *et al.*, 1979; Frazer and Shope, 1979; Dittmar *et al.*, 1979; Igarashi *et al.*, 1981). CHIK virus was first isolated by Ross (1956) and associates in South Mainland Tanzania during investigations on an epidemic of an apparently new disease, and has also been

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isolated from man and mosquitoes in Uganda, Congo, Thailand and India (Casals and Clarke, 1965). The East African epidemic was characterized by sudden onset, saddle-back fever, maculopapular rash, and severe arthralgia (Robinson, 1955). In Southeast Asia, the virus infection sometimes resulted in hemorrhagic manifestations. In this paper we described the application of the ELISA to measure antibody titers against CHIK virus using human sera collected in the Republic of Kenya. The possibility of the cross-reacting alphavirus prevalence was discussed on the basis of the study result and cross-reactivity by mouse antisera.

MATERIALS AND METHODS

Virus: CHIK virus, African strain was originally obtained from Dr. S. Ahandrik of the Virus Research Institute, Bangkok, Thailand. The stock of seed virus was prepared as previously described (Igarashi and Fukai, 1969). The infectivity was assayed on BHK21 cells by plaque formation under methyl cellulose overlay (Hashimoto *et al.*, 1971).

Cells: The host cells for virus growth were BHK21, clone 13 (MacPherson and Stoker, 1962), with cell growth medium of 10 % calf serum in Eagle's medium (Eagle, 1959).

Purification of the virus: The method is essentially as described before (Igarashi *et al.*, 1970). Cell growth media were removed from confluent sheets of BHK21 cells in 10 cm Petri dishes and the cells were infected with 0.5 ml/dish of 1:10 diluted seed virus. After 2 hours of adsorption at 37°C, the cells were covered by the maintenance medium (2 % calf serum in Eagle's medium) using 10 ml/dish, and were incubated at 37°C in 5 % CO_2-atmosphere. Infected culture fluid was harvested when cytopathic effect was observed over 70 % of cell sheets. Following procedures were performed at 4°C. The fluid was centrifuged at 2500 rpm for 15 min. To the supernatant (approximately 500 ml) was added an equal volume of saturated ammonium sulfate, pH 7.2. The mixture was kept for 15 min, and was centrifuged at 10,000×g for 15 min. The supernatant was removed and the precipitate was dissolved in 15 ml of STE (0.1 M NaCl, 0.01 M Tris-HCl, 0.001 M EDTA, pH 7.6) and was centrifuged at 2500 rpm for 15 min. The supernatant was layered on 4 ml of 15 % sucrose in STE and was centrifuged at 37,000 rpm for 90 min in an SW 41 rotor of Beckman L5-50 ultracentrifuge. The supernatant was removed and the pellet was resuspended in 0.5 ml of STE by sonication. The suspension was layered on top of a 15-30 % sucrose grandient in STE and was centrifuged at 37,000 rpm for 90 min. Fractions were collected by ISCO gradient fractionator, model 640, and peak fractions of OD_{254} were pooled and an equal volume of medium 199 containing 0.02 % gelatin was added as stabilizer (Takaku *et al.*, 1968). Aliquots were distributed in vials and stored at −70°C until required for use.

Test sera : Human serum samples were obtained from Kisumu, Nakuru, Nairobi, and Nyeri areas in the Republic of Kenya. These locations were shown in Fig. 1. Mouse antisera against several alphaviruses were prepard by 5 successive intraperitoneal inoculations of brain homogenate prepared from infected mice. These sera were stored at $-20°C$.

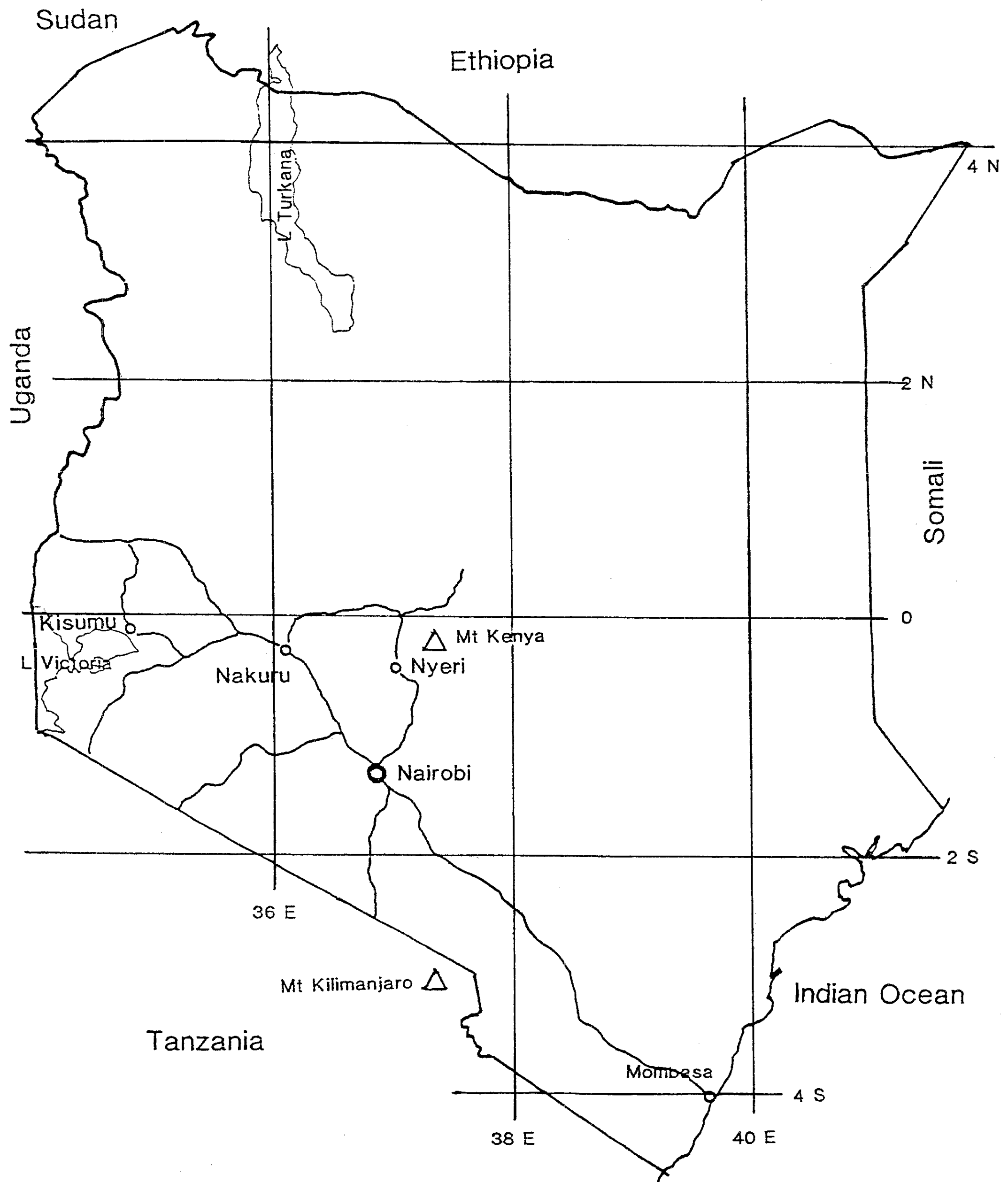

Fig. 1. Sampling place of human sera in the Republic of Kenya.

ELISA procedures: The indirect method of Voller *et al.* (1976) was followed with slight modifications (Igarashi *et al.*, 1981). Stock virus was diluted 1:200 for Ig-ELISA or 1:50 for IgM-ELISA with coating buffer (0.05 M carbonate-bicarbonate, pH 9.6) and then distributed into wells on plastic U-shaped microplate (Greiner Labortechnick) using 100 μl/well. The plates were incubated at 4°C overnight and the wells were emptied and washed 3 times with PBS-Tween (phosphate buffered saline containing 0.05% Tween 20 and 0.01% NaN_3, pH 7.2) for 3 min each. The plates were kept at −70°C until required for use. Before use the plates were washed once again as above. To the antigen-coated plates, standard positive and negative sera in increasing 2-fold dilutions were added using 100 μl/well. Test sera diluted 1:100 or 1:1000 were distributed to the remaining wells using 100 μl/well. The plates were incubated at 37°C for 1 hour and were washed as above. Goat anti-human IgG (heavy and light chains) peroxidase conjugate diluted 1:1000 or goat anti-human IgM (μ-chain specific) peroxidase conjugate diluted 1:400 was distributed in all wells using 100 μl/well. These enzyme conjugates were the products of Cappel Laboratories, USA. The plates were incubated at 37°C for 1 hour and washed again. Substrate solution containing 0.5 mg of *o*-phenylene diamine dihydrochloride per ml and 0.02% H_2O_2 in 0.05 M citrate-phosphate buffer, pH 5.0, was distributed in all wells (100 μl/well). The reaction was allowed to proceed at room temperature for 1 hour in the dark and the reaction was stopped by adding 4 N H_2SO_4 (75 μl/well). Optical density at 450 nm was recorded by a Micro ELISA Autoreader, Dynatech Co. with reference wavelength of 630 nm. ELISA titer of positive standard serum was graphically determined as the reciprocal of the highest dilution at which the ELISA OD was more than twice the value of the negative standard serum at the same dilution. ELISA titers for the test sera were calculated by a computer system (Morita *et al.*, 1982).

HI and N tests: These tests were performed on 44 serum samples obtained from Nyeri area. The method of Clarke and Casals (1958) was followed with modification to microtiter system in the HI test. Fifty percent plaque reduction N test was used with probit chart method and 10−fold serial dilution of the test sera (Russell *et al.*, 1967).

Statistical methods: The methods described by Snedecor (1952) was followed.

RESULTS

Anti-CHIK ELISA titers in human serum specimens:

Relationship between Ig-ELISA and N or HI titers on the 44 serum specimens taken from Nyeri area is shown in Fig. 2. Samples which showed HI titers of 10 or more possessed the Ig-ELISA titer over 140, and those with N titer of 10 or more, with a single exception, showed the Ig-ELISA over 180. Accordingly, we arbitrarily chose the antibody positive limit of Ig-ELISA as 145, and in the case of IgM-ELISA the limit was tentatively set at 100 similar to the results with Japanese encephalitis virus anti-

bodies (Bundo *et al.*, 1981; 1982). Based on these criteria, there were many specimens with negative HI or N titers against CHIK virus and positive anti-CHIK ELISA titers. These specimens will probably represent the specimens with cross-reacting antibodies against some alphaviruses.

Table 1 shows the prevalence of anti-CHIK Ig-ELISA antibodies in serum specimens taken from various areas. Adults had higher antibody positive rates than children. One of the children from Nairobi, aged 3 month old, had Ig-ELISA comparable to the average adult titers. Nakuru had the highest positive rate of Ig-ELISA (92%), followed by Kisumu (90%), and then Nyeri (65%) among adults. On the other hand, anti-CHIK IgM-ELISA was positive in smaller number of specimens with 14% in Nakuru, followed by Nyeri (8%) and Kisumu (2%). Anti-CHIK IgM-ELISA positives were not found among children in all areas (Table 2).

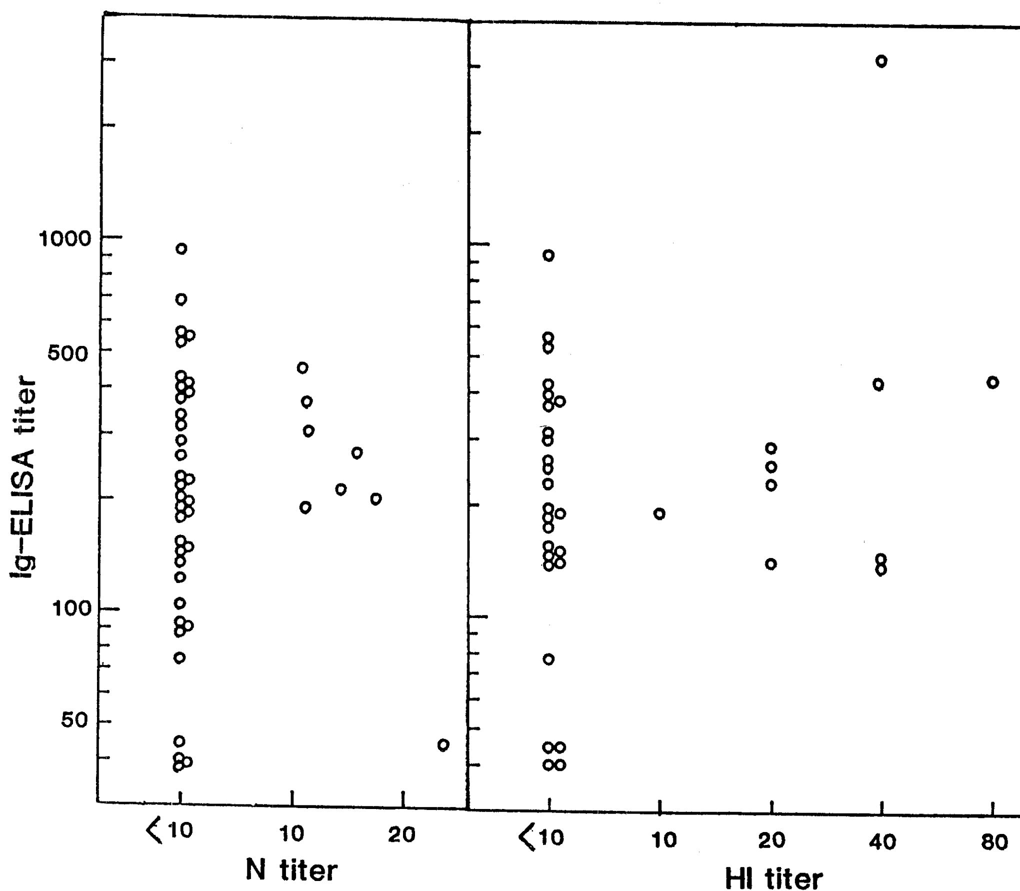

Fig. 2. Relationship between the Ig-ELISA and N- or HI titers against CHIK virus as measured for 44 serum specimens from Nyeri area.

Table 3 shows the geometrical mean titer (GMT) of Ig- and IgM-ELISA against CHIK. Kisumu area had the highest GMT of Ig-ELISA in adults and age-unknown groups. Nakuru was the second, followed by Nyeri. However, these difference were not statistically significant. The GMT of Ig-ELISA among children was low compared with adults, and GMTs of IgM-ELISA were low both in adults and children.

Cross-reactions by the ELISA among several alphaviruses:

Since there were many specimens with negative HI or N titers against CHIK still showing quite high Ig-ELISA against CHIK, we suppose that these specimens represent the cross-reaction among several related alphaviruses by the ELISA. Therefore, the cross-ELISA tests were performed with the following alphaviruses using purified virus as

Table 1. Number of specimens and sntibody positive rates as mesured by Ig-ELISA against CHIK virus

Sampling area	Number of specimens tested				Number of positives (%)		
	Total	Adults	Children	Age-unknown	Adults	Children	Age-unknown
Kisumu	67	49	—	18	44(90)	—	12(67)
Nakuru	92	89	3	—	82(92)	2(67)	—
Nyeri	128	101	27	—	66(65)	5(18)	—
Nairobi	50	—	50	—	—	3(6)	—
Total	337	239	80	18	192(80)	10(13)	12(67)

Posititives: Ig-ELISA over 145

Table 2. Antibody positive rates as mesured by Ig-ELISA against CHIK virus

Sampling area	Number of positives (%)		
	Adults	Children	Age-unknown
Kisumu	1 (2)	—	0 (0)
Nakuru	12 (14)	0 (0)	—
Nyeri	8 (8)	0 (0)	—
Nairobi	—	0 (0)	—
Total	21 (9)	0 (0)	0 (0)

Number of specimens tested is the same as in Table 1

assay antigens and mouse hyperimmuse sera. The viruses used were: Semliki Forest (SF), Mayaro (MAY), Bebaru (BEB), eastern equine encephalitis (EEE), western equine encephalitis (WEE), Sindbis (SIN), Getah (GET) viruses. These viruses were also grown in BHK21 cells and were purified similar to CHIK virus and used as antigens in the tests. Table 4 summarizes the results of cross-ELISA using purified alphaviruses as antigens and mouse hyperimmune antisera. The figures were normalized taking the titer observed for the homologous antiserum-virus combination as 100. Antiserum against CHIK showed significant cross-reactions to SF, MAY, and BEB. On the other hand, CHIK virus cross-reacted to antisera against WEE, SIN, and GET.

Table 3. GMT with standard deviation (both in log scale) of Ig- and IgM-ELISA against CHIK virus

Sampling area	Ig-ELISA			IgM-ELISA		
	Adults	Children	Age-unknown	Adults	Children	Age-unknown
Kisumu	2.74±0.46	—	2.76±0.65	1.43±0.23	—	1.45±0.26
Nakuru	2.54±0.30	2.24±0.15	—	1.61±0.32	1.63±0.38	—
Nyeri	2.24±0.34	1.93±0.28	—	1.57±0.34	1.43±0.30	—
Nairobi	—	1.81±0.23	—	—	1.26±0.04	—

Table 4. Cross-reactions of mouse antisera against several alphaviruses as measured by the Ig-ELISA (% ratio of heterologous to homologous)

Anti-serum	Antigens							
	SF	MAY	BEB	EEE	WEE	SIN	GET	CHIK
SF	100	391	254	27	31	27	31	4
MAY	21	100	63	7	5	1	1	2
BEB	1	15	100	0	10	1	0	1
EEE	0	138	340	100	6	13	0	9
WEE	0	219	119	143	100	15	0	47
SIN	11	100	534	36	0	100	0	81
GET	1859	283	317	10	28	13	100	47
CHIK	28	24	59	0	0	0	0	100

Abbreviation of the virus is described in the text.

DISCUSSION

The occurrence of arbovirus antibodies is influenced by the behaviour of vectors, including the ecological setting of breeding, their pattern and range of mobility, biting habits and longevity. The frequency and duration of exposure of humans to the viruses are thus influenced by the use of insecticides or protective clothings or insect screens (Downs, 1976). In this study, children as classified under 10 years, had lower levels of Ig-ELISA against CHIK than adults. The result agrees with the findings during the 1958 epidemic of CHIK in Thailand, in which 88 % of hospitalized cases were children under 10 years (Nelson, 1960). A 3 month old child with high Ig-ELISA against CHIK in Nairobi probably represents the result of maternal antibodies, because its IgM-ELISA titer was low. It appers that Kisumu had been invaded by the virus related to CHIK in the past, because its antibody positive rate is high in Ig-ELISA but low in IgM-ELISA. Nakuru and Nyeri, on the other hand, appear to have experienced the infections rather recently, because of higher positive rates of IgM-ELISA, especially in Nakuru.

Many specimens with negative HI or N antibodies against CHIK still possessed quite high Ig-ELISA against CHIK. The result could be explained by the existence of some other alphaviruses which cross-react with CHIK by the ELISA but not by the HI or N test. Cross-reactivity of alphaviruses by the ELISA was also shown by Frazier and Shope (1979). O'nyong-nyong (ONN) virus, which was not available during this study, was shown by Geser *et al.* (1970) to be so closely related to CHIK that the result by the HI with these two antigens were almost similar. So that the cross-reacting alphavirus responsible to the finding in this report will not be ONN. Antibody prevalance of CHIK or ONN as determined by the HI by Geser *et al.* (1970) appear to be lower than the antibody positive rate of anti-CHIK Ig-ELISA as shown in this report. It is difficult to use ELISA for measuring antibody titers specific to a given virus, for example CHIK, when cross-reacting alphaviruses coexist. However, the method increases the rapidity to eliminate "negative" sera from a given study specimens. Further differentiation can then be supplemented with other serological methods.

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免疫酵素測定法（ELISA）を用いたケニアの人血清のチクングンヤウイルスに対する抗体価の測定

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ケニア共和国のキスム，ナクール，ナイロビ，ニエリで採血した人血清337検体について，微量間接 ELISA 法によりチクングンヤ（CHIK）ウイルスに対する抗体価を測定し，抗 CHIK-免疫グロブリン(Ig) と IgM 抗体の比較を採血地点及び成人と子供について行なった．すべての採血地点において成人は子供に比べて Ig-ELISA 価の幾何平均値及び抗体保有率共に高かった．CHIK に対する血球凝集抑制又は中和抗体を保有していないが CHIK に対し有意な Ig-ELISA 抗体価を示す検体が多数存在した．この結果は，採血地点において CHIK と交叉反応を示す別のアルファウイルスが存在することを示唆すると思われ，このことはマウスの免疫血清を用いた交叉反応によっても推察される．

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