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INVESTIGATION OF CRIMEAN-CONGO HEMORRHAGIC FEVER AND HEMORRHAGIC FEVER WITH RENAL SYNDROME IN GREECE

Annual Report

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During a 12-month period, from April 1988 through April 1989, human serosurveys for Crimean-Congo hemorrhagic fever (CCHF) and Hemorrhagic Fever with Renal Syndrome (HFRS) were conducted in sereral counties of Greece. New cases of HFRS were diagnosed, the ELISA IgM capture method was applied for the early diagnosis of the disease 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 the disease. (cyn. b. Disease distribution; Disease Vectors)

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TABLE OF CONTENTS

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 12-month period, from April 1988 through April 1989, human serosurveys for Crimean-Congo hemorrhagic fever (CCHF) and Hemorrhagic Fever with Renal Syndrome (HFRS) were conducted in sereral counties of Greece. New cases of HFRS were diagnosed, the ELISA IgM capture method was applied for the early diagnosis of the disease 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 the disease.

B. HEMORRHAGIC FEVER WITH RENAL SYNDROME (HFRS)

B1. Human serosurvey.

During the period, April 1988 through April 1989, 630 blood samples were collected from residents of 6 counties of Greece (Rodopi, Xanthi, Kilkis, Ioannina, Trikala, and Arcadia) (Fig 1). Rodopi, Xanthi and Kilkis counties were selected against for the serosurvey because of their proximity to the Greek-Bulgarian borders, and loannina county because of its proximity to Greek-Albanian borders. The Bulgarian Rodopi area as well as the Albanian Epirus area, are highly endemic areas for HFRS and CCHF as reported by Bulgarian and Albanian scientists. In contrast, previous serosurveys and attempts to diagnose HFRS cases showed that Rodopi and Xanthi areas are not HFRS endemic areas. Additionaly, human serosurveys were also conducted in Gramos village (Kastoria county) and in Theodoriana village (Arta county) where HFRS cases were occured (Fig 1). One hundred fourteen blood samples were obtained from the residents of Theodoriana village and 86 from the residents of Gramos village.

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 830 examined individuals 475 were males and 355 females. The occupational distributions is shown in Table 1. Of 830 individuals, 446 were farmers, 83 wood-cutters, 66 farmers and occasionaly wood--cutters, 199 shepherds, and 36 farmers and occasionaly shepherds.

All human sera collected from April 1988 to April 1989, were Kept

at -20°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 were considered as positive if characteristic cytoplasmic fluorescence was detected at the 1:16 dilution.

In previous serosurvens conducted in Greece, between 1982 and 1988, a total number of 3,968 human sera were examined by IFA test. The overall antiboly prevalence rate was 4% with a range 0 to 14%. The highest percentage occured in areas where clinical cases of the disease were diagnosed. Moreover, seropositives were detected in 24 out of 26 counties (in total of 54 Greek counties) where serosurveys were conducted.

Analysis of the data showed that the males are more trequently infected than females, and that the most common groups infected are between 31 to 60 years.

The results of the current study which was conducted in five counties are shown in Tables 2 and 3. Nineteen seropositives were found in total, living in 5 counties. The highest prevelance of seropositivity (4,5%) occured in loannina county where most of the HFRS clinical cases were diagnosed. For the first time, seropositives were found in Arcadia county, which is in Peliponisos (South Greece). This finding supports the hypothesis that Hantaan virus is pread all over Greece. Male individuals are more frequently infected than females, (ration 3:1 respectively) and the high risk occupations are wood-cutters, farmers and shepherds (Table 1). Howerer, it is difficult to interpret the occupational distribution of the individuals used in this serosurvey. The residents of small villages are mainly farmers but occasionaly then work also as shepherds and woodcutters.

The results obtained from serosurveys conducted in Theodoriana and Gramos villages, where human HFRS cases were occured, are shown in Tables 4, 5 and E. Nineteen seropositives (17,4%) and 15 seropositives (17,4%) were found in a total of 114 (Theodoriana village) and 86 (Gramos village) individuals, respectevely. These prevelances of seropositivity are the highest which were found so far in Greece. Male individuals are more frequently infected than female. In a total of 19 infected individuals in Theodoriana village, 14 were male and 5 female (ratio male to female 3:1 respectively). The age group at high risk is 41 to 60 years and farmers are more frequently infected than shepherds.

B2. Disease

Acute and convalescent-phase sera were collected during the period April 1988 to April 1989, from 376 patients whose illness was clinically diagnosed as either HFRS or leptospirosis or pyrexia of unknown origin or pyrexia of unknown origin with elevated liver enzymes (SGOT, SGPT) and from 11 patients with pyrexia and hemorrhagic manifestations (Table 7). The patients were residents of various parts of Greece and were hospitalized in local hospitals or refered to the University clinics of Thessaloniki and Ioannina for specialized 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^{o}C untill tested by indirect immunofluorescence antibody (IFA) test for both IgG and IgM specific to Hantaan virus antibodies. All sera taken from patients suspected of having HFRS were also examined by enzyme-linked immunosorbent assay

(ELISA IgM capture) for the detection of specific IgM antibodies to Hantaan virus. Sera were test 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. The method IgM ELISA capture was described elsewhere (Anual Report 1987-1988). Also all the sera obtained from patients suspected of HFRS and leptospirosis were examined by IFA test for both IgG and IgM specific andibodies to Puumala, Urban Rat and Porogia viruses.

The medical records of patients with serologically confirmed HFRS were also reviewed. The diagnosis of HFRS was serologically confirment in five of the examined patients whose clinical findings were in accordance with the symptomatology of the disease. Two of the patients was residents of Arta county, 2 of Kastoria county and 1 of Trikala county (Fig 2). The results of the serological diagnosis by both IFA and ELISA IgM capture tests are shown in Tables 8. In all patients the disease was severe with abrupt unset, fever, flushing over the face and neck, confuctival injection acute abdominal pain, vomiting, hemorrhagic manifestations and acute renal insufficiency. Four of the patients survived and one died during the diguric phase of the disease.

B3.1. Attempts at Hantaan virus isolation from captured rodents.

Two areas (Gramos and Theodoriana) were selected as field work sites for trapping rodents. In these areas HFRS cases were occured during 1988 through 1989. Small mammal traps were set up within and around

huts and in fields and forests surrounding the huts. Five hundred traps were used in Gramos area (for four days) and also the same number of traps were used in Theodoriana area for four days. Only 9 small mammals were trapped and all of them in the area of Theodoriana. The small number of the live-trapped rodents was due to the previous extermination which was conducted in these areas by the shepherds and farmers using rat-poison.

The IFA test showed only one rodent with antibodies to Hantaan virus (titer 1:32), (Table 9). Spleen, lungs and kidnen samples of the positive roodent 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-slids were negative for Hantavirus antigen. After 15 days, the same procedure was performed and again all the prepared spot-slids when examined were negative for Hantavirus antigen. The small number of the trapped rodents was due to the use of rat-poison for the extermination of the rodents in the area just after the information that a lethal disease is endemic in these areas. Unfortunately, the residents of these areas did not reveal this information during our field work in these areas.

C. CRIMEAN - CONGO HEMORRHAGIC FEVER (CCHF)

C1. Human serosurvey

During the period April 1988 through April 1989, 630 human blood samples were collected. These blood samples were the same one which were used. Additionaly, human serosurvens were also conducted in Gramos village and Theodoriana village. One hundred fourteen blood samples were obtained from the residents of Theodoriana village and 86 from the residents of Gramos village (Fig. 4). All the obtained blood samples were examined by IFA (IgG, IgM) and ELISA (IgG, IgM) tests. the antigen for the IFA tests was prepared by dropping 50% Vero G-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 cheracteristic cyto-fluorescence was detected at dilution 1:4. For the ELISA test, two antigens were used. One, CCHF nucleocapsid antigen, which was provided by Dr J. Smith (USAMIID, Ford Detrick) and another, which was prepared in our laboratory, which was supernantent of Vero E-6 cells infected with CCHF virus strain IbAr200. The results of the current study which was conducted in five counties are shown in Table 1, whereas, the results obtained from Theodoriana and Gramos village are shown in Table 4. In total 630 blood sampls taken from the residents of 5 counties, 7 (1,1%) were seropositives living in 4 out of 5 counties where serosurveys were conducted. The highest seropositivity was found in Kilkis (3,9%) and in Ioannina (4,5%) counties (Fig. 4). Analysis of the age and sex distribution of the infected individuals reveals that the age group of 41-60 is at the

higher risk (Table 3) and males are more aften infected than females, ratio male to female 2:1 respectively. Serosurveys conducted in Theodoriana and Gramos villages revealed that Gramos village is a high endemic area of CCHF virus and the seropositivity is this area was found 15,1% and is the highest seropositivity in Greece (Table 4). This area, Gramos, is a mountainous area, (Kurst) close to Greek-Ablanian borders in an altitute of 2300m. The Kurst of this area has the same enviromental conditions with the Albanian one, which is a endemic CCHF area.

Analysis of the age and sex distribution of this area revealed that young indeviduals aged 10 to 30 are infected by the virus whereas the high risk age group is 51 to 60 (Table 6). Males are more often infected than females, ratio male to female 2:1 respectively. The IFA and ELISA titers of seropositives are shown a in Table 10. It is the first time, since 1982 where such high titers were found in humans against CCHF virus. All the postitive and negative sera when examined by IFA and ELISA test for the detection of specific IgM antibodies against CCHF virus were negative. The results obtained from the serosurvey conducted in Theodoriana village are shown in Table 4. In this are the seropositivity was found 4,2% and analysis of the age distribution showed that young individuals aged 11 to 20 are infected by the virus whereas the high risk age group is 51 to 60 (Table 5). Again, it was found that males were more often infected than females, ratio males: females 2:1 respectively.

C2. Animal serosurvey

During the period April 1988 through April 1989, 515 blood samples were obtained from goats whose the herds were in different counties of

Greece (Fig. 5)

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 Drama county (close to Greek-Bulgarian borders), Lesbos island (close to Turkey), Kozani county (close to Greek-Jugoslavian borders) and in Attiki county. It is very interesting that in Drama county, which is bordering Bulgaria, 151 blood samples were obtaind from 7 herds which were pastured at seven different areas of the county. Surprising, antibodies against CCHF virus were found only in one herd. In a total of 24 examined goats of this herd, 4 (16,5%) were found serpopositives. This herd was pastured very close to Bulgarian borders.

C3. CCHF Disease

Four hundred eleven blood samples (223 single, 173 paird) were collected from patients of CCHF endemic areas (Ioannina, Kastoria, Veria and Halkidiki) as well as from hospitals close to Bulgarian borders (Drama, Xanthi, Rodopi).

These blood samples were taken from patients with disease resembling CCHF (25), from patients with pyrexia of unknown origin (113), from patient with influenza like disease (181) from patients with pyrexia of unknown origin and elevated liver enzymes (SGOT, SGPT), (85) and from patients with pyrexia with hemorrhagic manifestations (7). All the obtained sera were examaned 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 (Kastoria, Drama, Arta). The identification of the ticks was made by the veterinatians 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 Visology and Enviromental Microbiology, Oxford, England). Upon identification, pools were essembled to contain 10-15 ticks of the same species (Table 12), labelled with the date and place of the collection. The pools were stored at -70^{O}C untill used. For virus isolation, 75% of the pooled ticks were used where at the 25% was stored in -70%.

b. Virus isolation

Pooled ticks were ground in a mortar and PBS buffer pH 7.2 enriched with 1% bovine serum albumin (Faction V) and penicillin/streptomycin 1% was added to make up a 10% suspension. After centrifugation at low speed the supernantant was inoculated in Vero G-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. To date no virus has been isolated from the collected ticks. If seens that the Vero E-6 cells are not sensitive for primary isolations of CCHF virus. Attempts will be made to isolate the virus from the rest of the collected ticks using another cell line i.e SW14. We avoid to use suckling mice for isolation of CCHF virus because we have not a P4 contaminant.

D. COMMENTS

It is difficult to interpret the significance in antibody prevalence by occupation because the residents of small villages are mainly farmers but sometimes work as shepherds and wood cutters. However, according to our data, it appears that farmers and wood cutters are at increase risk to infection with Hantaan and C-CHF viruses, whereas shepherds are more likely to acquire C-CHF virus than in general population. Thus far, our data do not allow us to determine the public health importance. Demonstration of antibodies in humans indicates previous viral infection but does not indicate past illness. It is obvious that a C-CHF virus or an antigemically close related to C-CHF virus, is endemic in Greece and infects humans. The high antibody prevalene in humans in certain areas as well as the antibody relative high prevalence in animals support this hypothesis. Also, the anti-CCHF antibody IFA titers in humans were found very high (1:512) specially in areas bordering south Albania where CCHF virus is endemic and severe human cases occur annualy. In contrast, the greek serospositive persons had no recollection of an illness clinically resembling a serious type of Crimean-Congo hemorrhagir fever. During April 1988 through April 1989, blood samples from patients living in high CCHF greek endemic areas bordering south Albania were sent to our laboratory for serodiagnosis. These patients had disease resembling CCHF or influenza like disease, pyrexia with elevated liver enzymes (SGOT, SGPT) and pyrexia with unknown origin. None of these patients was found to be infected with CCHF virus. All our data, suggest that the antibodies

detected in greek humans present infection with a C-CHF virus or with a virus antigenically related to it.

E. Publication from April 1988 through April 1989

1. Seroepidemiological survey for antibodies to Arboviruses in Greece. A. Antoniadis, S. Alexiou-Daniel, N. Malisiovas, J. Doutsos, Th. Polyzoni, J.W. LeDuc, C.J. Peters and G. Saviolakis. Arch. of Virology (in press).

Table 1: Occupational distribution of the infected individuals by Hantaan and C-CHF viruses.

Occupation	Five counties survay*	Gramos	Theodoriana	
Farmers	402	11	33	446
Wood-cutters	83			83
Farmers and Wood-cutters	66			66
Shepherds	43	75	81	199
Farmers and shepherds	36			36
TOTAL	630	86	114	830

*Rodopi, Xanthi, Kilkis, Ioannina and Arcadia counties

Table 2: Geographic distribution of individuals infected by Hantaan and C-CHF viruses.

County	No of tested individuals	Hantaan positives	C-CHF positives
Rodopi	119	2	1
Xanthi	115	3	1
Kilkis	102	4	2
Kastoria (Gramos)	86	15	13
Ioannina	198	9	3
Arta (Theodoriana)	114	19	5
Arcadia	96	1	0
	830	53(6.3%)	25(3.0%)

Table 3: Age distribution of Hantaan and C-CHF positive individuals.

Age	No of tested individuals	Hantaan	C-CHF
0-10	38	0	0
11-20	56	0	0
21-30	94	1	0
31-40	71	2	1
41-50	115	5	2
51-60	121	6	2
61-70	117	3	1
>70	18	2	1

Table 4: Results of serosurveys conducted in areas where human HFRS cases were diagnosed

County-village	No of tested individuals	Hantaan positives	C-CHF positives
Arta-Theodoriana	114	19	5
Kastoria-Gramos	86	15	13

Table 5: Age distribution of infected individuals in Theodoriana village

Age	No of tested individuals	Hantaan positives	C-CHF positives
0-10	11	0	0
11-20	27	0	1
21-30	10	1	0
31-40	4	1	0
41-50	14	4	0
51-60	23	10	4
61-70	18	2	0
>70	7	1	0
TOTAL	114	19(16.1%)	5(4.2%)

Table 6: Age distribution of infected individuals in Gramos village

Age	No of tested individuals	Hantaan positives	C-CHF positives
0-10	8	0	0
11-20	18	0	1
21-30	10	1	1
31-40	6	1	0
41-50	10	3	3
51-60	17	8	6
61-70	13	1	1
>70	4	1	1
TOTAL	86	15(17.4%)	13(15.1%)

Table 7: Serologically comfirmed HFRS cases from April 1988 through April 1989.

Syndrome	No of patient examined	HFRS cases	Deaths
Pyrexia of unknown origin	196	0	0
HFRS and Lepto-spirosis like disease	89	5	1
Influenza like diseases	95		
Pyrexia with hemorrhagic manifestation	18	0	0
Pyrexia of unknown origin with elevated liver enzymes	56	0	0
TOTALS	454	5	1

Table 8: Antibodies to Hantaan virus in 5 patients with HFRS (IFA and ELISA tests).

Patients' cod No	Day of illness	IFA titers IgG	IFA titers IgM	ELISA titers IgM
1	6	1:2048	1:8192	1:51100
2	8	1:8192	1:16384	1:102200
3	7	1:4096	1:8192	1:51100
4	5	1:2048	1:4096	1:102200
5	10	1:8192	1:16384	1:817600

Table 9: Small mammals captured near Theodoriana village (Arta county).

Species	No of trapped	No of positives	IFA titer
Apodemus flavicollis	5	1	1:64
Apodemus sylvaticus	2	0	-
Unidentified	2	0	-

Table 10: IFA titers of CCHF positive individuals living in Theodoriana and Gramos villages.

Cod. No	Village	Age	IFA titer (IgG)*
12	Theodoriana	60	1:8
27	Theodoriana	20	1:16
29	Theodoriana	53	1:4
78	Theodoriana	58	1:128
86	Theodoriana	59	1:16
118	Gramos	43	1:8
120	Gramos	52	1:16
124	Gramos	50	1:64
128	Gramos	18	1:64
138	Gramos	55	1:8
140	Gramos	64	1:4
147	Gramos	60	1:16
148	Gramos	51	1:512
160	Gramos	47	1:128
163	Gramos	73	1:32
170	Gramos	58	1:128
173	Gramos	26	1:256
191	Gramos	55	1:128

* All sera were examined by IFA and ELISA tests for the detection of specific IgM antibodies. None of them was found positive.

Table 11: Antibodies to C-CHF virus in animals pastured in 10 counties of Greece.

County	No of goats examined	No of seropositives IFA	No of seropositives ELISA
Pella	46	0	0
Chania	57	0	0
Kilkis	35	0	0
Katerini	22	0	0
Thessaloniki	81	0	0
Thasos island	26	0	0
Drama	151*	4	5
Attiki	34	1	1
Lesbos island	38	8	10
Kozani	25	2	2
TOTAL	515	15	18

Table 12: Tick species collected from April 1988 through April 1989.

Tick species	No of Pools	Animal species
Rhipicephalus sanguineus	2	Goat
Rhipicephalus bursa	8	Goat
Ixodes gibbosus	11	Goat-sheep
Hyalomma ana. anetolicum	10	Goat-sheep
Unindentified	9	Goat-sheep
TOTAL	40	

Fig. 1: Counties where serosurveys were conducted for antibodies to Hantaan virus during April 1988 through April 1989.

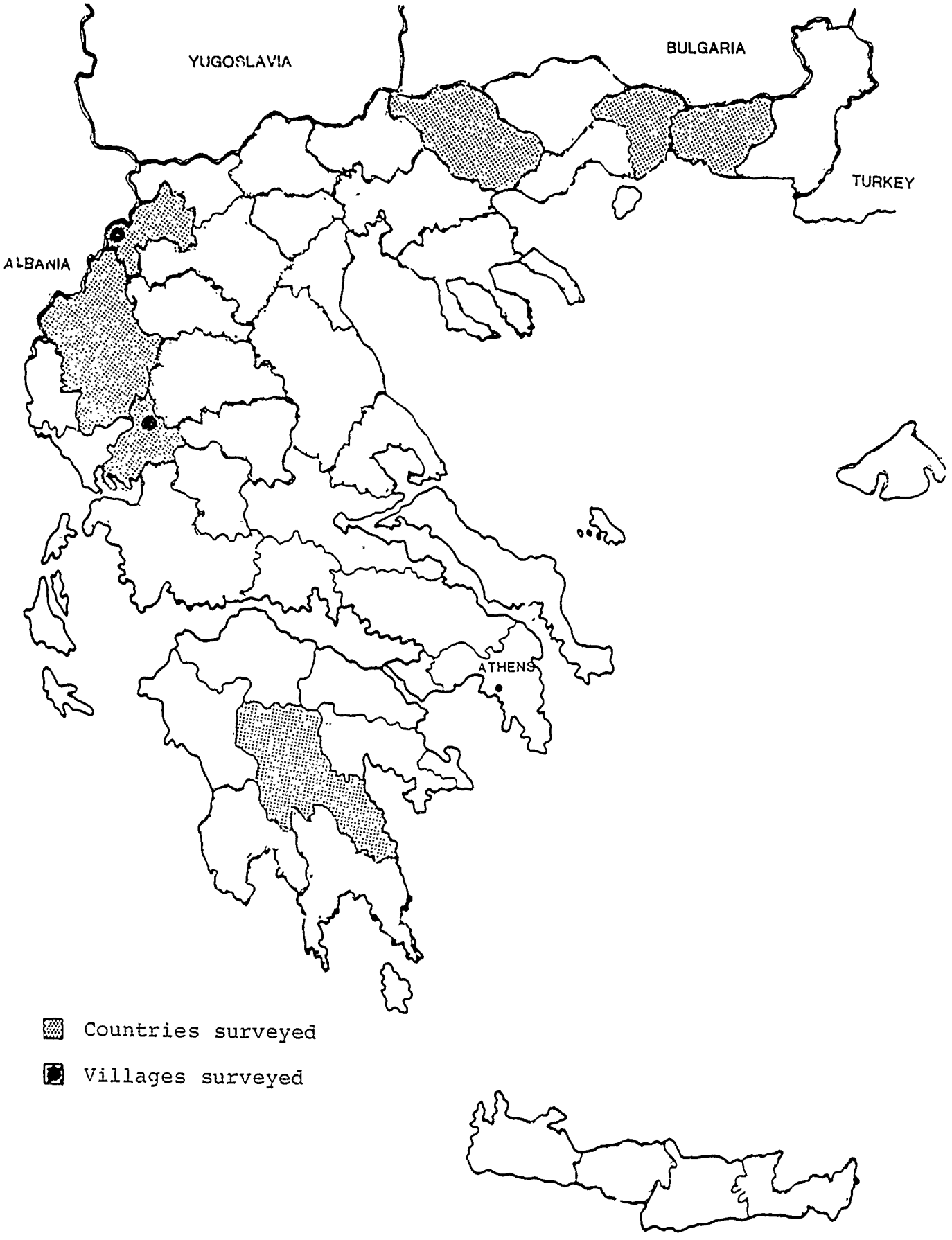

Fig. 2: Counties where HFRS cases were occured during April 1988 through April 1989

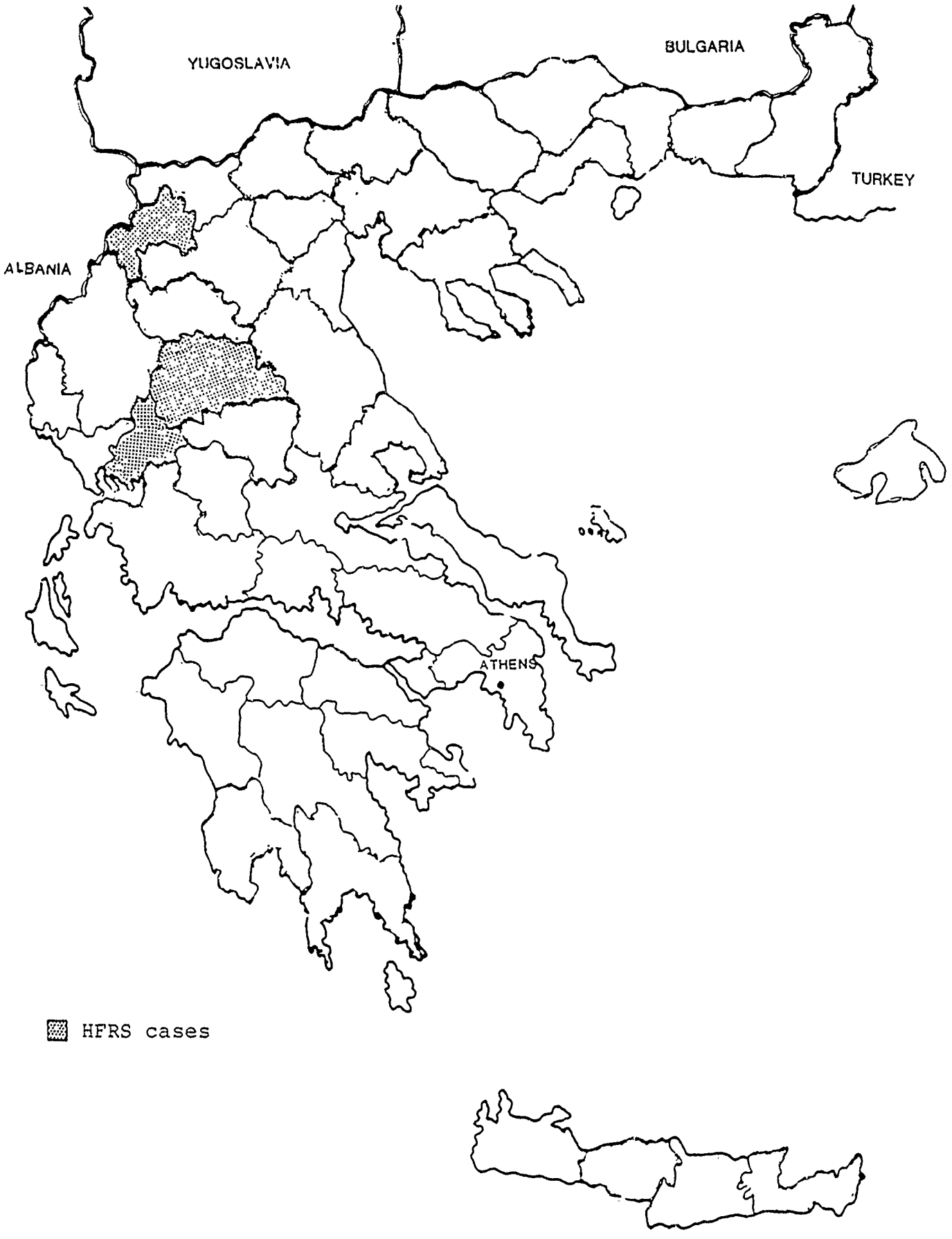

Fig. 3: Counties where field-work was condudted for live small mammals trapping

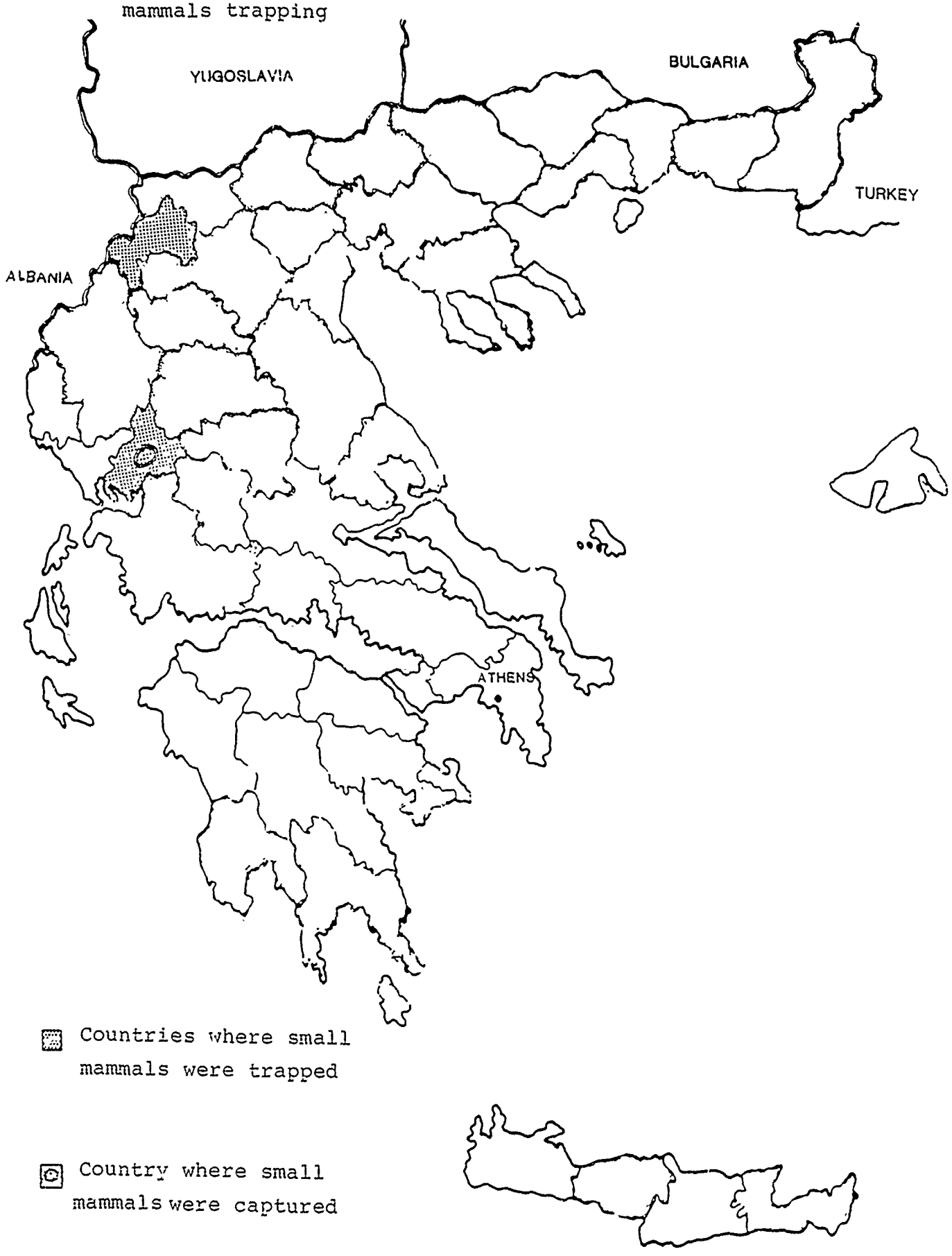

Fig. 4: Counties where human serosurveys were conducted for antibodies to C-CHF virus during April 1988 through April 1989.

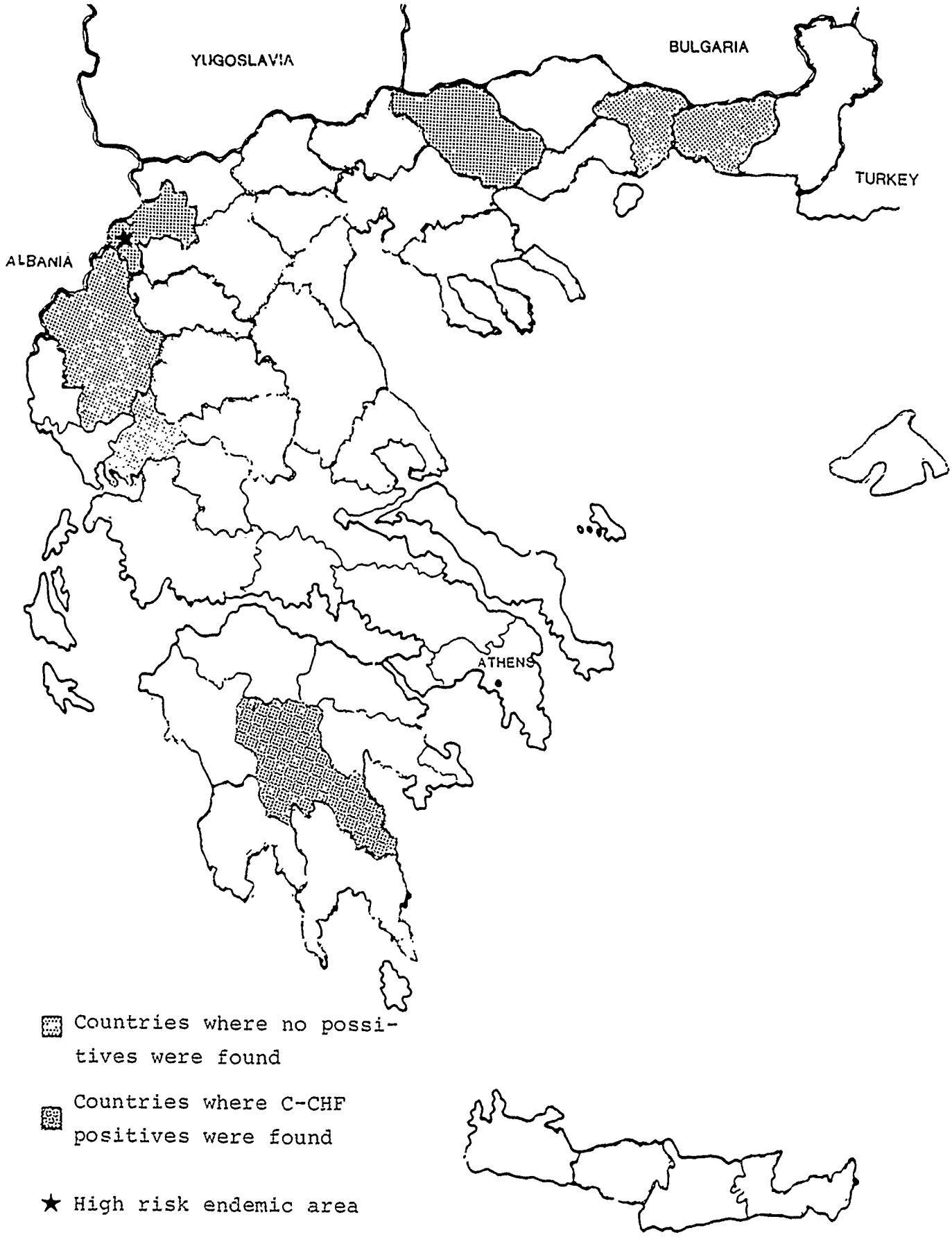

Fig.5: Animal serosurveys for antibodies to C-CHF virus conducted in 10 counties of Greece duting April 1988 through April 1989