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HEMORRHAGIC FEVER WITH RENAL SYNDROME IN GREECE

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Antibodies to Hantaan virus were detected in 31 out of 1679 individuals tested, and eight human HFRS cases were serologically diagnosed. The disease was severe and one of the patients died during the oliguric phase. Antibodies to CCHF virus were found in humans and animals and inapparent human CCHF infection was confirmed in a CCHF endemic area.

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**FOREWORD**

For the protection of human subjects, the investigator (s) have adhered to policies of applicable Federal Law 45CFR46.

In conducting research using animals, the investigator (s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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## **A. INTRODUCTION**

During a 14-month period, from April 1989 through June 1990, human serosurveys for Crimean-Congo hemorrhagic fever (CCHF) and Hemorrhagic fever with renal syndrome (HFRS) were conducted in several counties of Greece. New cases of HFRS were diagnosed and small mammals were trapped for Hantaan virus isolation

The study on Crimean-Congo hemorrhagic fever virus included identification of new endemic foci, animal serosurvey, collection of ticks for virus isolation, and attempts to diagnose human CCHF cases.

## **B. HEMORRHAGIC FEVER WITH RENAL SYNDROME (HFRS)**

**B1. Human serosurvey.** During the period of April 1989 through June 1990, 1657 blood samples were collected from residents of 14 counties of Greece (Evros, Rodopi, Xanthi, Drama, Serres, Pella, Halkidiki, Kozani, Kastoria, Florina, Arcadia, Thasos island, Hios island, and Iraklio (Krete island)). (Fig. 1, Table 1). Additionally, human serosurveys were also conducted in Gramos and Theodoriana villages where last year (1989) serosurveys were also conducted. One hundred and two blood samples were obtained from the residents of Theodoriana village and 83 from the residents of Gramos village. These two villages were resurveyed in order to investigate human HFRS and CCHF inapparent infections during one year interval. All blood

samples were identified according to age, sex, occupation, previous travel history (mainly abroad) and residence of the donors

Analysis of the epidemiological data on the sex distribution showed that out of 1657 individuals examined 945 were males and 712 females. The occupation and age distribution are shown in Tables 2 and 3 respectively. All human sera collected from April 1989 to June 1990, were kept at  $-20^{\circ}$  C until tested for anti-Hantaan virus antibody by IFA test with goat anti-human fluorescence immunoglobulin. The spot slides contained Vero E-6 cells, approximately 50% of them infected with the 76-118 strain of Hantaan virus prototype. The sera was considered as positive if characteristic cytoplasmatic fluorescence was detected at the 1:16 dilution.

The results of this study which was conducted in 14 counties of Greece are shown in Table 1. Thirty nine seropositives were found in total number of individuals tested, living in all surveyed counties. The highest prevalence of seropositivity (7,4%) occurred in Kastoria county where many HFRS clinical cases were diagnosed in the previous years. For the first time, seropositives were found in islands of Aegean Sea. These islands (Hios and Thasos) are very close to the Turkish mainland (Asia Minor). This finding supports the hypothesis that Hantaan virus is also endemic in Asia Minor, infecting humans.

Like our previous surveys, the current survey shows that male individuals are more frequently infected than females, (ratio 3:1 respectively) and the high risk occupations are wood-cutters, farmers and shepherds (Table 2). However, as it

was mentioned in our previous reports it is difficult to interpret the occupational distribution of the individuals used in our serosurveys because the residents of the small villages in Greece are mainly farmers but occasionally they work as shepherds and wood-cutters.

Comparison of the results obtained from the serosurveys conducted in Theodoriana and Gramos villages in 1989 and 1990 revealed that 3 individuals, two males and one female, had been infected by Hantaan virus during the period 1989-1990 but they did not develop any clinical symptom (Table 0). This finding supports the hypothesis that human inapparent infections by Hantaan virus occur in Greece.

**B2. Disease.** Three hundred-eighteen acute and convalescent-phase blood samples were examined for specific diagnosis of HFRS during the period April 1989 to June 1990. Sixty-seven of the 318 patients had pyrexia of unknown origin, 79 disease resembling HFRS and Leptospirosis, 106 with influenza-like disease, 21 with pyrexia and hemorrhagic manifestations and 45 with pyrexia of unknown origin with elevated liver enzymes (SGOT, SGPT,  $\gamma$ GT) (Table 4). The patients were residents of various parts of Greece and were hospitalized in local hospitals or were referred to the University clinics of Thessaloniki and Ioannina for specific diagnosis and treatment.

Samples of the patient's serum, single or paired, were examined on the day of arrival in the laboratory or kept in  $-20^{\circ}\text{C}$  until tested by indirect immunofluorescence antibody (IFA) test and enzyme-linked immunosorbent assay (ELISA IgM capture) for both IgG and IgM specific to Hantaan virus

antibodies. Sera were tested at two-fold dilutions (initial dilution 1:16) by IFA test with fluorescein-labelled goat anti-human immunoglobulin on spot-slides containing Vero E-6 cells. Approximately 50% of the cells were infected with the 76-118 strain of prototype Hantaan virus. All sera obtained from patients suspected of HFRS and Leptospirosis were also examined by IFA test for both IgG and IgM specific antibodies to Puumala, Urban Rat, and Porogia Viruses.

The diagnosis of HFRS was serologically confirmed in 8 of the examined patients whose clinical findings were in accordance with the symptomatology of the disease. Six of the patients were residents of Ioannina county, 1 of Karditsa county and 1 of Arta county (Fig. 2). The results of the serological diagnosis by both IFA and ELISA IgM capture are shown in Table 5.

All patients developed the severe form of the disease with abrupt onset, high fever, flushing over the face and neck, conjunctival injection, acute abdominal pain, hemorrhagic manifestations and acute renal insufficiency. Seven of the patients survived and one died during the oliguric phase of the disease.

Twenty six out of 67 HFRS patients in Greece were residents of 12 villages in Pindos mountain. This mountain is a kurst in North western Greece and it is a continuation of the Albanian kurst where many HFRS cases occur yearly. According to this observation, it seems that Pindos mountain is a high HFRS endemic area which covers a large part of Western Greece from north to south (Fig. 3).

**B3. Attempts for Hantaviruses isolation.** Thirty nine small mammals were trapped in three selected areas where previously human cases of HFRS had occurred. Antibodies to Hantaan virus were detected by the IFA test in two small mammals of the species *Apodemus flavicolis*. Lung tissues from the 2 seropositive rodents were dissociated with a mechanical blender, and virus isolation was attempted following procedures described in previous annual reports.

The IFA tests detected antibodies to the Hantaan virus in the two rodents with titers 1:32 and 1:64, (Table 7). Spleen and kidney samples of the positive rodents as well as those of the negative rodents, were inoculated in Vero E-6 cells for virus isolation.

Inoculated flasks were incubated for 15 days at 37°C then cells were suspended, passed to fresh flasks, and 10-well spot slides prepared and examined for characteristic hantavirus cytoplasmic fluorescence by using reference antibodies to Hantaan virus. All the prepared spot-slides were negative for Hantavirus antigen. After 15 days, the same procedure was performed and again all the prepared spot-slides when examined were negative for Hantavirus antigen.

### **C. CRIMEAN - CONGO HEMORRHAGIC FEVER (CCHF)**

**C1. Human serosurvey.** During the period April 1989 through Jyne 1990, 1657 human blood samples were collected. These blood samples were the same which were used for HFRS serosuevey.

Additionally, human serosurveys were conducted in Theodoriana and Grammos villages where serosurveys were also conducted one year before. One hundred blood samples were obtained from the residents of Theodoriana village and 87 from the residents of Grammos village (Table 4). All the obtained blood samples were examined by IFA (IgG, IgM) tests. The antigen for the IFA tests was prepared by dropping 50% Vero E-6 cells infected with CCHF virus strain IbAr200 on a 12 circle printed slides and fixing with aceto. The sera were considered as positive if characteristic cytofluorence was detected at dilution 1:4. For the ELISA test, two antigens were used. One, CCHF nucleocapsid antigen, which was prepared in our laboratory, (supernatant of Vero E-6 cells infected with CCHF virus strain IbAr200). The results of the current study which was conducted in 14 counties are shown in Table 2, whereas, the results obtained from Theodoriana and Grammos villages are shown in Table 4. In a total 1657 blood samples taken from the residents of 14 counties where serosurveys were conducted, the highest seropositivity was found in Xanthi (2,9%) and in Kozani (2,6%) counties (Table 1). Analysis of the age and sex distribution of the individuals reveals that the group of 44-50 is at the higher risk (Table 3) and males are more often infected than females, ratio male to female 3:1 respectively. The second serosurvey conducted in Theodoriana and Grammos villages during 1990 revealed that human inaparent infections by CCHF virus were occurred during the one year interval. Two individuals age 45 and 56 years respectively, showed a rise in antibodies to C-CHF virus without any clinical symptom.

**C2. Animal serosurvey.** During the period April 1989 through June 1990, 365 blood samples were obtained from goats and 350 from sheep whose the herds were in different counties of Greece (Table 8).

All the obtained sera were examined by IFA and ELISA tests using the same procedures as in human sera. Antibodies against CCHF virus were found in all counties surveyed. Antibodies against C-CHF virus by both IFA and Elisa tests were found in goats and sheep of all herds which were pastured at the four counties (Table 8). The highest percentage of seropositives (5,3%) was found in Kastoria county where the highest human seropositives was also found in humans during a serosurvey a year ago. According to our results it seems that goats in Greece are more often infected than sheep.

**C3. CCHF Disease.** Two hundred forty-three blood samples (45 single, 148 paired) were collected mainly from patients of CCHF endemic areas (Kastoria, Xanthi, and Evros,). Blood samples were taken from patients with pyrexia of unknown origin (67), from patient with influenza like disease (106), from patients with pyrexia of unknown origin and elevated liver enxymes (SGOT, SGPT), (45), from patients with pyrexia with hemorrhagic manifestations (21) and from patients whose disease was resembling Congo-Crimean hemorrhagic fever (3). All the obtained sera were examined by IFA and ELISA test for serodiagnosis. None of the patients was found to be infected by the CCHF virus.

### **C3. Attempts for virus isolation from ticks.**

#### **a. Tick collection**

Ticks were collected from sheep and goats which were pastured in counties where CCHF human and animal seropositives were found (Grammos, Xanthi, Evros). The identification of the ticks was made by the veterinaries of the Animal Infectious Diseases Department, Veterinary School, Aristotelian University of Thessaloniki. A small number of the collected ticks was also sent for identification to Dr P. Nuttal (Institute of Virology and Environmental Microbiology, Oxford, England). Upon identification, pools were essembled to contain 10 - 15 ticks of the same species (Table 9), labelled with the date and place of the collection. The pools were stored at -70°C until used. For virus isolation 50% of the pooled ticks were used for virus isolation and the remaining 50% were stored in -70.

#### **b. Virus isolation**

Pooled ticks were ground in a mortar in PBS buffer (PH 7.2, 1% bovine serum albumin (Faction V), 1% penicillin/streptomycin) to make a 10% suspension. After certifugation at low speed the supernatant was inoculated in Vero E-6 cells for virus isolation. Seven days leter, spot-slides were prepared from the inoculetel Vero E-6 cells and IFA were performed using mouse positive serum for the detection of CCHF virus. Todate no virus has been isolated from the collected ticks. It seems that Vero E-6 cells are not sensitive for primary isolations of CCHF virus. Attempts have been made for CCHF isolation from the rest of the collected ticks in the SW14 cell line but the results were also negative. Suckling

mice were not used for isolation of CCHF virus because a P4 containment facility is not available in our laboratory.

#### **D. COMMENTS**

**D1.** Hemorrhagic fever with renal syndrome is endemic in Greece. Thus far serologically confirmed cases have been occurred in many counties specially of Northern Greece. According to our data, a hyperendemic HFRS area exists. This area is the Hellenic Kurst which is a continuation of the Albanian one. Human cases of HFRS occur yearly in the Hellenic Kurst. These cases can be appear as sporadic cases and / or as outbreaks. Almost one third of the HFRS cases which were diagnosed in Greece occurred in small villages of the Hellenic kurst. (Figure 3).

**D2.** Antibodies against CCHF virus in humans were found in residents of 12 out of 14 counties surveyed. In contrast, thus far, no human disease was serologically diagnosed. Serosurvey conducted in Theodoriana and Grammos villages in 1989 revealed that in these villages the percentage of seropositive individuals was very high. A second survey which was conducted in 1990 in the two villages, showed that in an interval of 12 months, an individual was infected by C-CHF virus in 1989 his antibody titer to C-CHF virus was <1:4 where as, in 1990, one year after, his antibody titer was 1:128. This person had no recollection of an illness clinically resembling a serious type of Crimean hemorrhagic fever. This finding suggests that C-CHF human infections occur in Greece but the infection does not result on serious disease or even more the infection could be inaparent.

This may be due to the fact that the C-CHF virus which exists in Greece (AP 92 strain) is not a hot C-CHF virus strain. Studies done by Jonathan Smith in Fort Detrick, showed on molecular basis that the Greek CCHF (AP92) strain has antigenic differences from other hot C-CHF strains.

**E. Publication from April 1989 through June 1990**

1. Seroepidemiological survey for antibodies to Arboviruses in Greece. (1990)

A. Antoniadis, S. Alexiou-Daniel, N. Malisiovas, J. Doutsos, Th. Polyzoni, J.W. LeDuc, C.J. Peters and G. Saviolakis. Arch. of Virology [Suppl. 1]: 277-285.

Table 1 Antibody to Hantaan and C-CHF viruses in health residents of Greece: geographic distribution.

County	Total tested	Hantaan		C-CHF	
		No of positives	% positives	No of positives	% positives
Evros	45	2	(4,4%)	1	(2,2%)
Rodopi	266	3	(1,2%)	4	(1,5%)
Xanthi	68	1	(1,4%)	2	(2,9%)
Drama	416	2	(0,4%)	4	(0,9%)
Serres	35	0	(0,0%)	0	(0,0%)
Pella	173	2	(1,1%)	3	(1,7%)
Halkidiki	99	1	(1,0%)	1	(1,0%)
Kozani	76	3	(3,9%)	2	(2,6%)
Kastoria	85	6	(7,0%)	2	(2,3%)
Florina	122	7	(5,7%)	3	(1,6%)
Arcadia	98	1	(1,0%)	1	(1,0%)
Chios	71	1	(1,4%)	0	(0,0%)
Thasos	92	1	(1,0%)	1	(1,0%)
Iraklio	41	1	(2,4%)	0	(0,0%)
Total	1679				

**Table 2 Occupation distribution of residents who were infected by Hantaan and C-CHF viruses.**

Occupation	No of sera tested	No positives	Hantaan positives	C-CHF
Farmers		978	6	2
Wood-cutters		116	10	2
Farmers and wood-cutters		205	10	9
Sheperds		32	2	4
Farmers and sheperds		216	2	6
Others		110	1	1
Total		1657	31	24

**Table 3: Antibody to Hantaan and Crimean-Congo hemorrhagic fever viruses in residents of Greece by age. (Survey conducted from April 1989 through June 1990).**

Age (years)	No of Sera examined	Hantaan no (%)	C-CHF no (%)
0-10	75	1 (1,3%)	0 (0%)
11-20	193	0 (0%)	1 (1%)
21-30	226	3 (1,3%)	1 (1,3%)
31-40	252	9 (3,5%)	4 (1,5%)
41-50	265	8 (3%)	7 (2,6%)
51-60	308	5 (1,6%)	9 (2,9%)
61-70	296	3 (1%)	1 (0,3%)
> 70	42	2 (4,7%)	1 (4,7%)
Total	1657	31	24

**Table 4 Results of the first and second serosurvey conducted in Theodoriana and Grammos villages during 1989 and 1990 respectively.**

County - Village	No of tested individuals		Hantaan No of positives		C-CHF No of positives	
	1989	1990	1989	1990	1989	1990
Arta - Theodoriana	114	101	19	16	5	4
Kastoria - Grammos	86	87	15	14	13	12

**Table 5 Serologically confirmed HFRS cases from April 1988 through June 1990.**

Syndrome	No of patients examined	HFRS cases	Deaths
Pyrexia of unknown origin.	67	0	
HFRS and Leptospirosis like disease.	79	7	1
Influenza like disease.	106	1	
Pyrexia with hemorrhagic manifestations.	21	0	
Pyrexia of unknown origin with elevated liver enzymes.	45	0	
<b>Total</b>	<b>318</b>	<b>8</b>	

**Table 6 Antibodies to Hantaan virus in 8 patients with HFRS (IFA and ELISA-IgM tests).**

Patients Cod No	Day of illness	IFA titers		ELISA titers
		IgG	IGM	IgM
131	6	1:4096	1:16384	1:102200
216	7	1:4096	1:8192	1:102200
230	5	1:2048	1:16384	1:204000
283	7	1:2048	1:4096	1:51100
283	8	1:8192	1:8192	1:102200
313	5	1:4096	1:16384	?
451	6	1:8192	1:4096	1:408800
687	9	?	?	1:817600

\* Strongly backround in the negative control dilutions.

\*\* Non-specific fluorescence in the negative control dilutions.

**Table 7 Small mammals captured near Grammos village.**

Species	No of trapped mammals	No of positives	IFA titer
Apodemus flavicolis	11	2	1:64, 1:64
Apodemus sylvaticus	10	0	
Mus musculus (in HFRS patients house)	15	0	
Unidentified	3	0	
<b>Total</b>	<b>39</b>	<b>2</b>	

**Table 8 Antibodies to C-CHF virus in goats and sheep pastured in 4 counties of Greece.**

County	No of goats examined	Positives		No of sheep examined	Positives	
		IFA	ELISA		IFA	ELISA
Kastoria (Grammos village)	112	6	8	65	4	5
Evros	82	4	4	95	2	3
Rodopi	116	5	7	103	2	4
Xanthi	55	2	3	87	2	3
<b>Total</b>	<b>365</b>	<b>17</b>	<b>22</b>	<b>350</b>	<b>10</b>	<b>15</b>

**Table 9 Tick species collected from April 1989 through June 1990.**

Tick species	No of pools	Animal species
Phipisephalus bursa	11	goat
Phipicephalus sanguineus	1	goat
Ixodes gibbosus	13	goat - sheep
Hyalomma sp.	9	goat - sheep
Unidentified	10	goat - sheep

Fig. 2: Counties surveyed for antibodies to Hantaan and OCHF viruses

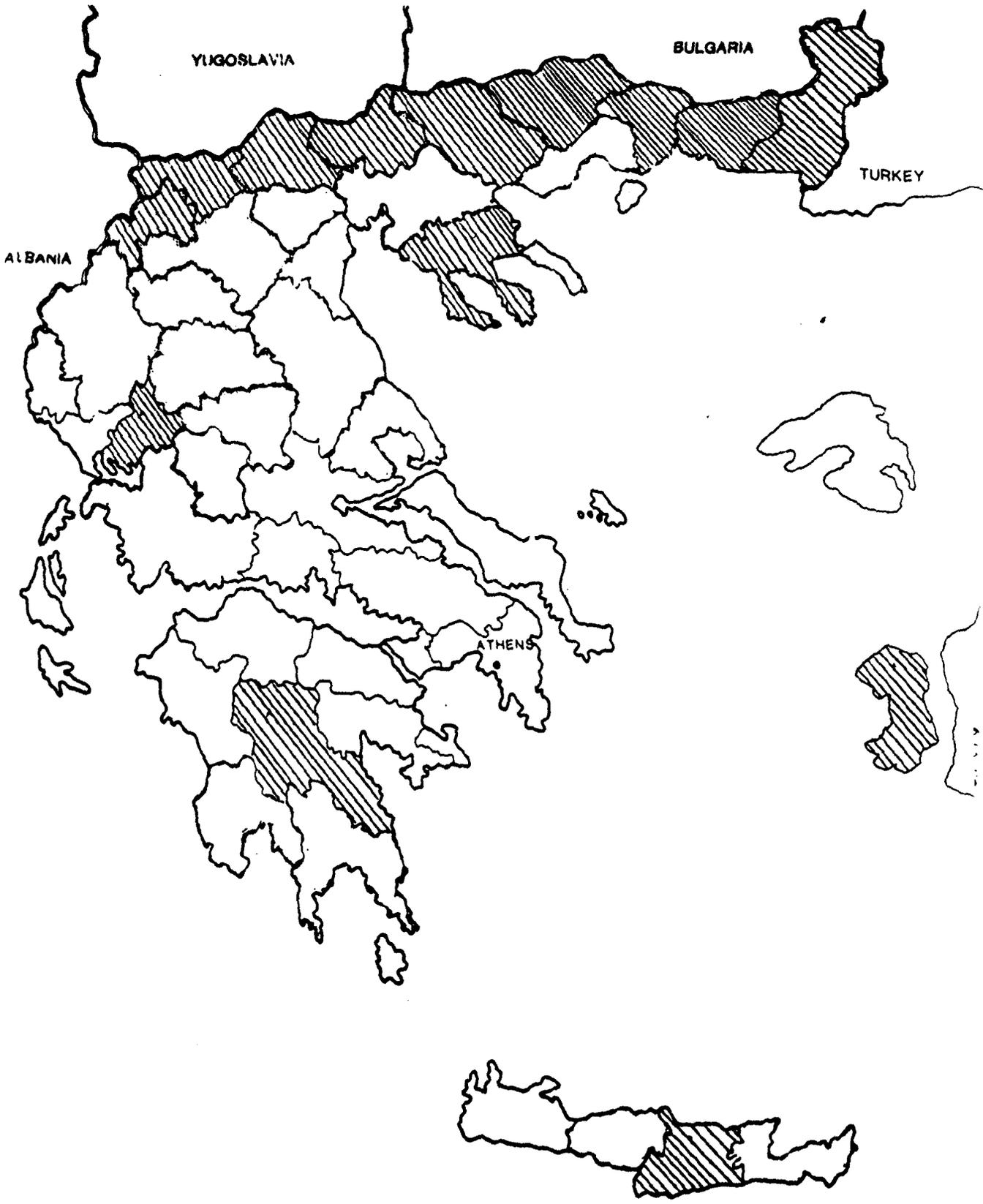
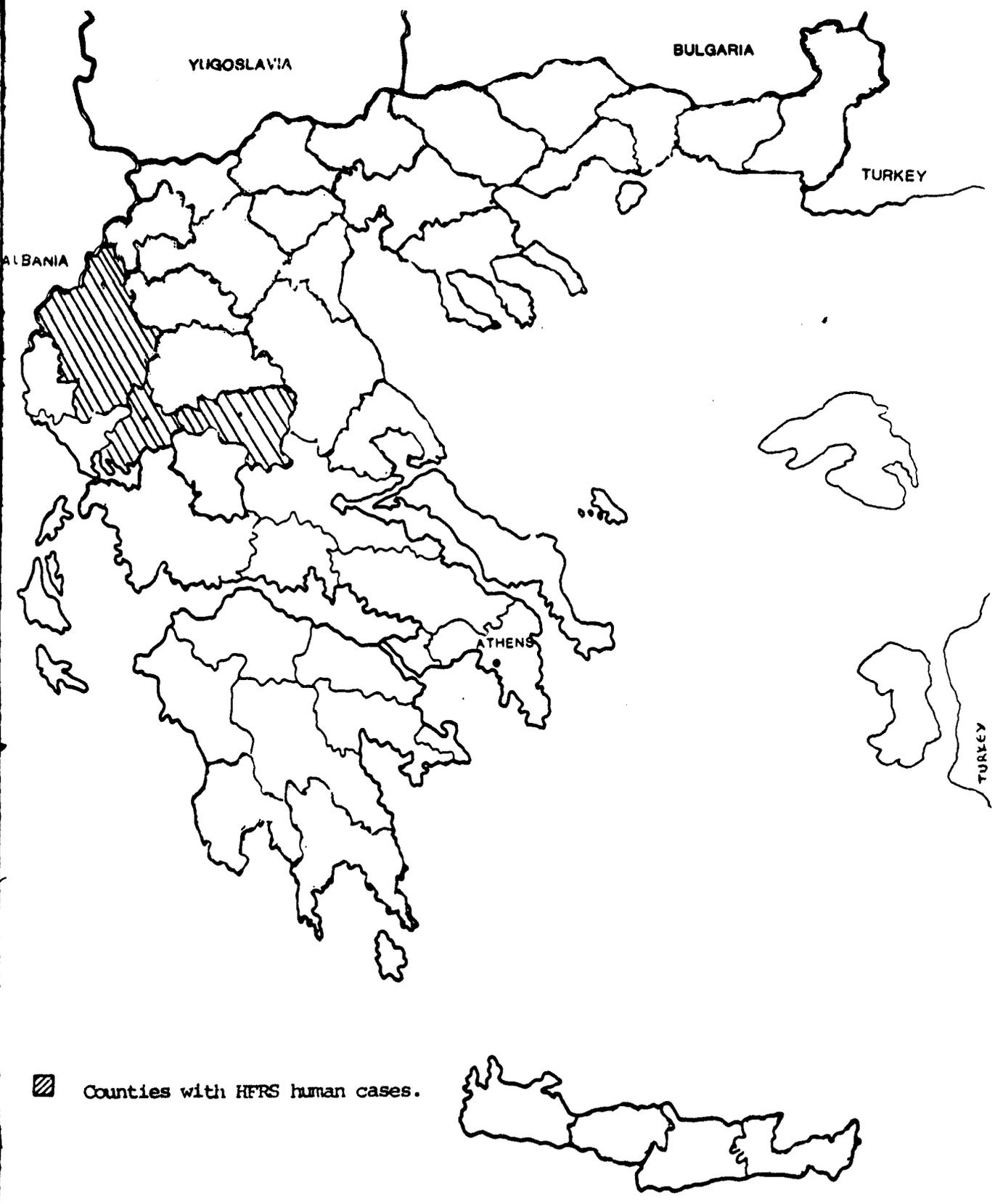


Fig. 2 : Map of Greece indicating the three counties where human HFRS cases were occurred.



▨ Counties with HFRS human cases.

Fig. 3: GEOGRAPHIC DISTRIBUTION OF HFRS IN THE HELLENIC KURST

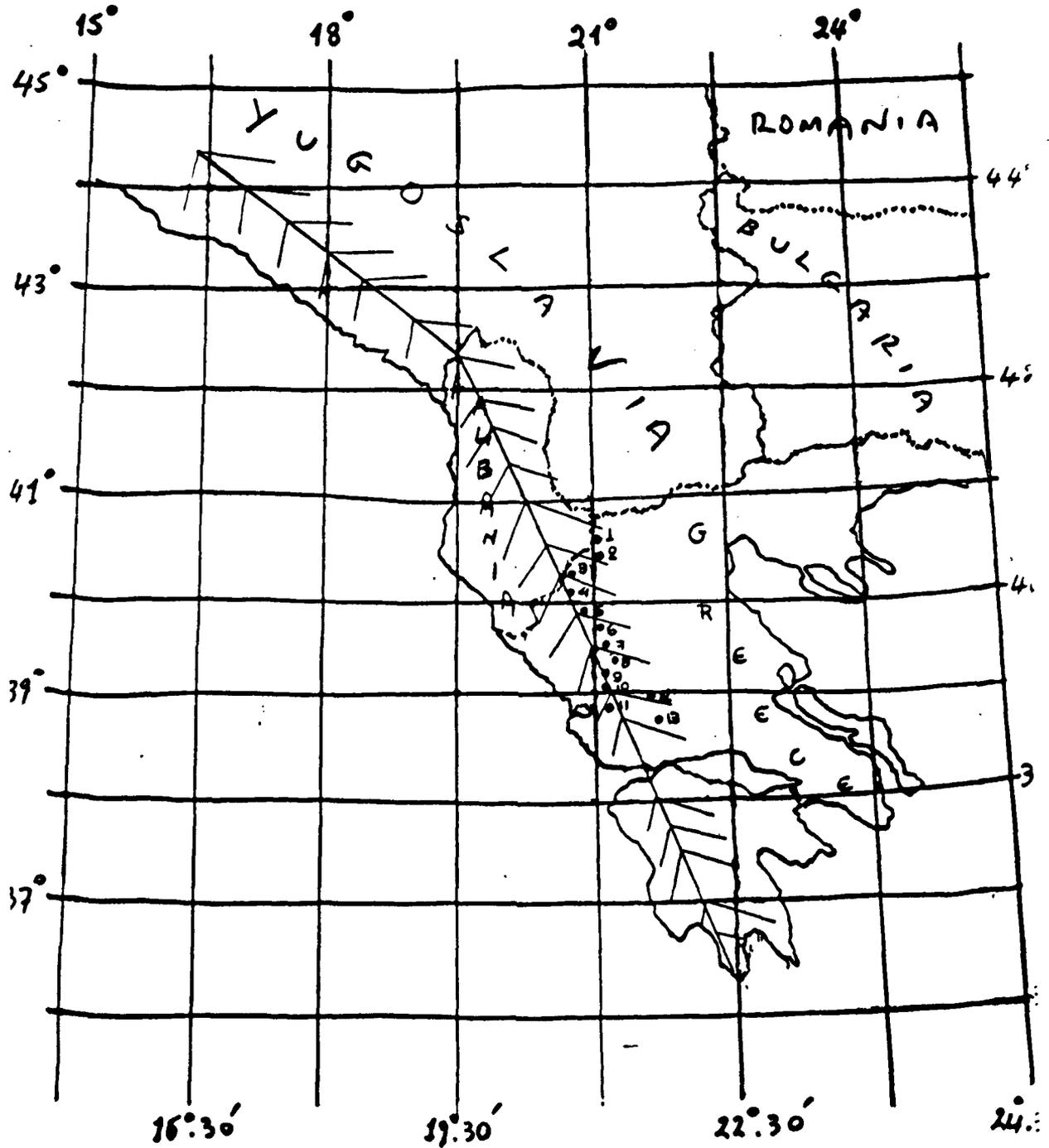


Fig. 3: GEOGRAPHIC DISTRIBUTION OF HFRS IN THE HELLENIC KURST

