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Continuous cell lines and immune ascitic fluid pools in arbovirus detection

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SUMMARY

Successive experiments led us to use two cellular systems, MOS61 (*Aedes pseudoscutellaris* cells) and Vero cells, among the continuous cell lines recommended by the WHO Collaborating Center for systematic research and isolation of arboviruses.

Virus detection in cell cultures is carried out with 7 mixtures containing 10 hyperimmune ascitic fluids made with the reference viruses. This technique enables the detection of 70 of the 80 arboviruses transmitted by mosquitoes in Africa and very easily detects arbovirus associations by using either monospecific or monoclonal immune ascitic fluids (dengue-1-2-3-4 and yellow fever viruses) used in the indirect immunofluorescence technique.

Key-words: Arbovirus; Detection, Isolation, Immune ascitic fluid, Vero and MOS61 cell lines.

INTRODUCTION

For approximately 30 years, the WHO Collaborating Center for Reference and Research on Arboviruses (CRORA) has been participating in the study of wild arbovirus cycles in Senegal. In such studies, teams of entomologists capture the mosquitoes, which are then grouped into monospecific batches of a maximum of 100 specimens before detection of the arboviruses.

Isolation techniques have developed with time; intracerebral inoculation of suckling mice was the only method at first, followed by intrathoracic inoculation of mosquitoes *(Toxorhynchites),* with research concentrated on yellow fever and dengue viruses (Rosen, 1981). At the present time, inoculation of sensitive cellular systems (quite often the continuous cell line of *Aedes pseudoscutellaris*) is used. Two systems are often used jointly: inoculation of suckling mice and the cellular system. Since 1989, detection in cellular systems has been carried out using pools of polyvalent immune ascitic fluid (PIAF). This technique can detect 70 of the 80 arboviruses transmitted by mosquitoes in Africa. The contribution of this new technique to arbovirus research is the subject of this paper.

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MATERIALS AND METHODS

Batches of mosquitoes

Material was collected at 3 sites (see map, fig. 1). In the Kedougou area (eastern Senegal), teams of ORSTOM entomologists carried out observations of yellow fever, dengue and Rift Valley fever vectors. In the Senegal River basin (Dakar Bango), the only aim was a better knowledge of the mosquitoes of this area. Catches were carried out monthly until June 1990, and all the mosquitoes were inoculated. In the Ferlo plain, systematic mosquito catches were carried out in two places (Yonofere and Barkedji) as part of an effort to discover the mechanisms which maintain Rift Valley fever virus in the area.

Virological techniques

For several years, the CRORA has recommended the use of a continuous mosquito cell line (*A. pseudoscutellaris:* MOS61) described by Varma *et al.* (1974) for the isolation of yellow fever and wild dengue viruses. This cell line was known to be very sensitive to epidemic dengue virus (Race *et al.,* 1979); it was also very sensitive to wild yellow fever virus, and it enabled the isolation of many strains (Varma *et al.,* 1975). It also allowed isolation of dengue viruses type 1, 2 and 4 from sporadic human cases in West Africa. The same technique has been applied to the Rift Valley fever virus (Digoutte *et al.,* 1983). Here it was applied to the isolation of viruses from pools of mosquitoes caught in the wild in systematic epidemiological surveys. This continuous cell line seems to be sensitive to many arboviruses, however, it is not sensitive to all the arboviruses isolated from mosquitoes in Africa. This fact led us to use a second cell system, that of Vero cells.

The method was tested experimentally using both cell systems with the reference strains of arboviruses (Brandt *et al.,* 1967). Specific immune ascitic fluids and polyvalent mixtures were tested successively on each virus. Low-level immune ascitic fluids were eliminated using indirect immunofluorescence assay. Polyvalent immune ascitic fluids diluted 1:10 must give clear immunofluorescence with the corresponding reference arbovirus. At present, 7 mixtures of

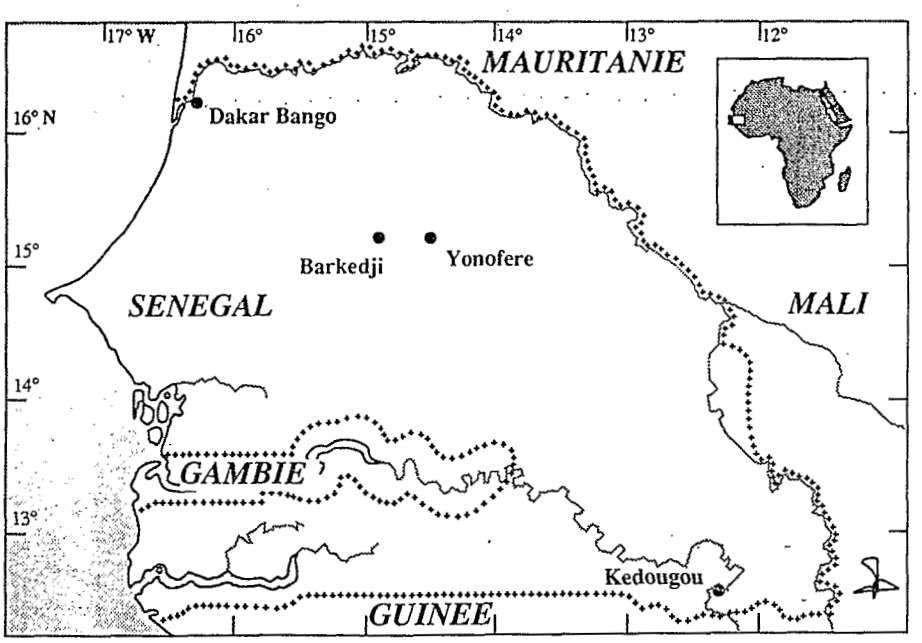

Fig. 1. Localities studied.

CRORA = WHO Collaborating Center for Reference and Research on Arboviruses.

PIAF = polyvalent immune ascitic fluid.

10 hyperimmune ascitic fluids made with the reference viruses are used for detecting arboviruses. Table I lists the pools of immune ascitic fluids and their composition. Mixtures 1-3 were used in 1989, mixture 4 was added in 1990; mixtures 1-6 were used for the first 6 months of 1991, after which the totality was used.

Viral identification is carried out with monoclonal antibodies for dengue and yellow fever viruses. For other viruses, we use the specific immune ascitic fluorescence titre and/or the classical methods after inoculation of suckling mice. Because of the necessary delays between mosquito catches, specific identification, constitution of batches and finally virological studies, only the results for 1989 and 1990 are definite; 1991 is still incomplete.

RESULTS AND DISCUSSION

Current results

In Barkedji, in 1990, 31,497 mosquitoes put in 407 pools gave 1 Babanki strain, 1 Bagaza, 35 West Nile and 17 mixed strains of West Nile + Bagaza; in 1991, 25,825 mosquitoes put in 776 pools gave 2 Babanki strains.

In Dakar Bango, in 1989, 344,901 mosquitoes put in 3,800 pools gave 7 Bagaza strains, 1 Bwamba and 1 Middelburg; in 1990, 145,070 mosquitoes put in 1,587 pools gave 1 Bagaza strain, 1 Bwamba, 1 Mpoko and 1 strain of a new virus of the Corriparta group (Ar D 66707).

In Kédougou, in 1989, 33,017 mosquitoes put in 654 pools gave 4 Bagaza strains, 1 Chikungunya, 28 dengue-2, 1 yellow fever, 1 Kedougou, 1 Ndelle, 1 Orungo, 2 Pongola, 2 Wesselsbron, 3 Yaounde and 11 Zika. The following mixed strains were identified: 1 dengue-2 + Chikungunya, 14 dengue-2 + Zika and 3 dengue-2 + Chikungunya + Zika. In 1990, 30,675 mosquitoes put in 497 pools gave 1 Bagaza strain, 1 Chikungunya, 19 dengue-2, 3 yellow fever, 5 Kedougou and 3 Zika.

In Yonofere, in 1989, 2,199 mosquitoes put in 105 pools gave 1 Wesselsbron strain and 1 West Nile. In 1990, 804 mosquitoes put in 44 pools gave 1 West Nile strain, 1 mixed strain of West Nile + Babanki and 1 mixed strain of West Nile + Bagaza.

Table I. Mixtures of immune ascitic fluids used in CRORA.

Mixture 1	Mixture 2	Mixture 3	Mixture 4
Babanki	Semliki-Forest	Chikungunya	Périnet
Middelburg	Ndumu	Yellow fever	Boteke
Bunyamwera	Wesselsbron	Ilesha	Nkolbisson
Simbu	Bagaza	Mpoko	Bouboui
Orungo	Uganda S	Okola	Zika
Palyam	Dengue-2	Rift Valley fever	Ngari
Tataguine	Dengue-4	Nyando	Pongola
Mossuril	West-Nile	Eret 147	Saboya
Bwamba	Kédougou	Bozo	Birao
O'Nyong-Nyong	Usutu	Igbo Ora	Shokwe

Mixture 5	Mixture 6	Mixture 7
Spondweni	Nkolbisson	Kamese
Yoka	Akabane	Bangoran
Botambi	Nola	Ingwavuma
Oubi	Odrenisrou	Ar Mg 966
Tanga	Gomoka	Bobia
Bangui	Tai	Dabakala
Yata	Pata	Ngoupe
Oubangui	Kindia	Somone
Acado	Andasibe	AR MMP 158
Dakar bat	Ndelle	Ar D 66707

Sensitivity and specificity of the isolation method using *A. pseudoscutellaris* cells

The isolation of many mixed strains (two viruses from the same mosquito pool) was new, leading us to verify the cell lines' sensitivity toward viral strains. First we compared these results with those obtained in 1987, when we found 11 mosquito pools infected both by yellow fever and Zika (see table II). Then we carried out an experiment to verify that preliminary dengue 2 infection increased MOS61 sensitivity to Zika virus. Finally, we inoculated suckling mice with all the negative mosquito pools from the genus *Aedes* and all the supernatants of positive cell cultures, to confirm Zika presence and identify it in another way, which confirmed previous identification by indirect immunofluorescence assay.

Yellow fever and Zika

In 1987, 799 mosquito pools of potential vectors for yellow fever were simultaneously inoculated into MOS61 cell lines and suckling mice. This test showed the high level of sensitivity of MOS61 cells toward the yellow fever virus: 115 strains isolated on MOS61 only, 5 on both systems and 1 only on suckling mice. For the Zika virus, the results were not the same: 18 strains isolated only on MOS61, 26 only on suckling mice and 15 on both systems; 11 pools contained both viruses. About one third of the Zika virus strains are lost with the exclusive use of MOS61, but it is a better system for yellow fever virus. For 9 cases out of 11, the yellow fever virus prevented Zika virus growth on MOS61 cells, but was easily recovered after inoculation of suckling mice.

Dengue-2 and Zika

One Zika virus strain was isolated exclusively after inoculation of suckling mice. Several assays showed that isolation using MOS61 cells was impossible. This strain, after passing via suckling mice, was inoculated into MOS61 cells, and the Zika virus was again found in the cell culture, which presented weak immunofluorescence and a non-cytopathic effect. A dengue-2 strain isolated from the mosquito pool was inoculated into the same cellular system, and the virus was again found with strong immunofluorescence and a non-cytopathic effect. The same dengue-2 strain was inoculated into MOS61 cells with the above Zika strain. In this case, we observed a strong cytopathic effect after day 4 with high-level Zika immunofluorescence. After 5 days the cell culture was destroyed.

Seven mosquito pools and their corresponding cell culture supernatants were inoculated into suckling mice. The results are given in table III. This experiment shows that a dengue-2 infection can mask a dual dengue-2/Zika infection, as well as a third virus: Chikungunya (Ar D 63272 pool). As previously described (Banerjee, 1969; Igarashi, 1979), the Chikungunya strain inoculated into insect cells (here *A. pseudoscutellaris*) loses its neurovirulence toward suckling mice (Ar D 63272 pool). On the other hand, this neurovirulence is preserved when MOS61 cells are

Table II. Comparison between AP61 cells and suckling mice for both yellow fever and Zika viruses.

		Yellow fever virus			
		Negative	PA	PSM	PA & PSM
Zika virus	Negative	630	104	1	5
" "	PA	16	2	0	0
" "	PSM	17	9	0	0
" "	PA & PSM	15	0	0	0

PA = positive on AP61 cell line; PSM = positive on suckling mice.

Table III. Mosquitoes and corresponding cell culture supernatant inoculation results.

No. of pool	Mosquito inoculation		Supernatant inoculation	
	MOS61	Suckling mice	MOS61	Suckling mice
Ar D 63272	Dengue-2	Negative	Dengue-2	Chikungunya
Ar D 63315	Dengue-2	Dengue-2	Dengue-2	Dengue-2
Ar D 63273	DEN-2 + Zika	Chikungunya	NT	Chikungunya
Ar D 63388	DEN-2 + Zika	Chikungunya	NT	Chikungunya
Ar D 65108	DEN-2 + Zika	Chikungunya	NT	Zika
Ar D 61727	Chikungunya	Chikungunya	Chikungunya	Negative
Ar D 65181	Dengue-2	Negative	Dengue-2	Dengue-2

NT = not tested.

coinfected with dengue-2 and Zika viruses (Ar D 63273 and Ar D 63388 pools). Infection with the dengue-2 virus seems to modify MOS61 cells in such a way as to facilitate the synthesis of two other virus components.

MOS61 cell sensitivity to other viruses

Using MOS61 cells, 235 mosquito pools, all of them of the same genus *Aedes* (potential yellow fever virus vectors), caught in Kedougou in 1989, tested negative. These pools were later inoculated into suckling mice, enabling five additional strain isolations: 1 Ndelle, 1 Zika, 2 Pongola and 1 Orungo, and proving that several viruses were not found with the mixtures 1, 2 and 3 used in 1989. This result led us to test MOS61 sensitivity towards all the known viruses isolated from mosquitoes in Africa, to enlarge the number of immune ascitic mixtures and to use a second continuous cell line system. At present, 70 virus strains are used to prepare 7 immune ascitic fluid mixtures. Among the 80 viruses isolated from mosquitoes in Africa, 10 do not grow on the two cellular systems (MOS61 and Vero): Palyam, Botambi, Oubi, Oubangui, Ingwavuma, Bobia, Dabakala, Ngoupe, Somone and AR MMP 158. On the other hand, 10 are detected thanks to the heterologous reaction in direct immunofluorescence, when their corresponding immune ascitic fluid is not present in the mixture.

In conclusion, the system used is a result of experiments that have led to successive increases in its sensitivity. This technique enables the detection of 70 of the 80 arboviruses transmitted by mosquitoes in Africa and easily detects arbovirus associations by using either monospecific or monoclonal immune ascitic fluids (dengue-1-2-3-4 and yellow fever viruses).

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Inoculation de lignées cellulaires en culture continue et utilisation de pools d'ascites immunes pour l'isolement des arbovirus

Pour la recherche et l'isolement des arbovirus, des expérimentations successives nous ont amenés à utiliser, parmi les lignées de cellules en culture continue dont l'utilisation est préconisée par l'OMS, les deux systèmes cellulaires MOS61 (cellules de *Aedes pseudoscutellaris*) et Vero. La détection des virus dans les systèmes cellulaires est réalisée à l'aide de 7 pools de 10 ascites hyperimmunes polyvalentes. Cette technique permet la mise en évidence de 70 sur 80 arbovirus transmis par les moustiques en Afrique, et permet de détecter très facilement les associations d'arbovirus grâce aux ascites immunes monospéci-

fiques ou monoclonales (dengue-1-2-3-4 et fièvre jaune).

Mots-clés: Arbovirus; Détection, Isolement, Ascite immune, Lignées cellulaires immune, Vero et MOS61.

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