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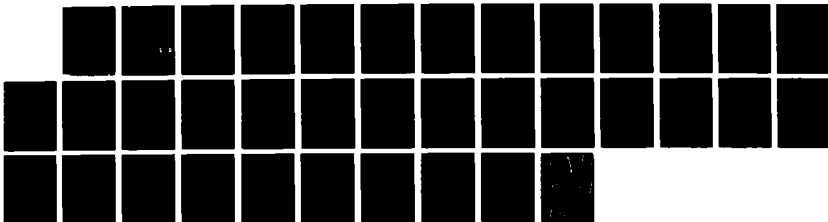
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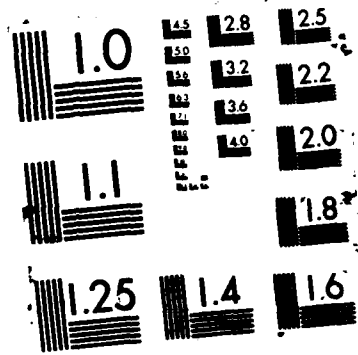
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STUDIES OF INFECTION AND DISSEMINATION OF RIFT VALLEY FEVER
VIRUS IN MOSQUITOES

ANNUAL REPORT

William S. Romoser

July, 1987

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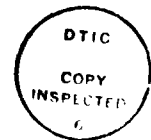
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SUMMARY

We are in the midst of a multimethod study of Rift Valley fever (RVF) virus in vector and hypothetical vector mosquitoes. We have found that (1) early dissemination and possible early or rapid transmission can occur in Culex pipiens; (2) evidence from several standpoints supports the idea of virus escape from the gut lumen in the region of the foregut-midgut junction; (3) the basal lamina may act as a midgut escape barrier or at least retard viral passage; (4) RVF virus is capable of infecting most tissues in the mosquito hemocoel and may exert its pathological effects both by a general depletion of energy stores and by interference with regulatory processes; (5) the fat body appears to be an important amplifying tissue; (6) in Aedes mcintoshi, the species strongly suspected of maintaining RVF virus transovarially in Kenya, the follicular epithelia and eggs are, at least in some cases, susceptible to infection.

FOREWORD

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. Introduction

A. Background

The literature pertinent to this project is extensively reviewed in Hardy et al. (1983) and in the original research contract proposal.

B. Objectives

The overall objective of this research is to contribute to our understanding of the epidemiology of Rift Valley fever. More specifically, our goal is to describe the dissemination, pathogenesis, and morphogenesis of RVF virus in vector competent and incompetent mosquitoes. Ultimately we hope to shed light on intrinsic factors which influence vector competence using RVF virus/mosquitoes as model systems.

C. Key Questions:

Specific questions we are addressing (pertinent both intraspecifically and interspecifically) include: (1) Why do some mosquitoes fail to develop midgut infections? ("midgut infection barrier"). (2) Why do some mosquitoes develop midgut infections, but fail to develop disseminated (systemic) infections? ("midgut escape barrier"). (3) Why do some mosquitoes with disseminated infections fail to transmit virus to their offspring or to vertebrate hosts? ("salivary gland barriers" and "ovarian barriers"). (4) What is the mechanism associated with "early dissemination" & "rapid transmission"? (5) What are the effects of temperature, viral dose, interrupted feeding, nutritional state, heredity, etc. on the operation of the various barriers?

D. Research Covered in This Report

In this report, I will summarize (1) earlier studies of RVF virus in Cx. pipiens using plaque assay (This study was carried out in collaboration with Dr. Michael E. Faran); (2) immunocytochemical studies of RVF virus infection and dissemination in Cx. pipiens, the species incriminated as the vector in the 1977 RVF epidemic in Egypt (Meegan et al., 1980); (3) immunocytochemical studies of field-collected Aedes mcintoshi, one of the hypothesized interepizootic maintenance species in Kenya (Linthicum et al., 1987); and (4) preliminary results of ultrastructural studies of RVF virus in Cx. pipiens.

II. Materials and Methods

A. Introductory Comments

We have applied or are planning to apply several methods for localizing viral infection in mosquito tissues. Each method has inherent advantages and disadvantages. Therefore the best approach is to use more than one method (Hardy, et al., 1983). To detect infectious particles in dissected organs and tissues, we are using plaque assay on Vero cells. To detect viral antigen, we are using the avidin-biotin-peroxidase complex (ABC) immunocytochemical technique for light level microscopy (Faran et al., 1986) and are planning to apply a monoclonal antibody/colloidal gold-protein A technique at the electron microscope level. To detect viral genome, we are involved in the development of a protocol for the application of a peroxidase-labelled complementary DNA probe to paraffin sections of whole mosquitoes. To detect whole virions and nucleocapsids, we are using standard transmission electron microscopy.

B. Plaque Assay

For viral assay, whole mosquitoes and mosquito parts are triturated in 1 mL of mosquito diluent (10% calf serum in Medium 199 with Hank's Salt and antibiotics) and tested for infectious particles by plaque assay on 2- to 4-day-old Vero cell monolayers (Gargan et al., 1983). The mean amount of virus ingested by a sample of mosquitoes taken immediately following each infectious blood meal represents the viral "dose" for a given experiment. When appropriate, legs are dissected and assayed separately in order to determine whether or not virus is present in the body cavity (hemocoel), i.e. whether or not virus has disseminated from the midgut.

C. ABC

The ABC technique was developed in 1981 (Hsu, et al., 1981). Faran, et al., 1986 adapted this very sensitive method for use with serial paraffin sections of formaldehyde fixed, whole mosquitoes. The technique is based on the use of primary antibody directed against viral antigen, followed by biotinylated secondary antibody directed against immunoglobulin from the vertebrate in which the primary antibody was formed. Finally, a complex of avidin and biotinylated peroxidase is applied. This complex binds with the biotinylated secondary antibody due to the great affinity between biotin and avidin. The location of the primary antibody/secondary antibody/ABC complex is then rendered visible by the addition of diaminobenzidine tetrahydrochloride (DAB), the oxidative polymerization of

which is catalyzed by peroxidase. The DAB polymer appears as a rusty brown precipitate. In our studies the "primary antibody" is actually a blend of monoclonal antibodies directed against RVF virus nucleocapsid protein and two envelope glycoproteins. These antibodies are provided by Cdr. James Meegan at USAMRIID.

To date we have applied the ABC technique 47 times and have achieved specific staining 47 times. Thus this technique as applied to localization of RVF viral antigen has been very reliable.

D. Complementary DNA

A biotinylated DNA probe complementary to the M segment of the RVF virus genome has been developed by Dr. Fred Knauert at USAMRIID. This probe has been applied successfully in the in situ detection of RVF virus genome in mouse liver sections. We are currently attempting to adapt this protocol for use with serial paraffin sections of mosquitoes.

E. Electron Microscopy

To prepare mosquito tissues for electron microscopy, tissues are (1) fixed in Karnovsky's solution (1.5% glutaraldehyde, 2.0 % formaldehyde in 0.1M phosphate buffer and 0.15M sucrose) for 1 1/2 to 2 hrs. at 0-4°C; (2) placed in buffered sucrose (15 min. to overnight); (3) post-fixed in 1.0% buffered osmium at 0-4°C for 1 hr.; (4) dehydrated by passing through an ethyl alcohol series; (5) placed in propylene oxide for 20 min., then 1:1 volumes of propylene oxide and resin (Epon 812; Araldite 502; DDSA; DMP-30) for 1 hr., the 1.0 mL of resin was added; (6) 3-24 hours later, tissues were embedded in aluminum foil pans and placed in an oven at 60°C for 48 hrs. Blocks of embedded tissues are cut on an ultramicrotome using a diamond knife, mounted on copper grids, and stained with uranyl acetate and lead citrate. Specimen grids are studied and electron micrographs taken with a Zeiss 110 transmission electron microscope.

F. Specific Investigations

1. RVF virus infection and dissemination in Culex pipiens

RVF tissue tropisms and the dynamics of infection and dissemination in Cx. pipiens were studied using the plaque assay and ABC techniques described above.

The studies which involved plaque assay of dissected organs were carried out prior to the funding of this contract, but are summarized here in order to provide a comprehensive perspective on the current status of this research. Mosquitoes were dissected on days 1-7 after an infectious blood meal. Plaque assays for RVF virus were performed on the legs, posterior midgut, ovaries, salivary glands, thoracic ganglia, and remaining organs and tissues (remnants). On days 7-12 and 14 after an infectious blood meal, mosquitoes were tested for their ability to transmit virus and then dissected. In addition, samples of mosquitoes were collected at relatively short time intervals following a viremic blood meal in order to test for the possibility of early dissemination. One group of individuals which had been given an infectious blood meal and was to be tested for "early dissemination" was vigorously shaken for several seconds in order to determine the possible effects of laboratory trauma on early dissemination.

For the immunocytochemical (ABC) studies, mosquitoes were infected by intrathoracic (IT) inoculation or by feeding on a viremic hamster. Orally infected mosquitoes received a mean dose of $10^{6.9}$ PFUs of virus ($n = 5$; range = $10^{6.7-7.1}$ PFUs). The Zagazig hospital strain (ZH 501) of RVF virus was used in our experiments. Ground legs provided information on the dissemination status of the virus and enabled us to compare plaque assay of dissected legs with immunocytochemical examination as criteria for determining dissemination status. Mosquitoes were incubated for various lengths of time at 26° C, fixed in 5-10 % formaldehyde, and stored in 70% ethyl alcohol until the time of paraffin infiltration. The details of infiltration with paraffin, preparation of serial sections, and application of the ABC technique are described in Faran, et al., 1986.

3. Electron Microscopy

For ultrastructural study, we prepared tissues from specimens of Culex pipiens fed on viremic hamsters and from specimens which were IT inoculated with RVF virus. Blood fed specimens were fixed at regular intervals up to 10 days following the blood meal and IT infected specimens were incubated for 3-7 days prior to fixation. All specimens were incubated at 26° C.

F. Aedes mcintoshi

During May and June of 1986, Aedes mcintoshi collected from an artificially flooded dambo at Sukari Ranch just outside of Nairobi, Kenya were shipped to USAMRIID. Specimens of this species were treated in three ways. (1) A

large number of of females were provided with a viremic blood meal from a hamster and then samples were killed and fixed in 5 % formaldehyde at different times following oral exposure to virus and stored in 70 % ethyl alcohol. (2) A sample of females was intrathoracically inoculated with virus, incubated for several days, fixed in 5 % formaldehyde and stored in 70 % ethyl alcohol. (3) Four specimens which had obtained a blood meal in Kenya were intrathoracically infected with virus, allowed to oviposit, incubated for several days and given an additional blood meal. These mosquitoes were killed and fixed when the eggs were fully developed. Mosquitoes from all three treatments were prepared for the subsequent application of the ABC immunocytochemical technique and microscopic examination.

III. Results & Discussion

A. Culex pipiens--Infection & Dissemination Studies

1. Summary of plaque assay results

Overall, 96.0% (380/393) of mosquitoes that took a viremic blood meal became infected. Dissemination rates averaged approx. 22% on days 1-14 and transmission rates approx. 33% on days 7-14. Transmission rates varied from 18.7% (3/16) on day 12 to 66.7% (2/3) on day 14. There were no significant differences in the viral titers of midgut samples among the nondisseminated infected (virus limited to alimentary canal), disseminated infected nontransmitting, and transmitting groups of mosquitoes. The sequence of infection of the organs and tissues studied appeared to be as follows: (1) midgut; (2) hemolymph, remnants; (3) ovaries, salivary glands and thoracic ganglia.

In one of our early experiments, 1 out of 20 mosquitoes had a disseminated infection 2 days after an infectious blood meal. On this basis, we tested for infectious virus at even earlier times following an infective blood meal. We found virus in the legs of 6 out of 50 (12 %) of a sample of mosquitoes tested as early as 4 hours after they ingested $10^{6.2}$ PFU of virus. We believe this is the first report of a bunyavirid virus disseminating in an insect after such a short incubation period. Trauma, simulated by vigorous shaking right after the viremic blood meal, did not affect either infection or dissemination rates.

2. Immunocytochemical studies

a. Infection & dissemination-- dynamics & rates

Excluding day 1, 112 of 137 or 81.8% of the specimens we studied became infected (Table 1). Mean infection rate was $81.5 \pm 14.8\%$ (range = 66.7 - 100 %). Although the average amount of RVF virus ingested by the mosquitoes used in this study was very close to the amount ingested in the plaque assay study ($10^{6.9}$ versus $10^{6.2}$ PFUs), the infection rate in these specimens was somewhat lower.

Excluding day 1, the overall dissemination rate (based on viral titration of legs and/or immunocytochemical examination) was 54 out of 112 infected specimens or 48.2% (Table 2). The mean daily dissemination rate was $49.9 \pm 20.9\%$ (range = 20.0 - 100.0%). Although these dissemination rates are not dramatically out of line with those in the viral titration study, they are slightly higher.

b. Patterns of midgut infection & escape of virus from the midgut

RVF antigen was detected in all midgut regions, i.e. the cardial epithelium; the tubular, anterior midgut; and the baglike, posterior midgut. Table 3 shows the relative frequency of infection of each of the 3 regions mentioned. Infections tended to be somewhat localized as opposed to covering broad areas.

The escape of virus from the alimentary canal has been assumed to occur from the midgut epithelial cells, especially in the posterior midgut since that is the destination of ingested blood. However, we believe virus also disseminates via cells at the foregut/midgut junction into the intussuscepted foregut (Figs. 1 & 2, IF). Following this route, virus would first infect the IF and associated sphincter muscle, then the hemolymph and/or the tissues of the diverticula/esophagous region, and finally the hemolymph.

Examination of patterns of RVF antigen distribution in the midgut and IF in association with information on dissemination status has provided the following pieces of evidence that support the hypothesis of virus escape via the IF (Table 4): (1) Six out of 72 specimens with non-disseminated infections on the basis of leg titration have been found to have antigen in the IF, indicating that this region can be infected with virus from the gut lumen. (2) Two of 57 individuals with disseminated infections have been found with antigen only in the IF, indicating that dissemination can occur from this site.

Table 1

Infection based on the presence of detectable RVF viral antigen in the intussuscepted foregut and/or midgut.

Days after Infective Blood meal	Sample Size	Number Infected*	Percent Infected
1	15	2+	-
2	10	7	70.0
3	15	10	66.7
4	10	9	90.0
5	10	5	50.0
6	10	9	90.0
7	9	7	77.8
8	10	9	90.0
9	10	7	70.0
10	10	9	90.0
11	10	9	90.0
12	10	10	100.0
13	10	10	100.0
14	10	9	90.0
21	3	2	66.7
Total	152	114	

*The "2" for day 1 is based on the fact that 2 specimens had virus in the legs, indicating a disseminated infection. In addition, one of these specimens had a clearly disseminated infection on the basis of antigen in the hemocoel.

Table 2

Dissemination based on the presence of infective RVF virus
the legs (plaque assay) or antigen in the intussuscepted
foregut or in tissues on the hemocoel-side of the midgut.

Days after Infective Blood meal	Number Infected	Number with Disseminated Infections	Percent of In- fected Specimens with Disseminated Infections
1	2+	2	-
2	7	2	28.6
3	10	4	40.0
4	9	4	44.0
5	5	1	20.0
6	9	5	55.6
7	7	2	28.6
8	9	3	33.3
9	7	5	71.4
10	9	5	55.6
11	9	4	44.4
12	10	5	50.0
13	10	6	60.0
14	9	6	66.7
21	2	2	100.0
Total	114	56	

Table 3

Distribution of RVF viral antigen in the midguts of orally infected mosquitoes.

Organ	RVF viral antigen-positive*
Cardia	49.1 % (26/53)
Anterior Midgut	76.4 % (42/55)
Posterior Midgut	83.3 % (45/54)

*Percent positive for RVF viral antigen (number positive/total number examined)

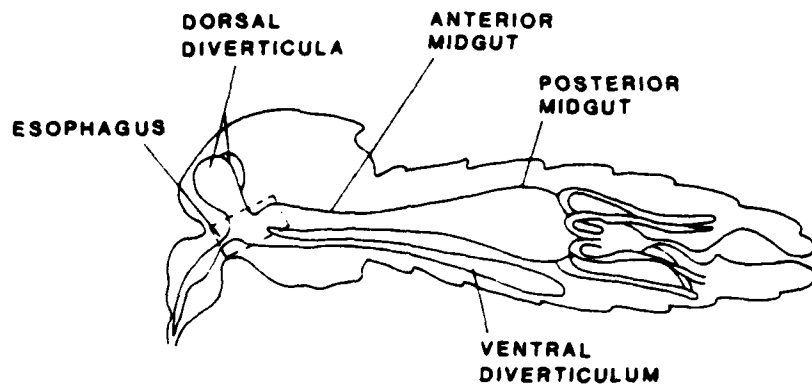


Fig. 1. Mosquito alimentary canal.

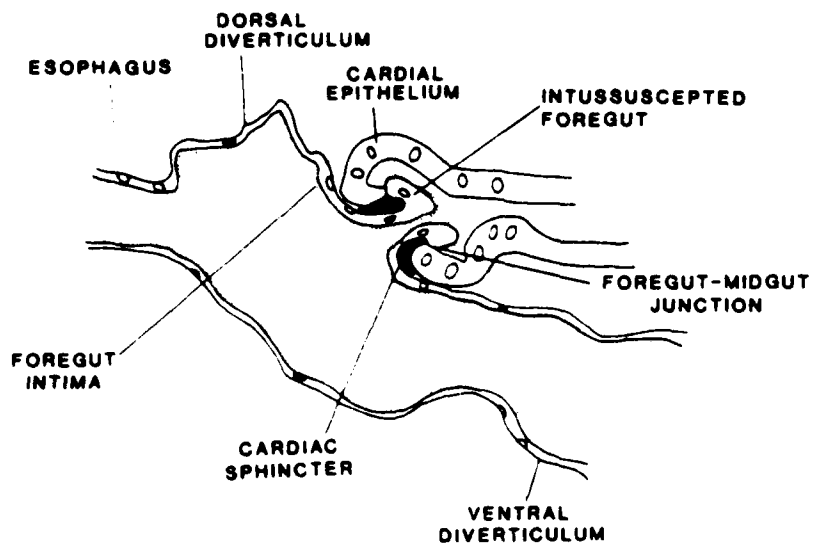


Fig. 2. Sagittal section in region of foregut/midgut junction (indicated by boxed area in Fig. 1).

Table 4

Patterns of RVF viral antigen distribution in the midgut and intussuscepted foregut of Culex pipiens orally exposed to RVF virus.

Antigen-positive region(s)*	% Nondisseminated (no. obs.)	% Disseminated (no. obs.)
1. IF only	0	3.5 (2)
2. IF/Ant. Mg.	2.8 (2)	8.8 (5)
3. IF/Post. Mg.	0	8.8 (5)
4. IF/Ant. Mg./Post. Mg.	5.5 (4)	61.4 (35)
5. Ant. Mg. only	18.1 (13)	1.7 (1)
6. Ant. Mg./Post. Mg.	41.7 (30)	15.8 (9)
7. Post. Mg. only	31.9 (23)	0
Total	100.0 (72)	100.0 (57)

*IF, intussuscepted foregut; Ant. Mg., anterior midgut; Post. Mg., posterior midgut.

Dissemination of RVF virus via the IF may be a very common occurrence (Table 4). Most individuals, 66 out of 72 or 91.7% of individuals with non-disseminated infections had antigen in the anterior and/or posterior midgut, but not in the IF. On the other hand, most individuals, 47 out of 57 or 82.5% of individuals with disseminated infections had antigen in the IF. That is a strong correlation exists between infection of the IF and dissemination.

We have also found several individuals with disseminated infections in which the anterior and/or posterior midgut were infected, but not the IF (Table 4), indicating that virus also escapes via the midgut epithelial cells.

As mentioned above, titration studies have shown that the occurrence of dissemination is independent of the ability of the midgut epithelial cells to support viral replication. This suggests the operation of an extra-cellular structure acting at times as a midgut escape barrier or at least retardant, possibly the basal lamina. Grimstad & Walker (personal communication) found a higher dissemination rate of LaCrosse virus in small versus large female Aedes triseriatus. Ultrastructural examination revealed that the smaller individuals had a much thinner basal lamina than the larger individuals. Their findings are consistent with the idea of a physical barrier external to the midgut epithelial cells, i.e. the basal lamina.

Many bloodfed mosquitoes we examined had a small amount of blood in the foregut diverticula immediately after feeding. Likewise blood in the diverticula has been reported to occur in other species. Thus, in addition to the infectious blood which passes through the IF region during the process of feeding, blood in the diverticula could be a source of infection in the IF region at a later time.

c. Distribution of virus following escape of virus from the midgut

Application of the ABC technique to serial paraffin sections has enabled us to follow the dissemination of virus beyond the midgut at a much more detailed level than has plaque assay of dissected tissues and organs, but the results generated by the two techniques have been consistent with one another.

On the basis of preliminary examination, several tissues (foregut, including the intussuscepted foregut; fat body; ganglia; salivary glands; epidermis; and ommatidia of the compound eyes) were selected and examined in order to evaluate the dynamics of dissemination of RVF virus from the

midgut. Data pertinent to these tissues have been examined in two ways: (1) calculation of a "dissemination index" for mosquitoes with disseminated infections for each day studied following an infective blood meal; and (2) accumulative percent infection as a function of time following an infective blood meal.

The dissemination index provided a way to estimate the relative extent of infection (antigen distribution) by day among individuals with disseminated infections. It was determined for each day following the infective blood meal by examining the following 12 tissues to determine how many had at least some RVF viral antigen-positive cells: salivary glands; ommatidia of the compound eyes; foregut; fat body in the head, in the thorax and in the abdomen; ganglia in the head, in the thorax, and in the abdomen; and finally epidermis in the head, in the thorax, and in the abdomen. The number of tissues which were antigen-positive were summated and divided by 12 to give the dissemination index. Thus, an index of 1.00 would indicate that all tissues used to calculate the index were antigen positive in a given specimen on a given day. Likewise, an index of zero would indicate no dissemination of virus on the basis of antigen distribution. It should be noted that we determined the dissemination index for all individuals which had disseminated infections on the basis of the presence of antigen or the presence of infectious virus in the legs.

Figure 3 presents the dissemination index for each day following the infective blood meal in all individuals with disseminated infections. Note that the extent of dissemination on any given day throughout the period of time examined varies considerably. Note also that for any given day there are almost no intermediate values. Since there are instances where the dissemination index is very low, even as late as day 12, these results appear to indicate that dissemination of RVF virus from the midgut occurs sporadically over a long period of time. The dearth of intermediate values is consistent with findings based on plaque assay of dissected organs and tissues that once RVF virus disseminates beyond the midgut, it rapidly infects the tissues in the hemocoel.

Since, as mentioned above, dissemination appears to occur sporadically over a long time period, we would expect to find, among any given day's sample, individuals which have had disseminated infections for varying lengths of time, ranging from those in which virus has just left the midgut to those in which virus has been present in the hemocoel for a long time. Therefore, at any given time after an infectious blood meal, one would expect the tissues "outside" of the midgut which tend to become infected first to have the highest frequency of infection, and those tissues which are among the last to become infected to have

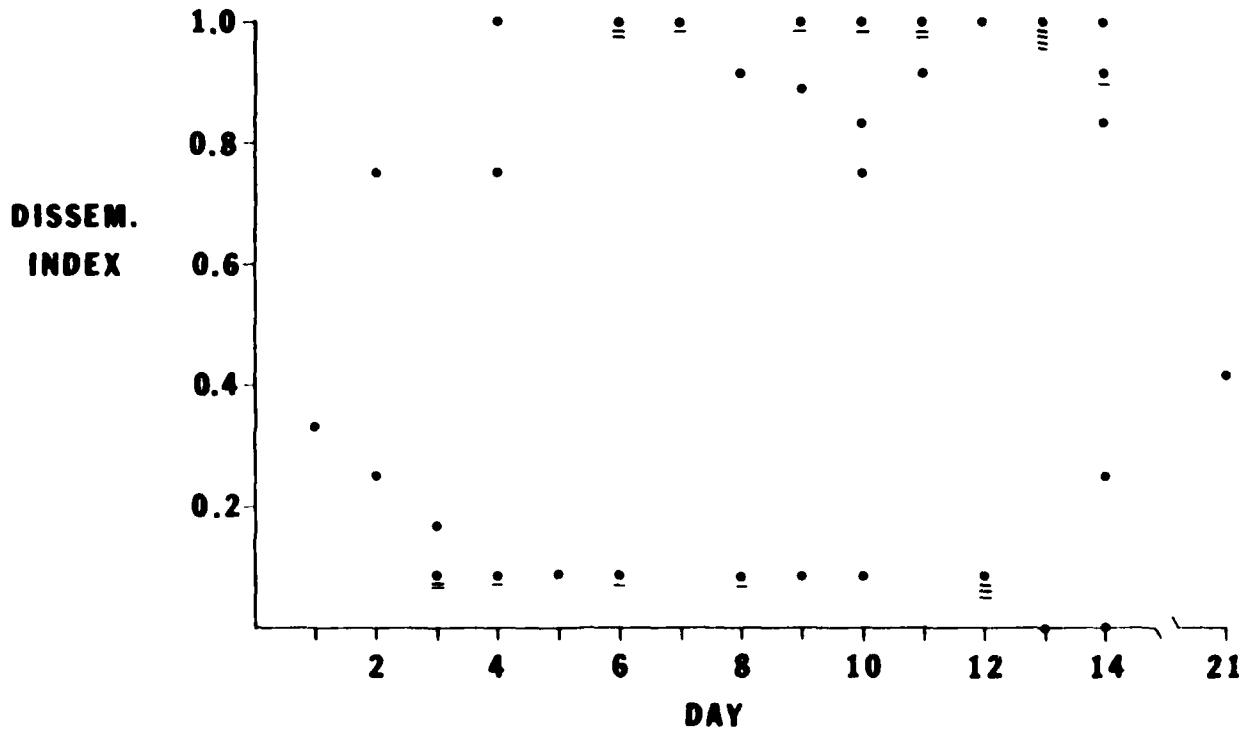


Fig. 3. Dissemination index and time following an infectious blood meal

the lowest frequency of infection. This, of course, assumes that all tissues are equally susceptible to infection. Such appears to be the case since the tissues included in this graph become 95-100 % infected in intrathoracically inoculated individuals (Tables 5-7). Displays of the relative frequency of infection of various tissues and organs in Figures 4 and 5 would thus seem likely to provide information regarding the sequence of infection.

Figure 4 presents the accumulative actual percent infection over time while Fig. 5 presents the accumulative percent infection over time based on the final frequency of infection of a given tissue/structure being taken as 100%. To avoid confusion in Fig. 5, only the curves for the IF, fat body and ommatidia are shown. The curves for the salivary glands, ganglia and epidermis are very close to those for the fat body and ommatidia. Fig. 4 facilitates comparison of actual percent infection among the tissues while Fig. 5 facilitates comparison of the rates at which the various tissues become infected. In Fig. 4 there is a consistent pattern by day 3, with the frequency of IF infection being considerably higher than the other tissues and organs, followed by the fat body, etc. The erratic nature of the curves prior to 3 days is due to small sample sizes. Figure 5 shows that the IF becomes infected consistently earlier than the other tissues, again followed by the fat body.

Applying the reasoning outlined above, Figures 4 and 5 suggest that the IF is infected first, followed by the fat body, and then remaining tissues. Three other points should be made relative to this graph. (1) These results are consistent with the IF as a route of virus escape from the midgut lumen and into the hemocoel; (2) Extensive infection of the fat body indicates that this relatively massive tissue may serve as a major amplifying tissue as suggested by Weaver (1986) in his recent paper on VEE virus in Culex taeniopus; and (3) The relative similarity of infection frequency (Fig. 4) between the salivary glands, ganglia, epidermis and ommatidia is further evidence that once virus escapes from the midgut and gains a foothold in the fat body, its spread to other tissues is rapid.

RVF virus mosquito tissue tropisms based on antigen distribution in the hemocoel are discussed in the following paragraphs.

Table 5 summarizes antigen distribution in the alimentary canal and permits comparison of results in orally infected and intrathoracically infected individuals. All parts of the alimentary canal examined become infected, the foregut tissues at a higher frequency than the hindgut tissues. Infection of both the foregut and hindgut involves virus which has escaped from the midgut. Note the fact that

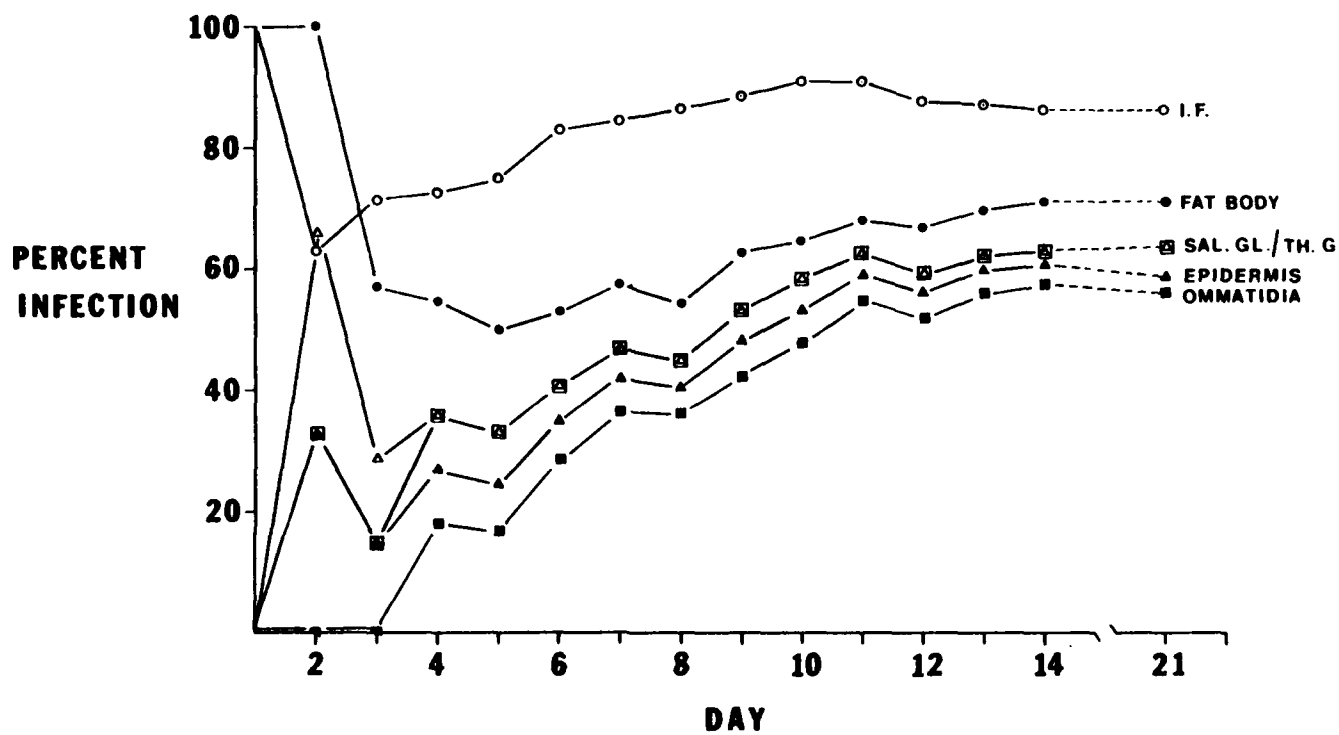


Fig. 4. Accumulative percent infection of selected tissues and organs following an infectious blood meal.

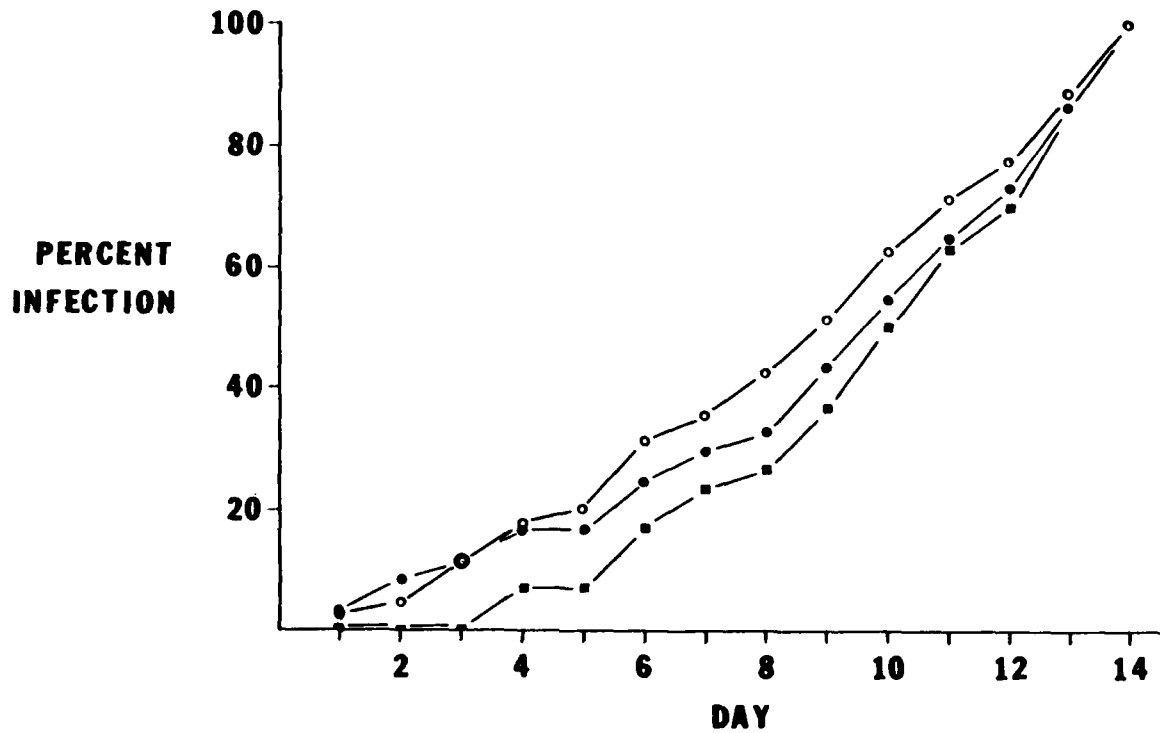


Fig. 5. Accumulative percent infection and time following an infectious blood meal with the final frequency of infection taken as 100%.

Table 5
Distribution of RVF viral antigen in the alimentary canal.

	Orally Infected	IT Infected
Esophagus	64.2 % (34/53)	100.0 % (21/21)
Ventral Diverticulum	73.6 % (39/53)	100.0 % (23/23)
Dorsal Diverticula	66.7 % (36/54)	100.0 % (22/22)
Intussuscepted Foregut	86.8 % (46/53)	95.0 % (19/20)
Cardia	49.1 % (26/53)	5.3 % (1/19)
Anterior Midgut	76.4 % (42/55)	17.4 % (4/23)
Posterior Midgut	83.3 % (45/54)	16.7 % (4/24)
Ileocolon	40.4 % (21/52)	55.6 % (10/18)
Rectal Epithelium	11.8 % (6/51)	56.3 % (9/16)
Rectal Papillae	6.0 % (3/50)	25.0 % (4/16)

*Percent positive for RVF viral antigen (number positive/
total number examined)

the esophagus, which is contiguous with the IF, shows a much lower infection frequency than the IF. This too is consistent with the IF route of escape from the midgut lumen. Since the esophagus is on the hemocoel-side of the IF, if virus usually infected the IF from the hemocoel, one would expect a higher, rather than lower, frequency of esophagus infection.

Note also in Table 5 the differences in infection frequency between orally and intrathoracically infected individuals. In the orally infected group a high infection rate is apparent in all foregut and midgut regions. However, in the intrathoracically (IT) infected group, midgut infection is very low, but foregut infection is 100%. This may indicate the existence of a barrier that somehow retards midgut infection from the hemocoel, but not foregut infection. The high frequency of infection of the foregut epithelium from the hemocoel shows that RVF virions move freely across the basal lamina associated with this tissue. This, coupled with the fact, mentioned above, that midgut cells support viral replication, regardless of dissemination status, again suggests the casting of the midgut basal lamina in a barrier role.

Table 6 summarizes antigen distribution in relatively massive organs and tissues. Although the differences are not strong, it is interesting to note that the frequency of infection in orally infected individuals tends to be greater in the head and thorax than in the abdomen. As with other observations, this is consistent with the earlier appearance of virus in the anterior regions of the hemocoel, i.e. the IF route hypothesis. It should also be noted that antigen in the ganglia was confined to the sheaths and cell bodies and not seen within the neuropile.

Table 7 summarizes antigen distribution in various parts of the salivary glands, all of which can become infected. Obviously, infection of the salivary glands is a prerequisite for transmission to occur. However, it is entirely possible that a particular salivary gland region must be infected before transmission can occur. This should be examined. As in our plaque assay studies, salivary gland infection, as indicated by the presence of viral antigen, was observed by day 2 following an infective blood meal, further evidence that it is possible for "early" transmission to occur.

Table 8 shows antigen distribution in the female reproductive tissues. The germaria, oocytes and nurse cells, and the follicular epithelia of Culex pipiens were not observed to contain antigen. The absence of viral antigen in these tissues is consistent with the fact that Culex pipiens appears not to transmit RVF virus

Table 6

Distribution of RVF viral antigen in various tissues*.

	Orally Infected	IT Infected
<u>Epidermis</u>		
Head	51.9 % (27/52)	100.0 % (23/23)
Thorax	58.5 % (31/53)	100.0 % (26/26)
Abdomen	47.2 % (25/53)	92.0 % (23/25)
<u>Fat Body</u>		
Head	66.0 % (35/53)	100.0 % (25/25)
Thorax	66.0 % (35/53)	100.0 % (25/25)
Abdomen	62.3 % (33/53)	95.8 % (23/24)
<u>Ganglia</u>		
Head	62.3 % (33/53)	92.0 % (23/25)
Thorax	62.3 % (33/53)	96.4 % (27/28)
Abdomen	47.1 % (24/51)	94.7 % (18/19)

*Percent positive for RVF viral antigen (number positive/
number examined).

Table 7

Distribution of RVF viral antigen in the salivary glands.*

	Orally Infected	IT Infected
Lateral Distal Lobes	54.7 % (29/53)	100.0 % (22/22)
Lateral Proximal Lobes	35.8 % (19/53)	71.4 % (15/21)
Median Distal Lobe	37.5 % (18/48)	89.5 % (17/19)
Median Proximal Lobe	33.3 % (16/48)	36.8 % (7/19)

*Percent positive for RVF viral antigen (number positive/
total number examined).

Table 8

Distribution of RVF viral antigen in the female reproductive tissues.*

	Orally Infected	IT Infected
Germaria	0 % (0/53)	0 % (0/15)
Oocytes/nurse cells	0 % (0/53)	0 % (0/15)
Follicular Epithelium	0 % (0/53)	0 % (0/15)
Follicular Relics	19.0 % (8/42)	0 % (0/1)
Ovariolo Sheath	3.8 % (2/53)	0 % (0/15)
Ovarian Sheath	7.5 % (4/53)	0 % (0/15)
Calyx	33.3 % (17/51)	28.6 % (4/14)
Lateral Oviduct	12.5 % (6/48)	30.8 % (4/13)
Common Oviduct	10.4 % (5/48)	20.0 % (2/10)
Genital Chamber	2.0 % (1/50)	30.0 % (3/10)
Accessory Gland	0 % (0/29)	0 % (0/8)
Spermatozoa	0 % (0/44)	0 % (0/10)

*Percent positive for RVF viral antigen (number positive/number examined).

transovarially (M. Turell, personal communication). On the other hand, apparent relics of follicular epithelia, the calyces and of the ovarioles, the oviducts, and the genital chamber are sometimes antigen positive in spots.

The female accessory gland (Table 8) was not seen to contain antigen. In addition, most of the mosquitoes we examined were inseminated, but in no case were spermatozoa positive for RVF antigen.

Both skeletal and visceral muscle appear to become infected (Table 9). Antigen distribution in skeletal muscle is peripheral to the region containing myofibrils. Antigen is often very difficult to detect in muscle tissue and additional light level and ultrastructural study will be necessary in order to determine the extent of infection. It is anticipated that ultrastructural study will likewise shed light on the infection of the tracheae and tracheoles which are also very difficult to interpret with the ABC technique.

As in humans infected with RVF virus, mosquitoes also suffer "retinitis", that is the ommatidia of the compound eyes often contain antigen (Table 9). Another sensory organ on the head, Johnston's organ is often positive for antigen (Table 9).

The tissues of the stomatogastric nervous system, hemocytes, and oenocytes were at times seen to contain antigen (Table 9).

What is the significance of RVF antigen distribution relative to the life of the mosquito? A number of recent studies have challenged the traditional view that arboviruses are not pathogenic in their arthropod hosts. For example, Turell, Gargan & Bailey (1985) described a reduced ability to refeed, and decreased fecundity, in Culex pipiens infected with RVF virus. Recently, Faran et al. (in press) demonstrated a decrease in survival of female Culex pipiens infected with RVF virus.

Given the extent of tissue and organ infection we have found in Culex pipiens infected with Rift Valley fever virus, such gross pathological effects are not too surprising. Certainly extensive infection of the fat body, a massive tissue, which plays a central role in vitellogenesis, must constitute an overall energy drain which would be reflected in reduced fecundity, and ultimately survival. In addition, infection of regulatory tissues and organs such as the ganglia of the central nervous system, neurosecretory cells in the brain, and the corpora allata could conceivably have detrimental effects on the overall functioning of a mosquito, even in the absence of widespread infection. It is clear that the area of mosquito arboviral pathology is wide open for study.

Table 9

Additional RVF viral antigen positive tissues.

Skeletal Muscle (myofibrillar regions negative)

Visceral Muscle

Ommatidia of Compound Eyes

Johnston's Organ

Corpora Allata

Ventricular Ganglion

Frontal Ganglion

Median Neurosecretory Cells

Hemocytes

Oenocytes

B. Aedes mcintoshi--Infection & dissemination Studies

Our studies of Aedes mcintoshi are somewhat preliminary. So far, the tissue tropisms based on antigen distribution appear to be similar to those found in Culex pipiens. However, the dynamics of tissue infection in the African species may be different. A clear and exiting difference between Ae. mcintoshi and Cx. pipiens, is the discovery of RVF antigen- positive follicular epithelia and an antigen-positive chorionated egg in an intrathoracically inoculated specimen of Aedes mcintoshi. This specimen had a blood meal in Kenya, had been allowed to oviposit in the lab at USAMRIID. It was then given a second blood meal and allowed to develop eggs. In addition, other specimens with RVF virus antigen-positive follicular epithelia have been seen in slides of Ae. mcintoshi. The RVF antigen-positive egg showed possible pathology, i.e. apparent coalescence of yolk granules. It is possible that the apparent pathology is related to environmental conditions (temperature ? humidity ?) and that under some conditions transovarial transmission occurs, while under other conditions, pathology occurs. Thus, infected eggs, embryos or first instar larvae may die. These individuals would be undetected by plaque assay of pooled samples. In any case, this finding provides additional support for the hypothesis that Aedes mcintoshi is involved in the endemic maintenance of RVF virus by vertical transmission (Linthicum et al., 1987).

C. Ultrastructural Studies

Although our ultrastructural studies have only recently begun, we have found putative virions in the sphincter muscle associated with the IF, in the ventral diverticulum and apparently adsorbed to, and within, the salivary glands. The observation of virions in the IF and in the ventral diverticulum is consistent with the hypothesis that RVF virus escapes from the gut lumen via the IF. In the salivary glands, virions were observed within cisternae of either or both smooth endoplasmic reticulum and Golgi bodies of two transmitting mosquitoes. This suggests that viral replication occurs in salivary gland cells.

IV. Studies in Progress

A great deal of time and effort has been spent during the past year preparing specimens for study. Therefore, we have a large backlog of fixed and infiltrated specimens as well as immunocytochemically stained (ABC) tissues already mounted on slides, that will require further work. Studies currently in progress and for which specimens are

already prepared include the following: (1) Immunocytochemical (ABC) and viral titration studies of the salivary glands of IT infected Aedes aegypti [work at USAMRIID has indicated the probable operation of a "salivary gland barrier" in this species (M. Turell, personal communication)]; (2) Immunocytochemical study of the effects of blood-feeding on infection of the midgut from the hemocoel of IT infected Culex pipiens; (3) Immunocytochemical (ABC) study of RVF viral tissue tropisms in field-collected Kenyan Culex spp.; (4) Immunocytochemical study of RVF virus infection and dissemination in orally infected Ae. fowleri (slides have been prepared from progeny of mosquitoes collected in Senegal); (5) Immunocytochemical (ABC) study of transovarial transmission of RVF virus in four field-collected, ground-pool-breeding, species of Kenyan Aedes; (6) Immunocytochemical survey of field-collected (Kenya) male and female Ae. mcintoshi for the presence of natural RVF viral infections and search for evidence of transovarial transmission (i.e. infected males and infected females collected as pupae); (7) Immunocytochemical (ABC) study of "salivary gland barrier" in Anopheles sp.; (8) Use of a cDNA probe for the in situ detection of RVF viral genome in paraffin sections of mosquitoes; (9) Ultrastructural study of RVF virus morphogenesis in Cx. pipiens; (10) Immunocytochemical study of RVF virus tissue tropisms in the tick Hyalomma truncatum.

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