

OBSERVATIONS ON NATURAL AND LABORATORY INFECTION OF RODENTS WITH THE ETIOLOGIC AGENT OF KOREAN HEMORRHAGIC FEVER*

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Abstract. Studies were conducted to define the natural host range of the Korean hemorrhagic fever (KHF) agent in South Korea, and to identify colonized rodents susceptible to this infection. Eight species of field rodents were captured in areas of Korea endemic for KHF and their tissues were examined by immunofluorescence for the presence of KHF antigen. One hundred and fourteen of 817 *Apodemus agrarius coreae* captured between 1974 and 1978 had one or more positive organs. No positive organ was found in 239 rodents of the other seven species examined. Two hundred and thirty-eight specimens of *Apodemus agrarius jejuensis* captured on Jeju Island, an area thought to be free of disease, were also negative. Attempted laboratory infection of nine species of rodents captured in the field but maintained in the laboratory was successful only in the two subspecies of *Apodemus*. The 46 specimens of *A. a. jejuensis* tested in this manner were all uniformly susceptible to infection as determined by immunofluorescence. Serial sacrifice of experimentally infected *A. a. jejuensis* revealed viremia of short duration terminating on day 10 postinfection. In contrast, other tissues of this animal, including lung, kidney, liver and parotid gland were positive on day 10 and remained so through the 100-day observation period. When 12 species of colonized laboratory rodents were inoculated with KHF agent five were found to develop KHF antibody by indirect immunofluorescence and two, *Calomys callosus* and *Apodemus agrarius ningpoensis*, developed detectable KHF antigen in their tissues.

The first successful isolation and propagation of the etiologic agent of Korean hemorrhagic fever (KHF) was described in a previous communication.¹ The presence of KHF agent-specific antigen in frozen sections of various tissues of *Apodemus agrarius coreae*, a vesper mouse indigenous to en-

demical areas of disease on the Korean peninsula, was demonstrated utilizing the indirect fluorescent antibody (IFA) technique and human KHF convalescent sera. Numerous isolations of the agent have been made from rodents of this species, and successive passage of the agent in a subspecies, *Apodemus agrarius jejuensis* captured in areas free of natural disease, has been reported.^{1,2}

Characterization of the agent is now in progress and will be the subject of a later report. Preliminary results, however, indicate that a small proportion of the infectious agent population will pass membrane filters of 100 nm average pore diameter (APD). In these same experiments, infectious tissue suspensions of $\geq 10^6$ *Apodemus* ID₅₀/ml failed to pass 50-nm APD membrane filters.^{2,3} Other experiments have shown the infectious entity, which is insensitive to a wide range of antibiotics, to be fully sensitive to organic lipid solvents and nonionic detergents.^{2,3} Thus, the etiologic agent of KHF appears to be a medium-size, enveloped virus.

The present report describes more fully labo-

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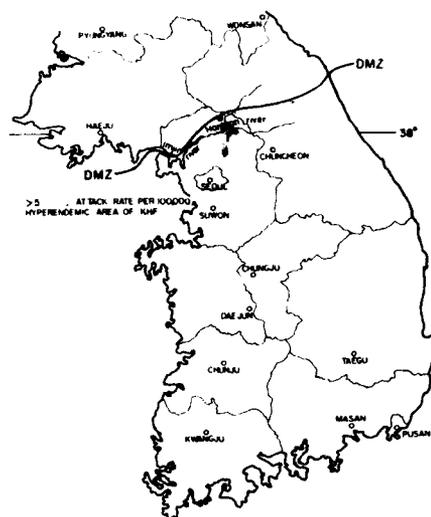


FIGURE 1. Location of the small farm villages (stars) studied within the hyperendemic zone.

ratory observations made on natural and laboratory-induced infection in *Apodemus*, attempts to isolate the agent of KHF from other species of rodents indigenous to South Korea, and studies of KHF agent infection of colonized laboratory and captured rodents maintained in the laboratory.

MATERIALS AND METHODS

The techniques of agent identification and titration were essentially similar in both laboratories and have been previously described.¹ The IFA technique for demonstration of KHF-specific antigens utilizing frozen thin sections of animal tissue and human KHF convalescent sera were

TABLE 2
Natural Korean hemorrhagic fever agent infection of wild rodents captured during the years 1974-1978

Species	No. IFA positive/ no. examined
<i>A. a. coreae</i>	114/817
<i>A. a. jejuensis</i>	0/238
<i>Microtus fortis pelliceus</i>	0/103
<i>Crocidura lasiura</i>	0/71
<i>Clethrionomys rufocanus regulus</i>	0/35
<i>Cricetulus triton nestor</i>	0/9
<i>Mus musculus yamashinai</i>	0/17
<i>Micromys minutus ussuricus</i>	0/3
<i>Tamias sibiricus asiaticus</i>	0/1

identical. Fluorescein isothiocyanate (FITC)-conjugated anti-rodent species sera were obtained from various commercial sources and were standardized and diluted to contain eight units of antiglobulin for the antibody tests.¹ Attempts to experimentally infect colonized rodents at USAMRIID, which included the studies reported on *Calomys callosus*,⁴ *Sigmodon hispidus*, *Microtus montanus*, the MHA hamster (Charles River Breeding Labs Inc., Wilmington, MA), strain 13 guinea pigs (Crest Caviary, Raymond, CA), and a portion of the mouse and rabbit data, were conducted with the fifth *Apodemus* passage of KHF agent, strain 76-118, which was isolated from rodents captured in Songnaeri, Korea, in 1976. The remaining studies were all conducted at the Korea University College of Medicine Laboratory, Seoul, Korea, and utilized the fifth through eighth *Apodemus* passage of this same KHF strain. The 10% suspension of lung tissue from the fifth *Apo-*

TABLE 1
Distribution of Korean hemorrhagic fever antigen in the tissues of *A. a. jejuensis* after intramuscular inoculation of 10^5 *Apodemus ID₅₀**

Tissue examined	No. with antigen/3, by days								
	5	7	10	13	21	34	50	76	100
Blood	0	3	3	0	0	0	NT†	NT	NT
Lungs	0	0	3	2	3	2	2	2	2
Kidneys	0	0	3	2	2	3	1	1	1
Liver	0	0	3	2	3	1	1	1	1
Parotid	0	0	3	2	2	1	1	1	1
Bladder	0	0	3	0	0	0	1	0	0
Spleen	0	0	0	0	0	0	0	0	0

* Strain 76-118 passage 8. Three animals were killed and examined for the presence or absence of antigen on the indicated days.

† NT, not tested.

demus passage of strain 76-118 utilized at USAMRIID contained approximately $10^{3.5}$ infectious units/ml. The seventh passage suspension, the principal stock utilized in the laboratory in Korea, contained $10^{5.2}$ infectious units/ml. Experimentally infected rodents were inoculated intramuscularly (IM) or intralung (IL) with undiluted agent suspensions unless otherwise indicated in the text.

Some of the earlier studies at USAMRIID included pretreatment of rodents with cortisone acetate in an attempt to increase their susceptibility to the KHF agent. The procedure was as described by Imam et al.⁵ for hamsters. Rodents were treated with cortisone acetate (0.2 mg/g body weight) in a divided dose schedule over 4 days beginning one day prior to inoculation with infectious material.

Captured rodents utilized in studies of the natural host range or attempts at experimental infections were obtained in the manner and at the study sites previously described.¹ Briefly, these study sites are six small farm villages located in close proximity to the 38th North parallel and the Hantaan River (within the shaded area shown on the map in Fig. 1). The area is rural and mountainous; capture sites included both cultivated fields and areas of scrub vegetation.

Normal (low probability of natural infection) *A. a. coreae* and *A. a. jejuensis* for agent isolation and titrations were obtained by capture from Chin and Jeju islands, respectively. Natural disease of man or rodent has not been found on either of these islands, both of which are south of the 35th parallel, north latitude. The colonized species of *Apodemus* described in the text, *Apodemus agrarius ningpoensis* (Taiwan), *Apodemus speciosus* (Japan) and *Apodemus peninsulae* (Japan) were obtained from Dr. K. Tsuchiya.

RESULTS

Further studies in *A. a. coreae*

It had been previously indicated that KHF-specific antigen could be detected in lung, kidney, parotid gland, bladder, liver and submaxillary gland, but not spleen tissue of captured naturally infected rodents.¹

A similar distribution of antigen in tissue was shown in experimentally infected *Apodemus* captured on the mainland; however, it was impossible to know with certainty that these rodents had not been naturally infected. In that study, KHF an-

TABLE 3
Experimental infection* of colonized rodents with Korean hemorrhagic fever (KHF)

Species	KHF antigen in tissues No. IFA +/no. examined*	Clinical signs	IFA antibody
Mouse			
Baby, ICR	0/88	-	+
Weanling/adult, ICR	0/21	-	+
C57BL/6	0/10	-	-
C-DBA/2N	0/10	-	-
Rat			
White (S/D)	0/15	-	+
White, Wistar	0/20†	-	+
<i>S. hispidus</i>	0/10	-	-
Hamster			
Baby Syrian	0/8	-	-
Adult Syrian	0/17	-	-
Adult MHA	0/10	-	-
Chinese	0/4	-	-
Guinea pig			
Hartley strain	0/6	-	+
Strain 13	0/4	-	+
Rabbit, New Zealand white	0/12	-	+
Mongolian Gerbil	0/6	-	-
<i>Microtus montanus</i>	0/8	-	-
<i>A. speciosus</i>	0/4	-	-
<i>A. peninsulae</i>	0/4	-	-
<i>C. callosus</i>	8/16	-	-/?
<i>A. a. ningpoensis</i>	5/5	-	+

* Intramuscular inoculation with 8,000 *Apodemus* ID₅₀ of KHF strain 76-118 passage 7; 3-week results.

† Blood subinoculated in *A. a. jejuensis* was infectious on days 7-11 postinoculation of rats.

tigen was shown to persist in lung tissue for at least 69 days. This study has been repeated in *A. a. jejuensis* experimentally infected with eighth passage virus, and the period of observation was extended to 100 days (Table 1). The agent was detected in blood from days 7 to 10. Other tissues became positive at this time, and IFA-detectable antigen persisted in lung, kidney, liver and parotid glands through the 100-day observation period. Again, as in the previous study, the rodents did not demonstrate any clinical signs of illness. The most intense fluorescence was observed in lung tissue. The infectious entity from lung tissues of infected *A. a. coreae* has been carried through 26 successive passages. A 5% lung tissue suspension from the 11th passage in this series was found to contain $10^{7.2}$ infectious units/0.3 ml.

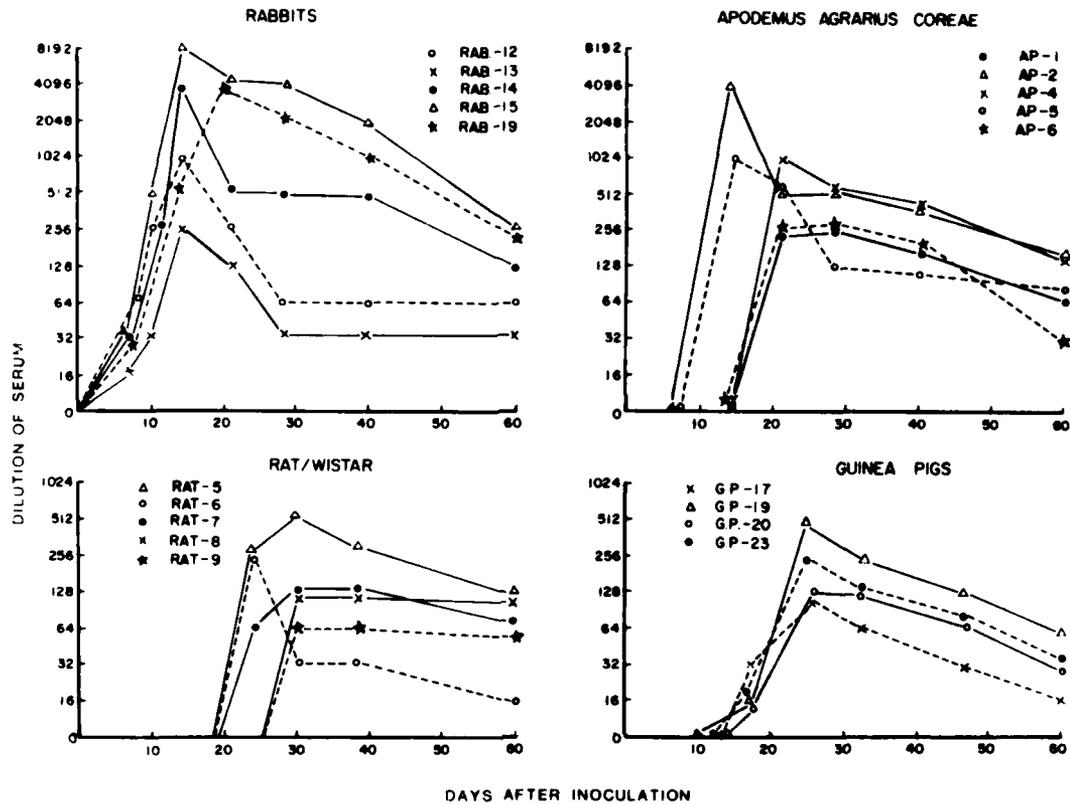


FIGURE 2. Immunofluorescent antibody responses to Korean hemorrhagic fever agent after inoculation into animals.

Examination of other rodents captured in the endemic area

Attempts to implicate rodents other than *Apodemus* as natural hosts of KHF have been uniformly unsuccessful. A total of 239 rodents of seven different species collected in the endemic area from 1974–1978 has been examined and found to be negative. In contrast, 13% of more than 800 *A. a. coreae* collected during the same period from the same collection sites were found to be positive. A list of species and numbers of rodents captured and examined for evidence of KHF infection during this period is found in Table 2.

KHF infection of colonized rodents

Previous attempts to establish KHF infection in several species of colonized laboratory rodents were unsuccessful. The criterion for infection was development of specific IFA-detectable antigen in tissue sections of the test host 21 days postinocu-

lation with 1,000 ID_{50} of *Apodemus*-passaged agent. This study has been expanded to include several more species of colonized rodents; the criteria of infection have been extended in some instances to include development of IFA-detectable antibody. The complete list of rodents studied and the results are shown in Table 3.

As can be seen, *A. a. ningpoensis* and *C. callosus* developed IFA-detectable antigen in tissue sections. *C. callosus*, a South American rodent and the reservoir rodent host for Machupo virus,⁴ was not uniformly susceptible. Several attempts to adapt the agent of KHF to this host by successive passage of IFA-positive lung tissue suspensions failed. The second passage of KHF agent in weanling *Calomys* resulted in four of four animals infected, but in retrospect this appears to have been a fortuitous event in that subsequent trials have resulted in approximately 50% infection rates regardless of passage level. Infection of *C. callosus* is similar, however, in several aspects to

that in *Apodemus*. Lung is the most likely tissue to be positive and provides the most uniform and intense staining by IFA. As in *Apodemus*, detectable antigen peaks about 21 days postinoculation, persists for long periods of time and the animals do not become clinically ill. Pretreatment of *C. callosus* with cortisone acetate had no apparent effect on the infection success rate, and did not affect the final outcome. Postinfection antibody in *Calomys* was not demonstrated in these experiments. It is not clear at this time whether the FITC-conjugated antiglobulins utilized to attempt to demonstrate antibody were inadequate or whether this rodent does not develop IFA-detectable antibody in response to infection. Anti-mouse, anti-rat, anti-hamster and anti-*Calomys* FITC conjugate globulins were utilized; however, the anti-*Calomys* conjugate was of uncertain quality. In contrast, *Apodemus* develop demonstrable antibody detectable with FITC anti-mouse conjugate; high titers of circulating IFA antibody are present in serum at the same time the IFA detectable antigen can be demonstrated in lung or other tissues.

Limited experience with *A. a. ningpoensis* is very encouraging in that all five animals tested to date have developed IFA detectable antigen in test tissues. Confirmation of the uniform susceptibility of this colonized host awaits the availability of more animals in the newly established colony in Korea.

Several species of colonized rodents tested in these studies also developed IFA-demonstrable KHF antibody in response to inoculation with KHF infectious material. These demonstrated infections, although inapparent (no KHF IFA detectable antigen in tissues and no clinical signs) are of importance because these species provide alternate sources of nonhuman antibody. The New Zealand white rabbit is particularly useful in this regard in that the antibody response is prompt and relatively high titered. Four of five rabbits inoculated IM with 8,000 *Apodemus* ID₅₀ of *Apodemus*-passaged agent developed KHF IFA titers of $\geq 1:1,024$ from 14 to 21 days postinoculation (Fig. 2). Antibody titers fell off rapidly (≥ 4 -fold) by 60 days, and all five animals developed significant (≥ 4 -fold) and rapid rises in antibody titer when challenged a second time at 80 days. Similar data for guinea pigs, the Wistar strain of white rats, and *A. a. coreae*, are shown. It is important to note that viremia was demonstrated in the Wistar rat on days 7-11, even though KHF-detectable antigen could not be demonstrated in

TABLE 4
Experimental infection* of captured rodents maintained in the laboratory with Korean hemorrhagic fever (KHF)

Species	KHF antigen in tissues No. IFA +/no. examined	Clinical signs	IFA antibody
<i>A. a. coreae</i>	42/50	-	+
<i>A. a. jejuensis</i>	46/46	-	+
<i>C. t. nestor</i>	0/16	-	-
<i>M. f. pelliceus</i>	0/4	-	-
<i>C. r. regulus</i>	0/4	-	-
<i>T. s. asiaticus</i>	0/3	-	-
<i>R. norvegicus</i>	0/16	-	+
<i>Mus musculus</i>	0/5	-	-
<i>C. lasiura</i>	0/3	-	-

* Intramuscular inoculation with 8,000 *Apodemus* ID₅₀ of KHF strain 76-118 passage 7; 3-week results.

the lungs or kidneys. This is the only species in which viremia was detected when lungs or kidneys were negative.

Experimental infection of wild captured rodents

Several species of wild rodents captured at the various study sites were returned to the laboratory and maintained for varying periods of time in clean animal rooms. These rodents, including the two subspecies of *A. a. coreae* captured on the mainland or Chin Island and *A. a. jejuensis* captured on Jeju Island, were inoculated with KHF infectious material in an attempt to produce experimental infection as described for the colonized laboratory rodents. Results are shown in Table 4. The animals were inoculated IM with 8,000 ID₅₀ of seventh *Apodemus* passage KHF, strain 76-118. Lungs and kidneys were removed from sacrificed animals and examined for IFA-detectable antigen on day 20. There was uniform susceptibility of *Apodemus* captured on Jeju Island. *Rattus norvegicus* was the only captured rodent other than the *Apodemus* which showed evidence of experimental infection with KHF. Infection of *R. norvegicus*, however, was detectable only by the demonstration of IFA-detectable antibody post-inoculation.

DISCUSSION

The purpose of these studies was 2-fold: 1) to determine the natural rodent host range of KHF agent on the South Korean peninsula; and 2) to

find a colonized rodent that was an adequate substitute in the laboratory for the wild captured *A. a. coreae* and *A. a. jejuensis*. With regard to the former goal, it appears that the variants of the *Apodemus* mouse are the only significant reservoir rodent hosts of the disease in the rural areas of the study sites. The findings that *R. norvegicus* is susceptible to infection suggests that it may be a temporary host and that perhaps other species may play a reservoir role in suburban or city environments where *Apodemus* are less common inhabitants.

Either or both of these species may have played a role in the serious outbreaks of KHF-like illness that occurred in Osaka, Japan, during the early and middle 1960s.^{6,7} *Apodemus* species native to Japan are not found in any significant numbers in metropolitan areas such as Osaka City: *Rattus* species which can be infected, warrant investigation as a link in transmission of the disease to man if not as potential reservoir host.

The second goal, that of finding a colonized laboratory rodent susceptible to KHF, is of major importance to this work. Attempts to colonize *A. a. coreae* or *A. a. jejuensis* have not been successful. Preliminary results with *A. a. ningpoensis* indicate that this animal may fill this role. If this species proves to be uniformly susceptible, it will greatly reduce the difficulties of these studies. The restriction to use of captured rodents has obvious disadvantages, including expense. Very recently a few captured *A. a. jejuensis* were induced to breed in the laboratory. These rodents are only now in the second generation, but offer hope that this very susceptible species may eventually be available for use as a colonized animal.

Although these studies were not designed to determine the mode of transmission of KHF, the data in Table 1 indicate that blood-sucking arthropods are probably not good candidates as disseminators of infection. The short viremia versus the long maintenance of the infectious entity in other tissues suggests the possibility that one principal mechanism of transmission may involve contamination of foodstuff, water or bedding and soil by excretion of infectious body wastes or fluids and possibly dissemination by aerosol. It is important to point out that work in infected animal rooms is not without considerable risk. The recent experience of Japanese workers at Tohoku Uni-

versity hospital, where infection of 14 professional members of the staff with KHF was traced to an animal room,¹⁰ is not an isolated incident. Several other medical school facilities within Japan have had similar experiences.¹⁰ Further, when Wistar rats from Japan were brought to the laboratory in Korea for study, four members of the staff in Seoul subsequently became infected. Thus, it behooves investigators undertaking studies of KHF agent to observe every safety precaution when entering and working in infected animal rooms, including utilization of protective masks, gloves and clothing.

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