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DIFFERENTIATION BETWEEN TWO AFRICAN ARENAVIRUSES (LASSA AND MOZAMBIQUE) BY PLAQUE ASSAY

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Summary. — The biological characteristics of Lassa and Mozambique viruses were compared by plaque assay. Both viruses produced plaques under the same conditions, however, Lassa virus plaques measured on average 2 mm in diameter being twice as large as the Mozambique virus plaques. By plaque reduction tests, cross protection was demonstrated between both viruses showing that Lassa and Mozambique viruses are distinct but related.

Key words: african arenaviruses; Lassa virus; Mozambique virus; plaque assay

Introduction

Lassa and Mozambique viruses belong to the LCM complex (Old world) of arenaviruses family. Lassa virus was originally isolated from a human in Nigeria (Buckley and Casals, 1970). Numerous other isolates were recovered from human cases as well as from *Mastomys natalensis*, *Rattus rattus* and *Mus minutoides* rodents trapped in Nigeria, Sierra Leone and other West African countries (Demartini *et al.*, 1975; Wulff *et al.*, 1975; Johnson, 1985).

Mozambique virus, first isolated from *M. natalensis* rodents trapped in Mopeia, Central Mozambique (Wulff *et al.*, 1977), was subsequently recovered from *M. natalensis* and *Aethiops chrysophilus* rodents captured near Que Que and Chiredzi, Zimbabwe (Johnson *et al.*, 1981). Mozambique virus in complement fixation (CF) and immunofluorescence (IF) tests was shown to be immunologically related, but probably not identical with Lassa virus (Wulff *et al.*, 1977).

Using monoclonal antibodies prepared against LCM virus, Buchmeier *et al.* (1981) distinguished between Lassa and Mozambique viruses. In this study, five monoclonal anti-NP antibodies, which reacted with LCM, Lassa and Mozambique viruses, and a single anti-G2 monoclonal reacting with LCM and Mozambique viruses only, were isolated. Later Kiley *et al.* (1981) reported that the anti-G2 antibody binds to the G2 protein of both LCM

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and Mozambique viruses, indicating that the LCM and Mozambique cross--active sites reside on the G2 protein of both viruses. Studies by Monath *et al.*, (1974) and Jahrling *et al.* (1985) indicate that Lassa virus strains from different West African countries are readily distinguishable by neutralization tests.

Gonzales *et al.* (1982), reported the isolation of Mobala virus, a new Lassa--related arenavirus, from *Praomys* spp. rodents trapped in the Central African Republic (CAR). Based on serological tests, Gonzales (1984) reported that Ippy virus was Lassa-related arenavirus. Ippy virus was first isolated in CAR in 1970 from a rodent *Arvicanthis* sp. Digoutte and Pajot (Berge, 1978). Although limited serologic studies have shown the presence in humans of antibodies to Mozambique virus in Mozambique and to Mobala virus in the CAR, none of either Mozambique virus or Mobala virus has been associated with any acute infection similar to Lassa fever disease.

Nonetheless, these observations emphasise the need for the development of a simple laboratory technique and reagents for rapid and precise diagnosis of arenavirus infections in order to distinguish between the new African arenaviruses. In this report, we have compared both Lassa and Mozambique viruses with regards to their plaque-forming characteristics and serological reactions in the plaque reduction neutralization tests (PRNT).

Materials and Methods

Cell cultures. A stable African green monkey kidney cell line (Vero) was used. The cells were prepared by the Tissue Culture and Media section of the Centers for Disease Control (CDC), Atlanta. Cells were grown in either 30 ml or 250 ml flasks.

Growth, maintenance and overlay media. Vero cells were grown in Eagle's Minimum essential medium (MEM) supplemented with 5 % inactivated foetal calf serum (FCS); and maintained in Eagle's MEM supplemented with 2 % inactivated FCS.

The two overlay media used for the plaque assay were prepared as follows: First overlay medium: 2× Basal minimum essential medium (BME) 81.50 ml, 100× Glutamine 2.00 ml, 7 % Sodium bicarbonate sol. 3.14 ml, 1 mol/l HEPES buffer 3.36 ml, inactivated FCS 8.00 ml, and 100× antibiotics 2.00 ml to a total of 100.00 ml (solution A). Then 2 g agarose (Seakem®; Marine Colloids, Rockland, ME) was dissolved in distilled water (100 ml) and sterilized for 10 min (solution B). Both solutions were kept at 45 °C, and mixed in equal amounts before use. The second overlay medium was made up of 1 % Difco® Noble Agar in Hanks BSS, autoclaved for 10 min and kept at 45 °C. Prior to use, neutral red was added to final concentration of 0.0825 g/l.

Test diluent (TD). For all virus and serum dilutions, the diluent was made up of Hanks BSS, HEPES buffer (10 mmol/l) and a 10 % normal fresh guinea pig or human serum stored at −70 °C. Fresh human serum was obtained from a commercial source. Both guinea pig and human sera were found negative for Lassa virus by immunofluorescence (IF), complement fixation (CF) and NT.

Virus strains. The histories of the three virus strains (2 Lassa strains and 1 Mozambique strain) are shown in Table 1. All work with Lassa and Mozambique viruses and their immune ascitic fluid was carried out in the CDC Maximum Containment laboratory, a P4 facility for handling highly pathogenic agents.

Immune ascitic fluids (IAF) were prepared as described by Wulff *et al.* (1978). In brief, adult hamsters were inoculated with a 20 % suckling hamster brain suspension of either Lassa or Mozambique virus. The virus suspension was mixed in a 1 : 1 ratio with Freund's complete adjuvant, (FCA) each hamster receiving 0.5 ml intraperitoneally. Sarcoma TG 180 was then administered to the hamsters and the ascitic fluid harvested between day 25 and 28 of inoculation of the virus-FCA mixture. For the production of hyperimmune ascitic fluid, each hamster received

Table 1. Origin and passage history of Lassa and Mozambique virus strains

Virus (strain) origin	Laboratory number	Passage history	Stock titre (PFU/ml)
Lassa (Pinneo) Nigeria	800521	MP(1), V(9)	2.3×10^6
Lassa (Josiah) Sierra Leone	802134	V(4)	2.0×10^6
Mozambique (Mozambique)	802851	MP(5), V(4), PPV(3)	6.3×10^6

MP — adult mouse passage (passage number in brackets);
V — Vero cells; PPV — plaque purified in Vero cells

intraperitoneally 0.2 ml aliquots of the appropriate virus-FCA mixture on days 0, 7, 23 and 42, and 0.1 ml aliquots on days 49 and 63. Following the inoculation of sarcoma TG-180, hamsters were drawn the ascitic fluids between day 75 and day 84. Each of IAF had a titre of 1.512 in FA tests with homologous virus.

Plaque assay. Monolayer cultures of Vero cells were prepared in 5 ml flasks by seeding with 100,000 Vero cells in 10 ml of growth medium. After cells became confluent (within 48—72 hr), the growth medium was removed and each culture inoculated with 0.2 ml of virus preparation. Virus adsorption took place at 37 °C for 1 hr, thereafter, 10 ml of the first overlay was added to each flask. The overlay was allowed to set for 15—20 min before incubation in inverted position at 37 °C. The second overlay (5 ml) was added on incubation day 4; results were read after further 24 hr.

Plaque reduction neutralization test (PRNT) was performed either with constant serum to varying virus (CS-VV) dilution or with constant virus to varying serum (CV-VS) dilution. The details of these tests have been previously described (Tomori *et al.*, 1986). Briefly, for the CS-VV method, uninactivated serum was kept at a constant dilution of 1 : 5 and aliquots of the serum dilution were reacted in equal amounts with each of a 10-fold serially diluted virus preparation. The final serum dilution was therefore 1 : 10. In the CV-VS method, each aliquot of serial two-fold dilutions of uninactivated serum was reacted with an equal volume of a virus preparation equivalent to a dose of 100 PFU.

Each virus-serum mixture was then incubated in a 37 °C water bath for 1 hr with intermittent shaking. Replicate Vero cell cultures were each inoculated with 0.2 ml of the virus-serum mixture, which was allowed to adsorb at 37 °C for 1 hr. Following adsorption, the first overlay was added and the test completed as earlier described for the plaque assay.

We considered a reduction in plaque counts of 80 % as significant. The log of neutralization index (LNI) of a serum tested by the CS-VV technique was calculated as the difference between the log PFU in control negative serum and the log PFU in test serum. By the CV-VS technique, the serum antibody titre was calculated as the reciprocal of the highest dilution of the test serum reducing the number of test virus dose by 80 %.

Results

Plaque characteristics

Vero cell cultures were inoculated with a calculated dose of 100 PFU of either Lassa or Mozambique virus. The kinetics of plaque formation by Mozambique virus were similar to those earlier described for Lassa virus (Tomori *et al.*, 1986). No plaques were seen on day 1, and the maximum number of clearly defined and discrete plaques were seen on day 4 and day 5 of inoculation. Mozambique plaques measured on the average 1.0 mm in diameter, half the size of Lassa virus plaques which were 2.0 mm in diameter (Fig. 1).

Table 2. The constant virus-varying serum cross-plaque reduction neutralization test between Lassa and Mozambique virus strains

Virus	Titres of immune ascitic fluids to		
	Lassa (Pinneo)	Lassa (Josiah)	Mozambique
Lassa (Pinneo)-800521	**128**[a]	32	32
Lassa (Josiah)-802134	32	**64**	16
Mozambique-802851	8	8	**64**

Antibody titres are the reciprocal of IAF dilutions neutralizing at least 80 % of test virus dose (100 PFU). Homologous titres in bold type.

Cross PRNT with Lassa and Mozambique viruses

The results of the PRNT using the CV-VS method are shown in Table 2. One shot hamster IAF prepared against Lassa (Pinneo) virus had a homologous neutralizing antibody titre of 128; against Lassa (Josiah) virus strain and Mozambique virus, the titres were 32 and 8, respectively. The homologous antibody titre of the 1-shot Lassa (Josiah) IAF was 64, with corresponding titres of 32 and 8 against Lassa (Pinneo) and Mozambique viruses, respectively. In the reaction against the homologous virus, 1 shot Mozambique IAF had a titre of 64, and reacted at a titre of 8 against each of the Lassa (Pinneo) and Lassa (Josiah) virus strains.

Similar results with no significant differences in titres were obtained when 6 shot hamster hyper-immune ascitic fluids replaced the 1 shot immune fluids in the CV-VS neutralization tests.

For the CS-VV technique only the one shot immune hamster ascitic fluid was used; and the results are presented in Table 3. The LNI of Lassa (Pinneo), Lassa (Josiah) and Mozambique IAFs in reactions with corresponding (homologous) viruses were 2.5, 2.0 and 1.9, respectively. With the exception of Mozambique IAF which had LNI of 1.0 against Lassa (Pinneo) virus, all other IAFs showed a LNI of < 1.0 in reactions against heterologous viruses.

Table 3. The constant serum-varying virus cross-plaque reduction neutralization

Virus	Log (PFU) of neutralization index of immune ascitic fluids to		
	Lassa (Pinneo)	Lassa (Josiah)	Mozambique
Lassa (Pinneo)-800521	**2.5**[a]	0.5	1.0
Lassa (Josiah)-802134	0.8	**2.0**	0.8
Mozambique-802851	0.2	0.3	**1.9**

LNI is the difference between the log PFU of virus preparation in the presence of norman serum and log PFU of virus preparation in the presence of tested serum. Homologous titres in bold type

Discussion

We present data showing differences in the plaque size produced by Lassa and Mozambique viruses under the same experimental conditions. In the PRNT, Mozambique virus IAF show a greater capacity to neutralize Lassa virus strains than the Lassa virus strains had for Mozambique virus. Our observations are in agreement with the results of other in vitro tests employing either the IF or in the PRNT (Buchmeier *et al.*, 1981; Jahrling, 1986).

In in vivo experimental infection Kiley *et al.* (1979) and Walker *et al.*, (1982), Lassa disease was prevented in rhesus monkeys by prior infection with low-pathogenic Mozambique virus. In rhesus monkeys, Lassa virus normally produces a disease clinically similar to the severe form observed in man (Stephen and Jahrling, 1979). It has been suggested (Kiley *et al.*, 1981) that the presence of an uncleaved glycoprotein on the Mozambique virus particle (but not on Lassa virus) may play a role for differential pathogenicity of the two viruses.

The two Lassa virus strains (one from Nigeria, and the other from Sierra Leone) show significant differences in cross-PRNT, especially with the CS-VV method. In another study, Jahrling (1986) further demonstrated that Lassa virus strains from Sierra Leone and Liberia are more closely related to each other, than either is to the Nigerian Lassa virus strain. Furthermore, in the same study, Jahrling (1986) showed that Mobala virus from the CAR was distinct from either the Lassa or Mozambique viruses.

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Explanation to Figure (Plate XXXI):

Fig. 1. Mozambique virus plaques (left) and Lassa virus plaques (right) in Vero cell cultures.