Order Number 9118041

The isolation of a Hanta-like virus from rats in Hawaii

Langford, Michael Joseph, Ph.D.

University of Hawaii, 1990

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

THE ISOLATION OF A HANTA-LIKE VIRUS

FROM RATS IN HAWAII

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOMEDICAL SCIENCES
(TROPICAL MEDICINE)

DECEMBER 1990

By

Michael Joseph Langford

Dissertation Committee:

Nyven Marchette, Chairperson
Robert Desowitz
Leslie Tam
Karen Yamaga
John Grove

ABSTRACT

Earlier studies in Hawaii by Diwan et al. (1985) detected antibody to Hantaan virus in rats and people although attempts to isolate an etiological agent were unsuccessful at that time. The purpose of this study was to confirm the serological evidence for a Hantaan related virus in Hawaii, to isolate the etiologic agent, characterize it, and determine its relationship to other hantaviruses. In this study 364/1272 (29%) feral rats, 163/420 (39%) laboratory rats, and 33/227 (14%)feral cats were found to have antibody to Hantaan virus (76-118) by indirect immunofluorescent antibody test (IFAT) at a titer of 1:32 or greater. Attempts to isolate virus from the lungs and spleen of seropositive rats were successful, and the virus was named Manoa virus.

This study has demonstrated a widespread prevalence of antibody to Hantaan virus by IFAT among peridomestic and laboratory Rattus species and feral cats in Hawaii. The antibody is not typical of antibody to other rat-associated hantaviruses and has a unique cross reactive pattern to other known hantaviruses by IFAT. By western blot analysis, the antibody was non-reactive to the major Hantaan virus proteins but was weakly reactive to some minor proteins.

Manoa virus is between 100 and 220 nm in size, it is deoxycholate sensitive, supernatant from infected monolayers contain 10^6 TCID/ml, and it causes mild cytopathic effect in Vero E-6 cells. Fine fluorescent cytoplasmic granules are detectable by IFAT early after inoculation, and they eventually fill the cytoplasm. Ultrastructurally there are numerous electron dense intracytoplasmic inclusions characteristic of the hantaviruses as well as numerous viral particles in thin section of infected Vero E-6 cells.

Manoa virus, isolated from Hawaiian peridomestic and laboratory rats with antibody to Hantaan virus, is Hantavirus-like but has unique characteristics that differ from those of other known hantaviruses. The known rat associated hantaviruses are all very similar and are strains of Seoul virus. The basic characteristics of Manoa virus suggest that it is a bunyavirus and possibly a Hantavirus although it does not appear to fit in the Seoul virus subgroup of rat hantaviruses. Current investigations suggest that Manoa virus may be a new and unique Hantavirus.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

KOREAN HEMORRHAGIC FEVER. During the Korean War (1950-1953) U.S. military physicians stationed in Korea first encountered cases of an acute febrile illness with hemorrhagic manifestations and renal involvement that was previously unknown to Western medicine. Beginning in the spring of 1951 United Nations troops began to become affected with an acute illness characterized by high fever, chills, prostration, headache, generalized myalgia, abdominal and back pain, and hemorrhagic manifestations to include petechia, ecchymoses, and scleral hemorrhages. Laboratory examinations revealed leukopenia followed by leukocytosis, thrombocytopenia, proteinuria, and azotemia. The course of this illness often led to cardiovascular instability, shock, renal failure, and death (Gajdusek, 1962; US Army Tech. Bull. TB MED 240, 1953).

The basic pathological lesion of this disease is endothelial cell damage or dysfunction, which is manifested by widespread capillary engorgement, diapedesis of erythrocytes, and focal hemorrhages involving many organs including the skin, serosal and mucosal linings, and the sclera. Altered capillary permeability manifests itself as interstitial and retroperitoneal edema (Siamopoulos et al., 1954). Lesions in vascular walls consist primarily of

increased endothelial cellularity. Virus specific immunofluorescence in vascular endothelium has been demonstrated (Kurata et al., 1985).

Pathological changes of the kidneys vary according to the stage of the disease. Early, there is congestion of the subcortical medullary vessels. Later, there is vascular congestion of intertubular spaces followed by progressive tubular degeneration. The kidneys become swollen, developing a pale cortex with an extremely congested and often hemorrhagic medulla. A distinctive feature of the kidney is hemorrhage at the junctional zone with extensive tubular necrosis. Necrosis of the pyramids may also occur. (Oliver and MacDowell, 1957).

By the end of 1951, 1016 cases had been reported with approximately 80 fatalities (Powell, 1954). By the end of 1954 more than 2400 UN troops had contracted the disease and many died (Lee, 1982).

This illness soon came to be known as Korean hemorrhagic fever (KHF). Physicians reported the clinical and pathologic features of this illness in detail and it was soon noted that these descriptions matched earlier ones by Russian physicians in Siberia (Smorodintsev et al. 1959) and by Japanese physicians in Manchuria (Ishii et al., 1942).

HEMORRHAGIC FEVER WITH RENAL SYNDROME (HFRS). Prior to the isolation of the causative agents of these nephropathies and

the acceptance of the term "hemorrhagic fever with renal syndrome" (HFRS) to describe them, these diseases were known by many different names. The most widely used names were Korean hemorrhagic fever (KHF) in Korea, epidemic hemorrhagic fever (EHF) in China, epidemic hemorrhagic nephrosonephritis in Russia, and nephropathia epidemica (NE) in Scandinavia.

The Russians called the disease by various names, including Churilov's disease, Far Eastern nephrosonephritis, epidemic hemorrhagic nephrosonephritis, and hemorrhagic fever with renal syndrome. The Japanese used names based on the geographical location of their cases such as Erhtaojiang disease (Nidoko fever), Heiho fever, Hulin (Korin) fever, Kokko (Kokuko) fever, and Songo (Sunwu) fever before settling on the term epidemic hemorrhagic fever (Yanagihara and Gajdusek, 1988).

Hemorrhagic fever with renal syndrome has been recognized for many decades in Asia, Eastern Europe, and Scandinavia (WHO, 1982). Clinical disease has been reported in Far Eastern and European USSR, the People's Republic of China, Korea, Japan, Sweden, Finland, Norway, Denmark, Hungary, Czechoslovakia, Bulgaria, Romania, Yugoslavia, Belgium, France, Greece, and the United Kingdom (Yanagihara and Gajdusek, 1988) (Table 1).

TABLE 1

GLOBAL DISTRIBUTION OF HFRS
(Yanagahara and Gajdusek, 1988)

Country	City or Region	Disease First Recognized	Serological Confirmation in Humans
USSR	Tula Primorye	1930 1935	1981 1978
China	Heilungjiang	1936	1980
Korea	Chorwon	1951	1976
Japan	Osaka	1960	1979
Sweden	Ostersund Skelleftea	1933 1933	1979
Finland	Lapland	1942	1980
Norway	Hamar	1948	1982
Denmark	Svendborg	1957	NR *
Hungary	Izbeg	1953	1985
Czechoslo-vakia	Trnava	1953	NR
Bulgaria	Stara Plania	1954	NR
Yugoslavia	Zagreb	1952	1982
Belgium	Brussels	1979	1983
Greece	Thessaloniki	1982	1982
France	Reims	1983	1983
UK	Sutton	1984	1984

* Serology not reported

HFRS continues to occur throughout the world and recent reports indicate that nearly 62,000 cases occurred in China during 1982 alone (Song et al., 1984). Incidence rates in

different Chinese provinces ranged from less than 2/100,000 up to 168/100,000 (Jiang, 1983). Eleven thousand cases of HFRS were reported to have occurred in the Soviet Union between 1978 and 1983, and the Republic of Korea continues to report several hundred cases per year with a mortality rate of about 5% (Lee, H.W, 1982).

Antibodies to Hantaan virus have been found in sera from numerous countries where clinical disease has not been recognized: Argentina, Bolivia, Brazil, Colombia, Canada, Burma, Thailand, the Philippines, Hong Kong, Malaysia, India, Egypt, Gabon, Kenya, Uganda, Benin, Burkina Fasso, the Central African Republic, and the United States (Yanagihara and Gajdusek, 1988)

ETIOLOGICAL AGENT OF HFRS. The similarity of clinical signs among these nephropathies had led to the speculation that they might also have a common etiology (Gajdusek, 1953, 1962). The diseases originally known as KHF and EHF are severe forms of HFRS while NE is a milder form. Recent studies indicate that human infection from Rattus-associated hantaviruses may cause a mild flu-like illness with fever, myalgia, flank pain, headache, and proteinuria (WHO, 1983).

Despite intensive efforts by Russian and Japanese biomedical scientists from 1938 to 1946, the etiologic agent of HFRS remained unrecognized. However, Russian and Japanese investigators were successful in reproducing HFRS

experimentally in humans by intravenous and intramuscular inoculation of filtered urine or blood taken from acutely ill HFRS patients. Experimental infection was also shown to provide protective immunity to subsequent parenteral challenge (Smorodintsev, et al., 1959; Ishii, et al., 1944).

Russian and Japanese scientists had noted the association of the disease with field rodents as potential vectors but were not able to reproduce the disease in experimental animals. During the Korean War, the US Army Commission on Hemorrhagic Fever also failed to experimentally reproduce the disease in any of a wide range of experimental animals and cell cultures (US Army Tech Bull TB MED 240, 1953).

The etiological agent of KHF was finally isolated from the lungs of the Korean striped field mouse, Apodemus agrarius corea in 1976 by Dr. Ho Wang Lee and his colleagues. They named the virus Hantaan virus (strain 76-118) after the Hantaan river in Korea (Lee et al., 1978). Hantaviruses are now known to be the causative agents of KHF and other related acute interstitial nephropathies in man now collectively known as hemorrhagic fever with renal syndrome.

RELATIONSHIP OF HANTAVIRUSES TO OTHER BUNYAVIRUSES. The initial isolation of Hantaan virus made possible the rapid

acceleration of Hantavirus research. Although Hantaan virus was initially isolated in laboratory rats, it was soon adapted to a line of African Green Monkey kidney cells (Vero E-6 cells). Using Hantaan virus infected Vero E-6 cells as a source of antigen, an indirect immunofluorescent antibody test was developed (Lee, H.W., et al., 1978; French et al.,1981). Several other methods have also been developed for the serological identification of infection: serum dilution plaque-reduction neutralization tests (Takenaka, et al., 1985), hemagglutination inhibition tests (Okuno, et al., 1986), complement fixation tests, immune adherence hemagglutination tests (Sugiyama, et al., 1984), enzyme-linked immunosorbant assay (Gavrilovskaya, et al., 1981), and solid phase radioimmunoassay (Tkachenko, et al., 1981 ,1982). The etiological agents of NE in Europe and Scandinavia (Lee et al., 1979a), and EHF in China (Lee et al., 1980) and Japan (Lee et al., 1979b) were shown to be antigenically related to Hantaan virus of Korea by IFAT. Morphological, biochemical, and molecular studies have also shown that the etiological agents of KHF, EHF, and NE are related to one another and are unique among the Bunyaviridae. Early studies indicated that the virus was a bunyavirus but was antigenically and biologically distinct from the then known genera of Bunyaviridae (Tsai et al., 1982). These agents are now placed in the newly defined genus of Hantavirus in the family Bunyaviridae (Hung et al.,

1983; McCormick et al., 1982; Schmaljohn and Dalrymple, 1983a; Schmaljohn et al., 1983b, 1985; White et al., 1982). Hantaan virus is now recognized as the prototype of the genus Hantavirus in the family Bunyaviridae.

THE FAMILY BUNYAVIRIDAE. Although the family Bunyaviridae is the largest family of the mammalian viruses, it was one of the last groups to be recognized (Fields et al., 1990). It currently contains over 200 viruses in five different genera: Bunyavirus, Hantavirus, Nairovirus, Phlebovirus, and Uukuvirus. Additionally there are several bunyaviruses which do not fit into any of the currently recognized genera.

In spite of the large number of bunyaviruses, relatively few are known to be pathogenic for man and/or other mammals. Some of those known to be pathogenic are: Rift Valley fever virus (Phlebovirus), Akabane virus (Bunyavirus), Cache Valley virus and California Encephalitis virus (Bunyavirus), Nairobi sheep disease virus (Nairovirus), Crimean-Congo hemorrhagic fever virus (Nairovirus), and Hantaan virus (Hantavirus).

Most bunyaviruses are arthropod-borne, replicating in and being transmitted between vertebrates by mosquitoes, ticks, sandflies, or Culicoides spp. vectors. Hantaviruses, however, are unique in that they are transmitted directly between mammalian hosts.

The history of bunyaviruses has been intimately associated with exploration and development of the tropics and they have often been associated with febrile illness. Many acute short term fevers in the tropics, often diagnosed as malaria, are probably due to viral infection. As noted by Downs (1975) the diagnosis of Malaria is a "great umbrella," encompassing not only Plasmodium infection but also a great many other diseases, primarily viral. Many of these febrile illnesses have been due to bunyavirus infection and are of a self limiting nature, thus patients recover and a proper diagnosis is never achieved.

During the 1950's The Rockefeller Foundation set out on a worldwide program to investigate arthropod-borne viral fevers (Theiler and Downs, 1973). Several other organizations also embarked upon similar missions at this time. Among these were: the University of California at Berkley; the Gorgas Memorial Laboratory, Panama; the U. S. Centers for Disease Control; the research unit of the U. S. Navy in Cairo; the U. S. Army Unit in collaboration with the Institute for Medical Research in Malaya; the Middle America Research Unit, Panama; the East African Medical Research Council in Uganda; the Institutes Pasteur in several parts of the world; and the governments of India, Brazil, South Africa, Trinidad, and Nigeria in collaboration with the Rockefeller Foundation (Shope, 1985). More than 25 bunyaviruses causing febrile human illness were isolated

from arthropods, wild animals, domestic animals, sentinel animals, and human beings. All of those causing uncomplicated fevers or fevers with rash are tropical in distribution. Most are focally distributed and do not cause large outbreaks of human disease. The distribution of bunyaviruses is dependent upon the distribution of their maintenance host and vector.

The typical illness is characterized by sudden onset, fever, chills, headache, myalgia, malaise and anorexia. Rash is occasionally seen. Duration of illness is usually 1-3 days with gradual recovery. Although there are no long term ill effects from illness caused by these viruses, they can be temporarily incapacitating. Few of the bunyaviruses causing limited fevers in man have resulted in human mortality and thus, there are few reports of histopathology in man.

The vector borne bunyaviruses viruses pathogenic for man fall into 3 categories: those transmitted by forest mosquitoes, such as the Group C agents; those transmitted by tropical anophelines, such as Tataguine, Bwamba, and Guaroa; and those transmitted by ticks, such as Dugbe. Heterologous cross-reactions by complement fixation, hemagglutination inhibition, neutralization, immunofluorescence, and ELISA are common within the groups (Shope, 1985).

The best studied of these are the group C bunyaviruses which contain 12 recognized serotypes: Apeu, Caraparu,

Gumbo Limbo, Itaqui, Madrid, Maratuba, Murutucu, Nepuyo, Oriboca, Ossa, Restan, and Vinces viruses. All are transmitted by culicine mosquitoes, all have come from tropical North, Central, or South America, most (10/12) cause human illness, most (8/12) have rodent hosts, and many (5/12) have marsupial hosts (Arthropod-Borne Virus Information Exchange, 1988).

Near Belem, Brazil seven related Group C viruses circulate in different ecosystems of the same forest and without interfering with each other (Woodall, 1979). Two group C viruses do however share an ecosystem (Caraparu and Itaqui) and have developed unique surface glycoproteins that enable them to do this. Although the viruses have identical nucleoprotein, they have different surface glycoproteins which are responsible for induction of virus neutralizating antibody formation and serotype specificity, and thus both viruses are able to coexist within the same rodent host and not interfere with the replication or transmission of each other (Shope, 1985).

Members of the family Bunyaviridae are spherical, generally 90-100 nm in diameter, enveloped, and have a tripartite RNA genome. The envelope is a lipid bilayer and is covered with glycoprotein peplomers. The genome consists of three segments of single stranded RNA, each within its own nucleocapsid. The genome segments are designated as large (L), medium (M), and small (S) RNA strands of 9 to 15,

3 to 6, and 1.2 to 2.4 kilobases respectively. Each RNA segment is linked at the ends by hydrogen bonds into a circular form. The genome is negative sense except for the genus Phlebovirus in which the S segment is ambisense (the 5' end is positive sense).

The virion contains four major proteins: a transcriptase (L, 150-200 kd), a nucleoprotein (N, 25-50 kd), and two glycoproteins (G1 and G2, 40-120 kd) which form the surface of the peplomers. Group and type specific epitopes for the bunyaviruses are expressed on the nucleoprotein and the glycoprotein peplomers respectively. (Fields, et al., 1990)

THE GENUS HANTAVIRUS. The hantaviruses possess all of the bunyavirus characteristics; they are enveloped viruses with helical symmetry and have a tripartite, negative sense, single stranded, RNA genome. They are somewhat pleomorphic and have a diameter of 78-200 nm (Hung, et al., 1985), are sensitive to deoxycholate and other lipid solvents, are stable at 4-20 C and rapidly inactivated, at temperatures above 37 C (Lee, P.W., et al., 1985; Lee, H.W., et al., 1982). Purified Hantaan virus has a buoyant density of 1.16-1.17 g/ml in sucrose and 1.20-1.21 g/ml in cesium chloride (Elliott, et al., 1984; Schmaljohn, et al., 1983). The L, M, and S segments have molecular weights of approximately 2.7 X 10^6, 1.2 X 10^6, and 0.6 X 10^6 daltons

respectively (Schmaljohn, et al., 1983). These genomic segments are enclosed in separate nucleocapsids, each surrounded by its own lipid envelope. The L segment is believed to encode a viral polymerase. The M segment has a single, long, open reading frame containing two non-overlapping genes encoding the two envelope glycoproteins, G1 and G2. The S segment has a single long open reading frame containing the gene for the nucleocapsid protein (Schmaljohn, 1988). The hantaviruses have also been shown to have a unique 3'-terminal nucleotide sequence (3'AUCAUCAUCUG) which is distinct from the other genera of Bunyaviridae (Schmaljohn, et al., 1985).

Four distinct ecological complexes distinguishable by plaque-reduction neutralization test are recognized within the genus Hantavirus (Schmaljohn, et al., 1985; Lee, P.W., et al., 1985): 1) Hantaan virus and the Korean striped field mouse, Apodemus agrarius corea (Lee et al., 1978); 2) Seoul virus and urban rats, Rattus rattus and R. norvegicus (Lee et al., 1982); 3) Puumala virus and the bank vole, Clethrionomys glareolus (Brummer-Korvenkontio et al., 1980); and 4) Prospect hill virus and the meadow vole, Microtus pennsylvanicus (Lee et al., 1985). These four virus groups can also be distinguished by monoclonal and polyclonal anti-sera and are recognized as subgroups by the American Committee on arthropod-Borne Viruses (Table 2).

TABLE 2

HANTAVIRUS COMPLEXES DEFINED BY HOST SPECIES			
Virus complexes	Natural Host	Distribution	Human Disease
Hantaan	Apodemus agrarius	Eastern Asia Eastern Europe	Korean hemorrhagic fever
Seoul	Rattus sp.	Worldwide	HFRS (mild to severe)
Puumala	Clethrionomys glareolus	Scandinavia, Europe, USSR	HFRS (mild)
Prospect Hill	Microtus pennsylvanicus	United States	Not known

Hantaan virus is the etiologic agent of the severe form of HFRS and occurs in Korea, China, and southeastern Siberia. The striped field mouse, Apodemus agrarius, is the rodent host for this virus. There are two seasonal peaks of human disease, one in spring and summer and the other in fall and winter. These peaks are associated with increased human contact with the rodent host. They occur during planting and harvesting of crops and, as winter approaches, when rodents seek shelter in buildings of human habitation.

After the initial isolation of Hantaan virus from Apodemus agrarius corea in Korea, additional strains were isolated from HFRS patients as well as from several other rodent species (Brummer-Korvenkontio et al., 1980; Lee et

al., 1982; Yanagihara et al., 1984; Song et al., 1983; Chumakov et al., 1981; Lee et al., 1985).

During studies of Korean hemorrhagic fever a mild form of HFRS was recognized in Seoul, Korea and other urban areas where Apodemus agrarius, the vector of KHF, did not exist. Urban R. rattus and R. norvegicus were shown to have antibody reacting to Hantaan virus, and a new serotype, Seoul virus, was subsequently isolated from rats seropositive to Hantaan virus. Seoul virus is the prototype of all known Rattus associated hantaviruses. The rat associated hantaviruses are now known to have a worldwide distribution although they have not always been implicated with human disease which raises the question as to whether human disease may be occurring outside currently accepted endemic areas. In the Americas and many other parts of the world where seropositive rats have been found clinical HFRS has not been recognized.

Hantaviruses have been isolated on numerous occasions from R. rattus and R. norvegicus in Korea (Lee et al., 1982), R. norvegicus in Brazil (LeDuc et al., 1985), R. norvegicus in US port cities (Tsai et al., LeDuc et al., 1982), and from laboratory R. norvegicus in Japan (Kitamura et al., 1983) and Europe (Dournon et al., 1984). Hantaviruses have also been isolated from peridomestic rats (R. rattus and R. norvegicus) in Japan, the People's Republic of China, and Thailand. Seropositive peridomestic

rats have also been detected in Egypt, Kenya, the Philippines, Burma, Hong Kong, Taiwan, Australia, Fiji, Papua New Guinea, Argentina, and Hawaii although a Hantavirus has not yet been isolated from these areas.

The public health significance of this still emerging zoonotic disease is not yet clear, but clinical disease may occur in high risk humans in contact with infected rats.

Puumala virus causes a mild form of HFRS and occurs in eastern Europe, Scandinavia and western USSR. The natural host for this virus is the bank vole, Clethrionomys glareolus. The incidence of human disease peaks in mid-to-late summer when people are active outdoors and in late fall to early winter when voles seek shelter from the elements (Brummer-Korvenkontio et al., 1980).

Prospect Hill virus occurs in the northeastern United States but has not been associated with human illness. The natural host for the virus is the meadow vole, Microtus pennsylvanicus. Antibody to this virus has been detected in mammalogists. (Lee et al., 1985)

Hantaviruses have also been isolated from the common house mouse, Mus musculus, (Leaky virus) in Texas (Baek et al., 1987), from a cat, Felis catus, in China (Zhao-zhaung et al., 1985) and, from the musk shrew, Suncus murinus, in China (Tang et al., 1985).

Other less well defined virus-rodent associations may also occur. Antibody reactive to Hantaan virus has been

found in Microtus californicus and Clethrionomys rutilus in Alaska, Peromyscus maniculatus in Minnesota (Yanagihara et al., 1984; Lee et al., 1982), and Peromyscus difficilis, P. californicus, Neotoma mexicana and N. cinerea in western and southwestern United States (Yanagihara et al., 1984).

Hantaviruses appear to be associated predominately with rodents in nature. Those so far studied are well adapted to their rodent hosts and do not appear to cause pathological alterations nor clinical disease. Hantavirus infections in rodents appear to be lifelong with persistent shedding of the virus in urine, saliva and feces (Lee et al., 1981a; Lee et al., 1986; Yanagihara et al., 1985a). Transmission between rodents is thought to occur primarily via inhalation of virus laden excretions and/or secretions. In the case of rats, transmission may also occur via bite wounds (Lee et al., 1981b; Dournon et al., 1984).

HUMAN DISEASE ASSOCIATED WITH HANTAVIRUS. Aside from the more classical KHF, EHF and NE forms of HFRS, Hantavirus related disease in humans may be somewhat ill-defined clinically. Previous investigations indicate that hantaviruses isolated from peridomestic rats may cause a mild flu-like illness with fever, myalgia, flank pain, headache, and proteinuria (WHO, 1983). They may also be associated with chronic kidney disease (LeDuc, unpublished communication).

HFRS should be considered in patients with an acute febrile illness, abdominal pain, transient oliguria, and accompanying indications of renal involvement such as proteinuria, and/or elevated creatinine levels not attributable to other causes. A serological diagnosis of HFRS can be made by demonstrating rising antibody titers between acute and convalescent serum samples or by the demonstration of IgM antibody in an acute serum sample (LeDuc et al., 1985).

Hantaan antibody in humans tends to occur primarily in adult males with occupational exposure to the natural rodent reservoirs. Among humans at increased risk are trappers, pest control personnel, and mammalogists; consequently, the disease is seen predominately in men between 20 and 50 years of age (Yanagihara et al., 1984). Serological surveys of humans have usually concentrated on those at increased risk (Yanagihara et al., 1985). Young children rarely develop HFRS even in endemic areas (Lahdevirta, 1971). Although human disease appears to be geographically limited, Hantavirus antibody has been found in humans outside traditionally defined endemic areas (Lee et al., 1981; Gibbs, 1982; Lee et al., 1981; Yanagihara et al., 1985).

The apparent absence of clinical disease attributable to several hantaviruses may be explained in several ways: 1) Some strains of Hantavirus may not be pathogenic for people. 2) People living in well developed countries probably have

minimal exposure to rodents and consequently have a low incidence of disease, 3) Clinical suspicion of this disease is probably very low to non-existent in non-endemic countries and so the disease may go unrecognized. 4) Patients with mild forms of the disease could easily recover from a hantavirus infection without the actual cause being determined, particularly since diagnostic tests are not readily available to most physicians.

Laboratory outbreaks of HFRS have been documented in the USSR, Korea, Japan, UK, and Belgium (Yanagahara and Gajdusek, 1988). Rats have been incriminated as the source of Hantavirus in laboratory outbreaks in Korea (Lee and Johnson, 1982), Japan (Umenai et al., 1979), Belgium (Desmyter et al., 1983), and the UK (Lloyd et al., 1984). Laboratory outbreaks in the USSR have incriminated Clethrionomys voles and Apodemus mice as the source of the Hantavirus (Casals, et al., 1966). Such outbreaks underscore the need for vigilance when working with experimentally or naturally infected wild or laboratory rodents.

The peridomestic rat-associated hantaviruses have been implicated in several outbreaks of human disease, occasionally with fatalities. Rattus rattus and R. norvegicus have been incriminated as the source of hantavirus infection in urban HFRS outbreaks in Korea and China (Lee et al., 1982). Preliminary studies in Hawaii by

Diwan et al. (1985) suggested that a Hantavirus is enzootic in all three species of rat present on the major islands of Hawaii. In 1982 8.1% (126/1552) of the rats trapped on the islands of Oahu, Maui, and Hawaii had antibody reactive with Hantaan virus. The highest prevalence of antibody, 17.7% (30/169), was in R. norvegicus the second highest prevalence, 7.2% (84/1159) was in R. rattus and the lowest prevalence, 5.9% (12/202) was in R. exulans.

Human seropositivity to Hantaan virus was demonstrated in Hawaii although there is no evidence of HFRS in Hawaii. Vector control personnel working as rat catchers for the Hawaii State Department of Health had an antibody prevalence to Hantaan virus of 30.5% (11/36) although none could recall symptomatology suggestive of HFRS. All efforts to isolate a Hantavirus from Hawaiian rats were unsuccessful at that time.

The widespread distribution of hantaviruses and their association to HFRS make the relationships of the less well studied hantaviruses to human disease a matter of important public health concern which deserves continued study. The primary purpose of the present study was to isolate the strain of Hantavirus present in the local rat population, to characterize and identify it, and to assess its potential for causing human infection.

MATERIALS AND METHODS

ANIMAL PROCEDURES. Capture and processing, tissue harvest, animal inoculation, and animal disposal.

Capture and Processing: Rats were trapped on the islands of Oahu, Kauai, Maui, and Hawaii using wire mesh "live traps". The traps were usually baited with fresh coconut, but other baits, such as shrimp, bread, oatmeal mixed with peanut butter, and bacon were also used.

Baited traps, distributed at dusk in locations determined to be of high rat activity, were checked each morning, and empty traps were sprung to prevent the capture of mongooses. Rats captured were transported to the Laboratory Animal Facility, University of Hawaii where they were anesthetized with carbon dioxide, weighed, sexed, bled by cardiac puncture and the sera collected and stored at -20° C until used for serological evaluation.

Laboratory rats were housed in and obtained from the Laboratory Animal Services, University of Hawaii. The animals were housed and maintained in four separate buildings on and off campus.

Cats were live trapped at Schofield Barracks, Fort Shafter and other military installations on Oahu by the pest

control division of the US Army and bled by personnel of the US Army Veterinary services.

Tissue Harvest: Lungs and spleens were harvested from freshly killed rats for virus isolation attempts. Carbon dioxide anesthetized rats were exsanguinated via cardiac puncture and then immediately dipped into a bath of betadine soap solution to wet down the hair and make organ harvest as aseptic as possible. Freshly killed rats were pinned to a dissection board in dorsal recumbency for organ harvest. A ventral midline incision was made from the mid-cervical region to the pubic symphysis and the body walls reflected laterally to expose the internal organs. Spleen and lungs were removed and placed in pre-labeled one dram glass vials. The vials were tightly capped and immersed in the gas phase of a liquid nitrogen refrigerator for quick freezing. Frozen tissues were stored at -65° C.

Animal Inoculation: Eight, seronegative, 5 week old, male, Sprague Dawley rats (Laboratory Animal Services, University of Hawaii) were caged as pairs in microfilter top cages and maintained under standard conditions at the University of Hawaii Laboratory Animal Services facility. One animal of each pair, identified by ear notching, was inoculated with the virus isolate for antibody production. The uninoculated

rat served as a control for contact transmission of the virus from the inoculated animal.

The inoculum was prepared by harvesting supernatant from infected Vero E-6 monolayers and centrifuging at 5000 rpm for 30 minutes at 4° C to remove cell debris. The supernatant was then stored in 1 ml aliquots at -65° C. Supernatant contained approximately 10^6 infectious units per ml. The inoculum was quick thawed in a 37° C water bath just prior to inoculation, and rats were injected bilaterally with 0.25 ml into the semitendinosus-semimembranosus muscle group.

Rats were anesthetized with carbon dioxide and bled by cardiac puncture at 4 and 6 weeks post inoculation. The blood was collected in red top serum tubes, allowed to clot, and sera collected and stored at -20° C.

Disposal: Rats were disposed according to the procedures of the Laboratory Animal Services, University of Hawaii. Remains were double bagged in empty paper sacks and stored at minus 20° C until disposal according to the established procedures of the University of Hawaii.

Antibody to Hantaan Virus in American Samoa: Human sera collected in American Samoa during 1985 for a Hepatitis study were supplied by Dr. Arwind Diwan and were tested by ELISA for antibody to Hantaan virus. The methods of village

selection and individual selection are unknown. Original serum collection numbered approximately 32,000, however, many of the Hepatitis B antigen positive sera were sent to CDC, Atlanta and thus were not available for testing. As it was impractical to test all samples, a random sequential sampling technique was employed in which every fifth serum was tested.

Sera were screened for antibody to Hantaan virus at a dilution of 1:32 and those with an O. D. > 0.100 by ELISA were retested in triplicate at 1:100 to determine seropositivity.

SEROLOGICAL TECHNIQUES. Indirect immunofluorescent antibody test (IFAT), high density particle agglutination assay (HDPA), and enzyme linked immunosorbant assay (ELISA).

Hantavirus Antisera: Antisera to Brazil, Seoul, Prospect Hill, Yugoslavia, Thailand, Leaky, Egypt, Puumala, Baltimore, and R22 (China) hantavirus strains were prepared in rats and were obtained from USAMRIID, Ft. Detrick, MD.

Indirect Immunofluorescent Antibody Test (IFAT): Indirect immunofluorescent antibody tests were performed using teflon coated spot slides of acetone fixed Vero E-6 cells infected with Hantaan virus (76-118, Salk Institute, Swiftwater, PA.)

to determine serological prevalence of infection with hantaviruses. Sera diluted in phosphate buffered saline at pH 7.4 (PBS) were screened at a dilution of 1/32 for antibody to Hantaan virus. Serum dilutions were incubated with the antigen for 30 minutes in a humidified chamber at 37° C, washed 3 times for 5 minutes each in PBS, air dried at room temperature, incubated for 30 minutes with fluorescein conjugated anti-rat IgG (heavy and light chain specific, Sigma, St. Louis, MO) in a humidified chamber at 37° C, washed, and air dried as above. The slides were then mounted with 10% glycerol in PBS, coverslipped, and examined for characteristic immunofluorescence using a Zeiss UV microscope with epiillumination and graded as follows:

- - : Absence of fluorescent cytoplasmic granules.
- + : Few weakly fluorescent cytoplasmic granules.
- ++ : Brightly fluorescent cytoplasmic granules fewer in number than positive controls.
- +++: Brightly fluorescent cytoplasmic granules as numerous as positive controls.

Positive and negative control sera were included in all tests.

High Density Particle Agglutination Assay (HDPA): The HDPA test, developed by Dr. Ho Wang Lee in collaboration with Dr. Tetsuo Tomiyama, University of Tokyo, Japan (Tomiyama and Lee, 1990), was provided courtesy of Dr. Ho Wang Lee at The

Institute for Viral Diseases, Korea University, Korea. The high density particle (HDP) used in this test is a composite particle having a silica core surrounded with a dyed layer covered with a second silica layer. The surface of the particle is covered with functional groups specifically designed for the adsorption of antigen or antibody. The particles have a density of 2.0 gm/ml and are 1.8 um in diameter.

The components supplied with this test are as follows:

1) Hantaan virus antigen sensitized HDP (lyophilized).
2) Control HDP, normal antigen sensitized (lyophilized).
3) Positive control sera (human anti-Hantaan sera).
4) Reconstituting buffer for HDP.
5) Serum diluent.
6) Microplate (A&T disposable plate with V-shaped wells.

Antigen sensitized and control particles, and positive control sera were reconstituted with 1 ml of reconstituting buffer 10-30 minutes prior to use. The titer of the positive control sera is reported to be 1:2560. Twenty-five microliters of the positive control serum diluted 1:80 and 25 microliters of test serum diluted 1:40 were placed into two wells each, 25 ul of control-HDP was put into one well of each serum and 25 ul of Hanta-HDP is put into the second

well of each serum, mixed thoroughly by tapping the sides of the plate, and incubated at room temperature for 40 minutes to overnight.

The agglutination patterns were interpreted as follows:

- : Particles concentrated in the shape of a button in the center of the well with smooth round outer margins.

+/-: Some of the particles concentrated in the shape of a button in the center of the well with the remaining particles agglutinated to uniformly cover the bottom of the well.

+ : Definitely large ring with filmy agglutination spread within the ring.

++ : Agglutinated particles spread out to uniformly cover the bottom of the well.

Enzyme Linked Immunosorbant Assay (ELISA): Human sera were tested by ELISA for antibody to Hantavirus antigen (Hantaan virus, strain 118-76) adsorbed to microtiter plate wells (Dynatech, Chantilly, VA). Antibody was detected using a horseradish peroxidase (HRPO) conjugated anti-human immunoglobulin (Accurate, Westbury, NY) to the captured hantavirus antibody and ABTS (Kirkegaard and Perry, Gaithersburg, MD) as the enzyme substrate. In the presence of the HRPO enzyme the ABTS is converted from a colorless substance to an intensely green substance with maximum light

absorption at 414 nm. Control wells adsorbed with noninfected cell extract were run in parallel. The OD at 414 nm in the test wells minus that in control wells was used to determine a "net" positive adjusted OD value.

Sera reacting at 1:32 were retested at 1:100, which is considered the minimum positive titer (Ksiazek, personal communication). Positive and negative control sera were included in all tests.

VIRUS ISOLATION. The method for isolation of hantavirus from rat tissues was adapted from LeDuc et al. 1984. Briefly, lungs and spleens from Hantaan virus antibody positive rats were aseptically removed, quick frozen in the gas phase of liquid nitrogen and held at -70° C until used for the virus isolation attempt. The tissues were quick thawed in a 37° C waterbath, macerated with a Stomacher blender (Temkar, Cincinnati, OH) in a 10% (weight/volume) suspension of Vero E-6 growth medium (Eagles minimum essential medium containing 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, 100 ug/ml streptomycin and 0.5% ug/ml fungizone), allowed to settle for 5-10 minutes to remove large tissue fragments, and the supernatant fluid inoculated onto nearly confluent monolayers of Vero E-6 cells in 25 cm^2 flasks at 0.5, 1.0, and 2.0 ml/flask. The inoculated cell cultures were

incubated in a humidified chamber at 37° C in 5% carbon dioxide.

At 14 day intervals, culture supernatant fluids were collected and stored at -70° C pending assay for infectious virus in Vero E-6 cells and the inoculated cell monolayers were either trypsinized or scraped to remove them from the flask. Cells were washed once in Vero E-6 growth medium and then passed onto fresh monolayers of 50-70% confluent Vero E-6 cells in 25 cm^2 flasks.

Cells from the inoculated flasks were also seeded onto 10 well spot slides and incubated overnight in a humidified chamber at 37° C in 5% carbon dioxide to allow the Vero E-6 cells to attach to the glass slides. The growth medium was removed from the spots with an aspiration needle, slides were air dried at room temperature with a blow drier and fixed in -70° C acetone for a minimum of 30 minutes. The presence of viral antigen within the cells was determined by IFAT using known positive Hantaan virus antisera and sera from locally caught seropositive animals. If no viral antigen was detected by day 60 post inoculation (four blind passages), virus isolation was considered to be unsuccessful and the culture was discarded.

VIRUS CHARACTERIZATION. Titration, size determination, deoxycholate sensitivity, buoyant density determination, electron microscopy, and western blot of viral antigens.

Titration: Virus stocks for titration were prepared by inoculating Vero E-6 cells with 100 ul of supernatant from previously infected Vero E-6 cultures, incubating in a humidified chamber at 37° C with 5% carbon dioxide and harvesting supernatant fluids on day 7 post inoculation. Supernatant fluids were centrifuged at 1000 rpm for 10 minutes to remove cellular debris, and 12 serial tenfold dilutions were made. One hundred microliters of each dilution was inoculated into 25 cm^2 flasks containing confluent monolayers of Vero E-6 cells and 6 ml of growth medium. Cell cultures were checked daily for a maximum of 14 days post inoculation and infection was determined by development of characteristic CPE and confirmed by IFAT using antisera from locally caught rats.

Size Determination: Supernatant fluid from an infected cell culture harvested on day 7 post inoculation containing 10^6 $TCID_{50}$/ml was sequentially passed through filters of 450, 220, and 100 nanometer porosities. After filtration, 100 ul of each filtrate was inoculated directly into 25 cm^2 plastic flasks containing confluent monolayers of Vero E-6 cells and 6 ml of growth medium. Cultures were incubated in a

humidified chamber at 37° C with 5% carbon dioxide for 14 days. Infection of cell cultures was determined by CPE and confirmed by IFAT using antisera from locally caught rats.

Deoxycholate Sensitivity Test: Virus was harvested from infected Vero E-6 cell monolayers on day nine post inoculation. Infected cells were dislodged from the flask by vigorous washing with the cell medium through a 14 gauge cannula on a 10 cc syringe. The resultant cell suspension was sonicated in a water bath type sonicator for 10 minutes to disrupt the cells and release the maximum amount of virus. The resulting suspension was then centrifuged for 10 minutes at approximately 575 x g to remove cell debris. The virus suspension containing 10^6 $TCID_{50}$/ml was diluted in phosphate buffered saline, pH 7.4 (PBS), containing 0.75% bovine albumin with or without 0.20% deoxycholate, at dilutions of 0 (reagent controls), 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Reagent controls and virus dilutions were incubated for one hour at 37° C and then 100 ul of the test suspension was inoculated into 25 cm^2 flasks containing confluent monolayers of Vero E-6 cells and 6 ml of growth medium. Uninoculated flasks of Vero E-6 cells were held as controls.

Flasks were incubated in a humidified chamber at 37° C with 5% carbon dioxide and held for up to 14 days. Virus growth was determined by CPE and confirmed by indirect IFA test using antisera from locally caught wild rats.

Buoyant Density Determination: Supernatant was harvested from 20 Vero E-6 flasks 5 to 7 days PI and processed by centrifuging at 5000 x g for 20 minutes to remove cell debris followed by ultracentrifugation at 100,000 x g for 180 minutes at 4° C to pellet the virus, and the pellet was resuspended in 1.0 ml of NTE buffer. Partially purified virus suspension from approximately 100 flasks were combined, ultracentrifuged at 100,000 x g for 180 minutes at 4° C, and the concentrated virus pellet resuspended in 1.0 ml of NTE buffer.

A sucrose gradient was prepared from a 60% stock solution of sucrose in NTE buffer. The stock solution was prepared by dissolving 77.2 gm of chemical grade sucrose (Sigma, St. Louis, MO) in NTE buffer to a total volume of 100 ml. Ten per-cent, 20%, 30%, 40%, and 50% solutions were made by diluting the 60% stock solution with appropriate volumes of NTE buffer. The solutions were sequentially layered in a 16 x 102 mm (18 ml) Beckman Ultra Clear ultracentrifuge tube (2.5 ml per layer with 60% solution on the bottom and 10% solution on the top) and allowed to diffuse overnight at 4° C. The virus preparation was layered over the sucrose density gradient, centrifuged at 100,000 g for 18 hours at 4° C in a Sorval ultracentrifuge and fractions of 50 drops each collected using a gravity fed 21 gauge vacutainer type needle inserted into the bottom of the centrifuge tube.

Two sucrose gradients were run in parallel, one was used to determine the density of each fraction and the other to determine which of the fractions contained infectious virus particles.

The refractive index of each fraction was determined on a refractometer (Bausch & Lomb ABBE-3L). The refractive index was used to determine the actual percent sucrose according to the Bausch & Lomb refractometer operator's manual and the specific gravity was determined using a table in Lange's Handbook of Chemistry, 1946 (pp 1317-1318). The specific gravities were confirmed by weighing 100 microliters of each fraction on a sensitive balance.

To locate infectious viral particles, 100 ul of each fraction was diluted with 900 ul of Vero E-6 growth medium and held at -70° C until inoculated onto confluent monolayers of Vero E-6 cells. One hundred microliters of each diluted fraction was inoculated into 25 cm^2 flasks containing confluent monolayers of Vero E-6 cells and 6 ml of maintenance medium. Inoculated flasks were held for 14 days post inoculation in a humidified chamber at 37° C with 5% carbon dioxide. The presence of infectious virus was determined by CPE and confirmed by indirect IFA test using antisera from locally caught seropositive rats.

Virus titers were determined by inoculating serial dilutions of the fractions containing onto confluent Vero monolayers in tubes and incubating for 14 days in a

humidified chamber at 37° C with 5% carbon dioxide. Tubes were examined daily for CPE and the presence of virus was confirmed by IFAT. Uninoculated controls were run in parallel for all assays.

The buoyant density for the isolate was determined from the specific gravity of the fractions containing the highest concentration of infectious virus particles.

Electron Microscopy: Confluent monolayers of Vero E-6 cells grown in 25 cm^2 flasks containing 6 ml of growth media were inoculated with 100 ul of supernatant from previously infected cell cultures and incubated for 5 to 7 days in a humidified chamber at 37° C with 5% carbon dioxide. The cells were harvested by repeated vigorous flushing of the monolayer with supernatant using a 14 gauge canula attached to a 10 cc syringe. The resulting cell suspension was centrifuged at 1500 rpm for ten minutes in a 15 ml conical centrifuge tube, supernatant fluid removed, and approximately 5 ml of 3% glutaraldehyde added to fix the cell pellet. After approximately two hours of fixation at 4° C the partially fixed cell pellet was freed from the tube and fixing continued overnight at 4° C. The fixative was replaced with Sorensen's phosphate buffer and the cell pellet stored at 4° C until further processing for transmission electron microscopy (TEM). The fixed cell pellet was dehydrated using ethanol and propylene oxide,

embedded in epoxy resin, thin sectioned using a glass knife, ultrathin sectioned using a diamond knife, and mounted on EM grids for viewing in a Zeiss electron microscope at Tripler Army Medical Center.

Western Blot Assay: Specificity of Ag-Ab reactions was determined by western blot assays. Western blots were performed using the MINI PROTEAN™ II DUAL SLAB CELL apparatus (Bio-Rad, Richmond, CA).

A 10% separating gel was made from a solution of 20.0 ml of acryl:bis in 12.0 ml of 1.88 molar TRIS at pH 8.8, 0.6 ml of 0.2 molar EDTA, 26.8 ml of glass distilled water, 0.03 ml of TEMED (Biorad, Richmond, CA), and 0.6 ml of 10% ammonium persulphate. The gel was cast in the apparatus using the following protocol: The glass plates were cleaned with detergent, wiped with isopropyl alcohol swabs, and dried with a lint free tissue. The glass separation strips were wiped clean with isopropyl alcohol swabs and the apparatus was assembled according to the instruction manual. The gel solution was prepared and degassed using negative air pressure prior to adding ammonium persulphate. Using a 25 ml pipette, the gel solution was placed between the plates in a steady stream in order to minimize bubble formation in the gel. The gel was overlaid with approximately 1/2 inch distilled water to allow a smooth surface to form and aid in loading the antigen. The gel was

allowed to polymerize for approximately one hour at room temperature.

The separating gel was overlaid with a 4% stacking gel made by mixing 2.5 ml acryl:bis in 1.88 ml 1.0 molar TRIS pH 6.8, 0.15 ml 0.2 molar EDTA, 10.15 ml glass distilled water, 0.0075 ml TEMED (Biorad, Richmond, CA), and 0.15 ml 10% ammonium persulphate. The stacking gel was degassed with negative pressure prior to adding the ammonium persulphate. The 1/4 to 1/2 inch stacking gel was overlaid with approximately 1/2 inch of glass distilled water to allow a smooth surface to form, and the gel allowed to polymerize at room temperature for 15-30 minutes.

Western blots were made of Hantaan virus (76-118), Vero E-6 cells from Fort Detrick, Manoa virus, and local Vero E-6 cells. Each antigen was diluted in a SDS-PAGE running buffer and heated at 100° C in a water bath for 5 minutes. Two hundred microliters of antigen in running buffer was added to the gel and run at 150 volts for 60 minutes to separate the proteins in the gel. The separated protein antigens were then trans-blotted to a nitrocellulose sheet for 15 minutes at 12 volts.

The nitrocellulose sheets containing the separated proteins were blocked overnight with PBS containing 5% powdered milk, washed in PBS, cut into 2 mm strips, dried, and stored at 4° C until used.

Antigen bearing strips were incubated with antisera diluted 1:50 in PBS containing 5% powdered milk and 5% normal goat serum at 4° C overnight. The strips were washed with PBS containing 0.1% Tween-20, incubated for 2 hours at room temperature with peroxidase labeled, affinity purified, goat antibody to rat IgG heavy and light chains (Kirkegaard & Perry, Gaithersberg, MD) diluted 1:1000 in PBS containing 5% powdered milk and 5% normal goat serum. The strips were washed with PBS containing 0.1% Tween-20, and developed for 5 minutes with DAB (3,3'-Diaminobenzidine tetrachloride/Nickel chloride) substrate for HRP (ZYMED, So. San Francisco, CA).

STATISTICAL ANALYSIS OF SEROLOGICAL DATA FROM FERAL RATS. Statistical evaluation of the feral rat serological data was performed using multiple logistical regression with weight a continuous variable, and sex and location of capture coded as 0,1 design variables. The calculations were performed with the University of Hawaii Medical School/School of Public Health VAX computer system using SAS statistical analysis program PROC CATMOD.

A regression coefficient obtained through multiple logistical regression is the logarithm of the odds ratio of a group for a particular outcome (seropositivity) as compared to a baseline group. The odds ratio is calculated

from the estimate of the analysis of individual parameters (odds ratio = e^{-b}, b = estimate of analysis of individual parameters for the effect in question) and the 95% confidence interval is calculated as $e^{-b +/- (1.96 \times s.e.)}$. The computer program PROC CATMOD inverts the classes for the dependent variable, so the regression coefficients must also be multiplied by -1 (SAS, 1988)

In the "maximum likelihood analysis" the likelihood is the probability of a sample expressed as a function of the parameters determined using fixed data (that data obtained in the course of this study) (Browmlee, 1965). The likelihood ratio test compares the likelihood of a set of data, assuming the null hypothesis is true, to the likelihood of the data after estimating the parameters. When the sample size is reasonably large, there is a convenient large sample approximation: $-2 \log_e (L_1/L_0)$ is equal to $-2 \log_e L_1 - (-2 \log_e L_0)$, which is approximately distributed as Chi-Square with the degrees of freedom equal to the number of parameters estimated (Brownlee, 1965).

RESULTS

SEROLOGICAL STUDIES.

Prevalence of Antibody to Hantaan Virus in Rats and Feral Cats: The IFAT was used to screen sera from 1272 feral rats, 420 laboratory rats, and 229 feral cats for antibody to Hantaan virus (76-118).

All sera were tested initially at a dilution of 1:32 and the degree of fluorescence rated as described in the methods section. Among feral rats tested; 71% were negative, 14% were +, 12% were ++, and 3% were +++. Among laboratory rats tested; 61% were negative, 19% were +, 14% were ++, and 6% were +++. Among feral cats tested 86% were negative, 9% were +, 4% were ++, and 1% were +++ (Table 3).

The large number of animals tested made it impractical to titer each serum sample, and only a sample or the sera were titrated. Because a + grading was considered the titration endpoint only sera graded ++ and +++ were titrated. Rat sera graded ++ at 1:32 had an average reciprocal titer of 129 with a standard deviation of 108 and a range of 32 to 512. Rat sera graded +++ at 1:32 had an average reciprocal titer of 330 with a standard deviation of 230 and a range of 64 to 1024. Cat sera graded ++ at 1:32 had an average reciprocal titer of 202 with a standard deviation of 73 and a range of 64 to 256 (Table 4).

TABLE 3

PREVALENCE OF ANTIBODY TO HANTAAN VIRUS IN HAWAIIAN RATS AND CATS by IFAT			
Test Results	Feral Rats	Lab Rats	Feral Cats
Negative	908/1272 (71%)	257/420 (61%)	196/227 (86%)
+	175/1272 (14%)	79/420 (19%)	20/227 (9%)
++	154/1272 (12%)	57/420 (14%)	10/227 (4%)
+++	35/1272 (3%)	27/420 (6%)	3/227 (1%)
Total Positive	364/1272 (29%)	163/420 (39%)	33/227 (14%)

TABLE 4

RECIPROCAL TITERS OF IFAT GRADED ++ AND +++ AT 1:32				
Grade	Average Reciprocal Titer	Standard Deviation	Range	N Value
++ (Rats)	129	108	32-512	113
++ (Cats)	202	73	32-256	13
+++ (Rats)	330	230	64-1024	42

The overall seropositivity rates were similar for the 3 species of rat tested (28-30%), although individual rates varied from island to island . Overall seropositivity rates in feral rats were 28% for R. rattus, 29% for R. norvegicus, and 30% for R. exulans. The individual island rates for all species ranged from: 21-38%. The antibody rates for individual species varied from 26% on Hawaii to 37% on Kauai and Oahu for R. rattus, from 17% on Oahu to 67% on Maui for R. norvegicus, and from 24% on Maui to 50% on Kauai for R. exulans (Table 5).

TABLE 5

FERAL RATS WITH ANTIBODY TO HANTAAN VIRUS BY IFAT

Location	R. rattus	R. norveg.	R. exulans	Total
E. Hawaii	75/338 * (22%)	14/76 (18%)	1/6 (17%)	90/420 (21%)
W. Hawaii	71/252 (28%)	11/49 (28%)	23/65 (35%)	105/366 (29%)
Hawaii (Unspec.)	53/167 (32%)	16/28 (57%)	7/28 (25%)	76/223 (34%)
Subtot.	199/757 (26%)	41/153 (27%)	31/99 (31%)	271/1009 (27%)
Kauai	11/30 (37%)	4/10 (40%)	1/2 (50%)	16/42 (38%)
Maui	35/107 (33%)	4/6 (67%)	8/34 (24%)	47/147 (32%)
Oahu	29/78 (37%)	1/6 (17%)	0/0 NA	30/84 (36%)
Total	274/972 (28%)	50/175 (29%)	40/135 (30%)	364/1282 (28%)

* Number with antibody @ 1:32/Number tested (Percent)

Species and sex had no significant effect on antibody prevalence. The effect of location was compared to the rate of seropositivity for west Hawaii (Table 6).

TABLE 6

ODDS RATIOS FOR EFFECTS ON SEROPOSITIVITY AMONG FERAL RATS WITH ANTIBODY TO HANTAAN VIRUS BY IFAT

Effect	Odds Ratio	95% Confidence Interval	Probability
Sex (Male)	1.13	0.88-1.45	0.3373
R. nor.	1.02	0.71-1.45	0.9273
R. exulans	1.07	0.72-1.59	0.7362
E. Hawaii	0.68	0.49-0.94	0.0191
Hawaii	1.29	0.90-1.84	0.1693
Maui	1.18	0.78-1.78	0.4274
Oahu	1.38	0.84-2.27	0.2062
Kauai	1.53	0.79-2.96	0.2087

Since only 1/5 locations (east Hawaii) appeared to have a significant effect ($P < .05$) on seropositivity, a likelihood ratio test of variation among species was performed to determine the likelihood that location did actually have an effect on seropositivity. The difference between the -2 $\log_e$ likelihood of location only and location plus species is 0.21 with 2 degrees of freedom ($P = 0.90$). This indicates that it is very unlikely that location actually had a significant effect on the prevalence of antibody to Hantaan virus.

Antibody to Hantaan virus appears to be widespread in the feral cat population on Oahu. Feral cats tested had an overall seropositivity rate of 14% by IFAT. Cats from 8 locations were tested although the majority came from only 3 locations (206/229) (Table 7).

Sentinel rats from the University of Hawaii Laboratory Animal Facility were tested for antibody reactive with Hantaan virus by IFAT, and 30/111 (27%) had antibody at 1:32 or greater (Table 8). Notably all of the seropositive rats came from Snyder Hall, one of the four facilities screened. seropositivity rates varied from 13% to 55% in different rooms in the Snyder Hall facility (Table 9). After measures were instituted to rid Snyder Hall of the presumptive Hantavirus (use of filter top cages, restricted movement of animal handlers, etc.) sentinel animals were again screened and 4/38 (11%) were seropositive; two from a single room at Snyder Hall and two from a single room at Leahi Hospital (a previously uninfected facility) (Table 8).

TABLE 7

FERAL CATS WITH ANTIBODY TO HANTAAN VIRUS BY IFAT	
Location	Seropositivity Rate
Aliamanu Mil. Res.	6/80 (7%) *
Fort De Russy	0/4 (0%)
Fort Kamehameha	1/1 (100%)
Fort Ruger	0/7 (0%)
Fort Shafter	6/19 (32%)
Schofield Barracks	18/107 (17%)
Tripler AMC	1/7 (14%)
Wheeler AFB	0/2 (0%)
Total	32/227 (14%)

* see footnote Table 5

TABLE 8

SENTINEL RATS WITH ANTIBODY TO HANTAAN VIRUS BY IFAT		
Building	Initial Survey	After Cleanup
Snyder Hall	30/75 (40%) *	2/20 (10%)
Biomedical Bldg.	0/18 (0%)	0/7 (0%)
Cancer Center	0/10 (10%)	0/7 (10%)
Leahi Hosp.	0/8 (0%)	2/4 (50%)
Total	30/111 (27%)	4/38 (11%)

* See footnote Table 5

TABLE 9

LABORATORY RATS (R. norvegicus) WITH ANTIBODY TO HANTAAN VIRUS (BY IFAT) AT SNYDER HALL	
Room Number	Seropositivity rate
506	9/28 (32%) *
515	4/30 (13%)
516	20/57 (35%)
517	83/151 (55%)
519	16/43 (37%)
Total	132/309 (43%)

*See footnote Table 5

Antigenic Relationships Among Hantaviruses: Hantavirus specific rat antisera cross reacted with rat associated hantaviruses and to a lesser degree with the non-rat associated hantaviruses by IFAT (Table 10a, 10b). Prospect Hill and Puumala viruses reacted slightly with each other but only poorly with other hantaviruses. There did not appear to be good correlation between the strength of 2-way reactions (Table 10a, 10b). On the basis of the IFAT, the known hantaviruses can be grouped into three groups: 1) the large Hantaan virus-rat virus group, 2) Puumala and Prospect Hill virus group, and 3) Leaky virus.

Hawaiian feral and laboratory rat sera that were seropositive to Hantaan virus by IFAT assay were tested for reactivity to other known hantaviruses by IFAT. The tests were done at a single serum dilution of 1:128 because of the

TABLE 10a

CROSS REACTIONS AMONG HANTAVIRUSES BY IFAT (RECIPROCAL TITERS)					
Rat Anti-Sera	Virus Antigen in Vero E-6 Cells				
	Hantaan	Seoul	Thailand	Balti-more	Brazil
Hantaan	8192	1024	1024	512	512
Seoul	4096	8192	4096	4096	4096
Thai.	2048	4096	8192	4096	4096
Balt.	4096	8192	2048	8192	2048
Brazil	2048	4096	4096	2048	8192
Egypt	1024	4096	4096	2048	4096
Yugo.	4096	1024	512	512	1024
Pr. H.	128	64	<32	<32	<32
Puumala	<32	<32	<32	<32	<32
Leaky	1024	256	256	256	256

TABLE 10b

CROSS REACTIONS AMONG HANTAVIRUSES BY IFAT (RECIPROCAL TITERS)					
Rat Anti-Sera	Virus Antigen in Vero E-6 Cells				
	Egypt	Yugo-slavia	Prospect Hill	Puumala	Leaky
Hantaan	1024	2048	256	512	2048
Seoul	4096	2048	1024	512	512
Thai.	4096	1024	256	512	512
Balt.	2048	1024	512	1024	2048
Brazil	2048	1024	512	1024	1024
Egypt	4096	512	128	256	512
Yugo.	1024	2048	512	1024	2048
Pr. H.	<32	64	2048	1024	128
Puumala	<32	<32	256	1024	<32
Leaky	256	128	256	1024	4096

shortage of Hantavirus spotslides. Although reaction patterns were somewhat variable, sera were strongly reactive with Brazil and Egypt strains, and most sera were also reactive with Prospect Hill and Thailand viruses. Sera were non-reactive or weakly reactive with Baltimore, Puumala, Seoul, Yugoslavia, and Leaky viruses (Table 11a and 11b).

TABLE 11a

REACTION OF HAWAIIAN RAT SERA HAVING ANTIBODY TO HANTAAN VIRUS AT 1:32 WITH DIFFERENT HANTAVIRUSES AT 1:128

Virus Antigen	Animal Number					
	8736	136R1	124R18	8739	14867	17216
Hantaan	+++	+++	-	++	++	+++
Seoul	-	-	-	+	+	++
Thailand	+	+	+	++	+	+
Baltimore	-	-	-	-	-	++
Brazil	+++	++	++	+++	+++	+++
Egypt	+	++	+	+++	+++	+++
Yugoslav.	-	-	-	-	-	-
Prospect	+	+	-	++	++	++
Puumala	-	-	-	-	-	+
Leaky	-	-	-	-	-	-

TABLE 11b

REACTION OF HAWAIIAN RAT SERA HAVING ANTIBODY TO HANTAAN VIRUS AT 1:32 WITH DIFFERENT HANTAVIRUSES AT 1:128						
Virus Antigen	Animal Numbers					
	15188	15194	218R15	218R15	232R31	23247
Hantaan	+++	+++	++	++	+	+
Seoul	++	-	-	+	+	-
Thailand	+++	++	+	+	+	++
Baltimore	++	+	-	-	-	-
Brazil	+++	+++	++	++	++	+++
Egypt	++	++	++	++	++	++
Yugoslav.	+	-	-	-	-	-
Prospect	+++	++	++	++	++	+++
Puumala	-	-	-	+	++	-
Leaky	-	+	+	+	+	+

High Density Particle Agglutination Assay (HDPA): The HDPA, using Hantaan virus (76-118) antigen, was used to evaluate specific rat antisera to several known hantaviruses. Hantaan virus antiserum and all of the rat associated hantavirus anti-sera (Seoul, Thailand, Baltimore, Brazil, Egypt, Yugoslavia, China, and Houston) reacted strongly. Antiserum to Leaky virus (isolated from a mouse) reacted weakly; and antisera to Prospect Hill and Puumala virus (both from voles) did not react (Table 12).

TABLE 12

REACTION OF SPECIFIC HANTAVIRUS ANTISERA TO HANTAAN VIRUS BY HDPA

Rat Anti-Sera	Agglutination Pattern			
	-	+/-	+	++
Hantaan				X
Seoul				X
Thailand				X
Baltimore				X
Brazil				X
Egypt				X
Yugoslav.				X
China				X
Houston				X
Leaky			X	
Prospect	X			
Puumala	X			
Total	2/12 * (17%)	0/12 (0%)	1/12 (8%)	10/12 (83%)

* Number positive/number tested (Percent)

Mouse ascitic fluid monoclonal antibodies to five different nucleocapsid proteins did not react (Table 13).

TABLE 13

REACTION OF MAF MONOCLONAL ANTIBODIES WITH HANTAAN VIRUS BY HDPA				
MAF Monoclonal Antibody	Agglutination Pattern			
	-	+/-	+	++
DC03-AB07 *	X			
K-HC02-BH11	X			
HP-AD01-BD06	X			
H-13-11E10-1-1	X			
K-JD04-AC06	X			

* Specificity determined by patterns of reactivity with different hantaviruses

None of 17 sera from locally obtained feral and laboratory rats that were seropositive to Hantaan virus by IFAT at 1:32 reacted strongly to Hantaan virus in the HDPA test; only 6/17 (35%) reacted weakly and 2/17 (12%) were questionable but considered negative (Table 14). Only one of 36 State of Hawaii vector control rat catcher personnel was reactive in the HDPA test compared to 13 that were seropositive by IFAT to Hantaan virus (Table 15).

TABLE 14

REACTION OF LOCAL RAT SERA POSITIVE TO HANTAAN VIRUS BY IFAT TO HANTAAN VIRUS BY HDPA					
Rat ID Number	Titer by IFA	Agglutination Pattern			
		-	+/-	+	++
Rattus rattus	512		X		
R. rattus	>1028	X			
R. norveg. (Lab)	512	X			
R. norveg. (Lab)	512	X			
R. norveg. (Lab)	512	X			
R. norveg. (Lab)	256		X		
R. norveg.(Lab)	512	X			
R. rattus	128	X			
R. rattus	512			X	
R. rattus	256			X	
R. rattus	512			X	
R. rattus	128			X	
R. norvegicus	512			X	
R. rattus	512			X	
R. rattus	512	X			
R. rattus	512	X			
R. rattus	512	X			
Total		9/17 * (53%)	2/17 (12%)	6/17 (35%)	0/17 (0%)

* See footnote Table 12

TABLE 15

REACTION OF HAWAII STATE RAT CATCHERS SERA TO HANTAAN VIRUS BY IFAT AND HDPA	
By Indirect IFA Test	13/36 (36%) *
By HDPA Test	1/36 (3%)
By Both IFA and HDPA Test	0/36 (0%)

* See footnote Table 12

Antibody to Hantaan Virus in American Samoa: None of the 4292 sera from human residents of American Samoa had detectable antibody to Hantaan Virus by ELISA.

VIRUS ISOLATION. Virus was isolated from lung and spleen tissues of 13/24 rats (1/3 feral Rattus rattus and 12/21 laboratory Rattus norvegicus) all of which were seropositive to Hantaan virus (76-118) by IFAT (Table 16). Virus was detected in Vero E-6 cells by cytopathic effect (CPE) and confirmed by IFAT using sera from local rat 232-89-R47 at 1:100 dilution. Rat 232-89-R47 had a titer of 1:512 to Hantaan virus (76-118) by IFAT.

Initially tissues were prepared for co-cultivation with Vero E-6 cell cultures by trituration with a scalpel blade in a petri dish. No virus was isolated in two attempts and this method was abandoned. Subsequent attempts used a hand tissue grinder to triturate the tissue and virus isolation was made in 4/9 attempts. The most efficient method, however, utilized a Stomacher blender (Temkar, Cincinnati, Ohio) and resulted in 9 presumptive virus isolates from 12 attempts.

CPE in Vero E-6 cells was characterized by increased definition of cell borders when viewed by light microscopy, decreased cell density, increased cell death, and detachment of the monolayer from the plastic flask, usually beginning at the periphery.

TABLE 16

VIRUS ISOLATION FROM RATS SEROPOSITIVE TO HANTAAN VIRUS BY IFAT			
RAT ID #	Reciprocal Titer	Species	Virus Isolated
228-89-R29	128	R. norvegicus	No
228-89-R21	256	R. norvegicus	No
228-89-R46	126	R. norvegicus	No
228-89-R48	512	R. norvegicus	Yes
228-89-R50	256	R. norvegicus	Yes
228-89-R110	128	R. norvegicus	No
117-89-R1	256	R. rattus	No
130-89-R1	64	R. rattus	No
138-89-R2	64	R. rattus	Yes
232-89-R3	128	R. norvegicus	Yes
232-89-R17	128	R. norvegicus	Yes
232-89-R21	128	R. norvegicus	Yes
232-89-R22	64	R. norvegicus	Yes
232-89-R27	128	R. norvegicus	Yes
232-89-R29	256	R. norvegicus	Yes
232-89-R31	512	R. norvegicus	No
232-89-R33	256	R. norvegicus	No
232-89-R39	64	R. norvegicus	No
232-89-R41	256	R. norvegicus	Yes
232-89-R43	64	R. norvegicus	Yes
232-89-R44	512	R. norvegicus	Yes
232-89-R47	512	R. norvegicus	Yes
232-89-R50	256	R. norvegicus	No

VIRUS CHARACTERIZATION. Two presumptive virus isolates were initially selected for characterization: One from a feral R. rattus (Isolate #8 from rat #138-89-R2) and the other from a laboratory R. norvegicus (Isolate #14 from rat #232-89-R44). Neither isolate reacted with standard reovirus antiserum by IFAT (Table 17). Antibody to Mycoplasma pneumonia did not react with either virus, and culture in Mycotin RS medium (Hana Biologics Inc., Alameda, CA) failed to detect any mycoplasma contamination.

Neither isolate reacted with any of the specific antisera from ten hantaviruses (Table 18). Both reacted in a similar manner to sera from locally caught rats and cats that were seropositive to Hantaan virus (Table 19). Since both isolates had similar reactivity patterns in these tests it was concluded that they were probably strains of the same virus and the isolate from R. norvegicus 232-89-R47, designated Manoa virus, was selected for further study.

TABLE 17

REACTIONS OF MANOA VIRUS ISOLATES TO REOVIRUS AND MYCOPLASMA ANTISERA BY IFAT		
Test Sera	Isolate #8	Isolate #14
Anti-Reovirus	Neg	Neg
Anti-Mycoplasma	Neg	Neg
232-89-R47 *	Pos	Pos

* Serum from a local rat positive to Hantaan virus

TABLE 18

REACTION OF MANOA VIRUS ISOLATES TO HANTAVIRUS ANTISERA BY IFAT		
Antisera	Isolate #8	Isolate #14
218-89-R15 *	Pos **	Pos
232-89-R47 *	Pos	Pos
Hantaan	Neg	Neg
Seoul	Neg	Neg
Thailand	Neg	Neg
Baltimore	Neg	Neg
Brazil	Neg	Neg
Egypt	Neg	Neg
Yugoslavia	Neg	Neg
Prospect Hill	Neg	Neg
Puumala	Neg	Neg
Leaky	Neg	Neg

* See note Table 17
** POS = Reaction of ++ to +++
NEG = No Fluorescence

TABLE 19

REACTION OF MANOA VIRUS TO SERA FROM LOCAL RATS AND CATS SEROPOSITIVE TO HANTAAN VIRUS BY IFAT AT 1:32

Sera #	Species	Iso. #8	Iso. #14	Hantaan
T-1121	R. rattus	+	++	+++
T-1265	R. rattus	++	++	+++
T-1301	R. rattus	++	++	+++
T-1340	R. rattus	++	++	+++
T-1382	R. rattus	-	-	+++
T-1417	R. norveg.	++	+++	+++
T-1553	R. rattus	+	+	+++
RIRL-62	R. rattus	+	+++	+++
T-474	R. rattus	+	-	+
T-549	R. rattus	+++	++	+++
T-840	R. rattus	+++	+++	+++
T-859	R. rattus	+++	+++	+++
T-904	R. rattus	-	-	+++
T-933	R. exulans	+	-	+++
228-89-R48	R. nor. (lab)	+	++	+++
228-89-R50	R. nor. (lab)	+++	+++	++
232-89-R31	R. nor. (lab)	+	+	+++
232-89-R44	R. nor. (lab)	++	+++	+++
232-89-R47	R. nor. (lab)	+++	+++	+++
15188	R. rattus	-	-	+++
17332	R. rattus	+++	+	++
188-89-C3	Felis catus	+++	+++	+++
195-89-C6	F. catus	+	+	+++
164-89-C1	F. catus	++	++	+++
170-89-C1	F. catus	+	+	++

Virus titer: A stock of the ninth Vero E-6 passage of Manoa virus contained 10^6 TCID/ml (Table 20).

TABLE 20

TITRATION OF MANOA VIRUS IN GROWTH MEDIA FROM INFECTED VERO E-6 MONOLAYERS IN 25 cm^2 FLASKS				
Sample	Day 3	Day 5	Day 7	Day 10
Control	Neg	Neg	Neg	Neg
10^{-2}	Pos *	Pos	Pos	Pos
10^{-3}	Pos	Pos	Pos	Pos
10^{-4}	Pos	Pos	Pos	Pos
10^{-5}	Pos	Pos	Pos	Pos
10^{-6}	Neg	Neg	Pos	Pos
10^{-7}	Neg	Neg	Neg	Neg
10^{-8}	Neg	Neg	Neg	Neg
10^{-9}	Neg	Neg	Neg	Neg
10^{-10}	Neg	Neg	Neg	Neg
10^{-11}	Neg	Neg	Neg	Neg
10^{-12}	Neg	Neg	Neg	Neg

* Presence of virus determined by characteristic CPE and the presence of viral antigen detected by IFAT.

Size: The size of Manoa virus was determined by sequentially passing supernatant media from Manoa virus infected cultures (passage 9) through filters of decreasing porosities and inoculating confluent monolayers of Vero E-6 cells with the filtrates. Infection of monolayers was determined by CPE and indirect IFA test. The infectious agent was able to pass through 450 nm and 220 nm filters but was retained by the 100 nm filter (Table 21).

Deoxycholate Sensitivity: Infectivity of Manoa virus (passage 18) in Vero E-6 monolayers was eliminated by pre-incubation with deoxycholate. Equivalent fractions of the virus preparation incubated with deoxycholate-free control reagents retained infectivity at dilutions up to 10^{-4} (Table 22). This is interpreted to mean that Manoa virus has an envelope containing essential lipids.

Buoyant Density: Rate zonal sedimentation of Manoa virus in 10 to 60% sucrose gradients produced two distinct opaque bands and an indistinct area of haziness surrounding the lower band (Figure 1). Most of the virus was in the fractions containing between 41% and 46% sucrose suggesting that the buoyant density of Manoa virus is between 1.18 and 1.20 gm/ml (Table 23).

TABLE 21

DIFFERENTIAL FILTRATION OF MANOA VIRUS	
Filter Size	Virus growth as determined by CPE and IFA
Not Filtered	Yes
450 Nanometers	Yes
220 Nanometers	Yes
100 Nanometers	No
Uninoculated Control	No

TABLE 22

DEOXYCHOLATE SENSITIVITY OF MANOA VIRUS		
Dilution of Inoculum	Infective Virus when treated WITH Deoxycholate	Infective Virus when Treated WITHOUT Deoxycholate
10^{-2}	No	Yes
10^{-3}	No	Yes
10^{-4}	No	Yes
10^{-5}	No	No
Control	No	No

Control: Virus negative inoculum

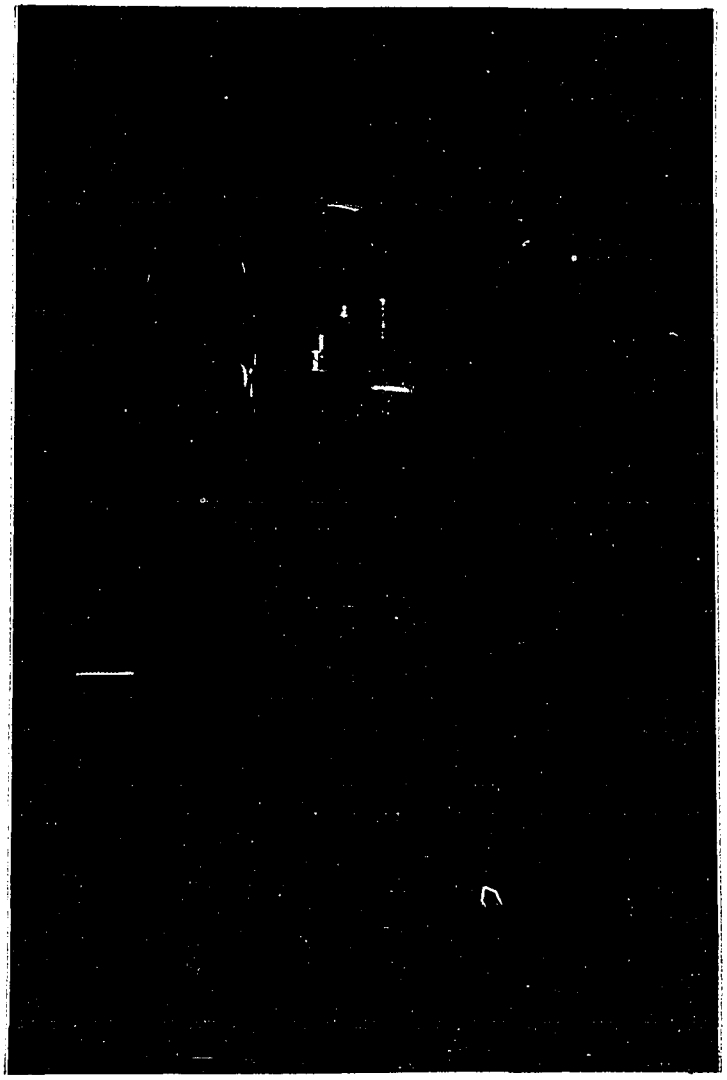

FIGURE 1. Rate zonal sedimentation of Manoa virus preparation in 10-60% sucrose gradient demonstrating two distinct opaque bands and an indistinct area of haziness surrounding the lower band.

TABLE 23

BUOYANT DENSITY DETERMINATION OF MANOA VIRUS USING RATE ZONAL SEDIMENTATION IN A 10-60% SUCROSE GRADIENT				
Fraction	Refractive Index	Per-Cent Sucrose	Density (gm/ml)	$TCID_{50}$ per ml
1	1.4354	57%	1.27	No Virus
2	1.4345	57%	1.27	No Virus
3	1.4332	56%	1.26	No Virus
4	1.4294	54.5%	1.25	No Virus
5	1.4206	50%	1.23	No Virus
6	1.4184	49%	1.22	No Virus
7	1.4161	48%	1.22	No Virus
8	1.4134	47%	1.21	10^{-1}
9	1.4105	46%	1.21	10^{-3}
10	1.4075	44%	1.20	10^{-3}
11	1.4042	42%	1.19	10^{-4}
12	1.4012	41%	1.18	10^{-4}
13	1.3986	39%	1.17	10^{-1}
14	1.3952	37.5%	1.16	10^{-1}
15	1.3908	35%	1.15	10^{-1}
16	1.3871	33%	1.14	No Virus
17	1.3836	31.5%	1.13	No Virus
18	1.3801	29.5%	1.12	No Virus
19	1.3764	27.5%	1.12	No Virus
20	1.3726	25%	1.10	No Virus
21	1.3689	23%	1.09	No Virus
22	1.3652	21%	1.09	No Virus
23	1.3609	18%	1.07	No Virus
24	1.3568	16%	1.06	No Virus
25	1.3532	13.5%	1.05	No Virus
26	1.3495	11%	1.04	No Virus

TABLE 23 (continued)

BUOYANT DENSITY DETERMINATION OF MANOA VIRUS USING RATE ZONAL SEDIMENTATION IN A 10-60% SUCROSE GRADIENT				
Fraction	Refractive Index	Percent Sucrose	Density (gm/ml)	$TCID_{50}$ per ml
27	1.3455	8.5%	1.03	No Virus
28	1.3425	6.5%	1.02	NO Virus
29	1.3397	4%	1.01	No Virus
30	1.3383	4%	1.01	No Virus
31	1.3394	4%	1.01	No Virus

Ultrastructure of Manoa Virus Infected Cells: Transmission electron microscopy (TEM) of Manoa virus infected Vero E-6 cells revealed intracytoplasmic granulofilamentous and granular inclusions. The granolofilamentous inclusions were the most frequently observed type of inclusion. They were variable in size (250-1500 nm) and number per cell (1-15), round to oval in shape, with a nonencapsulate regular border (Figure 2). They consisted of electron dense granules which were often linearly arranged and sometimes appeared as filaments.

The purely granular inclusions were fewer in number (0-3/cell) were smaller in size (100-500 nm), had less well defined borders, were somewhat irregular in shape, and consisted of electron dense granules that were more loosely arranged than granules in the granulofilamentous type inclusions (Figure 3).

Presumed viral particles were noted within the cytoplasm of some infected cells. They were round, approximately 55 nm in diameter and consisted of an electron dense core separated from an electron dense nucleocapsid by an electron lucent ring. They appeared to be in close association with some of the granular type inclusions (Figure 3), and probably represent non-enveloped incomplete virions.

FIGURE 2. Electron micrograph of Manoa virus infected Vero E-6 cells demonstrating numerous electron dense inclusions. X11,500.

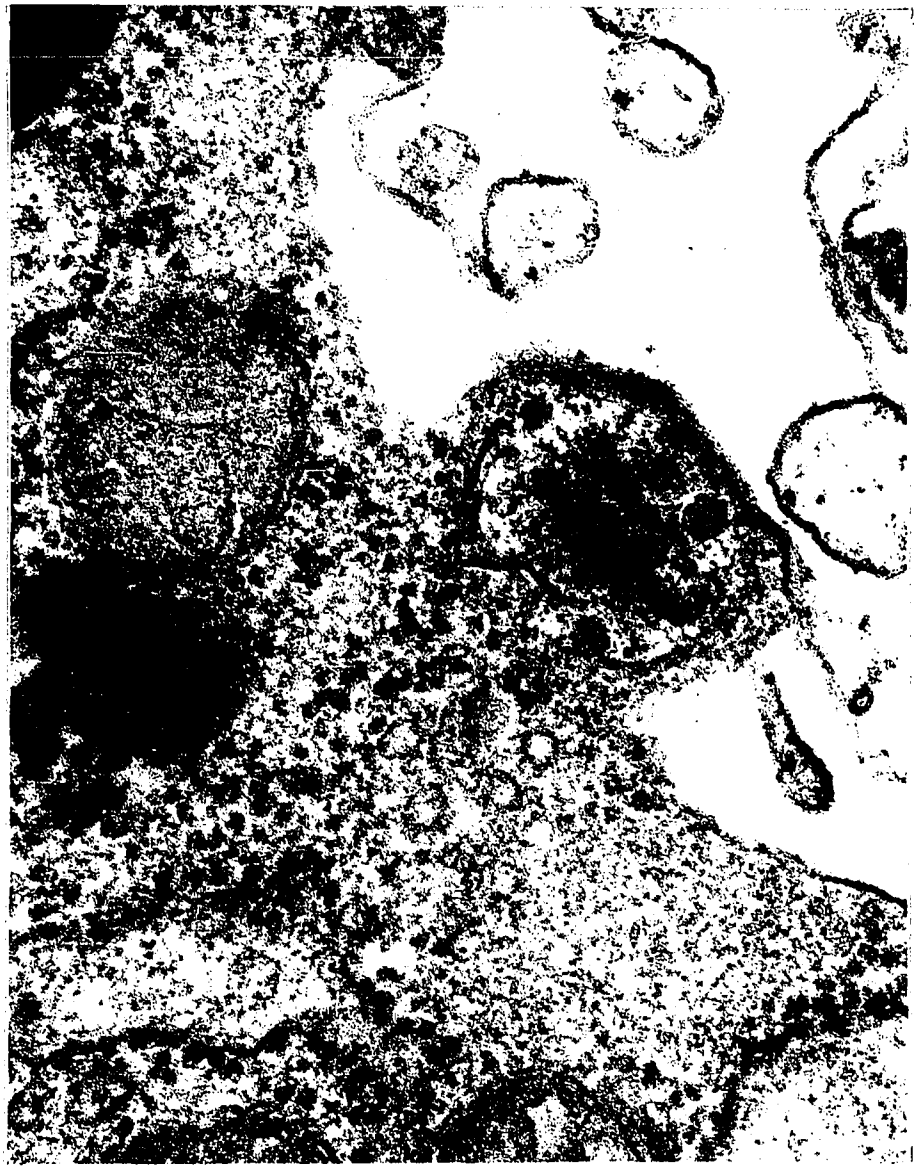

FIGURE 3. Electron micrograph of the cytoplasm of a Manoa virus infected Vero E-6 cells containing a granular type inclusion in association with several viral particles. X117,500.

Western Blot Analysis of Major Viral Antigens: The major antigens of Manoa virus and Hantaan virus (76-118) were revealed by western blot analysis of infected Vero E-6 cell preparations. Non-inoculated Vero E-6 cells were used as controls. Antisera to Manoa virus and several hantavirus strains as well as sera from two locally caught rats reactive to Hantaan virus by IFAT and serum from one "normal" rat without Hantaan virus antibody (obtained from USAMRIID, Ft. Detrick, MD) were tested on the antigen blots.

Antiserum to Hantaan virus strongly reacted with 50 K, 100 K, and 150 K proteins of homologous virus (Fig. 4, lane 10). Weaker reactions with 33 K, 35 K, and 40 K proteins were also observed. Brazil, Seoul, Yugoslavia, Thailand, Egypt, Baltimore, R22 (China), and Leaky virus anti-sera also were strongly reactive to the 3 major Hantaan virus proteins. Brazil, Seoul, Yugoslavia, Thailand, Egypt, and R22 (China) virus antisera were also weakly reactive to 33, 35, and 40 K proteins. Prospect Hill, Puumala and Manoa virus anti-sera were non-reactive to the Hantaan virus proteins. Sera from the "normal" rat reacted weakly with the 50 K protein.

Sera from two locally caught rats reactive to Hantaan virus by IFAT reacted weakly to an 84 K Hantaan virus protein, but not to any of the three major or three minor proteins. One of the two also reacted to a 42 K protein and

the other reacted very weakly with a 63 K protein not detected with homologous antisera.

Manoa virus antisera reacted strongly to a homologous 50 K and a 200 K protein (Fig. 5, lane 10). Brazil, Prospect Hill, Yugoslavia, Leaky, Thailand, Egypt, Baltimore, and Hantaan virus antisera also reacted with the 50 K protein of Manoa virus antigen and Puumala virus anti-sera reacted weakly with the 50 K protein. R22 (China) virus anti-sera reacted with the 60 K, 70 K, and 77 K proteins but only very weakly to the 50 K protein. Seoul virus antiserum was non-reactive with Manoa virus antigen.

Sera from the two locally caught rats reactive to Hantaan virus by IFAT were strongly reactive to the 50 K protein of Manoa virus antigen. Serum from one of the rats also reacted very strongly with 40 K and 200 K proteins. Serum from the other rat reacted strongly with an additional 4 proteins and moderately with an additional 5 proteins between the 46 K and 200 K markers (Fig. 5, lane16).

The "normal" rat serum was non-reactive to the Hantaan virus preparation but reacted weakly with a 44 K protein in the Manoa virus preparation.

FIGURE 4. Western Blot of Hantaan virus (76-118) reacted with several Hantavirus specific antisera, sera from local rats seropositive to Hantaan virus and Manoa virus antisera: 1) Brazil antisera, 2) Seoul antisera, 3) Prospect Hill antisera, 4) Yugoslavia antisera, 5) Thailand antisera, 6) Leaky antisera, 7) Egypt antisera, 8) Puumala antisera, 9) Baltimore antisera, 10) Hantaan antisera, 11) "Normal" rat sera, 12) China antisera, 13) Local rat #232-89-R47 sera, 14) local rat #228-89-R50 sera, 15) Manoa antisera (#3).

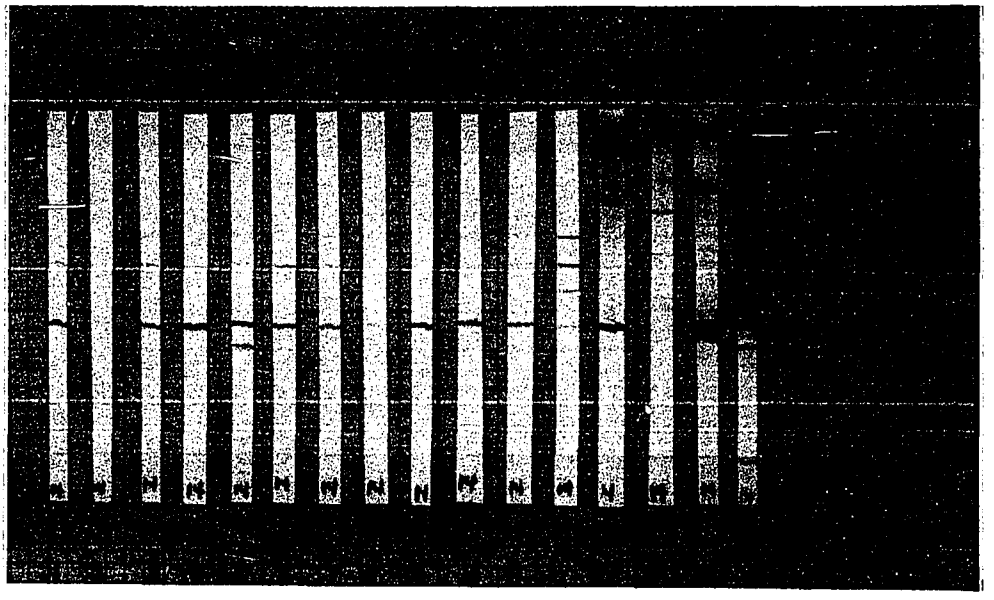

FIGURE 5. Western Blot of Manoa virus reacted with several Hantavirus specific antisera, sera from local rats seropositive to Hantaan virus and Manoa virus antisera: 1) Brazil antisera, 2) Seoul antisera, 3) Prospect Hill antisera, 4) Yugoslavia antisera, 5) Leaky antisera, 6) Thailand antisera, 7) Egypt antisera, 8) Puumala antisera, 9) Baltimore antisera, 10) Hantaan antisera, 11) "Normal" rat sera, 12) China antisera, 13) Manoa antisera (#2), 14) Manoa antisera (#3), 15) Local rat #228-89-R50 sera, 16) Local rat #232-89-47 sera.

DISCUSSION

The family Bunyaviridae consists almost entirely of viruses transmitted to vertebrates via arthropods, the major exception being the hantaviruses which are transmitted directly from vertebrate to vertebrate. There are at least 227 recognized bunyaviruses, the vast majority of which were isolated during exploratory surveys of arthropods, birds, reptiles, amphibians, and mammals in Latin America, Southeast Asia, and Africa (Bishop and Shope, 1979). Newly recognized bunyaviruses are regularly added to this list as the result of active exploration for new viruses from people, wild and domestic animals, and arthropods. Bunyaviruses have generally been isolated in suckling mouse brains, Vero cells, or insect cell cultures.

The bunyaviruses are divided into 4 genera: Bunyavirus, Hantavirus, Nairovirus, and Phlebovirus. However, not all bunyaviruses fit into these established genera and many remain unclassified.

Although rodents have been linked to HFRS for more than 50 years (Yanagahara and Gajdusek, 1988), the causative agent was not actually isolated until 1976 when Lee and Lee isolated Hantaan virus from the lungs of the Korean striped field mouse (Apodemus agrarius corea) in Korea (Lee and Lee, 1976). This breakthrough soon made serological diagnosis of

infection possible and led to the discovery that hantaviruses are not only widespread within several different rodent genera but also that the geographic distribution of the hantaviruses is worldwide, extending far beyond the endemic regions of HFRS.

Rodents belonging to the superfamily Muroidea and the families of Muridae (genera: Apodemus, Rattus, Mus) and Arvicolidae (genera: Clethrionomys and Microtus) serve as the principal reservoirs for the hantaviruses (Yanagihara, 1990). The rat-associated hantaviruses (Seoul virus subgroup) have a worldwide distribution, and in the United States numerous strains have been isolated from R. norvegicus captured in New Orleans, Houston, Philadelphia, and Baltimore (LeDuc et al., 1984). The apparently non-pathogenic (for humans) Rattus-derived hantaviruses isolated from within the United States are nearly indistinguishable from those causing human disease in Korea and Japan (Dantas et al., 1987; Schmaljohn et al., 1985; Lee et al., 1985; Sugiyama et al., 1984). Rats with antibody to Hantaan virus have also been trapped in New York City, San Francisco, Columbus, and the state of Hawaii although a virus has yet to be isolated from these areas (Tsai et al., 1985; Diwan et al., 1985; Yanagihara et al., 1985). In Maryland antibodies to Hantaan virus have also been detected in cats (Felis catus) (Childs et al., 1988).

This study has demonstrated a widespread prevalence of antibody to Hantaan virus by IFAT among peridomestic rats (R. rattus, R. norvegicus, and R. exulans), laboratory rats (R. norvegicus), and feral cats (F. catus) in Hawaii. Hantaan virus was the standard antigen used for detection of Hantavirus antibody. The Hantavirus genus prototype, Hantaan virus, probably does not exist in Hawaii as it is highly virulent and there is no evidence of HFRS on these islands. The serological reactions to Hantaan virus demonstrated in humans and other animals from Hawaii are presumed to represent antibody to a Hawaiian agent that is antigenically related to Hantaan virus.

The overall prevalence of antibody in peridomestic rats was similar for all three species tested (28-30%) and higher than those reported by Diwan et al., 1985 (6-18%). The increased prevalence may be due to the grading system used to determine seropositivity in this study. As detailed in the methods section, sera were screened at 1:32 and weak fluorescence (+) considered positive in this study is generally not considered to be a positive reaction by other workers (LeDuc, personal communication). However, since weak fluorescence (+) was seen only in groups of animals which also contained animals with strong fluorescence (++, and/or +++), we have accepted weak fluorescence (+) as indicating low antibody titer but still a positive reaction. Other possible explanations for the difference in antibody

prevalence noted may simply be due to sampling differences or to an actual increase in prevalence of infection among rats since 1982 when the original study was performed.

Infection with hantaviruses tends to be localized since transmission between vertebrates is by direct contact via their secretions and/or excretions. The percentage of seropositive animals, by species and location, was more variable than the overall seropositivity rate and varied between 17% and 67%, thus suggesting the virus is focally distributed throughout the islands of the Hawaiian chain (Table 5).

Statistical analysis of local feral rats with antibody to Hantaan virus indicated that species, sex, and location of capture had no significant effect on seropositivity. Using multiple logistical regression to analyze the data did suggest that rats from east Hawaii mat be at lower risk than those from west Hawaii ($P = 0.019$); however, by using the method of maximum likelihood analysis it was shown that location was very unlikely to have an effect on seropositivity ($P = 0.90$). Since no attempt was made in this study to sample all habitats or to obtain a completely representative or random sample the statistical analysis may not be valid. However, these data do establish that all three species of rat from all islands tested do have antibody to Hantaan virus by IFAT.

Feral cats with Hantaan virus antibody appear to be widespread on Oahu with an overall prevalence rate of 14%. All of the cats tested came from one of several military reservations on Oahu, most (107) from one facility. Seropositive feral cats were found only on residential facilities. Absence of seropositive cats on the two non-residential facilities (Ft. De Russy and Ft. Ruger) may suggest some association between residential neighborhoods and seropositivity to Hantaan virus; however, the number of animals collected from these two facilities was too small to be significant (Table 7).

In light of the high seropositivity rates of feral rats in Hawaii and the numerous reports of Hantavirus outbreaks in laboratory facilities (Kitamura et al., 1983; Dournon et al., 1984) it was decided to examine rats from the laboratory animal facility at the University of Hawaii. The initial examination was of stored sera collected from sentinel rats used in the four facilities managed by the laboratory animal service (Table 7). Although all of the animals had originally come from a common commercial source, only one facility (Snyder Hall) had sentinel rats with antibody to Hantaan virus by IFAT.

The overall prevalence of antibody in sentinel rats from Snyder Hall was 40%. Seropositive sentinel rats were present in all rooms tested with seropositivity rates in individual rooms varying from 13% to 55% (Table 8). The

overall seropositivity rate in the rat rooms in Snyder Hall was 43%. These results suggest a local source of infection as seropositive animals were not found in the other three buildings. Peridomestic rats appear to be the most likely source of infection for the laboratory rats.

Snyder Hall is a relatively open facility and feral rats have been found within the area where laboratory rats are housed. Laboratory rats were housed in open top cages kept in close proximity to one another on open racks and thus feral rats within the facility would have access to the tops of these cages, sharing food and contaminating the cages with their secretions and excretions. Behavioral studies utilizing wild caught feral rats that share a common test area with the laboratory rats were also performed in Snyder Hall and may have provided an opportunity for cross contamination between feral and laboratory rats. Once a hanta-like virus became established within the colony the close proximity of the open top cages would easily allow for aerosol transmission of the agent and rapid spread within the colony.

Based upon serological evidence it was assumed that there was a Hantavirus in the rat colony and that it was being transmitted via the aerosol route as are the other hantaviruses. In an attempt to rid the facility of this agent, several measures were taken: 1) all rats in infected rooms were replaced with non-infected rats; 2) rat breeding,

which had previously taken place in all rooms, was limited to a single non-infected room; 3) animal handling personnel were given additional training to inform them of the nature of the problem and instructed in preventative measures to reduce their risk of exposure and the risk of further spreading the infection throughout the laboratory animal colony; 4) the movement of people in and out of rat rooms was limited to minimize personnel contact between rooms; 5) and the use of filter top cages was instituted in the feral rat rooms to prevent the spread of aerosolized virus among cages. Efforts to eliminate the presumptive Hantavirus from the colony were largely successful (Table 8), supporting the suspicion that the agent in question was infectious in nature. The Leahi hospital infection, noted for the first time after these measures had been instituted to eliminate the virus from Snyder Hall, was suspected to have been an extension of the Snyder Hall infection as rats had been moved from Snyder Hall to Leahi Hospital, at the request of an investigator.

In an attempt to more fully characterize the Hantavirus antibody in Hawaiian rats a series of serological studies were performed. The first of these studies evaluated the levels of cross reactivity between hantaviruses and their specific antisera by IFAT and was performed to establish a baseline for relationships among hantaviruses (Table 10a and 10b).

Serological reactions between Hantavirus antisera and several known hantaviruses demonstrated variable reactivity and many one-way reactions. Hantaan virus antiserum reacted with all of the hantaviruses tested, as would be expected, since Hantaan virus antiserum is used for the initial detection and identification of hantavirus isolates. In heterologous reactions, it reacted most strongly with those rat-associated hantaviruses known to be pathogenic for man suggesting a closer relationship with the pathogenic hantaviruses than with those that are non-pathogenic.

The rat-associated hantaviruses were closely related by IFAT with heterologous reactions generally within one or two two-fold dilutions of homologous reactions. Such a pattern of reactivity suggests that these hantaviruses are more closely related to one another than to any of the non-rat hantaviruses. These antisera also reacted well with Hantaan virus indicating that they are also closely related to the prototypic virus.

The vole-associated hantaviruses had reaction patterns quite different from the other hantaviruses and numerous one-way reactions were noted. Although all of the specific antisera tested reacted with Prospect Hill and Puumala virus Prospect Hill antisera reacted with only five of the other hantaviruses (it was non-reactive to four rat-associated hantaviruses), and Puumala specific antisera was reactive only with itself and Prospect Hill virus. Prospect Hill

antisera reacted only weakly with the non-vole hantaviruses and not at all with Baltimore virus suggesting it is quite different from the rat-associated hantavirus which exists in close geographical proximity. Puumala antisera was non-reactive to all non-vole hantaviruses and only moderately reactive to Prospect Hill virus thus demonstrating a distant relationship to the other hantaviruses by IFAT.

A similar pattern of reactivity was noted by the HDPA utilizing Hantaan virus as the antigen. All of the rat-associated Hantavirus antisera agglutinated the particles suggesting their relatedness to Hantaan virus. The two-vole associated hantavirus antisera did not agglutinate particles suggesting that they are more distantly related to Hantaan virus than are the rat-associated hantaviruses. The agglutination pattern of Leaky virus antisera was intermediate between that of the rat-associated and the vole-associated virus, suggesting that it is not as closely related to Hantaan virus as the rat-associated hantaviruses but more closely related than the vole-associated hantaviruses. The patterns of reactivity in this study suggest grouping of the hantaviruses to be much the same as that established by other workers using different methods (Table 2). Hantaan virus stands on its own although it appears to be more closely related to the rat-associated hantaviruses than to the other hantaviruses. The rat-associated hantaviruses all group together well although the

pathogenic strains seem to be more closely related to Hantaan virus than the non-pathogenic strains. The vole-associated viruses are more closely related to each other than to the other hantaviruses and Puumala virus appears to be the most distantly related of the two. Leaky virus appears to be more closely related to Hantaan virus than to the other hantaviruses and remains non-grouped.

Sera from local rats with antibody to Hantaan virus by IFAT had a unique pattern of reactivity to the other hantaviruses. It reacted most strongly to the Brazil and Egypt hantaviruses, moderately with Prospect Hill virus, and poorly with the other hantaviruses (Table 11). Such a pattern suggests that the Hawaiian agent is more closely related to the New World and northern African strains than to those from Eurasia. This may also have other implications and suggests that the rats in Hawaii may not all have come from the west. The lack of reactivity with other rat-associated hantaviruses (Seoul, Baltimore, and Yugoslavia) indicate that the Hawaiian agent does not fit neatly into the Seoul grouping with the other known rat-associated hantaviruses and may be unique in this respect.

In an attempt to further clarify this unique reactive pattern Hantavirus specific antisera and local sera seropositive to Hantaan virus were tested by a newly developed HDPA (Table 14). Using local rat sera from animals with antibody to Hantaan virus by IFAT it was noted

that about 35% reacted at about the same level as Leaky virus but that the rest were non-reactive. This suggests that the Hawaiian virus may fit somewhere between Leaky virus and the vole-associated hantaviruses as to relatedness to Hantaan virus.

The IFAT appears to be more broadly cross-reactive than the HDPA assay, and thus more sensitive in detecting antibody to any of the Hantavirus group. Only one of 13 Hawaii rat catchers seropositive to Hantaan virus by IFAT was also positive by HDPA.

The isolation of a presumptive Hantavirus did not turn out to be as difficult as had been anticipated. Triturating of tissues with a scalpel blade in a petri dish was generally unsatisfactory. Triturating with either a hand tissue grinder or a "Stomacher blender" proved very satisfactory and using these method we attained an isolation rate of over 50%. Trituration of the tissues in a "Stomacher blender" was the most effective method for virus isolation, it is faster than the hand tissue grinder and less likely to introduce contamination into the preparation.

Hantavirus infection in Vero E-6 cells is classically determined indirectly by IFAT since the known hantaviruses do not produce CPE in Vero E-6 cells. Spot slides are made from inoculated Vero E-6 cell cultures at weekly intervals and the IFAT performed using rat antisera to Hantaan virus to detect the presence of hantavirus antigen within the

cytoplasm of the cells. Several investigators have attempted to isolate the Hawaiian virus using this method with negative results (Diwan, personal communication). In the present study, in addition to the Hantaan virus antisera, we also used serum from a local rat seropositive to Hantaan virus by IFAT to test for virus isolation.

Vero E-6 cells infected with Manoa virus developed specific intracytoplasmic fluorescence detectable with the antibody positive local rat serum but not with Hantaan virus antiserum. If we had relied only on Hantaan antiserum to detect virus growth it is unlikely that we would have detected Manoa virus in the cell cultures.

After passing Manoa virus approximately six times at two week intervals a mild form of CPE developed over a period of six to 12 days PI. CPE is not characteristic of the known hantaviruses which are purported not to cause CPE in Vero E-6 cell cultures. The CPE noted in Vero E-6 cell cultures suggests that Manoa virus is not typical of the other hantaviruses and may not fit well into the Hantavirus genus although this is not a definitive test.

Since all of isolates tested appeared to be the same based on immunofluorescent patterns and CPE, a single isolate from rat # 232-89-R44 was selected for detailed analysis and tentatively given the name "Manoa virus". The possibility that the isolates were reovirus, commonly found in rodents, was ruled out by light microscopy, demonstrating

the absence of characteristic intracytoplasmic inclusions in infected Vero E-6 cells and by lack of reactivity with reovirus specific antisera by IFAT.

The possibility that the isolates were mycoplasma were also ruled out. Ultrastructural evaluation of Manoa virus infected Vero E-6 cells using TEM showed the isolate to have the unique morphological characteristics of a virus. Attempts to isolate mycoplasma in culture and to identify mycoplasma in cell cultures with mycoplasma antisera also yielded negative results.

A lack of reactivity of Hantavirus specific antisera to Manoa virus by IFAT suggests that it may not be a hantavirus (Table 18). It would certainly be unique if it were a Hantavirus and yet did not cross react with any of the Hantavirus specific antisera tested against it. However, there are several one-way reactions between Prospect Hill and Puumala antisera to many of the known hantaviruses (Tables 10a and 10b), and thus this is not a definitive test. It may be significant that, of the hantaviruses tested, Prospect Hill virus was one of those to react to local sera positive for antibody to Hantaan virus by IFAT (Table 11).

Reactivity of 27 local sera reactive to Hantaan virus by IFAT to two Manoa virus isolates (one from a feral R. rattus and the other from a laboratory R. norvegicus) were nearly identical (Table 19) and thus were determined to

probably be the same virus. In only two of the sera tested was there a strong preference for one of the two isolates. Three of the Hantaan virus positive sera tested did not react with either of the isolates, suggesting that there is probably another Hanta-like virus present in Hawaii that may not be closely related to the Manoa virus.

Manoa Virus is 100 to 200 nanometers in diameter (determined by differential filtration), is sensitive to deoxycholate indicating that it has an envelope containing essential lipids, and has a buoyant density between 1.18 and 1.20 in sucrose. These results indicate that Manoa virus is almost certainly in the family Bunyaviridae but they are not definitive for the genus Hantavirus.

Ultrastructural examination of Manoa virus infected Vero E-6 cells suggests that Manoa virus belongs in the Hantavirus genus. The granulofilamentous and granular electron dense inclusions found in the cytoplasm of Manoa virus infected vero E-6 cells have been previously described (Hung et al., 1987). There are three distinctive types of viral inclusions (granular, granulofilamentous, and filamentous) in the cytoplasm of hantavirus-infected cells and their virus specificity was verified by immune colloidal gold and immunoperoxidase labeling. The inclusions are thought to represent either aggregates of virions or accumulations of virus specified precursors. The inclusion bodies were common morphological markers for the 13 strains

of hantaviruses studied by Hung et al., some of which were isolated from patients with HFRS as well as from free roaming and laboratory animals in China, Korea, and Japan.

Dr. Hung considered these inclusions to be excellent morphological markers of hantavirus infection since they have not been reported in any of the other Bunyaviridae except for Rift Valley fever virus (Phlebovirus). The presence of specific intracytoplasmic inclusions in Manoa virus infected Vero E-6 cells provides strong presumptive evidence that the morphogenesis of Manoa virus is similar to that of other known hantaviruses. However, the absence of reports of intracytoplasmic inclusions in other Bunyaviridae does not necessarily preclude their existence. There have been relatively few ultrastructural studies of bunyaviruses published.

Analysis of western blots of Hantaan virus proteins with Hantaan virus antiserum demonstrated three strongly reacting major viral proteins and three minor proteins. All of the rat-associated hantavirus antisera tested as well as the Leaky virus (mouse-associated) antisera were strongly reactive to the three major and three minor proteins demonstrating a close antigenic relationship to Hantaan virus. Prospect Hill, Puumala, and Manoa virus antisera did not react with any of the Hantaan virus proteins, suggesting a more distant relationship to the prototypic agent, although the non-reactivity of Manoa virus antisera may be

explained by the fact that antibody titers by IFAT were quite low and the technique for raising the antisera had not been optimized.

Sera from two local rats seropositive to Hantaan virus by IFAT did not react with any of these major or minor Hantaan virus proteins but did react weakly with other minor proteins. This suggests the Hawaiian agent has a closer antigenic relationship to Hantaan virus than either of the vole-associated hantaviruses but a more distant relationship than Leaky virus or any of the rat-associated hantaviruses tested. Although these results appear to conflict with those of the Manoa virus antisera raised in laboratory rats, they may be explained by the much higher titers of the local rat sera for Hantaan virus by IFAT.

Analysis of western blots of Manoa virus proteins using Manoa virus antisera demonstrated two strongly reacting proteins. However, the sera from local feral rats seropositive to Hantaan virus by IFAT reacted strongly not only to the major viral proteins demonstrated by the Manoa virus antisera but also to numerous other viral proteins, again suggesting that the raising of Manoa virus antisera needs to be optimized.

All of the other Hantavirus antisera with the exception of Seoul virus antisera reacted with one of the major Manoa virus proteins, although the reaction of Puumala and China antisera was weak. China virus antisera had a unique

pattern of reactivity, also reacting with three other viral proteins.

Results of the western blots of major viral proteins demonstrated a curious pattern of reactivity. Western blot analysis demonstrated that antibody in local rat sera was non-reactive to any of the major Hantaan virus proteins but was weakly reactive to proteins not noted in some of the other Hantavirus antisera. However, all of the rat-associated Hantavirus antisera tested were shown to be reactive to major and minor Hantaan virus proteins. This suggests that Manoa virus is distantly related, if at all, to the Seoul virus subgroup which includes all of the known rat-associated hantaviruses.

It was demonstrated that not all Hantavirus antisera are reactive with the Hantaan virus proteins by western blot analysis (Prospect Hill and Puumala virus antisera), suggesting that antibody to Hantavirus group antigen is either non-existent or below the threshold of this particular assay. Thus, the non-reactivity of Manoa virus antisera to Hantaan virus proteins does not necessarily preclude it from being a Hantavirus but suggests that if it is a Hantavirus it is only distantly related to the Hantaan virus subgroup as appears to be the case with the vole-associated hantaviruses.

Local sera seropositive to Hantaan virus by IFAT reacted strongly with Manoa virus proteins and mildly with

Hantaan virus proteins, suggesting that Manoa virus may be the agent inducing antibody to Hantaan virus by IFAT in local rats. The reactivity of the other Hantavirus antisera to Manoa virus proteins is notable in that over 80% reacted to a single major viral protein. Only Seoul and Puumala virus antisera were non-reactive to Manoa virus proteins. However, the serum from the "normal" rat supplied by USAMRIID, Fort Detrick, Maryland also reacted to this Manoa virus protein even though it was non-reactive to any Hantaan virus proteins or to either of the Vero E-6 cell proteins used as controls. This would suggest that the "normal" rats at USAMRIID are infected with an unrecognized contaminating agent which reacts to the Manoa virus 50 K protein and may explain the reactions of the other Hantavirus antisera as they may have been raised in these same "normal" rats. Antiserum to Manoa virus produced in seronegative laboratory rats reacted primarily to 50 K and 200 K Manoa virus proteins but not to the many minor proteins reacted to by the sera from local rats with antibody to Hantaan virus by IFAT. However, this serum was of low titer by IFAT, which may have affected the pattern of reactivity by the western blot. When the reactivity of local feral sera positive to Hantaan virus by IFAT are compared to each other and to Manoa virus antisera raised in laboratory rats, it appears that there is a wide variation in the specificity of antibodies reactive to proteins in the Manoa virus blot.

This suggests a variability in the strains of Manoa virus in Hawaii or variability in the development of antibody specificity in rats infected with Manoa virus as such rats appear to maintain the virus infection in the face of high antibody titers.

Results of this study indicate that the virus isolated from Hantaan virus seropositive Hawaiian peridomestic and laboratory rats is Hantavirus-like but has unique characteristics that differ from the other known hantaviruses. Known rat associated hantaviruses all appear to be very similar and have been found to be strains of Seoul virus. Manoa virus does not fit into this group.

The characteristics of Manoa virus that are unique for a Hantavirus suggest three possibilities: 1) more than one virus is present in the Hawaiian rat population and the virus isolated is not the one responsible for the production of antibody reactive to Hantaan virus, 2) this virus is not a Hantavirus but induces the production of non-neutralizing antibodies that cross react with several of the known hantaviruses, or 3) that this is a completely new Hantavirus.

In order to further define this virus other studies, such as cross neutralization tests to determine its relationship to other known hantaviruses must be done. However, due to the need for a class 3 biocontainment facility to perform these studies, they cannot be done in

Hawaii and it is anticipated that they will be performed at USAMRIID, Fort Detrick in the near future.

A definitive test to determine if Manoa virus is a Hantavirus would be the sequencing of the 3'-terminal nucleotide of the virus since all known hantaviruses have a unique sequence that is distinct from the other genera in the Bunyaviridae (Schmaljohn, et al., 1985). The level of homology would help to define the relationship of Manoa virus to the other hantaviruses. Manoa virus specific antibody also needs to be produced in sufficient quantity to allow immunoelectron microscopic techniques to be performed to further define the morphological characteristics of the virus and the specificity of the unique intracytoplasmic inclusions described in this study.

If Manoa virus is not a Hantavirus then its cross reactivity with other bunyaviruses needs to be ascertained to determine if this virus fits into one of the recognized groups or is actually a new subgroup of the Bunyaviridae. Preliminary work at USAMRIID Ft. Detrick, Maryland have demonstrated some reactivity of Manoa virus to Group C bunyavirus antisera (LaDuc, personal communication). The majority of the Group C bunyaviruses viruses were initially isolated near Belem, Brazil as was the Brazil strain of Hantavirus. This is quite interesting as this study has demonstrated that local rat sera with antibody to Hantaan virus also react strongly with Brazil virus by IFAT. This

suggests that there may be an unrecognized relationship between the Group C bunyaviruses and the hantaviruses or possibly that Manoa virus may fall somewhere between the two antigenically, or that as in Brazil there are two distinct bunyaviruses in the feral rats of Hawaii

As this appears to be a novel virus, nothing is yet certain about the pathogenesis, transmission, or growth dynamics of this virus in what appears to be its natural host, the Rattus species. The involvement of arthropod vectors is also yet to be defined and may serve as an important mode of transmission. Continuing studies should help to elucidate these undefined subjects.

The geographic distribution and the involvement of other species, including man, are also areas of considerable interest. The development of an easy to use serological test, such as HDPA, would be invaluable in helping to define these parameters.

BIBLIOGRAPHY

Artropod-Borne Virus Information Exchange. 1988

Baek, L. J., H. L. Amyx, R. Yanagihara, C. J. Gibbs Jr., and D. C. Gadjusek. 1987. Epizootiology of Hantavirus infections in the United States: isolation of a new Hantaan related virus from Mus musculus. Abstracts of the Pacific Sci. Assoc. 16th Congress 1:33.

Bishop, D. H. L., and R. E. Shope. 1979. In: Compresensive Virology, Vol. 14, edited by H. Frankel-Conrat and R. R. Wagner. pp 1-156. Plenum, New York

Brownlee, K. A.. 1965. Statistical Theory and Methodology in Science and Engineering, 2nd ed.. pp 1-590. John Wiley & Sons, Inc., New York

Brummer-Korvenkontio, M., A. Vaheri, T. Hovi, et al. 1980. Nephropathica epidemica: detection of antigen in bank voles and serologic diagnosis of human infection. J. Infect. Dis. 152:826-829.

Casals, J., H. Hoogstraal, K. M. Johnson, et al. 1966. A current appraisal of hemorrhagic fevers in the U.S.S.R.. Am. J. Trop. Med. Hyg. 15:751

Chen, L. B. and L. J. Hao. 1988. Identification of the antigenic components of Hantaan virus reacting with rabbit antisera and sera from patients with hemorrhagic fever with renal syndrome. J. Infect. Dis. 157:1264-1267.

Childs, J. E., G. W. Korch, G. A. Smith, et al. 1985. Geographical distribution and age related prevelence of antibody to Hantaan-like virus in rat populations of Baltimore, Maryland USA. AM. J. Trop. Med. Hyg. 34:385-387.

---, ---, G. E. Glass, et al. 1987. Epizootiology of Hantavirus infection in Baltimore: Isolation of a virus from Norway rats and characteristics of infected rat populations. AM. J. Epid. 126:55-68.

---, G. E. Glass, G. W. Korch, and J. W. LeDuc. 1988. The ecology and epizootiology of hantaviral infections in small mammal communities of Baltimore: a review and synthesis. Bull. Soc. Vector Ecol. 13:113-122.

Chumakov, M. P., I. N. Gavrilovskaya, V. A. Boiko, et al. 1981. Detection of hemorrhagic fever with renal syndrome (HFRS) in the lungs of bank voles Clethrianomys glariolus and redbacked voles Clethrionomys rutilus trapped in HFRS foci in the European part of the U. S. S. R., and serodiagnosis of their infection in man. Arch. Virol. 69:295-300.

Colhoun, J. B.. 1963. The ecology and sociobiology of the Norway rat. U. S. Government Printing Office, Washington, D. C..

Dantas, J. R. Jr., Y. Okuno, O. Tanishita, et al. 1987. Viruses of hemorrhagic fever with renal syndrome (HFRS) grouped by immunoprecipitation and hemagglutination inhibition. Intervirology. 27:161-5.

Diwan, A. R., H. W. Lee, R. W. Yanagihara, et al. 1985. Seroepidemiology of Hantaan virus in the state of Hawaii. in Hemorrhagic Fever with Renal Syndrom, Bender, T.R., A. R. Diwan, and J. S. Raymond Eds., University of Hawaii School of Public Health, Honolulu, Hawaii. pp26-30.

Desmyter, J., J. W. LeDuc, K. M. Johnson, et al. 1983. Laboratory rat associated outbreak of hemorrhagic fever with renal syndrome due to Hantaan-like virus in Belgium. Lancet 2:1445-1448.

Dournon, E., B. Moriniere, S. Matheron, et al. 1984. HFRS after a wild rodent bite in the Haute-Savoie and risk of exposure to Hantaan-like virus in a Paris laboratory. Lancet 1:676-677.

Downs, W. G.. 1975. Malaria, the great umbrella. Bull. N. Y. Acad. Med.. 51:984-990.

Elliott, L. H., M. Kiley, and J. McCormick. 1984. Hantaan virus: identification of virion proteins. J. Gen. Virol. 65:1285.

French, G. R., R. S. Foulke, O. A. Brand, et al. 1981. Korean hemorrhagic fever: propigation of the etiological agent in a cell line of human origen. Science 211:1046-1048.

Gavrilovskaya, I. N., N. Apekina, E. Gorbachkova, et al. 1981. Detection by enzyme-linked immunosorbant assay of haemorrhagic fever with renal syndrome in lung tissue of rodents from European U.S.S.R.. Lancet 1:1050.

Gajdusek, D. C.. 1953. Acute infectious hemorrhagic fevers and mycotoxicosis in the Union of Soviet Socialist Republics. Medical Science Publication Number 2, Army Medical Service Graduate School, Walter Reed Army Medical Center, Washington, D.C..

---. 1962. Virus Hemorrhagic Fevers. Special reference to hemorrhagic fever with renal syndrome (epidemic hemorrhagic fever). J. Pediatr. 60:841-857.

Gibbs, C. J. Jr., A. Takenaka, M. Franko, et al. 1982. Seroepidemiology of Hantaan virus. Lancet II 1406-1407.

Hung, T., S. M. Xia, T. X. Zhao, et al. 1983. Morphological evidence for identifying the viruses of hemorrhagic fever with renal syndrome as candidate members of the Bunyaviridae family. Arch. Virol. 78:137-144.

---, Z. Chow, T. Zhao, et al. 1985. Morphology and morphogeneis of viruses of hemorrhagic fever with renal syndrome (HFRS). I. Some peculiar aspects of the morphogenesis of various strains of HFRS virus. Intervirology 23:97-108.

---, S. Xia, Z. Chou, et al. 1987. Morphology and morphogenesis of viruses of hemorrhagic fever with renal syndrome. II. Inclusion bodies-ultrastructural markers of hantavirus-infected cells. Intervirology 27:45-52.

Ishii, S., K. Ando, N. Watanabe, et al. 1942. Studies on Songo fever. Jpn. Army Med. J. 355:1755.

Jiang, A. T.. 1983. A preliminary report of hemorragic fever with renal syndrome in China. Chin. Med. J. 96:265.

Kitamura, T., C. Morita, T. Komatsu, et al. 1983. Isolation of the virus causing hemorrhagic fever with renal syndrome (HFRS) through a cell culture system. Jap. J. Med. Sci. Biol. 36:17-25.

Kitano, M.. 1944. Studies on epidemic hemorrhagic fever. Manchoukuo Med. J. 40:191.

Kurata, T., T. Tsai, S. Bauer, et al. 1985. Suckling mouse model of Hantaan virus infection, in Hemorrhagic Fever with Renal Syndrome, Bender, T., A. Diwan, and J. Raymond Eds. University of Hawaii School of Public Health, Honolulu 60.

Lahdevirta, J.. 1971. Nephropatia epidemica in Finland. Ann. Clin. Res. 3(suppl. 8):1-154.

LeDuc, J. W., G. A. Smith, L. R. Bagley, et al. 1982. Preliminary evidence that Hantaan or a closely related virus is enzootic in domestic rodents. New Eng. J. Med. 307:624.

---, ---, and K. M. Johnson. 1984. Hantaan-like viruses from domestic rats captured captured in the United States. Am. J.Trop. Med. Hyg. 33:992-998.

---, ---, F. P. Pinheiro, P. C. Vasconcelos, E. S. T. Rosa, and J. I. Maiztegui. 1985. Isolation of a Hantaan-related virus from Brazilian rats and serologic evidence of its widespread distribution in South America. AM. J. Trop. Med. Hyg. 34:810-815.

Lee, H. W., P. W. Lee, and K. M. Johnson. 1978. Isolation of the etiological agent of Korean hemorrhagic fever. J. Infect. Dis. 137:298-308.

---, ---, J. Lahdevirta, and M. Brummer-Korvenkontio. 1979a. Aetiological relation between Korean hemorrhagic fever and nephropathia epidemica. Lancet 1:186-187.

---, ---, M. Tamura, T. Tamura, and Y. Okuno. 1979b. Etiological relation between Korean hemorrhagic fever and epidemic hemorrhagic fever in Japan. Biken J. 22:41-45.

---, G. R. French, P. W. Lee, L. J. Baek, et al. 1981a. Observations on natural and laboratory infection of rodents with the etiological agent of Korean hemorrhagic fever. Am. J. Trop. Med. Hyg. 30:477-482.

---, P. W. Lee, L. J. Baek, C. K. Song, and I. W. Seong. 1981b. Intraspecific transmission of Hantaan virus, etiological agent of Korean hemorrhagic fever, in the rodent Apodemus agrarius. Am. J. Trop. Med. Hyg. 30:1106-1112.

---. 1982. Korean hemorrhagic fever. Prog. Med. Virol. 28:96.

---, L. J. Baek, and K. M. Johnson. 1982. Isolation of Hantaan virus, the etiological agent of Korean hemorrhagic fever, from wild urban rats. J. Infect. Dis. 146:638-644.

---, and K. M. Johnson. 1982. Laboratory acquired infections with Hantaan virus, the etiologic agent of Korean hemorrhagic fever. J. Infect. Dis. 146:645-651.

Lee, P. W., D. C. Gajdusek, C. J. Gibbs Jr., and Z. Y. Xu. 1980. Aetiological relation between Korean haemorrhagic fever and epidemic haemorrhagic fever with renal syndrome in the Peoples Republic of China. Lancet 1:819-820.

---, C. J. Gibbs Jr., D. C. Gajdusek, and A. Svedman. 1981. Antibody to Korean hemorrhagic fever virus in man in parts of the world where hemorrhagic fever with renal syndrome is not known. Lancet II:256.

---, A. Svedmyr, D. C. Gajdusek, et al. 1981. Antigenic differences between European and east Asian viruses causing hemorrhagic fever with renal syndrome. Lancet II:256-257.

---, R. Yanagihara, M. C. Franko, et al. 1982. Preliminary evidence that Hantaan or a closely related virus is enzootic in domestic rodents. New Eng. J. Med. 307:624-625.

---, H. L. Amyx, R. Yanigahara, et al. 1985 Partial characterization of Prospect hill virus isolated from meadow voles in the United States. J. Infect. Dis. 152:826-829.

---, C. J. Gibbs Jr., D. C. Gajdusek, et al. 1985b. Serotypic classification of hantaviruses by indirecct immunofluorescent antibody and plaque reduction neutralization tests. J. Clin. Microbiol. 22:940-944

---, R. Yanagihara, C. J. Gibbs Jr., and D. C. Gajdusek. 1986. Pathogenisis of experimental Hantaan infection in laboratory rats. Arch. Virol. 88:57-66.

Lloyd, G., E. T. W. Bowen, N. Jones, and A. Pendry. 1984. HFRS outbreak associated with laboratory rats in UK. Lancet 1:1175-1176.

McCormick, J. B., D. R. Sasso, E. L. Palmer, and M. P. Kiley. 1982. Morphological identification of the agent of Korean Haemorrhagic fever (Hantaan virus) as a member of the Bunyaviridae. Lancet 1:765-767.

Myhrman, G.. 1951. Nephropathica epidemica. A new infectious disease in northern Scandinavia. Acta. Med. Scand. 140:52.

Oliver, J., and M. Macdowell. 1957. The renal lesion in epidemic hemorragic fever. J. Clin. Invest. 36:99.

Okuno, Y., K. Yamonishi, Y. Takahashi, et al. 1986. Haemagglutination-inhibition test for haemorrhagic fever with renal syndrome using virus antigens prepared from infected tissue culture fluid. J. Gen. Virol. 67:149.

Powell, G. M.. 1954. Hemorrhagic fever: a study of 300 cases. Medicine 33:99.

SAS Users Guide: Basics, Version 5. 1988. SAS Institute, INC. Cary, N. C..

Schmaljohn, C. S. and J. M. Dalrymple. 1983. Analysis of Hantaan virus RNA: evidence for a new genus of Bunyaviridae. Virology 131:482-491.

---, S. E. Hastey, S. A. Harrison, and J. M. Dalrymple. 1983. Characterization of Hantaan virions, the prototype virus of hemorragic fever with renal syndrome. J. Infect. Dis. 148:1005-1012.

---, ---, J. M. Dalrymple, et al. 1985. Antigenic and genetic properties of viruses linked to hemorrhagic fever with renal syndrome. Science 227:1041-1044.

Shope, R. E. 1985. Bunyaviruses. In: Fields Virology edited by B. N. Fields, D. M. Knipe, R. M. Chanock, et al. pp1055-1082. Raven Press, New York.

Siamopoulos, K., A. Antioniadis, N. Acritidis, et al. 1954. Symposium on epidemic hemorrhagic fever. AM. J. Med. 16:617.

Smorodintsev, A. A., V. Chudakov, and A. Churilov. 1959. Haemorrhagic Nephroso-nephritis. Pergamon Press, New York 124pp.

Song, G., C. S. Hang, X. Z. Qui, et al. 1983. Etiologic studies of epidemic hemorragic fever (hemorrhagic fever with renal syndrome). J. Infect. Dis. 147:654-659.

---, ---, H. X. Laio, et al. 1984. Antigenic differences between viral strains causing classical and mild types of epidemic hemorrhagic fever with renal syndrome in China. J. Infect. Dis. 150:889

Sugiyama, K., Y. Matsuura, C. Morita, et al. 1984. Determination of immune adherence HA of the antigenic relationship between Rattus and Apodemus-borne viruses causing hemorrhagic fever with renal syndrome. J. Infect. Dis. 149:472.

Takenaka, A., C. Gibbs, and D. Gajdusek. 1985. Antiviral neutralizung antibody to Hantaan virus as determined by plaque reduction technique. Arch. Virol. 84:197.

Tang, Y. W., Z. Y. Zhu, and T. F. Tsai. 1985. Isolation of HFRS virus from Suncus murinus an insectivore. Lancet 1:513-514.

Thieler, M. and W. G. Downs. 1973. The Arthropod-Borne Viruses of Vertebrates. Yale University Press, New Haven.

Tkachenko, E. A., A. Ivanov, T. Dzugurova, et al. 1981. Immunosorbant assays for diagnosis of haemorrhagic fever with renal syndrome. Lancet 2:257

---, T. Dzugurova, E. Leshchinskaya, et al. 1982. Serological diagnosis of haemorrhagic fever with renal syndrome in European regions of the U.S.S.R.. Lancet 2:1407

Tomiyama, T., and H. W. Lee. 1990. Rapid serodiagnosis of hantavirus infection using high density particle agglutination. Arch. Virol.. In Press.

Tsai, T. F., S. P. Bauer, D. R. Sasso, et al. 1982. Preliminary evidence that Hantaan or a closely related virus is enzootic in domestic rodents. New Eng. J. Med. 307:623-624.

---, ---, ---, et al. 1985. Serological and virological evidence of a Hantaan virus-related enzootic in the United States. J. Infect. Dis. 152:126-36.

Umenai, T., H. W. Lee, P. W. Lee, et al. 1979. Korean hemorrhagic fever in staff in an animal laboratory. Lancet 1:1314-1316.

U. S. Department of the Army. 1953. Epidemic hemorrhagic fever. Tech. Bull. TB MED 240, U. S. Government Printing Office, Washington, D. C. 23pp.

White, J. D., F. G. Shirley, G. R. French, et al. 1982 Hantaan virus, aetiological agent of Korean haemorrhagic fever, has Bunyaviridae-like morphology. Lancet 1:768-771.

Woodall, J. P.. 1979. Transmission of Group C arboviruses (Bunyaviridae). In: Artic and Tropical Arboviruses, edited by E. Kurstak. pp123-138. Academic Press, New York.

World Health Organization Regionl Office of the Western Pacific. 1982. Report of the working group on hemorrhagic fever with renal syndrome.

World Health Organization. 1983. Hemorrhagic fever with renal syndrome; memorandum from a W. H. O. meeting. Bull. W. H. O. 61:269-275.

Yanagihara, R., A. Svedmyr, H. L. Amyx, et al. 1984. Nephropathia epidemica: isolation and propagation of the causative virus in bank voles and Mongolian gerbils. Scandinavian J. Infect. Dis. 147:654-659.

---, R. Traub, D. C. Gajdusek, et al. 1984. Prospect Hill virus: serological evidence for infection in mammalogists. New Eng. J. Med. 310:1325-1326.

---, H. L. Amyx, and D. C. Gajdusek. 1985a. Experimental infection with Puumala virus, the etiologic agent of nephropathia epidemica, in bank voles (Clethrionomys glareolus). J. Virol. 55:34-38.

---, C. T. Chin, C. T. Weiss, et al. 1985. Serological evidence of Hantaan virus infection in the United States. AM. J. Trop. Med. Hyg. 34:396-399.

---. 1990. Hantavirus Infection in the United States: Epizootiology and Epidemiology. Rev. Inf. Dis.. 12,3:449-457.

Zhao-zhaung, L., H. Hao, and L. Yue-xian. 1985. Characterization of EHF virus isolated from cats in endemic area of Anhui province. Chin. J. Microbiol. Immunol. 5:81-83.