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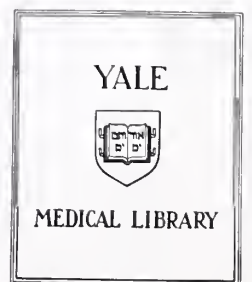
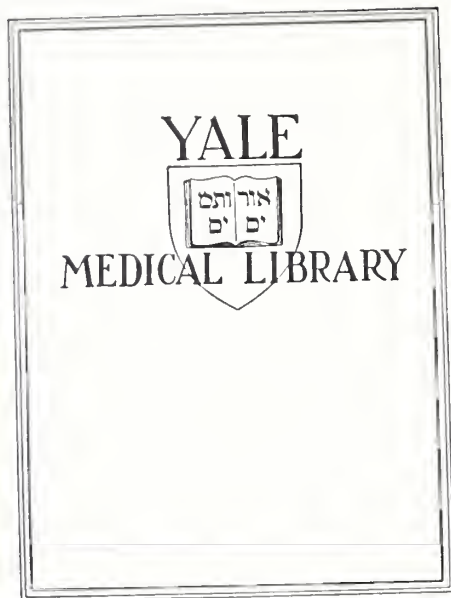
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HUMAN SEROLOGICAL SURVEY IN
LIBERIA OF SELECTED ARBOVIRUSES
AND OTHER VIRUSES OF VERTEBRATES



ALAN BRUCE BLOCH

1976



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Signature of Author

April 13, 1976
Date

Human Serological Survey in Liberia of Selected Arboviruses
and Other Viruses of Vertebrates

Alan Bloch


B.A., University of Connecticut, 1972

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Medicine

Department of Epidemiology and Public Health
Yale University School of Medicine

1976

To My Wife, Tricia



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Abstract

A serological survey was performed using 165 human serum samples collected in Liberia. These sera were tested against selected arboviruses and other vertebrate viruses. By complement-fixation (CF) test 5 positives for Lassa Fever virus were found; three of these were previously documented during the 1972 outbreak at Zorzor, Liberia. Complement-fixation and hemagglutination-inhibition (HI) antibodies against chikungunya were found in 21% of specimens; neutralization tests correlated well with CF and HI test results. CF tests for Bwamba virus were positive in 7% or 5 specimens; neutralization tests confirmed most of these positives. HI tests were done with four group B antigens: dengue II, West Nile, yellow fever, and Zika. Of these four agents, neutralization tests were done only against Zika virus. Sera which demonstrated HI patterns of Zika primary infection, successfully neutralized Zika virus. Thus there is good evidence for the existence in Liberia of Zika virus. No conclusions could be drawn about the presence in Liberia of Ilesha or Bunyamwera, because of the poor correlation between HI tests and neutralization tests. Serological test results were negative against Congo virus by CF test and against Mokola virus by CF test. A few sera were weakly positive against Ingwavuma by HI test, but these sera failed to neutralize virus.

Introduction

The past quarter century has witnessed the isolation of a large number of previously unknown viruses from arthropods and vertebrates. Of the 359 viruses in the 1975 edition of the International Catalogue of Arboviruses Including Certain Other Viruses of Vertebrates, only 35 were isolated during the first half of this century; the other 90% were discovered after 1950.¹ For each virus many questions need to be answered--the range of vectors and vertebrate hosts, the geographical distribution, and the potential for human infection and disease. In the region of West Africa, where a large number of arboviruses and other viruses has been isolated, these questions remain significantly unanswered for many viruses. This is especially true in countries without a virus research laboratory.

In one such country, Liberia, virus studies have been quite limited during the past quarter century. In a 1970 review by Bres of serological studies on arboviruses in Africa, there is reference to only two Liberian studies, both of which are unpublished.² One study by the Rockefeller Foundation Virus Laboratory analyzed 101 human sera from five cities and towns in western Liberia--Monrovia, Zorzor, Balahum, Voinjama, and Ghanta. Data suggested that infections with group A, group B, and Bunyamwera group viruses were common in Liberia. The second study was done in 1967 by Robin on 388 sera from Loffa County, in response to a yellow fever outbreak in the town of Salayea in which there were 5 survivors and 3 deaths. Yellow fever antibodies were found in: none of 19 sera in the age range 0-4 years; none of 153 sera in the age range 5-14 years; 2 of 65 sera (3%) in the age range 15-24 years; and 20 of 137 sera (14.5%) in the age range 25-59 years. As in the Rockefeller Foundation survey, antibodies were found to group A,

group B, and Bunyamwera group viruses.

One final study in Liberia consisted of an investigation in 1972 of a Lassa Fever outbreak at Curran Lutheran Hospital in the town of Zorzor in Loffa County. The outbreak involved 11 cases, of which 4 were fatal, giving a mortality rate of 36%. Lassa Fever virus is known to exist in several other West African countries, including Nigeria, where the first isolation was made in 1969 from humans, and also Sierra Leone. The ability of this virus to kill a high percentage of those infected, as demonstrated by outbreaks in each of these countries, makes further knowledge about this virus extremely desirable.

Thus one objective of the present survey in Liberia is to help ascertain how widespread the antibodies to Lassa Fever virus might be, especially among hospital workers for whom there may be an increased risk of exposure. Two other viral agents in the present survey have also been isolated from fatal human infections. One of these, Mokola, is a rabies-related virus which has been isolated from two children in Nigeria, one of whom died of a poliomyelitis-like illness.³ The second, Congo, has been isolated from humans in the Congo and Uganda. No human infections or human antibodies have been demonstrated in West Africa, although animals have demonstrated the virus.⁴ As in previous studies of Liberia, group A, group B, and Bunyamwera group viruses have been included in the present survey; viruses from several other groups have also been included.

Liberia lies on the west coast of Africa several degrees above the equator. Its area of 43,000 sq mi is about the size of Pennsylvania. There are three geographic zones: a 30-50 mile-wide strip of lowland along the 35 mi coast, characterized by mangrove marshes, lagoons, and tidal creeks; an interior tropical rain forest extending all the way to the inland border and characterized by hills and plateaus rising to elevations of 600-1000 feet; the Guinea Highland

in the far northern part of Liberia, where the forest is sparse and mountains rise to 5000 ft. The climate is hot and humid with temperatures in the 70's and 80's. There is a dry season and rainy season. Rainfall averages 175 in/yr along the coast and 75-100 in/yr inland.⁵

A 1971 survey estimated the population at 1,570,000. Seventy-two percent lived in rural areas (areas of 2,000 people or less), and 28 percent lived in urban areas.⁶ Large sections in the southeast third of Liberia and in the West in Loffa County are almost uninhabited. Population groups consist of 28 tribes, each with its own language, and also 15,000 descendants from black immigrants from the United States. The median age is 18.9 years, and the overall age distribution is typical of countries with high fertility and with a high proportion of young people. The general fertility rate (the annual number of live births per 1,000 women age 15-44 years) is 203. Agriculture is the main occupation for 3 out of 4 Liberians. Chief income for Liberia is derived from exports of iron ore and natural rubber.

Chief causes of morbidity and mortality include malnutrition, malaria, helminthic infestations, diarrhea, dysentery, tuberculosis, schistosomiasis, and leprosy. Trypanosomiasis has not been noted to be a significant problem. Cholera has occurred in recent years. Leading causes of childhood morbidity and mortality are prematurity, malnutrition, pneumonia and other respiratory infections, gastrointestinal infections, malaria, tetanus, measles, meningitis, and hepatitis. Overall statistics on morbidity and mortality are not readily available. However in 1970 it was estimated that 43% of all deaths were in children under one year of age. It was also estimated that 8 of 10 Liberians die at home, unattended by qualified medical personnel.⁷

Chapter One

Materials and Methods

Section One: Serum Collection and Survey Questions

Collections were made in Liberia from October through December, 1974 at four sites: ELWA Hospital (12 miles outside Monrovia), Maternity Center (Monrovia), Phebe Hospital (approx. 80 miles NE of Monrovia), and Curran Hospital at Zorzor (about 130 miles NNE of Monrovia). Thus an effort was made to collect samples from several separate locations in Liberia. Criteria for selection of people to bleed included a preference for hospital workers in order to answer questions about nosocomial viral infections. However an effort was also made to obtain blood from those not working in hospitals, but who lived in the region served by the hospital. Essentially all of the people in this latter category were hospital inpatients, admitted with various problems including obstetrical, surgical, or medical problems. All of the hospital personnel were in good health. Very few of the inpatients in the survey had febrile illnesses suggestive of an ongoing infectious process. Permission for venipuncture was granted by almost all hospital personnel. Blood samples from hospital inpatients were obtained along with the routine lab work. Consent for venipuncture in the outpatient clinic or in villages was extremely difficult to obtain, and sample collections were not pursued in these settings.

Blood was collected in 15cc vacutainer tubes via venipuncture of an alcohol-swabbed area. The tubes then stood upright at room temperature for 8-12 hours, and then the clot was removed with a sterile ap-

plicator stick. Remaining blood cells were removed by centrifugation for 10 min at about 1000 rpm, followed by removal of the supernatant serum with a sterile Pasteur pipette. By this time the serum was reasonably clean, with very few specimens showing any trace of hemolysis and none with any red blood cells visible. The sera which averaged 4-5cc were stored in sterile plastic screw-capped tubes inside a household freezer. At the end of December the 165 samples were placed in styrofoam-insulated boxes, driven to Robertsfield International Airport and stored in a freezer. They were subsequently flown via Pan American Airlines to J.F.K. Airport, stored in a freezer until customs clearance, and then shipped to the Yale Arbovirus Research Unit (YARU) in New Haven, Connecticut.

Questions asked of the blood donors included: name, sex, age, occupation, residences, past serious illness, especially with sore throat and difficulty swallowing. In certain cases, including those who claimed to have had Lassa Fever or similar illness, a more detailed account of illness was obtained.

Section Two: Serological Tests

Complement-fixation (CF) tests were done after the method of Casals with close adherence to his technique.⁸ An extensive and detailed description of this test may be found in Appendix I. YARU lyophilized viral antigens included: chikungunya, strain Ross-passage 170, prepared by Casals; Congo, strain K2/61-passage 4; Congo, strain U3010-passage 13, prepared by Casals; Mokola, strain IbAn 27377-passage 7; and Bwamba, strain M459-passage 85. These antigens were prepared by the sucrose-acetone method described by Clarke and Casals.⁹ In the unlikely possibility that the inactivated Lassa antigen (strain LP, <10 passages, obtained from Center for Disease Control in Atlanta) might be pathogenic, this CF test was done by Dr. Casals who has survived infection with this virus and is presumably immune to it.

Hemagglutination-inhibition (HI) tests were done by methods described by Clarke and Casals¹⁰ and by Casals¹¹ with close adherence to their techniques. A comprehensive description of the method may be found in Appendix II. Antigens used were the YARU lyophilized preparations obtained by the same sucrose-acetone method that was used in the CF tests and included: Bunyamwera, strain original -passage 49, prepared by Casals; chikungunya, strain Ross -passage 175; dengue II, strain TH36-passage unknown; Ilesha, strain KO/2 - passage 12; Ingwavuma, strain An 4165 - passage 8; West Nile, strain B 956 - passage 3; yellow fever, strain Asibi - passage 215; Zika strain MR 766 - passage 148.

The neutralization test (NT) was done after the method of Casals and was followed closely.¹² A full explanation of this technique may be found in Appendix III. The NT tests were done with the following lyophilized viruses: Bunyamwera, strain original - passage 46; Bwamba, strain M 459 -

passage 86; chikungunya, Ross strain - passage 176; Ilesha, strain
K0/2 - passage 12; Ingwavuma, strain An 4165 - passage 8; Zika, strain
MR 766 - passage 148. The calculation of LD_{50} was done after the method
of Reed and Muench. 13

Section One: Survey Information

Survey data and CF and HI test results from each individual who donated serum, are given in Appendix IV. A summary of the survey data is given in Table 1 and is briefly described below.

1. Location of serum collection. Of the 165 serum samples: 56% were obtained in Montserrado County, either at ELWA Hospital or at the Maternity Center; 23% were obtained in Bong County at Phebe Hospital; 21% were obtained in Loffa County at Curran Hospital in Zorzor. To identify locations in the survey, a map of Liberia is given on the next page.

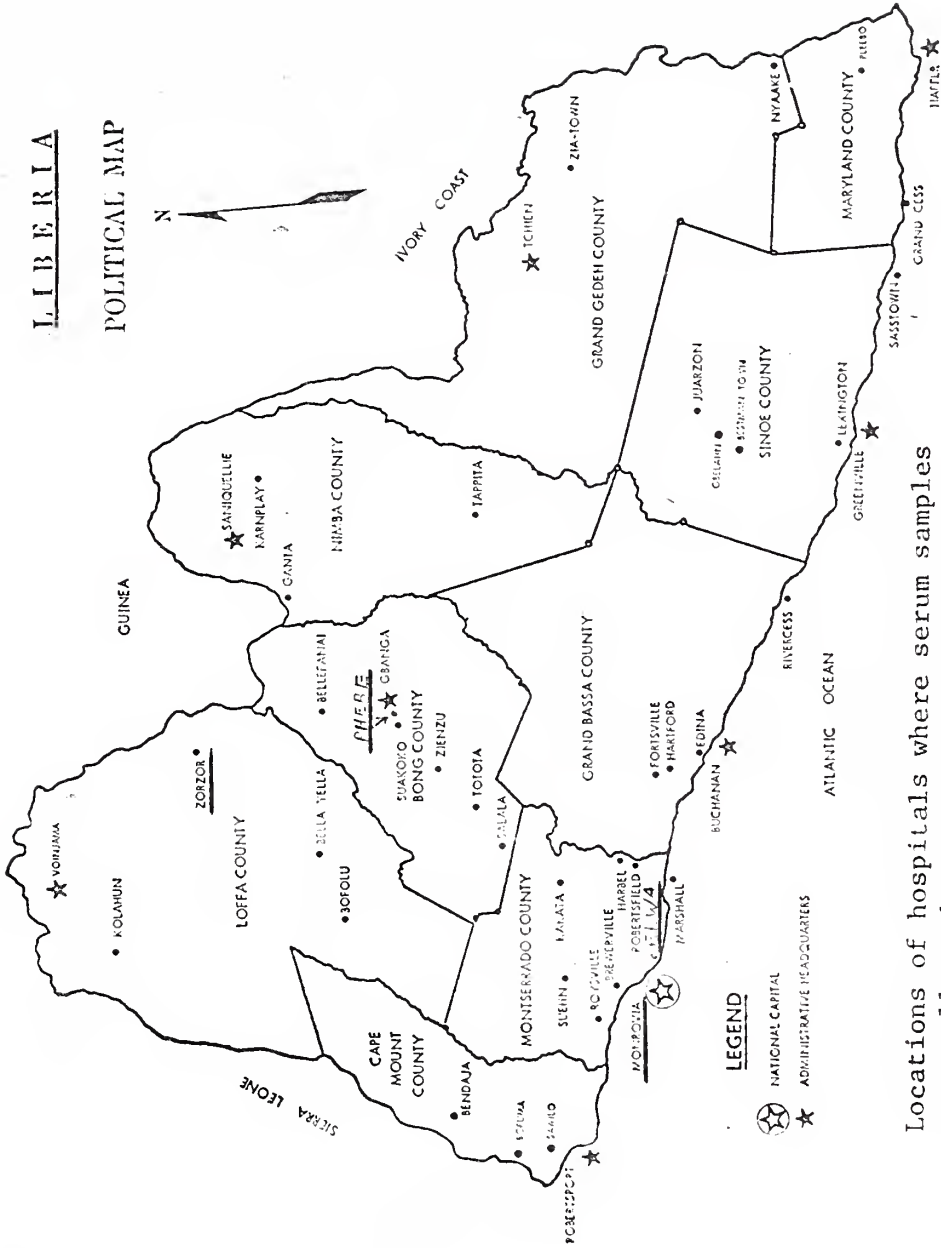
2. Sex. Thirty-one percent of samples were from males, and 69% were from females. The female predominance is due to an intentional effort to collect sera from many hospital personnel.

3. Occupation. Sixty-three percent of those surveyed were involved in health care delivery. The second-most predominant occupation was farming, practiced by a known 10% of those surveyed.

4. Age. The breakdown includes: under 20 years, 8%; 20-29 years, 39%; 30-39 years, 27%; 40-49 years, 14%; 50 years and older, 8%. None of those in the survey were under twelve years. The reason for so few sera from those under 20 years, is the added difficulty in obtaining such samples. As mentioned earlier, serum collection in villages and outpatient clinics was met with poor compliance. Inpatient pediatrics consisted primarily of children under five years of age. Because of the technical difficulty involved in obtaining a sufficient quantity of serum with the equipment available, no attempt was made to collect sera from the pediatric ward. A percentage distribution of the population of Liberia by age and sex for 1971 is given in Table 2.

LIBERIA

POLITICAL MAP



Locations of hospitals where serum samples were collected are underlined.

5. Residence. Of all those surveyed: 64% had spent some part of their lives in Montserrado County; 36% had lived in Loffa County; 28% had lived in Bong County. The other six counties were represented by less than 15% of those surveyed.

6. Serious illness with sore throat and dysphagia. Only eight people or 5% of those surveyed, gave this past history.

In the last four categories answers were obtained from 85-90% of those from whom serum was obtained. Thus there remain 10-15% of people for whom survey information was incomplete.

Table 1

Summary of Survey Information

I Location of Serum Collection	Number of Sera	Percentage
ELWA Hospital near Monrovia	61	37 %
Phebe Hospital near Gbarnga	37	23 %
Curran Hospital at Zorzor	35	21 %
Maternity Center in Monrovia	32	19 %
Total	165	100 %
II Sex	Number of People	
Male	51	31 %
Female	114	69 %
Total	165	100 %
III Occupation	Number of People	
Midwife	38	23 %
Midwife Student	24	15 %
Nurse	27	16 %
Other Health Care Workers	15	9 %
Total Number of Health Care Workers	104	63 %
Farmer	17	11 %
Housewife and mother ¹	13	8 %
Other	9	6 %
Unknown	20	12 %
Total	165	100 %
IV Age ²	Number of People	
Unknown	6	3.5 %
<10	0	0 %
10-19	13	8 %
20-29	64	39 %
30-39	45	27 %
40-49	23	14 %
50-59	5	3 %
≥60	9	5.5 %
Total	165	100 %
V Residence ³	Number of People	
Montserrado County	106	64 %
Bong	46	28 %
Loffa	60	36 %
Grand Bassa	17	10 %
Cape Mount	12	7 %
Nimba	18	11 %
Sinoe	10	6 %
Grand Gedeh	4	2.5 %
Maryland	7	4 %
Other		
USA	11	7 %
Sierra Leone	8	5 %
Ivory Coast, Guinea, Ghana, Sudan,		
Italy, England, France, India	1 each	.6 %
Majority of life outside West Africa, i.e.,		
total number of non-West Africans	7	4 %
VI Serious Illness with Sore Throat, Dysphagia	8	5 %

Footnotes

1. The number of housewives and mothers was derived in part from patients admitted for obstetrical and gynecological reasons.
2. In a number of cases the patient did not know his exact age. Thus the age was estimated as best as possible.
3. All residences during lifetime of each person are included in the numbers given. Thus a person who lived in several places is listed as having resided in each one. It must be stated that there were about 15-20 people for whom some or all lifetime residences were not obtained.

Table 2

Percentage Distribution of the
Liberian Population by Age
and Sex 1971

Age Groups	Both Sexes	Male	Female
All Ages	100.0	100.0	100.0
0-1 yr.	3.1	3.3	2.0
1-4 yrs.	13.6	14.2	13.2
5-9 yrs.	14.7	15.2	14.2
10-14 yrs.	10.2	11.4	9.1
15-19 yrs.	8.6	8.5	8.8
20-24 yrs.	7.8	6.2	9.4
25-29 yrs.	9.1	7.4	10.8
30-34 yrs.	7.2	6.5	7.9
35-39 yrs.	6.6	6.6	6.7
40-44 yrs.	4.9	5.1	4.6
45-49 yrs.	3.8	4.3	3.3
50-54 yrs.	3.1	3.4	2.8
55-59 yrs.	1.9	2.3	1.6
60-64 yrs.	1.9	2.0	1.7
65 years & over	3.4	3.8	3.1

Source: Liberia, Ministry of Planning and Economic Affairs,
Economic Survey of Liberia 1971.

A summary of the CF tests for the five antigens assayed is given in Table 4. The results with each antigen are briefly described below.

1. Lassa Fever. Of the 165 sera five were positive for Lassa Fever. A comprehensive sketch of these five which includes a description of illness, is given in Tables 3-A and 3-B. Three of the people were females previously documented as positives by Dr. Thomas Monath during a Lassa Fever outbreak in 1972 at Curran Hospital in Zorzor, Loffa County.¹⁴ In two of these females the illness was severe, while in the third it was relatively mild. A fourth female claimed severe illness while at Zorzor in November, 1969. These four women were working as midwives at the time of illness. All described a clinical picture compatible with Lassa Fever as known to date, except that the one who was ill in 1969 described yellow sclera as part of her illness. The fifth positive was a 19 year-old female studying midwifery at Phebe Hospital in Bong County. The first 18 years of her life were spent at Koindu, Sierra Leone, about 50 miles east of the site of the 1972 Lassa Fever epidemic at Panguma, Sierra Leone.¹⁵ She denied ever having been seriously ill.

2. Congo. There was one possible positive by CF. The serum was non-specific, but it manifested a stronger fixation of complement in the serum wells than in the control wells. Subsequent NT test of this serum by Dr. Casals was negative. (Congo virus, strain IbAr 10-200 passage 13, prepared by Casals).

3. Mokola. No positive sera were found.

4. Chikungunya. Although sera were screened at dilutions of 1:4 and 1:8, end-point titrations were not carried out. Twenty-five sera

(15% of all sera) were positive.

5. Bwamba. End-point titrations were carried out, and the titers are listed in Table 4. Eleven samples (7% of all sera) were positive.

Table 3-A

Data on People with Positive CF Test for Lassa Fever

Name	Lusu	Josephine Foryon	Martha Vankpne	Phebe Hallowanger	Jetta Momolu
Serum Number	14	33	82	92b	178
Sex	Female	Female	Female	Female	Female
Age	36	19	26	35	28
Occupation	Midwife, 5 yr	Midwife	Midwife, 5 yr	Midwife, 4 yr	Midwife, 8 yr
Residences by county	Loffa ¹ , 32 yr Montserrado, 4 yr	Koindu, SL ² , 18 yr Bong, 1 yr	Loffa, 7 yr Bong, 19 yr	Loffa, 12 yr Mulbergs ³	Montserrado, 20 yr Loffa, 8 yr
Duration at Zorzor	3 yrs, 1967-70	None	7 yrs	Not obtained, most likely 6 yrs	8 yrs, 1964-72
CF Titers by Monath ⁴	Not at Zorzor during outbreak of Lassa Fever	Never lived at Zorzor	Serum drawn: 4/10/72 1:32 4/20/72 1:16	Serum drawn: 4/10/72 1:4 6/12/72 1:32	Serum drawn: 4/15/72 <1:4 ⁵ 6/12/72 1:64
CF Titer by Casal ⁶	Serum drawn: 11/6/74 2/4	Serum drawn: 11/18/74 2/4	Serum drawn: 11/17/74 2/4	Serum drawn: 11/18/74 2/4	Serum drawn: 12/26/74 2/4
Details of Illness		Denies ever having serious illness			
Place of Illness	Zorzor		Zorzor	Zorzor	Zorzor
Date of Illness	November, 1969		Not ill ⁷	Onset 3/21/72	Onset 3/20/72
Length of Illness	Two weeks		Few days	Four weeks	Not obtained

Footnotes

1. Loffa county contains Zorzor, the site of the 1972 Lassa Fever outbreak.
2. Until one year ago Miss Foryon had spent all her life at Koindu, Sierra Leone, which is 50 mi. E of Panguma, the site of an outbreak of Lassa Fever in 1972.
3. Liberian town, county not obtained.
4. Values of CF titers were obtained from Dr. Monath through correspondence. Since no value for the titer of antigen was mentioned, only the serum titer is indicated.
5. Jetta Momolu is most likely the individual named Jetty Ziegler in Dr. Monath's study, who was married to Francis Momolu.
6. CF titers are recorded as: the reciprocal of the serum titer/ the reciprocal of the antigen titer. CF titers are recorded only for sera with "3+" or "4+" fixation of complement. A preliminary screening of all sera at a 1:8 dilution of antigen, showed only two positives, namely sera # 33 and 92b, both with titers of 4/8. A final screening with a 1:4 dilution of antigen was run on about 10 highly suspicious sera; and it showed the above five positives. It is noteworthy that in the final screening the endpoints for sera # 33 and 92b, namely 2/4, were below those of the preliminary screening, namely 4/8.
7. Although Dr. Monath's communication mentions that Martha Vankpne was not ill, this lady related the above account of illness. Dates for the onset of illness for Phebe Hallowanger and Jetta Momolu were obtained from Dr. Monath.

Clinical History of People with Positive CF Test for Lassa Fever¹

Name	LS	JF	MV	PH	JM
General Symptoms					
Fever	+ + ²	Denies ever having serious illness.	+	+	+
Chills	+		+	+	+
Rigors	+		0	+	+
Prostration	+		+	+	+
General Aches	+		+	+	+
Headache	+		+	+	+
Anorexia	+		0	+	+
Nausea	+		+	+	+
Vomiting	+		0	+	+
Specific Symptoms					
Sore throat	+		+	+	+
Difficulty swallowing	+ +		+	+	+
Coryza	0		+	+	+
Stiff neck	+		0	0	0
Agitation	+		0	0	0
Delirium	+		0	0	0
Coma	+		0	0	0
Reddened Eyes	0		0	+	0
Sclericterus	+		0	0	0
Hemorrhage/bruising	0		0	0	0
Rash	0		0	0	0
Lymphadenopathy	+		0	Hands, arms	0
Respiratory Difficulty	+		0	0	0
Urinary Difficulty	0		0	0	0
Other	Hearing difficulty		Backache	Diarrhea	Oliguria Tinnitus
Laboratory					
Malaria smear	0		+	Not known	Not known
Low white blood count	+		+	Not known	Not Known

¹Clinical findings are personal accounts, not documented by records. Only in the fourth and fifth patients was the morbid episode documented by acute and convalescent CF tests which showed >fourfold rise in titer for Lassa.

2. + + = symptom present and very severe.

+ = symptom present or low white blood count or positive malaria smear.

0 = symptom absent or negative malaria smear.

Table 4

CF Test Results Correlating Antigen with Degree of Seroreactivity¹

	Negative CF Test	Positive CF Test ²	Percent Positive
Lassa	160	Total of 5 sera at titer of 2/4	3%
Congo	164	Possibly one serum at a titer of 2/8 ³	<1%
Mokola	165	0	0%
Chikungunya	140	Total of 25 sera at the following screening titers: One serum at 4/8 Fifteen sera at 8/8 Nine sera at 8/12	15%
Bwamba	150	Total of 11 sera at the following titers: Three sera at 2/4 Seven sera at 4/4 One serum at 8/4	7%

Footnotes

1. Each box indicates the number of serum samples out of a total of 165. For Bwamba only 161 sera were assayed because four sera had already been used up.
2. CF tests are recorded as: the reciprocal of the serum titer/ the reciprocal of the antigen titer. CF titers are recorded only for sera with readings of "3+" or "4+" fixation of complement. Values represent end-point titrations for all antigens except chikungunya in which only a screening was done with 1:4 and 1:8 serum dilutions.
3. This serum sample was non-specific; but it manifested stronger fixation of complement in the serum wells than in the control wells.

Section Three: Hemagglutination-Inhibition Test Results

A summary of the HI tests for the 8 antigens assayed is given in Table 5. Selected aspects of the HI tests with the group B antigens are portrayed in Tables 6-A, B, and C. The results with each antigen are briefly described below. The serum titers listed below are adjusted for inhibition of hemagglutination of 8 units of antigen. Serum titers of " $<1:10$ " indicate sera giving hemagglutination-inhibition with 2-4 units of antigen, which when adjusted to 8 units of antigen, give values $<1:10$.

1. Chikungunya. 34 sera (21% of total) had a titer of $<1:10$ or higher. Of these 26 sera (16% of total) had titers $\geq 1:20$.

2. Ilesha. 11 sera (7% of total) were positive, all with a titer of $<1:10$.

3. Ingwavuma. 6 samples (4% of total) were positive. All had titers $\leq 1:10$ except for one serum with a titer of $1:20$.

4. Bunyamwera. 32 samples (19% of total) had a titer of $<1:10$ or higher. Of these 6 sera (4% of total) had titers $\geq 1:20$.

5. Dengue II. 28 sera (17% of total) had a titer of $<1:10$ or higher. Of these 20 sera (12% of total) had titers $\geq 1:20$.

6. West Nile. 58 samples (35% of total) had a titer of $<1:10$ or higher. Of these 26 sera (16% of total) had titers $\geq 1:20$.

7. Yellow Fever. 51 sera (31% of total) had a titer of $<1:10$ or higher. Of these 30 sera (18% of total) had titers $\geq 1:20$.

8. Zika. 42 sera (25% of total) had a titer of $<1:10$ or higher. Of these 23 sera (14% of total) had titers $\geq 1:20$.

9. Group B Antigens. See Tables 6-A, B, C. The four group B antigens tested by HI were: dengue II, West Nile, yellow fever, and Zika.

For these four all except dengue II reacted with sera which showed positivity only to that one group B antigen (Table 6-A). No sera were positive only to West Nile at titers $\geq 1:20$. Nine sera were positive only to Yellow Fever at titers $\geq 1:20$. Four were positive only to Zika at titers $\geq 1:20$.

Table 6-B indicates the number of sera that were positive to only two of the four group B antigens. Table 6-C deals with "broad group B" reacting sera, i.e., those serum samples which were positive to all four group B antigens. Eighteen sera (11% of total) showed HI positivity to all four group B antigens at a titer of $<1:10$ or higher. Of these 13 sera (8% of total) showed positivity to all four antigens at titers $>1:20$.

Finally of the 165 serum samples, 94 had HI antibodies (titer of $<1:10$ or higher) to one or more of the four group B members. Thus 57% of those surveyed possessed group B antibodies.

Table 5
 HI Test Results Correlating Antigen with Degree of Seroreactivity¹

	Serum Dilutions											Total Positive	Total Positive $\geq 1:20$	Percent Positive $\geq 1:20$
	Negative	<1:10	1:10	1:20	1:40	1:80	1:160	1:320	1:640					
Chikungunya	131	0	8	7	9	6	3	1	0			34	26	16%
Ilesha	154	0	11	0	0	0	0	0	0			11	0	0%
Ingwavuma	159	4	1	1	0	0	0	0	0			6	1	<1%
Bunyamwera	133	22	4	4	1	1	0	0	0			32	6	4%
Dengue II	137	6	2	5	4	3	5	2	1			28	20	12%
West Nile	107	16	16	12	8	4	1	1	0			58	26	16%
Yellow Fever	114	12	9	12	14	4	0	0	0			51	30	18%
Zika	123	9	10	7	5	2	5	4	0			42	23	14%

Footnote

1. Each box indicates the number of serum samples out of a total of 165, inhibiting hemagglutination of 8 units of antigen at the serum dilution indicated at the top of the column. The column marked "Negative" refers to sera giving no hemagglutination inhibition at the initial 1:10 serum dilution. The column marked "<1:10" indicates sera giving inhibition to one or two dilutions with 2-4 units of antigen, which when adjusted to 8 units of antigen give values <1:10. Column marked "1:10" indicates sera giving hemagglutination inhibition at a 1:10 dilution, etc.

Tables 6 -A,B,C

Selected HI Test Results with Group B Antigens

Table 6 -A

Number of Sera Reactive Only to One of the Four Group B Antigens¹

	Serum Dilution						Total
	<1:10	1:10	1:20	1:40	1:80	1:160	
Positive only to Dengue II	0	0	0	0	0	0	0
Positive only to West Nile	12	6	0	0	0	0	18
Positive only to Yellow Fever	10	6	4	4	1	0	25
Positive only to Zika	3	2	1	1	1	1	9

Footnote

1. Each box indicates the number of serum samples out of a total of 165, inhibiting hemagglutination 8 units of the indicated group B antigen at the serum dilution indicated at the top of the column. The column marked "<1:10" indicates sera giving inhibition to one or two dilutions with 2-4 units of antigen, which when adjusted to 8 units of antigen, give values <1:10. Column marked 1:10 indicates sera giving hemagglutination at a 1:10 dilution, etc. The four group B antigens are: dengue II, West Nile, yellow fever, and Zika.

Tables 6 -A,B,C

Selected HI Test Results with Group B Antigens

Table 6 -B

Number of Sera Reactive to Only Two of the Four Group B Antigens¹

	Number of Sera
Reactive to Dengue II and West Nile	2
Reactive to Yellow Fever and Zika	1
Reactive to West Nile and Yellow Fever	4
Reactive to West Nile and Zika	6

Footnote

1. Each box indicates the number of serum samples out of a total of 165, inhibiting hemagglutination of 8 units of the indicated group B antigens. Serum titers for these reactions range from "<1:10" upward. See Table 5 for a listing and explanation of these serum dilutions. While other combinations are possible, no other combinations for reactions to only two of the four group B antigens were observed. The four group B antigens are: dengue II, West Nile, yellow fever, and Zika.

Tables 6 -A,B,C

Selected HI Test Results with Group B Antigens

Table 6 -C

Number of Sera Reactive to All Four Group B Antigens ¹		Percent
Total number of sera in which serum titer was >0 for all 4 Group B antigens	18	11%
Total number of sera in which serum titer was \geq 1:20 for all 4 Group B antigens	13	8%

Footnote

1. Each box indicates the number of serum samples out of a total of 165, inhibiting 8 units of each of the four group B antigens. Serum titers for reactions in the upper box range from "<1:10" upward. Serum titers for reactions in the lower box (which is a subset of the upper box) range from 1:20 upward. See Table 5 footnote for listing and explanation of serum dilutions.

Section Four: Neutralization Test Results

Neutralization tests were done with six viruses in order to confirm the results of the CF and HI tests. The six NT's are displayed in Tables 7-A through 7-F. All six tests had adequate and valid controls. Tables 8-A through 8-F correlate the results of each NT test with the corresponding CF and/or HI tests. These correlations are described below. A non-protective serum is here defined as one in which there are 0-2 survivors from the original litter of 8 mice. A protective serum is defined as one in which there are only 0-2 deaths attributable to the virus from the original litter of eight mice. A partially protective serum is defined as one which does not fall into the other two categories.

1. Zika. (Tables 7-A and 8-A). The 3 sera positive by the HI test to Zika virus but not to other group B antigens neutralized Zika virus in mice; of 6 sera positive for Zika and for 1 or 2 group B antigens, 3 protected against Zika in the neutralization test and 3 did not. Eight sera positive to all 4 group B antigens failed to protect mice against Zika. Three sera negative to all 4 group B antigens and 3 positive to an antigen other than Zika, also failed to protect mice against Zika.

2. Bwamba. (Tables 7-B and 8-B). Of the six CF negative sera, two gave protection and four gave no protection from the virus. Of the eleven CF positive sera, eight were protective and three were not.

3. Ilesha. (Tables 7-C and 8-C). Of the six HI negative sera, five were protective and one was not. Of the eleven HI positive sera: nine neutralized the virus; one partially neutralized the virus; and one failed to neutralize the virus.

4. Ingwavuma. (Tables 7-D and 8-D). All five HI negative sera and all six HI positive sera failed to protect against the virus.

5. Chikungunya. (Tables 7-E and 8-E). All six sera shown to be negative by both CF and HI tests, failed to protect against the virus. All eleven sera shown to be positive by both CF and HI tests, protected against the virus.

6. Bunyamwera. (Tables 7-F and 8-F). Of the six HI negative sera: one was protective; one was partially protective; and four were non-protective. Of the thirteen HI positive sera: two were protective; two were partially protective; and nine were nonprotective.

Table 7-A

Neutralization Test: Zika¹

Litter Number ²	Virus Dilution ³	Serum or Ascitic Fluid ⁴	Deaths/Survivors ⁵	HI Dengue II ⁶	HI West Nile	HI Yellow Fever	HI Zika
07122	10 ⁻⁵	Liberian Human # 7	6/1				
23	"	" # 8	7/0	3	3	2	3
24	"	" # 9	8/0				
25	"	" # 11	1/7				
26	"	" # 12	7/1			1	1
27	"	" # 14	8/0			2	
28	"	" # 15	8/0				
29	"	" # 20	8/0	5	4	4	6
07130	"	" # 22	8/0	3	3	2	3
31	"	" # 23	8/0	5	3	3	3
32	"	" # 24	8/0	7	5	3	5
33	"	" # 27	8/0			3	
34	"	" # 31	8/0	5	4	3	4
35	"	" # 38	8/0	5	4	2	5
36	"	" # 81	0/3				5
37	"	" # 94	0/7				2
38	"	" # 103	1/6	0-1	1	1	6
39	"	" # 111	8/0	1	1		1
07140	"	" # 121	8/0		0-1		0-1
41	"	" # 123	8/0		1		1
42	"	" # 131	0/6		6		6
43	"	" # 139	8/0	6	1	3	5
44	"	" # 150	0/8				4
07145	"	Zika MIAF ⁷	0/8				
46	"	" "	0/8				
07147	"	NMAF ⁸	8/0				
48	"	" "	8/0				
07149	10 ⁻⁵	Titration ⁹	6/2				
50	10 ⁻⁶	"	4/3				
51	10 ⁻⁷	"	0/8				
52	10 ⁻⁷	"	0/8				
53	10 ⁻⁸	"	0/7				
54	10 ⁻⁹	"	0/8				

Footnotes

- Test was run from 5/20/75 to 6/3/75. Zika virus used in NT consisted of a wet pool, made from 10% baby mouse brain suspended in 7.5% bpa. Virus for the wet pool came from YARU lyophilized Zika, passage # 148, harvested 2/21/68 and freeze-dried 3/25/68.
- Each litter consisted of 3- or 4-day-old mice, eight in number, inoculated intracerebrally with app. 0.02cc.
- Dilutions of virus were made with 0.75% bpa.
- Virus-serum or virus-ascitic-fluid mixtures for inoculation were made by adding virus to an equal volume of serum or ascitic fluid, respectively.
- Mice which died before the viral disease began or under circumstances not possibly attributable to the virus, are not included in the determination of deaths or survivors.
- HI tests are recorded as the number of two-fold dilutions beginning at 1:10 inhibiting 8 units of antigen. Values recorded as 0-1 indicate sera giving inhibition to one or two dilutions with 2-4 units of antigen, which when adjusted to 8 units of antigen, give values <1:10. Values are otherwise not recorded when titer <1:10.
- Zika MIAF consisted of Zika mouse immune ascitic fluid, YARU lyophilized 8/27/66-9/28/66.
- NMAF consisted of normal mouse ascitic fluid, YARU lyophilized, R 16208, 7/10/70-7/22/70.
- Dilutions for titration were made by adding virus to an equal volume of the same NMAF described in footnote 8.
- These values are most certainly in error for the following reason. The ratio of deaths/survivors for litter # 07149 should have corresponded more closely to those for litters # 07147 and 07148, because they all were supposed to contain the same virus dilution of 10⁻⁵, along with the same NMAF. All dilutions for the titration were derived from a different 10⁻⁵ pool than the 10⁻⁵ pool used to inoculate all the other litters. The values for LD₅₀ and for the number of LD₅₀ for the NT, must therefore differ from those corresponding to the litters outside of the titration. The NT still has some validity, however, because the NMAF controls and the Zika MIAF controls are good.

Table 7-B

Neutralization Test: Bwamba¹

Litter Number ²	Virus Dilution ³	Serum or Ascitic Fluid ⁴	Deaths/Survivors ⁵	CF Bwamba ⁶
07157	10 ^{-3.8}	Liberian Human # 22	8/0	
58	"	" " # 24	2/6	
59	"	" " # 27	0/8	4/4
07160	"	" " # 46	8/0	
61	"	" " # 49	8/0	
62	"	" " # 51	1/7	
63	"	" " # 65	1/7	4/4
64	"	" " # 67	8/0	4/4
65	"	" " # 75	8/0	4/4
66	"	" " # 93	0/8	8/4
67	"	" " # 104	5/2	
68	"	" " # 129	1/7	4/4
69	"	" " # 136	0/8	2/4
07170	"	" " # 153	0/8	2/4
71	"	" " # 170	0/8	2/4
72	"	" " # 171	8/0	4/4
73	"	" " # 180	0/8	4/4
07174	"	Bwamba MIAF ⁷	1/7	
75	"	" "	2/5 ¹⁰	
07176	"	NMAF ⁸	8/0	
07177	10 ^{-3.8}	Titration ⁹	6/0	
78	10 ^{-4.8}	" LD ₅₀ = 10 ^{-5.7} .	7/0	
79	10 ^{-5.8}	" Number of LD ₅₀	2/4	
07180	10 ^{-5.8}	" for NT = 80.	4/3	
81	10 ^{-6.8}	"	0/8	
82	10 ^{-7.8}	"	0/8	

Footnotes

1. Test was run from 5/20/75 to 6/3/75. Bwamba virus used in NT consisted of a wet pool, made from 10% baby mouse brain suspended in 7.5% bpa. Virus for the wet pool came from a preliminary wet pool which came from YARU lyophilized Bwamba, passage # 86, 12/8/61.
2. Each litter consisted of 4- or 5-day-old mice, eight in number, inoculated intracerebrally with app. 0.02 ml.
3. See footnote 3, Table 7-A.
4. See footnote 4, Table 7-A.
5. See footnote 5, Table 7-A.
6. CF tests are recorded as: the reciprocal of the serum titer/ the reciprocal of the antigen titer. CF titers are recorded only for sera with readings of "3+" or "4+" fixation of complement. The titers recorded represent end-points.
7. Bwamba MIAF consisted of lyophilized NIH Polyvalent Bwamba mouse immune ascitic fluid, catalogue no. G 212-601-567.
8. NMAF consisted of normal mouse ascitic fluid, YARU wet frozen 5/16/74.
9. Dilutions for titration were made by adding virus to an equal volume of the same NMAF described in footnote 8.
10. Small size of the mice in this litter, suggests poor care by mother as cause of deaths.

Table 7-C

Neutralization Test: Ilesha¹

Litter Number ²	Virus Dilution ³	Serum or Ascitic Fluid ⁴	Deaths/Survivors ⁵	HI Ilesha ⁶
07183	10 ^{-4.3}	Liberian Human # 24	0/8	
84	"	" " # 56	0/8	1
85	"	" " # 67	0/8	1
86	"	" " # 69	1/7	
87	"	" " # 71	0/8	
88	"	" " # 73	1/6	
89	"	" " # 88	1/7	1
07190	"	" " # 92b	0/8	1
91	"	" " # 93	8/0 ¹¹	1
92	"	" " # 95	0/8	1
93	"	" " # 119	0/8	1
94	"	" " # 126	0/8	1
95	"	" " # 139	8/0	
96	"	" " # 143	0/8	1
97	"	" " # 145	0/8	1
98	"	" " # 153	1/7	
99	"	" " # 171	4/4	1
07200	"	Ilesha MIAF ⁷	0/8	
01	"	" "	0/0 ¹⁰	
07202	"	NMAF ⁸	8/0	
07203	10 ^{-4.3}	Titration ⁹	8/0	
04	10 ^{-5.3}	" LD ₅₀ = 10 ^{-6.2}	7/0	
05	10 ^{-6.3}	" Number of LD ₅₀	3/4	
06	10 ^{-6.3}	" for NT = 80.	4/4	
07	10 ^{-7.3}	"	0/8	
08	10 ^{-8.3}	"	0/8	

Footnotes

1. Test was run from 5/21/75 to 6/3/75. Ilesha virus used in NT consisted of a wet pool, made from 10% baby mouse brain suspended in 7.5% bpa. Virus for the wet pool came from YARU lyophilized Ilesha, passage # 12, R5964, 3/21/68.
2. Each litter consisted of 4-day-old mice, eight in number, inoculated intracerebrally with 0.02ml.
3. See footnote 3, Table 7-A.
4. See footnote 4, Table 7-A.
5. See footnote 5, Table 7-A.
6. See footnote 6, Table 7-A.
7. Ilesha MIAF consisted of YARU lyophilized Ilesha mouse immune ascitic fluid, WHO ATCC, R 3483, inact. 12/6/67.
8. See footnote 8, Table 7-B.
9. See footnote 9, Table 7-B.
10. Mother of litter died on 6th day of test.
11. Average survival time of this litter was 9.3 day, double that for all others litters with no survivors, which was 4-5 days.



Table 7-D

Neutralization Test: Ingwavuma¹

Litter Number ²	Virus Dilution ³	Serum or Ascitic Fluid ⁴	Deaths/Survivors ⁵	HI Ingwavuma ⁶
07209	10 ^{-4.3}	Liberian Human # 24	8/0	2
07210	"	" " # 26	8/0	
11	"	" " # 32	8/0	0-1
12	"	" " # 38	8/0	
13	"	" " # 67	8/0	0-1
14	"	" " # 77	8/0	
15	"	" " # 78	8/0	
16	"	" " # 80	7/0	
17	"	" " # 96	8/0	
18	"	" " # 115	6/2	0-1
19	"	" " # 119	7/1	0-1
07220	"	" " # 139	8/0	1
07221	"	Ingwavuma MIAF ⁷	0/7	
22	"	" "	0/8	
07223	"	NMAF ⁸	8/0	
07224	10 ^{-4.3}	Titration ⁹	8/0	
25	10 ^{-5.3}	" LD ₅₀ = 10 ^{-6.8}	8/0	
26	10 ^{-6.3}	" Number of LD ₅₀	6/2	
27	10 ^{-6.3}	" for NT = 320	7/1	
28	10 ^{-7.3}	"	0/8	
29	10 ^{-8.3}	"	0/8	

Footnotes

1. Test was run from 5/26/75 to 6/6/75. Ingwavuma virus used in NT consisted of a wet pool, made from 10% baby mouse brain suspended in 7.5% bpa. Virus for the wet pool came from YARU lyophilized Ingwavuma, passage # 8, harvested 4/18/68 and freeze-dried 4/23/68.
2. Each litter consisted of 4-day-old mice, eight in number, inoculated intracerebrally with app. 0.02ml.
3. See footnote 3, Table 7-A.
4. See footnote 4, Table 7-A.
5. See footnote 5, Table 7-A.
6. See footnote 6, Table 7-A.
7. Ingwavuma MIAF consisted of YARU lyophilized Ingwavuma mouse immune ascitic fluid, MG 42756, 3/21/66.
8. See footnote 8, Table 7-B.
9. See footnote 9, Table 7-B.

Table 7-E
Neutralization Test: Chikungunya¹

Litter Number ²	Virus Dilution ³	Serum or Ascitic Fluid ⁴	Deaths/ Survivors ⁵	CF Chikungunya ⁶	HI Chikungunya ⁷
07230	10 ^{-4.9}	Liberian Human # 7	8/0		
31	"	" " # 22	0/8	8/12	5
32	"	" " # 24	1/7	4/8	4
33	"	" " # 26	0/8	8/12	4
34	"	" " # 28	0/8	8/12	2
35	"	" " # 31	8/0		
36	"	" " # 38	0/8	8/12	5
37	"	" " # 52	8/0		
38	"	" " # 55	8/0		
39	"	" " # 66	8/0		
07240	"	" " # 87	0/8	8/8	4
41	"	" " # 96	0/8	8/8	5
42	"	" " # 97	8/0		
43	"	" " # 104	0/8	8/8	6
44	"	" " # 123	0/8	8/8	1
45	"	" " # 126	0/8	8/8	1
46	"	" " # 135	0/0	8/8	2
07247	"	Chikungunya MIAF ⁸	0/8		
48	"	" "	1/7		
07249	"	NMAF ⁹	7/0		
07250	10 ^{-4.9}	Titration ¹⁰	8/0		
51	10 ^{-5.9}	" LD ₅₀ = 10 ^{-6.6} .	6/1		
52	10 ^{-6.9}	" Number of LD ₅₀	3/5		
53	10 ^{-6.9}	" for NT = 50.	2/6		
54	10 ^{-7.9}	"	0/5		
55	10 ^{-8.9}	"	0/8		

Footnotes

1. Test was run from 5/27/75 to 6/9/75. Chikungunya virus used in NT consisted of a wet pool, made from 10% baby mouse brain suspended in 7.5% bpa. Virus for the wet pool came from YARU lyophilized Chikungunya, passage # 176, harvested 2/6/70 and freeze-dried 2/23/70.
2. Each litter consisted of 2-day-old mice, eight in number, inoculated intracerebrally with app. 0.02ml.
3. See footnote 3, Table 7-A.
4. See footnote 4, Table 7-A.
5. See footnote 5, Table 7-A.
6. CF tests are recorded as: the reciprocal of the serum titer / the reciprocal of the antigen titer. CF titers are recorded only for sera with readings of "3+" or "4+" fixation of complement. Titers recorded do not represent end-points but reflect only a screening with 1:4 and 1:8 dilutions of serum.
7. See footnote 6, Table 7-A.
8. Chikungunya MIAF consisted of lyophilized Chikungunya TH 35 mouse immune ascitic fluid, MG 43026, 4/20/66.
9. See footnote 8 on Table 7-B.
10. See footnote 9 on Table 7-B.

Table 7-F

Neutralization Test: Bunyamwera¹

Litter Number ²	Virus Dilution ³	Serum or Ascitic Fluid ⁴	Deaths/Survivors ⁵	HI Bunyamwera ⁶
07256	10 ^{-6.5}	Liberian Human # 21	8/0	
57	"	" " # 22	6/2	1
58	"	" " # 23	8/0	2
59	"	" " # 24	8/0	3
07260	"	" " # 31	8/0	1
61	"	" " # 32	8/0	1
62	"	" " # 35	8/0	
63	"	" " # 38	8/0	2
64	"	" " # 39	6/2	
65	"	" " # 42	8/0	2
66	"	" " # 93	4/4	
67	"	" " # 95	8/0	
68	"	" " # 97	5/3	1
69	"	" " # 122	8/0	0-1
07270	"	" " # 126	0/8	
71	"	" " # 139	8/0	2
72	"	" " # 145	4/4	0-1
73	"	" " # 153	0/8	4
74	"	" " # 164	0/8	0-1
07275	"	Bunyamwera MIAF ⁷	0/8	
76	"	" "	0/8	
07277	"	NMAF ⁸	8/0	
07278	10 ^{-6.5}	Titration ⁹	8/0	
79	10 ^{-7.5}	" LD ₅₀ =10 ^{-8.7}	8/0	
07280	10 ^{-8.5}	" Number of LD ₅₀	4/4	
81	10 ^{-8.5}	" for NT = 160	6/2	
82	10 ^{-9.5}	"	0/8	
83	10 ^{-10.5}	"	0/8	

Footnotes

1. Test was run from 5/28/75 to 6/9/75. Bunyamwera virus used in NT consisted of a wet pool, made from 10% baby mouse brain suspended in 7.5% bpa. Virus for the wet pool came from a preliminary wet pool which came from YARU lyophilized Bunyamwera, passage # 46, R 5882, freeze-dried 3/14/68.
2. Each litter consisted of 5-day-old mice, eight in number, inoculated intracerebrally with app. 0.02ml.
3. See footnote 3, Table 7-A.
4. See footnote 4, Table 7-A.
5. See footnote 5, Table 7-A.
6. See footnote 6, Table 7-A.
7. Bunyamwera MIAF consisted of YARU lyophilized Bunyamwera mouse immune ascitic fluid, 12/12/67.
8. NMAF consisted of YARU wet-frozen normal mouse ascitic fluid, Sarcoma 180, 5/1/69.
9. Dilutions for titration were made by adding virus to an equal volume of the same NMAF described in footnote 8.

Tables 8-A through F

Correlation of CF and HI Test Results with NT Test Results
On Selected Sera from the 165 Serum Samples

Table 8-A

Correlation of Group B HI Test Results with Zika NT Test Results¹

	Neutralization Test with Zika Virus			Total
	Non-protective	Partially Protective	Protective	
HI Negative to All Four Group B Antigens	3	0	0	3
HI Negative to Zika Antigen, but HI Positive to One Other Group B Antigen	3	0	0	3
HI Positive to Zika Antigen plus One or Two Other Group B Antigens	3	0	3	6
HI Positive to All Four Group B Antigens	8	0	0	8
HI Positive to Zika Antigen, but HI Negative to All Other Group B Antigens	0	0	3	3

Footnote

1. This table is derived from Table 7-A. Numbers in boxes indicate how many sera with the designated HI seroreaction responded in the indicated manner to challenge with the virus. A non-protective serum is here defined as one in which there are 0 to 2 survivors from the original litter of eight mice. A protective serum is defined as one in which there are only 0 to 2 deaths attributable to the virus from the original litter of eight mice. A partially protective serum is defined as one which does not fall into the other two categories.

Tables 8 -A through F

Correlation of CF and HI Test Results with NT Test Results
On Selected Sera from the 165 Survey Samples

Table 8 -B

Correlation of Bwamba CF Test Results with Bwamba NT Test Results¹

	Neutralization Test with Bwamba Virus			Total
	Non-protective	Partially Protective	Protective	
CF Negative to Bwamba	4	0	2	6
CF Positive to Bwamba with End-point Titer of:				
2/4 ²	0	0	3	3
4/4	3	0	4	7
8/4	0	0	1	1

Footnotes

1. This table is derived from Table 7-B. Numbers in boxes indicate how many sera with the designated CF seroreaction responded in the indicated manner to challenge with the virus. See Table 8-A, footnote 1 for definitions of "non-protective", "protective", and "partially protective".
2. See Table 7-B, footnote 6 for explanation of CF titers.

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Tables 8 -A through F

Correlation of CF and HI Test Results with NT Test Results
On Selected Sera from the 165 Survey Samples

Table 8 -C

Correlation of Ilesha HI Test Results with Ilesha NT Test Results¹

	Neutralization Test with Ilesha Virus			Total
	Non-protective	Partially Protective	Protective	
HI Negative to Ilesha	1	0	5	6
HI Positive to Ilesha	1	1	9	11

Footnote

1. This table is derived from Table 7 -C. See Table 8 -A, footnote 1, for definitions of "non-protective", "protective" and "partially protective".

Table 8 -D

Correlation of Ingwavuma HI Test Results with Ingwavuma NT Test Results¹

	Neutralization Test with Ingwavuma Virus			Total
	Non-protective	Partially Protective	Protective	
HI Negative to Ingwavuma	5	0	0	5
HI Positive to Ingwavuma	6	0	0	6

Footnote

1. This table is derived from Table 7 -D. See Table 8 -A, footnote 1, for definitions of "non-protective", "protective", and "partially protective".

Tables 8 -A through F

Correlation of CF and HI Test Results with NT Test Results
On Selected Sera from the 165 Survey Samples

Table 8 -E

Correlation of Chikungunya CF and HI Results with Chikungunya NT Results¹

	Neutralization Test with Chikungunya Virus			Total
	Non-protective	Partially Protective	Protective	
CF+HI Negative to Chikungunya	6	0	0	6
CF+HI Positive to Chikungunya	0	0	11	11

Footnote

1. This table is derived from Table 7-E. Numbers in boxes indicate how many sera with the designated CF and HI seroreactions responded in the indicated manner to challenge with virus. See Table 8 -A, footnote 1 for definitions of "non-protective", "protective", and "partially protective".

Table 8 -F

Correlation of Bunyamwera HI Test Results with Bunyamwera NT Test Results¹

	Neutralization Test with Bunyamwera Virus			Total
	Non-protective	Partially Protective	Protective	
HI Negative to Bunyamwera	4	1	1	6
HI Positive to Bunyamwera	9	2	2	13

Footnote

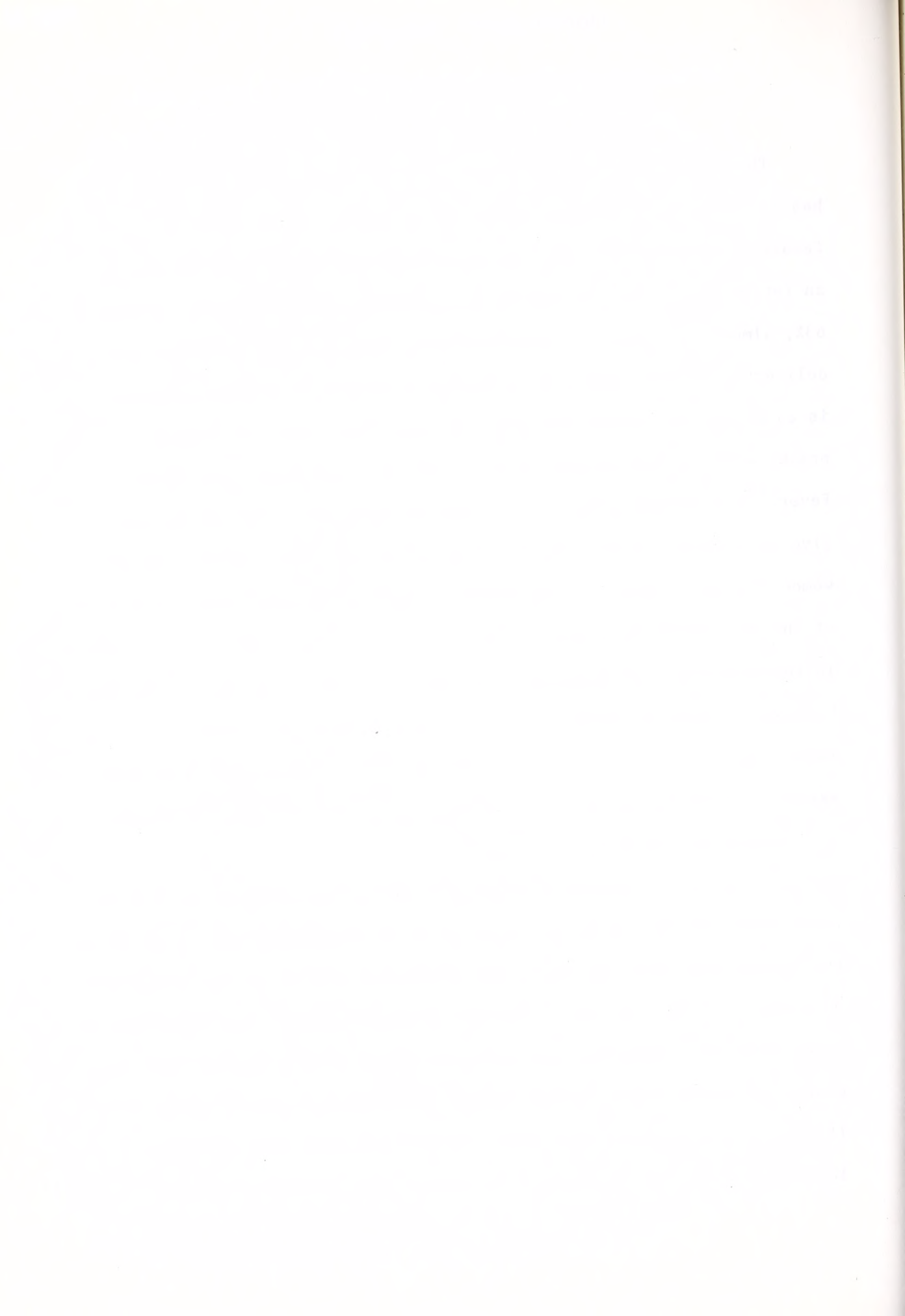
1. This table is derived from Table 7-F. See Table 8 -A, footnote 1, for definitions of "non-protective", "protective", and "partially protective".

Chapter Three: Discussion

Section One: Survey Population

The survey population, as described in chapter two, section one, has certain characteristics which set it apart. First there is a female predominance of 69%. As previously mentioned, this was due to an intentional effort to obtain sera from hospital personnel. Indeed 63%, almost two-thirds of those surveyed were involved in health care delivery. In one respect the weighted selection of hospital personnel is advantageous, namely for answering questions about nosocomial outbreaks of viral infections, such as have been documented for Lassa Fever.¹⁶ In another respect it is not advantageous, since it may not give an accurate representation of the viral exposures of both men and women who work outside of hospitals and who constitute the vast majority of the population of Liberia. The hospital sampling method is also skewed in representing less exposure to arboviruses than the general population because the work is inside, thus making the workers less accessible to insect vectors. At the same time the hospital sampling method is also skewed in representing more exposure to respiratory infections because of exposure to patients.

Age is another aspect of skewing of the survey. Eighty percent of those surveyed are within the range of 20 to 49 years of age. Only 8% of the people were under 20 years, and similarly, only 8% of the people were 50 years or older. As previously mentioned, the median age in Liberia is under 20 years. Therefore the survey is highly biased against this age group, and it is highly biased towards those who are over 20 years of age. It is entirely possible that viral exposure may have been different at the lower end of the age spectrum. One cannot also identify the age at which



infection occurs. Nor can one identify whether differences, if any, in age groups is due to longer exposure or to more intense exposure at one age. Finally in areas where there is a tendency to multiple infections by related viruses, one is more apt to find serological patterns of initial infections of that virus group in children, as opposed to adults.¹⁷

A final skewing of population concerns the fact that only three of the nine counties of Liberia, are substantially represented as areas of past or present residence. These counties are Montserrado, Bong, and Loffa. Therefore one should keep in mind that viral exposure may be different in the areas underrepresented by the survey.

In conclusion, because of the biased method of selection, the survey population is not a good random sample of the population of Liberia. Because of this and because even the most ideal survey may exclude people with positive findings, one must refrain from deciding definitively against the occurrence in Liberia of human exposure to any of the viruses for which no seropositivity was found. For the same reasons one must also refrain from using the survey to draw any final conclusions about the incidence in Liberia of human exposure to any viruses for which seropositivity has been demonstrated. The survey does not reflect the number at risk in the general population. For diseases of low incidence and those requiring special types of exposure, such as arboviruses, the only valid result is a positive one in the type of sample obtained; and even if positive, these results cannot indicate its extent or age/sex distribution.

Section Two: Serological Test Results

Prior to discussion of the test results with the Liberian sera, a few comments would be in order concerning the lifespan and specificity of CF, HI, and NT antibodies. Concerning the lifespan of these three types of antibodies, CF antibodies generally remain at high levels for the shortest length of time following infection.¹⁸ With respect to arboviruses it has been stated that CF antibodies remain only transiently at a high titer, often vanishing or dropping quickly to barely detectable levels within several months to a year after acute infection.¹⁹ However, there is definite evidence that CF antibodies to arbovirus infections may also last longer. In a five year follow-up on human cases during an epidemic of St. Louis encephalitis infection, Bond found CF antibodies to be still detectable at levels of $\geq 1:8$ in seven out of nine cases.²⁰ There is also evidence that for dengue virus they exist for as long as 20 years.²¹ HI antibodies are generally of longer duration than CF antibodies. However they do not remain at high levels for as long a time as do NT antibodies.²² Therefore if one uses CF and/or HI assays to screen sera, which indeed was the case with the Liberian sera, one must realize that a negative result will be obtained with those sera which no longer have detectable levels of CF and HI antibodies due to the passage of time but which nonetheless still have NT antibodies.

Besides lifespan one must consider the specificity of CF, HI, and NT antibodies. In general CF and HI antibodies are less specific than NT antibodies. Cross-reactions between related viruses are usually more pronounced by CF or HI tests and less pronounced by NT. However, "it has become increasingly apparent that overlaps by neutralization

test occur,.... The capacity of a serum to neutralize a virus is not necessarily proof of previous exposure of an individual virus, particularly in cases where successive exposure to several members of an antigenic group may result in wide cross-reactivity".²³ One must keep in mind that the definition of a group of arboviruses involves serological cross-reactivity of the member viruses with one another, usually by CF and HI tests, but also by NT.²⁴ Because of this overlap, serological diagnosis often requires the use of two or all three types of serological tests and the use of several or all closely-related group members known to exist in the geographic region of the survey.²⁵ However one may still not be able to arrive at a diagnosis. In this case the most that can be said, is that the person was infected with one or more members of that group of arboviruses. One must also keep in mind that positive serological results may be due to infection by an undiscovered virus which overlaps immunologically with agents used in the assay.

One must also consider the sensitivity of the CF, HI, and NT tests. It must be acknowledged, however, that no absolute standard is available to measure the sensitivity of these various tests for arbovirus infection.²⁶ The question of the relative sensitivity of these three tests must be posed for each individual virus separately, because one cannot assume that results for any given virus can be extrapolated accurately to another virus. Unfortunately, the question of relative sensitivity of these assays remains largely unanswered for many viruses, including many of the viruses in this survey. For one of the best - studied arboviruses, yellow fever, this question has been answered to some degree. For yellow fever the HI test has been shown to be more sensitive than

the CF test. In humans infected with this virus as a primary infection, HI antibodies usually appear before CF antibodies, reach higher geometric mean titers, and persist longer than CF antibodies.²⁷

It is really not possible in a quantitative manner to compare the sensitivity of CF and HI tests with the mouse neutralization tests, which were used in this Liberian survey. Only undiluted serum is used in the mouse NT test, whereas the serum is diluted out to end-point in the CF and HI tests. However because serum is diluted in tissue culture NT tests, it is possible to compare the sensitivity of these tests with those of the CF and HI tests. In general it may be stated that CF titers have a maximum of less than 1:1000 while simultaneous HI titers may be over 1:10,000.²⁸ NT titers may also be over 1:10,000 for 50% plaque reduction.

Another important consideration in screening sera, is the initial serum dilution that is used. The lower the serum dilution, the more likely will be the possibility that a positive test result will reflect the presence nonspecific complement-fixation or nonspecific hemagglutination-inhibition. Thus there is a disadvantage to having the initial dilution too low. If one uses a higher dilution as the initial titer, one will eliminate more of the falsely positive sera. However at the same time one will also screen out some weakly positive sera that reflect genuine viral infection. Thus one wants to choose an initial serum dilution that is neither too high nor too low. Nevertheless one must always be cognizant that a positive serological result may be due to nonspecific complement-fixation, nonspecific hemagglutination-inhibition or nonspecific neutralization. To be sure, attempts are made to remove the substances responsible for these reactions or to identify their presence by running controls. However these attempts are not always successful.

1. Lassa Fever.

Lassa virus is a member of the Tacaribe virus group which belongs to the genus Arenavirus. It is the only member of the Tacaribe group known to exist in Africa.²⁹ The virus has been isolated from humans in Nigeria, Sierra Leone, and Zorzor, Liberia. NT antibodies have also been identified in human sera from Guinea.³⁰ Consistent with the Tacaribe group, is the finding of the Lassa virus in rodents in Sierra Leone.³¹ There is no evidence to support transmission by arthropod vectors.³²

As previously mentioned, five CF positives for Lassa were found from the 165 sera in the survey (Chapter Two, Table 3). A preliminary CF screening of all sera was done using either a 1:4 or 1:8 dilution of antigen with 1:4 and 1:8 dilutions of serum. This initial screening revealed only two positives; among the negatives were sera from two persons who stated that they had Lassa Fever, confirmed by laboratory test. These were two of the CF positives from Monath's study in Zorzor.³³

A final retest by CF was done with serum samples from the following people: the two CF positives in the initial screening, the two CF negative in the initial screening who were positive in Monath's study, and all those in the survey who gave a history of serious illness with sore throat and dysphagia. In this final screening the antigen dilution was 1:4 and the serum dilutions were two-fold, beginning with undiluted serum, as opposed to beginning with 1:4 diluted serum in the initial screening. From this an additional three CF positives were uncovered, bringing the total to five. These three additional CF positive included the two who were CF negative in the initial screening who were positive in Monath's study; the third was a woman who gave a history of sore throat, dysphagia, and serious illness while working as a midwife at Zorzor in 1969. Thus

on the basis of retest, Casals states that these five sera are all clear-cut positives. Thus all 3 people in the survey who claimed to have Lassa Fever were CF positive; there were no people in the survey who claimed to have Lassa Fever but were CF negative. Approximately 10 samples were tested at this lower serum dilution of 1:2. One cannot help but speculate whether any additional CF positive sera might be uncovered by doing a final screening on the remaining 155 samples with serum dilutions below 1:4. However the relative paucity of antigen makes this study implausible for the present. One might also speculate whether additional positive sera might have been uncovered by using mouse brain Lassa antigen, instead of the Vero cell antigen, which is less sensitive. However, the Vero antigen is the only preparation now available.³⁴

One observation to be made from this assay, concerns the apparent rate of decline of Lassa CF antibodies (Chapter Two, Table 3). The three women documented by Monath as having Lassa Fever, were CF positive at serum dilutions of 1:32 or 1:64 between April and June of 1972.³⁵ Between October and December of 1974, when the sera for this survey were obtained, the titers recorded as 1:2 or 1:4. However, comparison of the CF titers by Monath with these recent titers cannot be entirely regarded as valid. Besides the possibility of variation in technique, there is the fact that the antigens were different. Monath used a mouse brain antigen, while the antigen used more recently by Casals is a Vero cell antigen. Casals has had occasion to run both antigens simultaneously on some sera, and he has found the mouse brain antigen to be more sensitive.³⁶ Since the sera collected by Monath in 1972 are still available at the Center for Disease Control in Atlanta, it would be worthwhile to do simultaneous CF tests on the paired sera, in order to minimize any variation in technique and any variation in antigen. One must bear in mind the possibility that some of those in the survey may have had Lassa Fever but are no longer identifiable, because of the disappearance of CF antibodies.

Another finding from the survey, is the absence of evidence for the occurrence of Lassa CF antibodies outside the areas for which the existence of the virus has already been documented. Besides the three women who were documented at Zorzor by Monath, a fourth CF positive in the survey was a woman who in 1969 at Zorzor, contracted a severe illness entirely compatible with all other cases of Lassa Fever, except for sclericterus. Since this woman spent her entire life in Loffa County except for the four years prior to survey, during which she lived outside Monrovia, it seems most likely that she contracted her infection in the area of Zorzor. Similarly the fifth CF positive in the survey was obtained from a woman who spent her entire life in Koindu, Sierra Leone except for the year prior to survey, during which she lived near Phebe Hospital in Bong County. This fact suggests that she contracted her infection while she lived in Koindu, which is 50 miles from the documented outbreak of Lassa Fever in Panguma, Sierra Leone.³⁷ To be sure these last two persons may have contracted their illness in other areas or from people who contracted their illness in other areas. However the majority of their lives were spent in areas already known to contain Lassa virus. One must keep in mind that even if all of the positives in this survey did occur in areas already known to contain Lassa virus, it is still entirely possible that the virus may exist in other areas of Liberia. To make any other conclusion would be unwarranted, because of: 1) the shewing of this survey population; 2) the limited number of specimens; 3) and the short lifespan of CF antibodies; and 4) the sensitivity of the test and the dilution used.

A significant observation from the survey is the wide spectrum of the degree of illness with Lassa Fever (Chapter Two, Table 3). Of the three cases documented by Monath, two were severly ill, and the third was only

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mildly ill, still being able to work. The fourth person who was also at Zorzor was seriously ill. The seropositive person from Sierra Leone denies ever being seriously ill. This is consistent with observations which show that subclinical infection or mild forms of the disease may occur.³⁸ Even if the illness is severe, the clinical onset is often very non-specific.³⁹ For these reasons the disease may elude recognition by health care workers and thereby lead to further propagation of the virus to other people. While discussing the clinical recognition of the disease, it is worth remarking that all four people from Zorzor described sore throat and difficulty swallowing as part of their illness. This is consistent with the findings of the three major epidemics described in the literature. In the 1970 epidemic in Jos, Nigeria 19 or 23 cases (83%) had pharyngitis.⁴⁰ In the 1972 epidemic in Panguma, Sierra Leone 9 of 11 cases (82%) had pharyngitis.⁴¹ In the 1972 epidemic in Zorzor, Liberia all 10 cases (100%) had pharyngitis.⁴² Most of these cases had severe sore throat; in turn most of the cases with sore throat also had dysphagia. In the Sierra Leone epidemic this pharyngitis was characterized by reddening of tonsillar and peritonsillar tissues, soft palate and posterior pharynx, with raised patches of yellowish exudate occasionally coalescing into a pseudo-membrane. Ulcerations resembling typical aphthous ulcers were seen in only one of the 11 patients in Sierra Leone.⁴³ To be sure sore throat, dysphagia and pharyngitis are not specific for Lassa Fever. Indeed these may be due to bacterial agents such as Streptococcus or other viral agents. However there is no data available by laboratory documentation on the incidence of any of these infective agents in Liberia. Nevertheless, it has been recommended that in a known or suspected area in West Africa, the diagnosis of Lassa Fever should be

considered "for any person with fever, generalized malaise, and myalgia, especially if sore throat or pharyngitis are present. Any adult patient who enters a hospital in West Africa with these symptoms and who is found to be leukopenic, should ideally be hospitalized at once in an isolation ward".⁴⁴ The presence of leukopenia would differentiate it from the usual streptococcal infection in which leukocytosis is characteristic.

One final comment about Lassa virus concerns its relationship to hospital contagion. Some of the documented outbreaks involved an index case being brought into the hospital, and here the disease was transmitted to other patients and to hospital personnel.⁴⁵ Because hospital work may afford increased opportunity to contract the virus from patients or from other hospital personnel, a deliberate attempt was made in this survey to collect as many sera as possible from those employed in health care delivery. Of the 104 health workers in the survey, five had CF antibodies to Lassa. The three positives documented by Monath were all midwives. The fourth claimed to be sick while working as a midwife at Curran Hospital in Zorzor. There is a good possibility, however, that the fifth CF positive person contracted her illness before she began health care work, i.e., while she lived at Koindu, Sierra Leone. Of the 61 samples obtained from those who were not connected with health care, none were found to be positive.

2. Congo.

Congo virus is a member of the Crimean Hemorrhagic Fever-Congo group, of which Congo is the only member known to exist in Africa.⁴⁶ The virus has been isolated from humans in the Congo and Uganda. It has also been isolated from Nigerian cattle and from ticks in Nigeria and Senegal.⁴⁷ Thus the virus is enzootic in West Africa, although the virus or antibodies to the virus have not been found in humans from this region. The results of the Liberian survey add no new findings. All sera were negative by CF test except for a questionable positive which was partially non-specific. Subsequent neutralization test of this sample was negative.

3. Mokola.

Mokola is a rabies-related virus which has been isolated from several humans in Nigeria, including one child who died of a poliomyelitis-like illness. The virus has also been isolated from shrews in Nigeria. Antibodies have been found in humans, cattle, sheep, goats, swine, bats, and shrews.⁴⁸ CF tests for this agent were completely negative in the Liberian survey.

4. Chikungunya.

Chikungunya is a member of the group A arboviruses, which belongs to the genus Alphavirus. Of the group A viruses six are known to exist in Africa: chikungunya, Middelburg, Ndumu, o'nyong nyong, Semliki Forest, and Sindbis.⁴⁹ All except Sindbis and Ndumu have been isolated in West Africa. Chikungunya, o'nyong nyong, and Sindbis viruses have been found in humans. NT antibodies have been found in human sera for Semliki Forest and Ndumu.⁵⁰ However human antibodies to Middelburg have been virtually absent.⁵¹ Human infections with Semliki Forest have been documented in laboratory workers, although such infections have not been documented in nature. These viruses are transmitted by culicine and anopheline mosquitoes.⁵²

Chikungunya virus has been isolated from humans in the West African countries of Nigeria and Senegal; it has also been isolated in East Africa and South Africa.⁵³ Using the HI test with a battery of group A antigens, it has been demonstrated that the first or "primary infection" of an animal or human with a group A virus, will produce a distinctive HI pattern with the different antigens which is diagnostic of infection with that virus. The pattern of HI antibodies resulting from primary group A infections is "so distinctive that it is the method of choice" for doing surveys for this arbovirus group. HI studies have shown that chikungunya and "to a slightly lesser extent", o'nyong nyong are the major group A infections of humans in Africa. These studies imply that chikungunya is present all over sub-Saharan Africa, including the West African countries of Liberia, Guinea, Ghana, and Nigeria. Liberian chimpanzees have also shown the same primary infection pattern by HI test.⁵⁴

It must be stated that o'nyong nyong is closely related to chikungunya virus.⁵⁵ Because convalescent sera from people exposed to o'nyong nyong

virus will protect against chikungunya virus, one cannot use the NT with chikungunya virus to distinguish chikungunya from o'nyong nyong. One reliable means of distinguishing between these two agents is by running serum samples in HI tests with both antigens and checking for a higher titer with one of them.⁵⁶ Since this has not been done in the Liberian survey, one must reserve final judgment as to how many of the positive tests in the survey might actually be due to chikungunya and how many might be due to o'nyong nyong. It must be kept in mind that "group A agents that are shown to be closely related by HI studies, are also, as far as available evidence goes, closely related by CF or neutralization tests."⁵⁷ So it is with chikungunya and o'nyong nyong.⁵⁸ Although o'nyong nyong has not been isolated from humans in West Africa, as is the case with chikungunya, this fact does not allow one the liberty of eliminating the possibility that this agent is responsible for some of the positive serologies with chikungunya.

As mentioned earlier, of the 165 sera in the survey, 24 sera were CF positive for chikungunya and 34 sera were HI positive for the same agent. The 24 sera which were CF positive were also HI positive. The fact that CF antibodies usually disappear more rapidly than HI antibodies, is the most likely explanation for the observation that 10 sera were HI positive but CF negative.

Of the 24 sera that were positive for chikungunya by both CF and HI tests, eleven were selected for the NT (Chapter Two, Table 8-E). All these sera neutralized chikungunya virus. Six sera which were negative for chikungunya by both CF and HI tests, were also selected for the NT; all six failed to neutralize virus. One significant conclusion that can be drawn, is the excellent positive correlation of CF and HI test results with NT results.

The data suggest very strongly that Liberian sera that are positive to chikungunya by CF and HI test, will also be positive to chikungunya by NT. Thus one may conclude that all of the 24 sera that are positive to chikungunya by both CF and HI test, would also be positive by NT. These 24 sera constitute 15% of all the sera collected in Liberia. If one also includes the 10 sera which were positive by HI test but negative by CF test, the total of 34 sera with antibodies to chikungunya, constitutes 21% of all sera collected in Liberia.

Several interesting correlations can be made between the survey population and the serological test results. (Table 9 on following page). The percentage of males with antibodies to chikungunya (31%) was twice that of females (16%). The percentage of farmers with antibodies (52%) was more than three times that for health care workers (15%). The percentage of those with antibodies at Phebe Hospital (38%) was more than three times that of Curran Hospital in Zorzor (11%) and more than ten times that of the Maternity Center in Monrovia (3%). Although these correlations are noteworthy, statistical analysis of the figures was not felt to be justified. The reason for this, is the highly biased method of selection in this survey, as explained in the first section of this chapter. Nonetheless these correlations suggest that exposure to the agent or agents producing antibodies to chikungunya, may be associated with activities more frequently undertaken by males, including farming; and exposure may be less frequent among health care workers. These correlations may also suggest a higher exposure rate in the region served by Phebe Hospital than in the region served by Curran Hospital. Also the exposure rate appears to be very low among the health care workers at the Maternity Center who have in general worked in that hospital for many years and lived in the urban setting of Monrovia for most of their lives. One

Table 9

Prevalence Rates of Chikungunya Antibodies

Characteristics	Number Tested	Number Positive ¹	Percent Positive
Liberian Survey, Total	165	34	21%
Sex			
Male	51	16	31%
Female	114	18	16%
Occupation			
Health Care Worker	104	16	15%
Farmer	17	9	52%
Other or Unknown	44	9	20%
Hospital			
Phebe	37	14	38%
Curran	35	4	11%
ELWA	61	15	25%
Maternity Center	32	1	3%
Age			
<10	0	-	-
10-19	13	4	31%
20-29	64	10	16%
30-39	45	4	9%
40-49	23	8	35%
50-59	5	2	40%
≥60	9	6	67%
Unknown	6	0	0%

¹Positive sera had CF titers $\geq 1:4$ and/or HI titers $\geq 1:10$. CF and HI test results were confirmed on selected sera by NT tests.

must keep in mind, however, that these conclusions may not be an accurate reflection of the true picture, due to the skewing of the survey population, which was mentioned at the beginning of the Chapter.

One last observation about chikungunya may be made by employing the following reasoning. If a person has antibodies to a virus, and if that person resided in only one location for his entire life, then most likely the responsible viral agent originated from the same location. Some exceptions to this generalization might be: exposure to the agent during a sojourn away from his residence; or importation into his residential area of an agent, for example, by an outside visitor. However, most likely that agent is indigenous to the person's local area. There were a number of people in the Liberian survey who had antibodies to chikungunya, and who also claimed only one lifetime residence. The counties in which these people resided were: Bong (sera #22, 23, 36, 42, and 47), Loffa (sera #87, 96, and 99), Nimba (serum #104), Grand Bassa (serum #126), and Montserrado (serum #169). Thus one might conclude that there is a reasonable possibility that the agent or agents producing antibodies to chikungunya, is present in these counties.

5. Bwamba.

Bwamba is a member of the group Bwamba, which belongs to the Bunyamwera supergroup, which belongs to the genus Bunyavirus. The Bwamba group consists of only two viruses, Bwamba and Pongola; both have been found only in Africa.⁵⁹

Bwamba has been isolated from humans in Nigeria, Uganda, and the Central African Republic.⁶⁰ Neutralization tests show human antibodies to Bwamba are widespread in Africa south of the Sahara, namely in Central, East, West, and South Africa.⁶¹ Isolations have not been made from lower vertebrates,⁶² although NT antibodies have been found.⁶³ The virus has been isolated from anopheline mosquitoes.⁶⁴

Pongola virus has not been isolated from humans or lower vertebrates.⁶⁵ However NT antibodies have been found in humans from the southern African countries of Angola, Botswana, Mozambique, and South Africa. The virus has been isolated from anopheline and culicine mosquitoes from countries as far north as the Central African Republic, Uganda, and Ethiopia. No isolations have been made in West Africa.⁶⁶

It must be emphasized that Bwamba virus and Pongola virus have much serological overlap.⁶⁷ By CF test the two viruses are related, although they are not identical.⁶⁸ "Due to the marked immunological overlap between Bwamba and Pongola viruses, the interpretation of the results of N tests is difficult."⁶⁹ Nevertheless it is possible to distinguish between them by using CF and neutralization tests. It must be acknowledged, however, that the tests performed on the Liberian serum samples are not sufficient to make any definitive identification.

With this in mind the results of the NT with Bwamba will be discussed (Chapter Two, Table 8-B). Of the eleven CF positive sera, eight were protective,

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and three were non-protective. One possible explanation for the failure of these three CF positive sera to neutralize virus, is that the CF antibodies resulted from infection with a virus related to Bwamba which was not able to neutralize Bwamba. This virus might be Pongola, which need not elicit NT antibodies to Bwamba.⁷⁰ This virus might also be an undiscovered relative. One must also consider that the end-point serum titer for these three sera, was 1:4, which may be too weak to be considered a definitely positive CF test. One final possibility is an error in technique; however, adequate controls were present for both the CF and neutralization assays.

Of the six CF negative sera used in the Bwamba NT, two were protective, and four were non-protective. The fact that CF antibodies disappear more quickly than NT antibodies, is the best explanation for the neutralization of virus by CF negative sera. Thus the CF screening test which was used to screen all the Liberian samples, failed to identify a number of sera with NT antibodies to Bwamba, making it impossible to state accurately what proportion of people possessed NT antibodies to Bwamba.

One last observation about Bwamba is based upon the rationale mentioned in the discussion of chikungunya. A couple of people with NT antibodies to Bwamba claimed only one lifetime residence, suggesting that the agent responsible for these antibodies is indigenous to their local areas. One such area is Bong County (serum #24), and another is Loffa County (serum #93).

6. Ingwavuma.

Ingwavuma is a member of the Simbu group of viruses, which belongs to the Bunyamwera supergroup. Nine members of the Simbu group have been reported in Africa: Ingwavuma, Nola, Sabo, Sango, Sathuperi, Shamonda, Shuni, Simbu, and Thimiri. The only one of these agents to be isolated from humans, has been Shuni.⁷¹ This isolation was made in Nigeria.⁷² Besides Shuni, Simbu is the only virus of the group for which human antibodies have been found. All of these viruses have been isolated from vertebrates and/or arthropods in West Africa, except for Nola, Simbu, and Thimiri.⁷³ The arthropod vectors for these agents are culicine mosquitoes and/or Culicoides flies.⁷⁴

As mentioned above, Ingwavuma has not been isolated from man. Neither have human antibodies been found. Virus isolations have been made from several species of birds from Nigeria, Central African Republic, and South Africa. The virus has also been isolated from culicine mosquitoes from the Central African Republic and South Africa.⁷⁵

The Liberian survey yielded no evidence to suggest the occurrence of Ingwavuma in humans (Chapter Two, Table 8-D). Six serum samples were weakly positive by HI test, but they were all negative by NT. While these weakly positive HI tests may imply exposure to a relative of Ingwavuma, one must keep in mind that HI titers of 1:10 are of questionable significance and should not be assumed to be clear-cut positives.⁷⁶

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7. Bunyamwera Group Members: Ilesha and Bunyamwera.

Ilesha and Bunyamwera are members of the Bunyamwera group which belongs to the Bunyamwera supergroup. Four viruses of the Bunyamwera group have been found in Africa: Birao, Bunyamwera, Germiston, and Ilesha. Arthropod vectors for these viruses are either anopheline or culicine mosquitoes.⁷⁷

Birao has been isolated only from mosquitoes in the Central African Republic. Significant serological survey work with Birao has not been done.⁷⁸

Bunyamwera has been isolated from humans in Nigeria, Uganda, Kenya, and South Africa.⁷⁹ Virus isolations and serological surveys indicate that Bunyamwera virus is widespread throughout Africa.⁸⁰

Ilesha virus has been isolated from humans in Nigeria, Cameroun, Central African Republic, and Uganda. NT antibodies have been found in humans but not in any other vertebrates. These antibodies came from people in Nigeria, Uganda, and the West Nile region.⁸¹

Germiston has been isolated in several countries of eastern and southern Africa, primarily from mosquitoes and hamsters. NT antibodies have been found in humans and lower vertebrates in the same regions of Africa. Although human infections with Germiston have occurred as a result of laboratory exposure, no naturally occurring human infections have been documented.⁸²

Bunyamwera, Ilesha, and Germiston are serologically interrelated in a complicated manner. By CF test Ilesha is more closely related to Bunyamwera than to most other group members, while Germiston is quite distinct from all other group members.⁸³

By HI test Germiston is closer to Bunyamwera virus than to other members of the group.⁸⁴ Sera with NT antibodies to Bunyamwera may con-

Tables and Figures

Table 1

Table 2

Table 3

Table 4

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Table 6

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Table 8

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Table 30

tain HI antibodies to not only Bunyamwera but also to Ilesha.⁸⁵ One means of resolving this overlapping of HI antibodies, has been to run each serum sample in HI tests with each of the three antigens--Germiston, Ilesha, and Bunyamwera. With those sera in which the titer for one of these three antigens was clearly higher than the titers for the other two antigens, a presumptive diagnosis could be made. However with those sera in which the titers for two or all three of these antigens were almost equal, no presumptive diagnosis was possible. "In extensive HI studies with Bunyamwera group antigens--and sera from West Africa, the Sudan, and Ethiopia--positive reactions were quite common. In most instances the HI patterns suggested previous infection with either Germiston or Ilesha virus. In only relatively few instances were the titers obtained with a Bunyamwera antigen higher than those observed with the other two--indicating that infections with Ilesha and Germiston viruses are far more common in these regions than infections with Bunyamwera."⁸⁶ These studies suggest that Ilesha is probably responsible for human infections in Liberia, as well as Ghana, Nigeria, and Ethiopia.⁸⁷

By NT there is little overlap between Ilesha and Bunyamwera.⁸⁸ However there is a very marked overlap between Germiston and Bunyamwera. Casals has demonstrated that mice which had been previously exposed to Bunyamwera virus were almost entirely resistant to intraperitoneal challenge with Germiston.⁸⁹

In conclusion it can be seen that the Bunyamwera group viruses are serologically interrelated in a complex manner. Since HI tests were not done on the Liberian sera with Germiston to complement the HI tests done with Bunyamwera and Ilesha, no presumptive diagnosis can be made on the basis of HI titer comparison.

7a. Ilesha.

The striking finding in the serological test results on Ilesha is the wide discrepancy between the HI and NT test results (Chapter Two, Table 8-C). Only 11 sera were found to be positive by HI test, all with a low titer of 1:10. In the NT test 9 of these 11 were protective and one was a partially protective. Thus most of the HI positive sera were positive by NT test. The surprising finding is that 5 out of 6 of the HI negative samples were also protectors. Thus most of the HI negative sera were also positive by NT test. It appears therefore that the proportion of NT positive sera is the same for HI positive and HI negative sera.

There are several possible explanations for these serological test results. One is that there truly is a high incidence of NT antibodies to Ilesha among the population of Liberia. If this is the case, then one must explain why HI antibodies were found in only 7% of the Liberian sera. Even if one acknowledges that some of the sera may have lost their HI antibodies due to the passage of time, it is highly unlikely that such a large proportion of the population would have lost their HI antibodies. Another discrepancy is the total absence of high HI titers, which one would anticipate, if most of the population truly have NT antibodies. As previously mentioned, HI titers of 1:10 are generally of questionable significance. It has been shown that humans can generate high antibody titers to Ilesha.⁹⁰ So one can exclude the inability of humans to form highly antibody titers.

A second possible explanation for this discrepancy, is that there may be a non-specific neutralizing substance in the sera.⁹¹ Such a substance could cause protection in a NT test, regardless of whether or not the serum contains true NT antibodies. A third explanation might be that

the HI technique was subject to error. This seems unlikely since HA titrations to determine the number of units of antigen present, were valid. Other controls were also valid. It is also possible that the Ilesha antigen is insensitive, i.e., is difficult to inhibit. One final possibility might be the presence in Liberia of a relative of Ilesha which has strong cross-over by NT test with very little cross-over by HI test.

7b. Bunyamwera.

Of the 13 HI positive sera out of the 165 samples in the survey, only two were protective by NT test; two were partially protective; and nine were non-protective (Chapter Two, Table 8-F). Of the six HI negative sera in the NT test, one was protective; one was partially protective; and four were non-protective. One very noticeable observation from these results, is that the ratio of protectors, partial protectors, and non-protectors, is almost the same for the HI negative and HI positive sera.

One might recall that in the discussion of Ilesha the ratio of protectors to non-protectors was also almost the same for the HI positive and HI negative sera. Similar to Ilesha, one possible explanation might be that all of the protectors and partial protectors were due to infection with Bunyamwera or a related group member. One might explain the fact that HI negative sera were protectors, as a consequence of the more rapid disappearance of NT antibodies. This might also be explained by a related virus which has cross-over of NT antibodies without much cross-over of HI antibodies. One might explain the fact that HI positive sera were non-protectors, as resulting from exposure to a virus related to Bunyamwera which would elicit HI antibodies to Bunyamwera but not NT antibodies. As with Ilesha a second explanation might be the presence of a non-specific neutralizing substance. A final explanation might be that the HI technique was subject to error. This seems unlikely, since HA titrations and other controls were valid.

One conclusion that might be drawn is that in the context of Liberia, the HI test for Bunyamwera is not very adequate for predicting how a serum sample will react in a NT test for Bunyamwera. If one eliminates those sera with an HI titer of $\leq 1:10$ as being of too low a titer to entertain as definitely positive, there are still sera which are negative by NT test

with HI titers of 1:20 (sera #23, 38, 42, and 139) and 1:40 (serum #24). The highest titer observed by HI test was 1:80 (serum #153), and this serum did turn out to be positive by NT test. One however cannot use this one serum sample to make a general statement that an HI titer of 1:80 or greater will correlate well with NT test positivity. One might have anticipated the possibility of a poor correlation between Bunyamwera HI and NT tests, since this has been observed previously with sera from Mozambique.⁹² The occurrence of viruses related to Bunyamwera could result in sera which would give HI positive but NT negative results. One might recollect that presumptive positives for Germiston and Ilesha have been shown to be more prevalent in Africa than presumptive serological positives for Bunyamwera. These other two agents can elicit HI antibodies to Bunyamwera.⁹³ One must also bear in mind that a positive NT test for Bunyamwera does not mean that Bunyamwera was the responsible agent: "Until more extensive studies have been made, the interpretation of the results of N tests with the Bunyamwera virus must be interpreted with caution."⁹⁴

8. Group B Arboviruses: Dengue II, West Nile, Yellow Fever, and Zika.

Group B arboviruses belong to the genus Flavivirus.⁹⁵ There are a total of 17 group B viruses in Africa.⁹⁶ Group B is divided into three subgroups, based upon the arthropod vectors involved.⁹⁷

The largest of the three subgroups is concerned with those viruses transmitted by mosquito vectors; these viruses have seldom been isolated from other arthropods. Most of these agents can cause disease in man, and they often do so in large outbreaks. Isolates have been obtained most commonly from man and birds, although isolations have been made from other categories of vertebrates.⁹⁸ African members of this subgroup include: Banzi, Bouboui, dengue I, dengue II, Ntaya, Spondweni, Uganda S, Usutu, Wesselsbron, West Nile, yellow fever, and Zika. All have been isolated from humans, except Bouboui, Ntaya, Uganda S, and Usutu.⁹⁹ Human serological tests have been positive for all of these except Usutu. Except for Banzi and Ntaya, all of these viruses have been isolated in West Africa. Of the four group B agents used in the Liberian survey, dengue II, yellow fever and Zika have been isolated from humans in West Africa. The West African isolates of West Nile do not include any from humans.¹⁰⁰

The second subgroup concerns group B viruses that are carried by ticks. Only one such virus has been isolated in Africa, namely Kadam.¹⁰¹ It has not been isolated from humans or other vertebrates. The tick is the only animal from which the virus has been isolated. It has been isolated only in Uganda.¹⁰²

The final subgroup of group B viruses consists of those agents for which an arthropod vector has not been identified.¹⁰³ Four members of this subgroup have been isolated in Africa: Dakar Bat, Entebbe Bat, Koutango, and Saboya.¹⁰⁴ None have been isolated from humans, and

positive human serologies have been found only for Dakar Bat virus in Senegal and for Saboya virus in Sierra Leone. All have been isolated from bats or rodents. All except Entebbe Bat virus have been isolated in West Africa.¹⁰⁵

At this point it is appropriate to discuss the interpretation of group B serological test results. To be sure, "the most definitive diagnosis of an infection is made if a virus is isolated and identified. Often, however, the diagnostician is confined to the study of pairs of sera taken at different times after the onset of illness."¹⁰⁶ Even less definitive than the above two situations, is the study of an unpaired serum sample taken at a time during which the patient may or may not have been ill, as is the case with the Liberian survey samples.

Besides the limitation imposed by the use of unpaired sera, a second diagnostic limitation on the Liberian survey stems from the notorious tendency of group B viruses for marked immunological overlap, which often times renders serological identification absolutely impossible. This is true especially of "secondary infections." A "secondary infection" may be defined as the second infection of a subject with agents from an arbovirus group, the agents responsible for these two infections being different from one another.¹⁰⁷ A person who has had a secondary infection with group B viruses will therefore have been exposed to two different group B viruses. The hallmark of a group B secondary infection, is that the serum is widely cross-reactive, possessing significant antibody levels to many group B agents. Heterologous antibody levels may even exceed homologous antibody levels.¹⁰⁸ There is nearly complete absence of specificity of HI and sometimes of CF antibodies, and the presence of heterologous NT antibodies may be quite marked. Therefore, after a secondary infection, it is extremely difficult, if not impossible, to make any specific serological

diagnosis.¹⁰⁹ It is therefore essential when doing serological survey work in a region where several group B agents are known to exist, to employ several group B antigens by CF or HI tests to determine whether the serum demonstrates a secondary infection. If such is the case, the only permissible conclusion is that the subject has been exposed to group B viruses; specific diagnosis of the responsible agents is not possible.¹¹⁰ Since Liberia is in a region where numerous group B agents are known to be present, four group B agents were employed in the HI tests with the serum samples, in order to identify secondary infections.

It is still possible, however, to draw some diagnostic conclusions about infections with group B viruses, since identification is possible with respect to "primary infections." A "primary infection" may be defined as the first infection of a subject with an agent from an arbovirus group.¹¹¹ A person who has had a primary infection with a group B virus will therefore have been exposed only once to only one group B virus. To be sure, the preferable way of diagnosing a primary infection, is to have paired sera taken at different times after the onset of illness and to demonstrate the de novo appearance of group B antibodies. However it is still possible to use an unpaired serum sample to identify group B primary infections and make a presumptive diagnosis of the responsible agent. The reason for this is that after a primary infection with a group B virus, the HI antibody pattern is characteristic for each virus and is diagnostic of previous infection with that agent.¹¹² With this pattern there is often the production of antibodies to other group B agents. The highest titer is obtained with homologous antibodies,¹¹³ and "the heterologous titers are higher to closely related viruses and lower to more distantly related agents."¹¹⁴ Thus in order to identify the patterns of

primary reactions it is necessary to employ a number of group B antigens by CF or HI test.¹¹⁵ A diagnosis by HI test of a primary infection and of the agent responsible can be made if there is a serum titer of $\geq 1:20$ and 8 units of antigen "with a fourfold or greater difference between it and the highest titer given by the serum against another antigen of the same group."¹¹⁶ It must be mentioned that while each group B agent has its own characteristic pattern for primary infection, this pattern may not be very commonly observed in West Africa, because of the presence of many group B agents, "... in the majority of localities studied a very high incidence, approaching 100% in many cases, of group B antibodies occurred. In only a relatively few cases could a diagnosis of specific infecting virus be made from the HI pattern."¹¹⁷

A comment upon the usefulness of neutralization tests with group B viruses is now appropriate. It should be stated that the NT is only diagnostic in situations shown to be primary infections by HI tests. "It is our experience that the results of all group B N tests must be interpreted with extreme caution, unless the HI pattern is typical. As the predominant HI pattern in tropical Africa is one typical of multiple infections it follows that most N tests must be interpreted with great care. A positive N test result with a given group B agent is consistent with, but by no means proof of, infection with that agent. These criticisms apply to all antibody survey using the N test in the Sudan and other tropical African countries."¹¹⁸ The reason for the limited diagnostic capability of the NT in situations other than primary infection pattern by HI test, is the marked cross-neutralization among group B agents, even after primary infections:¹¹⁹ "...an infection with any group B virus is followed by the development of neutralizing antibodies not only to the infecting agent but also to a greater or lesser extent to many (possibly all) other group B viruses. The extent to

which these heterologous antibodies are developed is largely unknown and, consequently, in antibody surveys utilizing the N test only, the results are often difficult or impossible to interpret."¹²⁰

The discussion of the Liberian serological test results with the four group B members will be limited primarily to Zika and yellow fever. Zika was the only group B member to be run by NT, and it has a fairly distinct HI pattern for primary infections. Yellow fever is the most famous and the most thoroughly studied arbovirus. In West Africa primary infection patterns with West Nile have seldom been seen, although group B antibodies are quite prevalent. Instead of primary infection patterns, HI antibodies to West Nile have almost always been part of patterns consistent with group B secondary infections. The NT with West Nile cannot be trusted to be diagnostic because, "In all the countries of Africa--with the possible exception of Egypt--other group B viruses are prevalent. Many of the positive N test results with the WN virus may in fact be due to overlap with other group B agents."¹²¹ A similar statement can be made about the N test for dengue in Africa.¹²²

8a. Zika.

The following quote will serve as an adequate introduction for the discussion of the Liberian serological test results for Zika. "In extensive HI studies with sera from West Africa, it was found that the great majority of sera gave a pattern non-diagnostic of a Zika infection. However in a small proportion of sera, the HI patterns were diagnostic. These were observed mainly in children or at times in adults in regions where the overall incidence of group B antibodies was low. Confirmation of the diagnosis made by HI studies was obtained by the use of N test with Zika virus, indicating a very close correspondence between the two tests. In the antibody survey studies in the RFVL-NY, using HI techniques and sera from Africa, we have good evidence that human infections are prevalent in the various countries of West Africa (Nigeria, Ghana, Liberia, and the Cameroons)..."¹²³

Upon comparison of Zika HI test results with Zika NT results in the Liberian survey (Chapter Two, Table 8-A), one notices that many of the sera which were HI positive, were NT negative. Half of the sera which were HI positive to Zika plus one or two other group B antigens, turned out to be negative by NT. While the neutralization tests for Zika were positive in the other half of these sera, one cannot state that the serological tests are diagnostic of Zika infection, because for some of these sera the HI patterns are not consistent with a primary infection. As previously mentioned, an HI pattern characteristic of a primary infection is necessary for serologic diagnosis with group B members. However one of these sera does fit the criteria for a primary infection pattern by HI test, since the titer for Zika is more than fourfold higher than the titers for the other antigens (serum #103). Thus with this serum sample, one may make a presumptive diagnosis of Zika infection.

Also tested were eight broad group B sera which were HI positive to all four of the group B antigens; such sera are characteristic of secondary or multiple infections with group B viruses. Very surprisingly all eight sera failed to neutralize Zika. To be sure, this failure of broad group B sera to neutralize Zika, cannot be construed to be a universal phenomenon of all sera with broad group B reactions. However the above results demonstrates that positivity by HI test for Zika, does not necessarily imply positivity by NT for Zika. It is noteworthy that with these broad group B sera, the HI titers for Zika were as high as 1:320 (serum #20); yet neutralization did not occur.

Another observation with Zika concerns those sera which were HI positive for Zika but were HI negative to all other group B antigens. These sera belong to an HI pattern which is characteristic of primary infections with Zika.¹²⁴ All of these sera neutralized Zika virus (Chapter Two, Table 8-A). As mentioned earlier in the discussion, a primary infection pattern by HI test, confirmed by NT, may be considered adequate to make a presumptive diagnosis of infection with a particular group B agent. One may therefore consider that all sera in the survey which fall into the same HI pattern, represent Zika infections.¹²⁵ A total of six sera in the survey with an HI titer of 1:10 or higher, fit this pattern (Chapter Two, Table 6-A). Two have titers of 1:10 (sera #11 and 168). To be sure, there is justification for disregarding these sera with titers of 1:10 as inconclusive, because of the low titer.¹²⁶ However the titers of the other four are: 1:20 (serum #106), 1:40 (serum #134), 1:80 (serum #150), and 1:160 (serum #81).

One might comment about the occurrence of a similar behavior with other group B members. Primary infections with other group B members are manifested by an HI pattern with antibodies elicited only for that given virus but for no others.¹²⁷ A similar primary infection pattern is known to exist

for yellow fever and for the 17D strain used for yellow fever immunization.¹²⁸

In the Liberian survey there were nine sera that were positive only to yellow fever with an HI titer of 1:20 or higher, but were negative by HI test to the three other group B antigens (Chapter Two, Table 6-A). One may thus assume that these nine sera represent actual cases of yellow fever or immunizations with the 17D strain. In the Liberian survey similar HI test results were not observed with dengue II or West Nile (Chapter Two, Table 6-A).

One other statement about Zika pertains to reasoning mentioned earlier in this Chapter to support the idea that if a person spends his entire life residing in one locality, any viral agent to which he has been exposed, most likely originated in the locality. One may state therefore that Zika most likely occurs in Montserrado County (sera #103 and 150).

In summary it may be safely concluded that on the basis of this survey, Zika virus is present in Liberia. This is consistent with previous serological surveys of Liberia. The exact proportion of the survey population exposed to Zika, cannot be determined, because except for primary infections, one cannot with reasonable certainty use HI or neutralization test results to arrive at a presumptive serological diagnosis.

8b. Yellow Fever.

The following quote will serve as an appropriate introduction to the discussion of yellow fever. "Our studies with sera from West Africa indicated that in the majority of localities studied a very high incidence, approaching 100% in many cases, of group B antibodies occurred. In only a relatively few cases could a diagnosis of specific infecting virus be made from the HI pattern. These were observed chiefly in children and in a few places in adults where the overall group B incidence was low. These studies indicated that YF infections were prevalent in Liberia, Ghana, Nigeria, and the Cameroons, the countries from which sera were obtained for this survey."¹²⁹

Because yellow fever immunization histories were not obtained from those surveyed, the scope of worthwhile investigation into yellow fever with the Liberian sera, was felt to be somewhat limited. As mentioned in the preceding paragraph, serological studies with Liberian sera have revealed HI patterns diagnostic of yellow fever. During the previous decade human cases of yellow fever in Liberia were diagnosed on the basis of clinical evidence and post-mortem examination.¹³⁰ At that time immunization campaigns were done. To be sure, all of the 10 foreigners in the present Liberian survey, and most certainly, some nationals have had immunization against yellow fever. Without immunization histories it is impossible to differentiate between HI and NT antibodies resulting from actual infection with yellow fever and antibodies resulting from immunization with the 17D strain. For this reason neutralization tests with this virus were not performed.

The subject of primary infection patterns for yellow fever by HI tests, has already been mentioned above in the discussion of Zika. One particular aspect of these primary infections, concerns sera possessing HI titers of 1:80

or greater. Such high titers occur either after immunization with the 17D strain in which case these high antibody levels persist only for a brief period of several weeks, or after an actual case of yellow fever in which case this high titer will often persist for a longer period of years.¹³¹ Only one serum sample in the survey fits these criteria, having a titer of 1:80 (serum #181). This person was a 28 year-old nurse at the Maternity Center who had lived for 19 years in Sinoe County before coming to Monserado County. Since she is in a position in which recent immunization may have been possible, it cannot be discerned without a vaccination history, what is the cause of her high titer.

One last comment about yellow fever concerns one individual who was HI negative to yellow fever but who was certainly immunized (serum #72). This person is an American who came to Liberia six years ago. Since yellow fever immunization is required to enter Liberia, she most definitely received the 17D vaccine. To be sure, HI antibodies to yellow fever may have been detectable in the past; and they may have dropped below detectable levels with the passage of time. However another possibility is the established observation that HI antibodies sometimes never develop after immunization.¹³²

8c. Other Group B HI Reactions.

One comment might be made about the broad group B sera which were HI positive to all four of the group B antigens (Chapter Two, Table 6-C). As previously mentioned, such sera are characteristic of secondary or multiple infections with group B viruses. Of the 18 broad group B sera in the survey, 16 were HI positive to Bunyamwera. Since there is no immunologic cross-over between group B viruses and Bunyamwera supergroup viruses, a possible explanation for this observation is that the people were resident in localities (rural, forest, semi-urban) in which vertebrate hosts and invertebrate vectors of several arboviruses were established. With many species of mosquitoes in such localities, some of which have been shown to be virus vectors, the possibility of acquiring infections by viruses of each of several groups will be increased.

One last comment in the discussion concerns the overall incidence of group B HI antibodies. As mentioned in the previous chapter, 57% of those surveyed possessed HI antibodies to one or more of the four group B members. While this constitutes a majority of the people in the survey, it does not approach the incidence of 100% which has been observed in many parts of West Africa. One must, however, refrain from concluding that this percentage is an accurate representation of the Liberian population as a whole. One must bear in mind that the survey population is skewed along certain lines. One must also remember that immunization with the 17D strain of yellow fever is a source of artificially generated group B antibodies.

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Appendix I

Complement-Fixation Test

Diluent for all tests consisted of veronal buffer diluted 1:5 with demineralized distilled water, having a pH of 7.3-7.4.

The hemolytic system consisted of an equivolume mixture of Baltimore Biologic Lab hemolysin held at a 1:400 dilution for 15 min prior to mixing and of Baltimore Biologic Lab sheep red blood cells held at a 2 1/2% dilution for 15 min prior to mixing. Prior to use the hemolytic system was allowed to stand for 15 min.

Complement consisted of Flow Laboratories lyophilized guinea pig complement which was rehydrated with the accompanying prepackaged solvent. This was then diluted 1:55 for the titration of complement.

Serum samples at dilutions of 1:4 and 1:8 were placed in a water bath at 60°C for 20 min to inactivate complement and non-specific reactants. Further serum dilutions were made from these. The same procedure was used to inactivate the immune ascitic fluid which served to confirm the identity of antigen in each CF test.

A. Titration of Complement.

A series of complement dilutions was prepared in 13x100mm test tubes, constituting the master set of complement dilutions which was used in the preliminary and final titrations of complement:

Tube #	1	2	3	4	5	6	7	8	9
C' 1:55 in cc	1.6	1.4	1.2	1.0	0.8	0.7	0.6	0.4	0.2
VB 1:5 in cc	1.4	1.6	1.8	2.0	2.2	2.3	2.4	2.6	2.8

Preliminary complement titration consisted of a series of 12x75mm test tubes, one for each master tube. To each tube was added: 0.3cc of the cor-

responding master dilution, 0.1cc of diluent, and 0.2cc of the hemolytic system. The tubes were shaken vigorously, incubated in a water bath at 37°C for 30 min with occasional shaking, and then allowed to stand at room temperature for one hour before reading. The smallest amount of complement causing complete hemolysis was considered as one unit of complement. Since the CF test proper required 2 units of complement per 0.2cc, the 1:55 dilution of complement was adjusted by further dilution, if necessary, to make 2 units/0.2cc.

B. Complement-Fixation Test Proper.

The basic test consisted of a 6 drop/well system, using 0.025cc drops into 96-well plastic trays. The first drop was serum in a dilution of 1:4 or varying two-fold dilutions thereof. The next two drops consisted of the 2 unit complement. The fourth drop consisted of antigen in varying dilutions. After addition of these 4 drops/well the trays were covered with celluloid sheets to prevent evaporation and stored overnight (12-18 hours) at 4°C. On the following day the trays were allowed to stand at room temperature for 15 min, and then the final two drops, the hemolytic system, were added with vigorous shaking on a vibrator. The trays were again covered with celluloid and incubated for 30 min at 37°C with one vigorous shaking after 10 min. The trays were then allowed to sit at room temperature for several hours before reading. Results were recorded as follows: complete hemolysis "0"; no hemolysis "4"; intermediate values of "+", "1", "2", "3".

The tests also included the following controls:

- 1) Assay of each human serum with the fourth drop in the system being normal mouse brain or veronal buffer. These controls served to screen out anticomplementary and mouse tissue reactive sera.

2) Final titration of complement in which the first three drops of the system were the master dilutions of complement, and the fourth was diluent. This served to monitor any change of the strength of complement overnight.

3) Assay of the antigen at varying dilutions (usually 4-fold from 1:4 up to 1:256) with varying dilutions (usually 2-fold from 1:4 up to 1:128) of mouse immune ascitic fluid specific for that antigen. This was done to confirm the identity of the antigen. Controls of normal mouse brain and veronal buffer were also used to insure that neither the antigen nor the immune-ascitic fluid were anticomplementary.

Appendix II

Hemagglutination-Inhibition Test

Goose erythrocytes for the test were collected in acid-citrate-dextrose, washed four times with a dextrose-gelatin-veronal solution, and thereafter standardized using a spectrophotometer by making a dilution with 0.15M NaCl as diluent to give an optical density between 0.43 and 0.47 at 490mu. To prevent microbial contamination merthiolate was added 1:10,000 by volume.

Solutions used in the HI test were: a) borate-saline at pH 9 (0.05M borate-0.12 NaCl, abbreviated BS); b) BS at pH 9 with 0.4% bovine plasma albumin (abbreviated BS-BPA); c) viral adjusting diluents (abbreviated VAD), consisting of buffers of various pH values from 5.9 to 7.0 prepared from varied proportions of mono- and di-basic sodium phosphate.

Preparation of Liberian serum samples involved first extraction with kaolin to remove nonspecific inhibitors of hemagglutination and secondly washing with goose erythrocytes to remove any natural agglutinins. For the kaolin extraction, 0.5cc of serum was placed in a 13x100mm test tube, to which was added 2.0cc of BS at pH 9 and then 2.5cc of a 25% suspension of acid-washed kaolin. The mixture was kept at room temperature for 20 min with occasional shaking, and then it was centrifuged at 2500 rpm x 20 min at 4°C. Subsequently 0.1cc of packed goose erythrocytes (packed by centrifugation of the standardized goose erythrocytes at 1500 rpm x 10-15 min at 4°C) was added to the supernatant, following which the test tube was stoppered and shaken gently. The tube was allowed to stand in an ice bath with occasional shaking for 20 min; and then it was centrifuged at 1500 rpm x 10

min at 4°C. The supernatant was pipetted off and stored at -20°C. This serum was considered 1:10 dilution BS at pH 9

Tests involved use of an automatic calibrated pipette to add aliquots of antigen, serum, solution, or erythrocytes into plastic trays containing 96 wells. A microtest was used as much as possible in order to conserve these ingredients. While the remaining description pertains to the microtest, the macrotest was done similarly but with 8 times more volume per aliquot, e.g., 0.2cc as opposed to 0.025cc.

A. Hemagglutination Test.

HA tests were done on each antigen in order to: 1) determine optimal pH for hemagglutination and 2) determine the titer of hemagglutination at optimal pH.

Lyophilized antigen was rehydrated with sterile water and diluted 1:5 with BS-BPA. This was allowed to stand overnight at 4°C to allow for dispersion of aggregates.

On the following day 0.025cc of antigen was placed into the first well of a row of 12 wells, each filled with 0.025cc of BS-BPA. The antigen was then serially diluted two-fold from 1:10 up to 1:5120. The twelfth well in the row was left as a control and was not used to dilute the antigen. The above procedure was repeated for as many rows as values of pH which were tested to determine the pH optimum. Values assayed ran from 5.9 to 7.0 in increments of 0.1. For any given antigen the values assayed were restricted to those of its characteristic pH range. To each row was then added the goose erythrocyte preparation diluted 1:24 with VAD of the proper pH. The test was allowed to stand at room temperature for several hours before reading. Hemagglutination was read as: "complete" if a uniform layer of red cells was present on the bottom of the well; "absent" if a small

centrally-located button of red cells was present; "partial" if the pattern was between that of complete and that of absent; "trace" if the button was not quite complete or centrally-located.

The pH value which gave complete hemagglutination at the highest titer was then chosen as the pH for the HI test, and the titer was then taken as the unit antigen. The control well at each pH served to insure that hemagglutination properties were absent from the goose cell-VAD mixture, as well as from the BS-BPA.

B. Hemagglutination-Inhibition Test.

Each HI test began with a preliminary HA test. A solution of antigen at a concentration 16 times that of the previously determined unit antigen was serially diluted two-fold, beginning at 1:2 up to 1:128; and the HA test was then done at optimal pH. If the highest titer showing complete hemagglutination was 1:8 or 1:16, it meant that the solution used for the serial dilutions contained 4 or 8 units of antigen, respectively, when diluted 1:2; and the HI test could be commenced using this solution. If the endpoint was more than 1:16 or less than 1:8, the solution was diluted or concentrated, respectively, to obtain the 4 to 8 units of antigen needed for the HI test.

In the actual HI test the kaolin-extracted, erythrocyte-washed serum was serially diluted two-fold beginning at 1:10 with BS-BPA as diluent. To each well an equal volume (0.025cc) of the 4-8 unit antigen was added. For each serum sample a control well of 0.025cc of serum without antigen was included. The trays were covered with a celluloid sheet to prevent evaporation and allowed to stand overnight (approx. 18 hours) at 4°C.

A small amount of the 4-8 unit antigen was kept in a test tube overnight at 4°C; and on the following day it was used in a final HA test executed in the same manner as the preliminary HA test. The purpose of this final HA test

was to ascertain whether the strength of the antigen in the HI test might have changed overnight.

After refrigeration the HI test was completed by the addition to each well of 0.05cc of the goose erythrocyte preparation diluted 1:24 with VAD of the optimal pH. Both the final HA test and the HI test were allowed to stand for several hours at room temperature before reading. For each serum sample the highest titer giving complete absence or "inhibition" of hemagglutination was recorded. If the number of units of antigen present in the final HA test was not 8, the HI titers for all the sera were corrected to correspond to the titers which would have been present had the HI test been done with 8 units of antigen. For instance, if the HA test showed 2 units of antigen, the serum titer was multiplied by 1/4. These corrected titers were then recorded by the number of the well corresponding to that titer:

Well #	1	2	3	4	5	6	7	8
Titer	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280

The control well for each serum sample was checked to insure that there were no hemagglutinating properties present after the kaolin-erythrocyte processing.

Appendix III

Neutralization Test

A. Preparation of Virus.

Each rehydrated virus was titrated in suckling mice; and the brains were harvested to prepare a wet pool of virus which, after being titrated, was used in the NT proper. The purpose of the wet pool was to boost the infective titer or LD_{50} of each virus and also to eliminate the mass of inactivated virus particles which accumulated in the process of lyophilization and which can divert serum antibodies from neutralizing active infectious virus particles and thereby give erroneous results. Methods for titration, for preparation of the wet pool, and for calculation of the LD_{50} are explained below.

Initial titrations with rehydrated YARU lyophilized virus, were done using 0.75% bpa (bovine plasma albumin diluted with 0.05M phosphate buffered saline at pH 7.2) to make 10-fold dilutions of virus from 10^{-3} or 10^{-4} up to 10^{-9} or 10^{-10} . These dilutions were placed in an ice bath and then injected in approximately 0.02cc amounts via intracerebral route into randomized 2- and 3-day-old mice, numbering 8 per litter. The mice were checked daily for two weeks for: sickness, paralysis, death, or absence. The LD_{50} was subsequently calculated.

For preparation of the wet pool four paralyzed mice from each virus were frozen at -60°C and subsequently thawed, following which their brains were: harvested, suspended in 8cc of 7.5% bpa, and homogenized with a glass homogenizer precooled to -20°C . The homogenate was then spun at 10,000 rpm x 45 min at 4°C . The homogenate was pipetted off and constituted an approx. 1:10 dilution of virus known as the wet pool. Part of this wet pool was used im-

mediately without prior freezing in a final titration while the remainder was stored at -60°C in aliquots of approximately 1cc each.

The LD_{50} constitutes the theoretical concentration which will kill 50% of inoculated subjects. The first step in its calculation involves using the titration to determine at each dilution, both the cumulative survivors from all lower dilutions and the cumulative deaths from all higher dilutions. The exponential integer for the LD_{50} was contained in the highest dilution at which the cumulative deaths outnumbered the cumulative survivors. The exponential fraction for the LD_{50} was determined by the following formula:

$$\frac{(A - B) (C + D)}{2 (AD - BC)}$$

in which

A=cumulative deaths at highest dilution at which deaths outnumbered cumulative survivors.

B=cumulative survivors at highest dilution at which cumulative deaths outnumber cumulative survivors.

C=cumulative deaths at lowest dilution at which cumulative survivors outnumber cumulative deaths.

D=cumulative survivors at lowest dilution at which cumulative survivors outnumber cumulative deaths.

As an example of how the LD_{50} is calculated, the following titration is given:

Virus Dilution	Deaths	Survivors	Cum. Deaths from all Higher Dilutions	Cum. Survivors from all Lower Dilutions
10^{-3}	8	0	49	0
10^{-4}	8	0	41	0
10^{-5}	8	0	33	0
10^{-6}	8	0	25	0
10^{-7}	8	0	17	0
10^{-8}	6	2	9=A	2=B
10^{-9}	3	5	3=C	7=D
10^{-10}	0	8	0	15

10^{-8} =the highest dilution at which cumulative deaths outnumber cumulative survivors and thus contains the exponential integer for the LD_{50}

The exponential fraction =

$$\frac{(A - B)(C + D)}{2(AD - BC)} = \frac{(9 - 2)(10)}{2(63 - 6)} = \frac{70}{114} = 0.6$$

Therefore $LD_{50} = 10^{-8} \times 10^{-0.6} = 10^{-8.6}$

B. Neutralization Test

The NT proper involved using the thawed wet pool with 0.75% bpa as diluent to make a concentration of virus 125 x the LD_{50} and then adding it in 0.2cc amounts to 0.2cc of each serum sample. The samples were then incubated in a water bath at 37°C for one hour, following which they were kept in an ice bath during the course of inoculation. Each virus-serum mixture was injected in a dose of approximately 0.02cc into randomized 2-, 2-, 4-, or 5-day-old mice, numbering 8 per litter. The mice were checked daily for two weeks for: sickness, paralysis, death or absence.

The completed NT for each virus consisted of five parts:

- 1) 125 LD_{50} of virus + CF or HI positive sera. Sometimes these were divided into strongly and weakly positive sera.
- 2) 125 LD_{50} of virus + CF or HI negative sera for that virus.
 - a) Sometimes the test included sera positive for other viruses of the same group, e.g., Zika negative but broad Group B positive, when doing NT for Zika.
 - b) Each test included sera which were CF or HI positive for antigens outside of the group, e.g., Ingwavuma negative but chikungunya positive when doing NT for Ingwavuma.
 - c) Each test included sera negative to all viruses assayed by CF or HI.

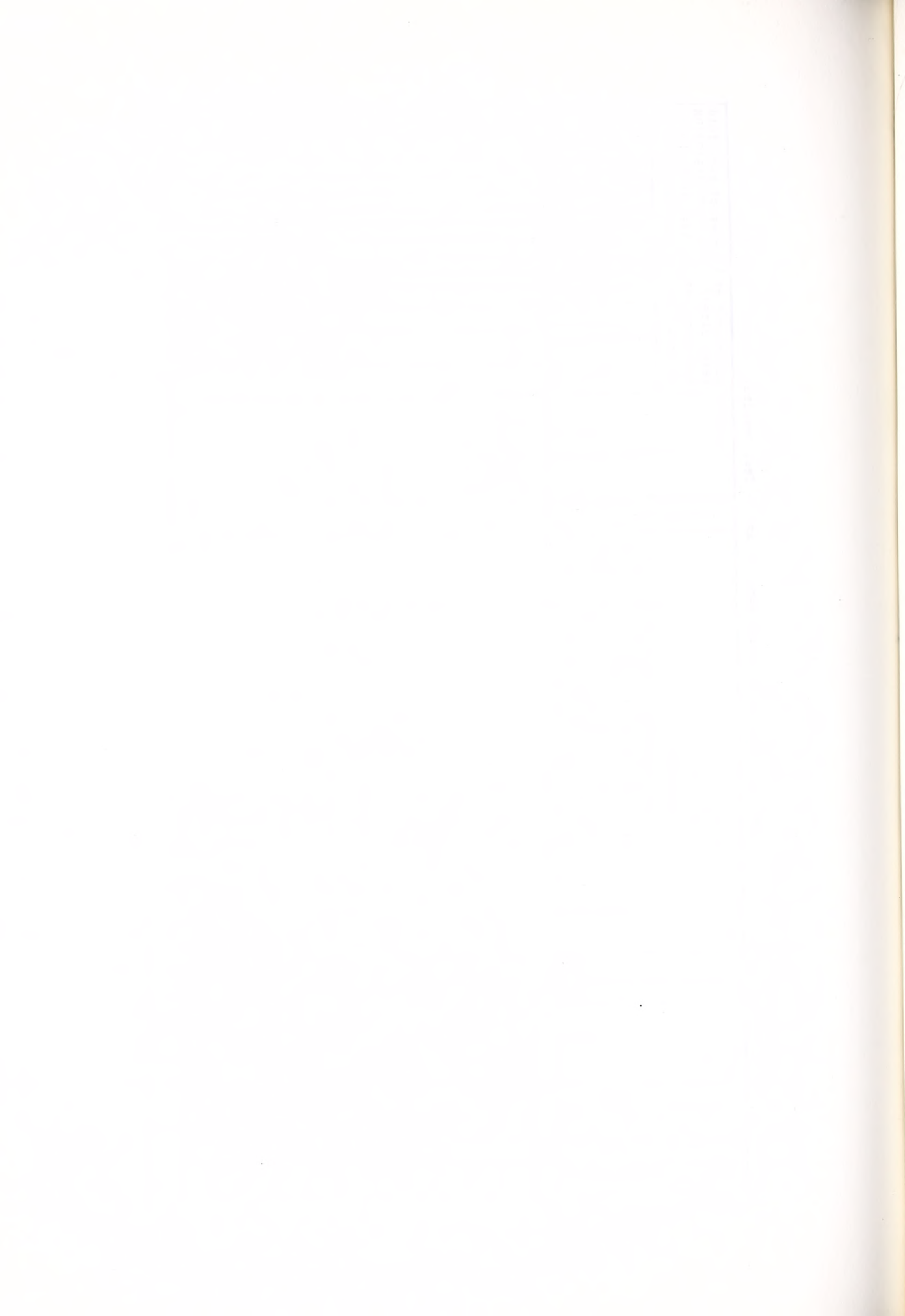
- 3) 125 LD₅₀ of virus + mouse immune ascitic fluid for that virus.
- 4) 125 LD₅₀ of virus + normal mouse ascitic fluid.
- 5) A titration of virus in 10-fold dilutions with 0.75% bpa as diluent, beginning at 125 LD₅₀ and ending at 0.0125 LD₅₀.

For example, Bwamba: LD₅₀ = 10^{-5.9}.

Dilutions were: 10^{-3.8}, 10^{-4.8}, 10^{-5.8}, 10^{-6.8}, 10^{-7.8}.

Parts 2, 3, and 4 were included to give adequate controls. While an ideal of 125 LD₅₀ per mouse was aimed for, it was realized that the actual value would most likely be other than this; and therefore a titration would be necessary to determine the LD₅₀ for the NT proper. From this the actual number of LD₅₀ doses per mouse could be determined:

$$\begin{aligned} \#LD_{50} \text{ doses for NT} &= \frac{125 \times LD_{50} \text{ for wet pool}}{LD_{50} \text{ for NT}} \\ &= \frac{\text{Dilution of wet pool virus used for NT}}{LD_{50} \text{ for NT}} \end{aligned}$$



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