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SEROEPIDEMIOLOGICAL SURVEY FOR CONGO-CRIMEAN HEMMORRHAGIC FEVER AND HANTAAN VIRUS

Final Report

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Serosurveys of discrete populations were conducted using immunofluorescence assays and enzyme-linked immunosorbent (ELISA) assays designed to detect antibodies to Crimean-Congo hemorrhagic fever (CCHF) virus and Hantaan-like viruses. Clinical cases of hemorrhagic fever with renal syndrome (HFRS) were confirmed using these assays. Individuals positive for CCHF were identified but no clinical cases were found. Hantaan-like viruses, the causative agents of HFRS, were isolated from various rodent species and their characterization undertaken. Attempts were made to isolate virus from humans.

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A. HANTAAN VIRUS

1. Patients and Disease

One hundred and eighty male and female farmers, shepherds and woodcutters were admitted to various General Hospitals of Thessaloniki and other General Hospitals located in the county capitals, with clinical diagnoses of leptospirosis, acute nephritis, or acute renal insufficiency. The diagnosis of HFRS was serologically confirmed in 28 of these patients by rising antibody titers (IgM and IgG) to Hantaan virus. In cases where only single blood samples were available, the determination of specific IgM antibodies to Hantaan virus in high titers confirmed the diagnosis. None of 110 patients with influenza-like disease or pyrexia of unknown origin were found to be infected by Hantaan virus.

Analysis of data concerning clinical signs and symptoms of the disease as reported in the patient's medical records is shown in Table I. Of 28 cases serologically diagnosed, (data of 23 cases analyzed) 3 (14%) died and 12 (54%) developed severe symptoms including flushing over face and neck, conjuctival infection, pneumonic infiltration, pulmonary edema, confusion, shock, and hemorrhagic manifestations. Among those severely ill, 8 (35%) required renal dialysis. The predominant symptoms in all patients were fever, headache, nausea, vomiting, and abdominal pain, while flushing of the face, conjuctival infection, pulmonary edema shock and hemorrhagic manifestations were only common in the severely ill patients. Proteinuria with microscopic hematuria and increased serum urea and creatinine were present in all patients. Additionally, an inapparent infection was serologically diagnosed in the wife of a severely ill patient by detection of high IgG (1:1024) and IgM (1:512), against Hantaan virus. Table II shows a comparison of the clinical characteristics among HFRS patients from Greece, Finland and Korea.

2. Human serosurvey

Previous serosurveys conducted in 12 counties of Greece in 1981 and 1983 examined 445 and 347 human sera respectively by the IFA test. An additional 1,796 human sera collected from another 9 counties during the period January 1985 till October 1986 (Grant period), were examined by IFA test. All sera were analyzed together and the results were summarized according to region and county of origin in which the serosurveys were conducted, as shown in Table III. The overall antibody prevalence rate was 4% with a range from 0 to 14%. The maximum percentage positive occured in areas where clinical disease was diagnosed.

Moreover, seropositives were detected in 15 of 22 countries, indicating that the virus is widespread in Greece. From the clinical cases and the serosurveys, the ratio of males to females infected is approximately 3:1. The first cases appeared in early May and cases were observed until late October. The highest risk age group ranged from 30 to 50.

5. Small mammal serosurvey

In 1984 a collection of small mammals was carried out in an endemic area, Tsepelovo, Epirus, and during 1985-86 further collections were made in four other areas, one endemic and three nonendemic. Apodemus flavicollis was common in fields adjacent to villages and in the surrounding mountains at all collection sites. High antibody titers were detected in four mouse sera (Table IV). Captured house rats (Rattus rattus) were common in villages and in a slaughter house of Thessaloniki. Two seropositive Rattus rattus were found (Table IV), both from Thessaloniki. To date, neither Apodemus agrarius, the host of Hantaan virus, nor Clethrionomys glareolus, the host of Puumala virus, was captured at any site sampled.

6. Serological identification of the virus

Serological identification of the virus responsible for HFRS in Greece was succeeded by examination of patients' sera by both IFA and PRN tests using Hantaan, Seoul, and Puumala viruses. As shown in Table V, IFA tests detected high titers to both Hantaan and Seoul viruses, with no significant differences observed between them, and relatively low titers to Puumala virus. In contrast, with PRN tests (Table VI) and the same sera, highest titers were usually observed with Hantaan virus, whereas the reactivity with both Seoul and Puumala viruses was substantially lower than that observed with the IFA test.

4. Virus isolation

So far, all attempts to isolate the virus from humans (patients), and from lung tissues of Apodemus flavicollis and Rattus rattus have been unsuccessful.

CONCLUSIONS

In Greece, HFRS is much more severe than nephropathia epidemica in Scandinavia and HFRS in Western Europe. Among the 23 cases studied, 12 (52%) were severely ill, 8 (35%) required renal dialysis, and 3 (14%) died. Additionally, shock, pulmonary edema, and hemorrhagic manifestations developed in 5, 2, and 3 patients respectively. The most prominent differences between nephropathia epidemica and Korean hemorrhagic fever concerns the mortality rates, which are approximately 0.5% and 10% respectively. However, mortality rates are not the only difference. Hemorrhagic manifestations are more frequent in KHF, as are some neurologic symptoms, pulmonary manifestations, and shock. The clinical findings from Greek patients suggest that the disease is more like the severe Asian form of HFRS, caused by Hantaan virus, than the milder nephropathia epidemica caused by Puumala virus.

Sera of the Greek HFRS patients, examined by the IFA test with Hantaan, Puumala, and Seoul viruses, showed equivalent titers against both Hantaan and Seoul viruses, but titers against Puumala virus were much lower, suggesting that this virus was not the infecting agent. In contrast, when the same sera were examined by PRN test with Hantaan, Puumala, and Seoul viruses, they showed little reactivity with Puumala or Seoul viruses, but high titers were seen to Hantaan virus. In general, the 50% reduction point was much higher than the 80% titers and in many instances 100% reduction was never reached, even in the initial 1:16 dilution. These results suggest that neither Puumala nor Seoul viruses were the infecting agents, and indicate that Hantaan, or a closely related but distinct virus may be the cause of HFRS in Greece.

Serological diagnosis of the disease can be made using the IFA test to detect IgM antibodies during the early days of the disease. The earliest diagnosis we made was on the fifth day of the disease (IgM=1:2, IgG=1:512), whereas, in a second blood sample taken 13 days later from the same patient, the IgM titer was 1:512 and the IgG titer was 1:16,000.

Hemorrhagic fever with renal syndrome in Greece appears in late May and lasts until early October with a peak in August. The disease is widespread in Greece and may appear as isolated cases or outbreaks, particularly when groups such as woodcutters, shepherds, and farmers are forced to spend the night outdoors or in temporary quarters, thereby being exposed to infected rodents. Man-to-man transmission apparently does not occur. None of the hospital staff taking care of HFRS patients were found to have antibodies against Hantaan virus.

One case only, the wife of a patient, had high antibody titers at the same time as her husband, who was seriously ill. When questioned, however, she claimed that she did not show any clinical symptoms in the past year. The infection was inapparent and the woman may had contacted the infection at the same site as her husband, since she was accompanying him to the woods.

Results from the serosurvey screening in the northeastern (Evros), and western (Crete and Corfu) counties revealed that the virus is spread throughout the country. Clinical cases were serologically confirmed in 6 counties. Two endemic areas can be classified in the high risk areas, Tsepelovo and Promahi. Additionally, 15 of 22 counties, where seropositives were found, can also be characterized as endemic areas of the virus (Fig.). The percentage of seropositives in the total number of sera examined was 4%, while the percentage in endemic areas was 14%. The age groups of 30 to 60 years were most frequently positive, with a significantly higher incidence among males. The male to female ratio was approximately 3:1.

The vertebrate host of the virus causing severe HFRS in Greece may be Apodemus flavicollis. Antibodies to Hantaan virus have only been found in this species, and it is found thoughout the Balkan peninsula.

In conclusion, HFRS is endemic in Greece, and the clinical manifestations of the disease more closely resemble Korean hemorrhagic fever than nephropathia epidemica or the western European form of HFRS. There is a broad spectrum of symptoms, and the infection ranges from inapparent to lethal. Further attempts must be made to isolate the virus from patients and rodents.

D. CCHF VIRUS

Blood samples from 2655 people were obtained from 22 of 54 counties in Greece: 13 in Northern Greece (Thrace, Macedonia, and Epirus states), 4 in central Greece (Thessalia state), 2 in southern Greece (Peloponisos state), 1 in Corfu island and 2 in Crete island. These sera were examined for antibody to CCHF by IFA and ELISA tests, with results as shown in Table VII. CCHF virus appears to be widespread throughout the country. Antibodies were found in 7 counties, but 3 counties (Imathia, Karditsa and Kilkis) appear to be natural foci of the virus because of the high percentage of seropositives.

In our initial attempts to confirm the existence of CCHF disease, we collaborated with several hospitals in northern Greece. 409 blood samples (single and paired) were taken from patients with clinical disease resembling CCHF, from patients suspected for leptospirosis, patients with pyrexia of unknown origin, patients with influenza-like disease, and from patients with pyrexia and elevated liver enzymes (SGOT, SGPT). The last two groups of patientswere examined in parallel for CMV, HSV Epstein-Barr, hepatitis A and hepatitis B infections. Unfortunately,none of the patients was found to have recently been infected by CCHF virus.

The sensitivity and specificity of the CCHF antibody assays are still under investigation, and we have conducted studies aimed at increasing the sensitivity of the IFA test. Best results were obtained by a modification of the "spot-slides" preparation as follows: Vero E-6 cells (instead of Vero) were infected with CCHF strain 10200. At six days post infection, the cells were harvested in growth media suspension for "spot-slide" preparation. Instead of leaving the prepared "spot-slides" to dry and then fixing,we let them attach and grow overnight at 37°. This procedure, previously attempted for Hantaan spot-slides, yielded flat rather than rounded cells, which enhanced our ability to visualize cytoplasmic fluorescence. With this mocification we screened 100 human sera previosly considered to be either negative or questionably positive, and detected 10 sera as definitively positive. We propose to use this "spot-slide" modification in our future studies.

Studying the sensitivity of IFA and ELISA techniques, we applied both tests to the same samples obtained for the epidemiological and diagnostic studies. We have found that ELISA is twice as sensitive as IFA; however, its specificity is currently unknown and this will be a focus of our continuing investigations.

On the basis of our previous work, we conclude that CCHF virus exists in Greece. The question of associated human disease remains open, and will be a major topic for investigation during our continued efforts.

TABLE I

Clinical symptoms and signs in 23 HFRS Greek patients

Symptoms and Signs	No. of Patients
Fever	23
Rigors	23
Headache	23
Malaise	23
Abdominal pain	22
Myalgia	21
Arthralgia	19
Vomiting	19
Backache	18
Flush over the face and neck	15
Conjunctival injection	15
Hypotension	15
Dulled sensorium	10
Confusion	10
Shock	5
Diarrhea	4
Pneumonic infiltration	3
Cough	3
Hemorrhagic manifestations	3
Pulmonary edema	2

Table II

COMPARISON OF CLINICAL CHARACTERISTICS
OF HFRS PATIENTS IN GREECE, FINLAND, AND KOREA

	Percentage of cases		
	Greece	Finland*	Korea**
Hemorrhagic manifestations			
Conjunctival injection	65	18	97
Petechial rash	1	12	95
Petechiae	15	36	98
Purpura	0	0	37
Hematoma	5	0	5
Hematemesis and melena			
Respiratory and circulatory symptoms			
Cough	13	6	31
Dyspnea	10	0	25
Pneumonic infiltrations	13	0	25
Pulmonary edema	8.5	0	5
Shock	21	0	10
Neurological symptoms			
Dizziness	50**	12	94
Blurred vision	ND**	12	52
Convulsions	0	0	9
Mortality rate	13	<1	5-20

TABLE III

ANTIBODY TO HANTAAN VIRUS IN HEALTHY RESIDENTS OF GREECE

Region, county	No. of sera positive/no. tested (% positive)		No. of HFRS cases
Thrace			
Evros	0/129		---
Rodopi	0/62		---
Xanthi	1/79	(1.2)	---
Macedonia			
Serres	0/42		---
Kilkis	6/149	(4.0)	1
Thessaloniki	1/191	(0.5)	---
Halkidiki	6/95	(6.3)	---
Pella	13/93	(14)	5
Imathia	3/185	(1.6)	---
Piera	2/100	(2.0)	---
Kastoria	0/62		1
Kozani	4/153	(2.6)	1
Thessalia			
Larissa	2/103	(1.9)	---
Karditsa	1/48	(2.0)	1
Magnesia	4/132	(30)	---
Trikala	0/0		1
Epirus			
Ioannina	22/282	(7.8)	13
Peloponnisos			
Messinia	0/20		---
Lakonia	1/45	(2.2)	---
Crete			
Hania	0/27		---
Iraklio	3/81	(3.7)	---
Corfu	4/95	(4.2)	---
Others	32/462	(6.9)	---
TOTAL	105/2635	(4)	23

*Blood bank: Residents of examined counties

TABLE IV

SMALL MAMMALS CAPTURED IN ENDEMIC AND NONENDEMIC AREAS IN GREECE TESTED FOR IFA ANTIBODIES TO HANTAAN VIRUS

Location	Species	No. of trapped	No. of positives[+]
Region:Epirus	Rattus rattus alexandrinus	41	---
County:Ioannina[*]	R. rattus frugivorus	10	---
Area:Tsepelovo	Apodemus flavicollis	23	2
	A. sylvaticus	1	
	Crocidura sp.	1	---
Region:Macedonia			
County:Pella[*]			
Area:Promahi	Apodemus flavicollis	19	2
	A. sylvaticus	4	---
	Mus domesticus	1	---
County:Serres[**]			
Area:Rice fields	Apodemus flavicollis	9	---
County:Thessaloniki			
Area:Rice fields	Apodemus sylvaticus	3	---
	A. flavicollis	9	---
County:Thessaloniki[**]			
Area:Slaughter house	Rattus rattus alexandrinus	15	2
TOTAL		136	6

[*]Endemic "high risk" areas

[**]Endemic areas

[+]IFA titers ranging from 1:64 to 1:2048

TABLE V

IFA ANTIBODY TITERS TO HANTAAN, SEOUL, AND PUUMALA VIRUSES IN GREEK HFRS PATIENTS' SERA BY DAY OF ILLNESS

Serum No.	Day of Illness	Hantaan	Seoul	Puumala
1	9	1024*	1024	16
2	17	8192	8192	256
3	8	8192	8192	256
4	18	4096	4096	256
5	5	1024	1024	<16
6	8	512	512	<16
7	14	512	<6	<16
8	7	1024	256	256
9	8	4096	1024	256
10	9	1024	256	256
11	10	2048	256	64
12	20	2048	512	64
13	10	2048	1024	32
14	28	4096	4096	64
15	10	4096	1024	64
16	9	1024	256	128
17	8	512	128	<16
18	12	8192	2048	128
19	7	4096	2048	128
20	10	4096	256	128
21	Inapparent	256	256	128
CONTROLS				
KHF patient serum		4096	256	64
Girard Point Rat serum		256	512	16
NE patient serum		128	64	512

*Reciprocal of highest dilution showing characteristic cytoplasmic fluorescence

TABLE VI

PRNT ANTIBODY TITERS TO HANTAAN, SEOUL AND PUUMALA VIRUSES IN GREEK PATIENTS' SERA BY DAY OF ILLNESS

Serum No.	Day of Illness	Hantaan	Seoul	Puumala
1	9	2048*	16	32
2	17	1924	16	<16
3	8	64	64	32
4	18	1024	64	<16
5	5	512	64	<16
6	8	128	<16	128
7	14			<32
8	7	4096	64	32
9	8	4096	<16	16
10	9	4096	<16	16
11	10			NT
12	20	128	16	256
13	10	1024	<16	<16
14	28			128
15	10	2048	<16	<16
16	9	NT	NT	NT
17	8	4096	256	1024
18	12	2048	<16	16
19	7	2048	16	16
20	10	4096	16	64
21	Inapparent	4096	64	32
CONTROLS				
KHF patient serum		2048	128	64
Girard Point Rat sera		256	2048	<16
NE patient serum		<16	<16	1024

*Reciprocal of highest dilution yielding ≥50% reduction of plaque dose (100 PFU)

TABLE VII

ANTIBODY TO CCHF VIRUS IN HEALTHY RESIDENTS OF GREECE

Region, county	No. of sera positive/no. tested (% positive) IFA	ELISA
Thrace		
Evros	0/129	0/129
Rodopi	0/ 62	0/ 62
Xanthi	1/ 79 (1,2)	1/ 79 (1,2)
Macedonia		
Serres	0/ 42	0/ 42
Kilkis	2/149 (1,3)	5/149 (3,3)
Thessaloniki	0/191	0/191
Halkidiki	0/ 95	0/ 95
Pella	9/ 93 (9,6)	17/ 93 (18,2)
Imathia	8/185 (4,3)	14/185 (7,5)
Pieria	0/100	0/100
Kastoria	0/ 62	0/ 62
Kozani	0/153	0/153
Thessalia		
Larissa	1/103 (0,9)	4/103 (3,8)
Karditsa	3/ 48 (6,2)	5/ 48 (10,4)
Magnesia	0/132	0/132
Trikala	0/ 0	0/ 0
Epirus		
Ioannina	1/282 (0,3)	2/282 (0,7)
Peloponnisos		
Messinia	0/ 20	0/ 20
Lakonia	1/ 45 (2,2)	2/ 45 (2,2)
Crete		
Hania	0/ 27	0/ 27
Iraklio	1/ 81 (1,2)	2/ 81 (2,4)
Corfu	1/ 95 (1,0)	2/ 95 (2,1)
Others	0/462	1/462 (0,2)
Total	28 /2635 (1,0)	50/2635 (1,9)

*Blood bank: Residents of examined counties

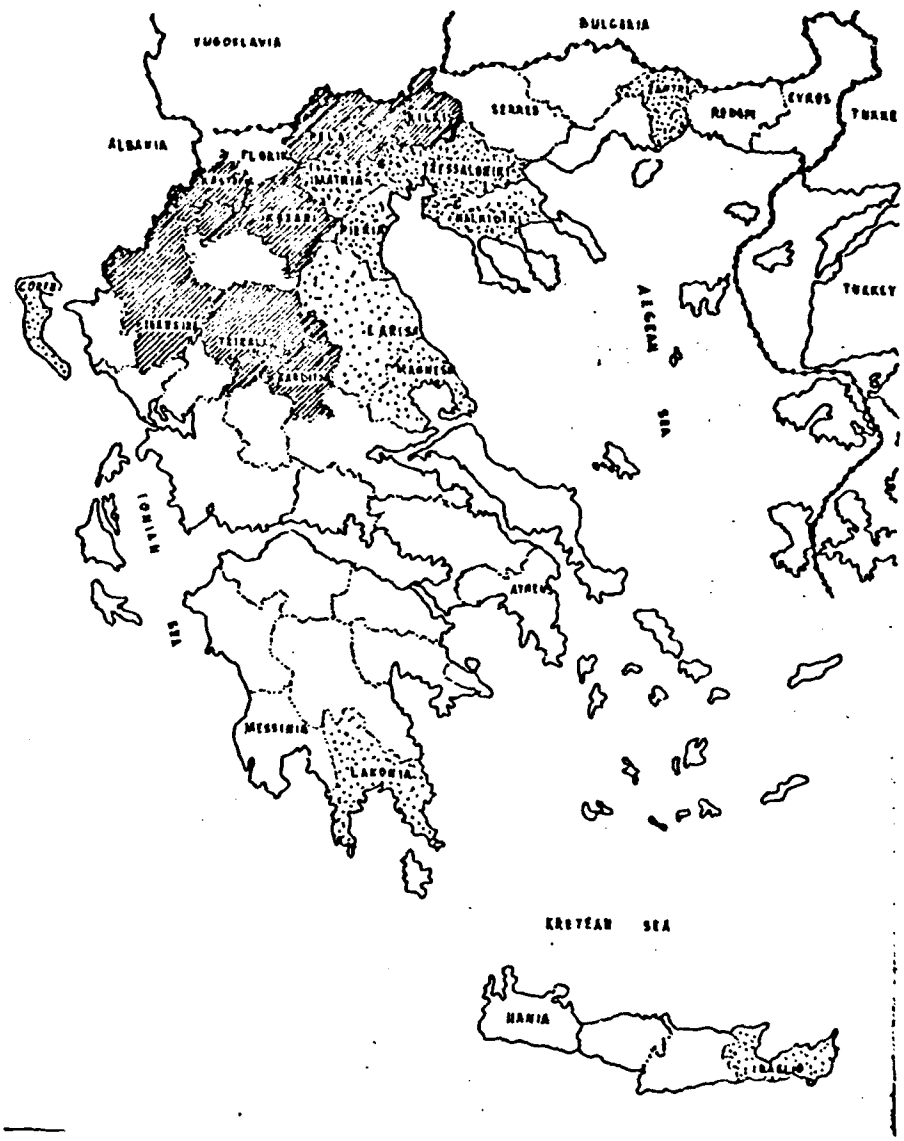

Figure 1 : HFRS distribution in Greece

HFRS cases serologically diagnosed

Antibody to Hantaan virus in healthy residents of Greece

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