Emergence of Crimean–Congo haemorrhagic fever in Greece

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Abstract

In the summer of 2008, the first case of Crimean–Congo haemorrhagic fever (CCHF) was observed in Greece. The laboratory diagnosis was established using nested RT-PCR and quantitative real-time RT-PCR. A high viral load and increased levels of cytokines were detected on the third day of illness and the patient died 7 days after the onset of symptoms. Nucleotide sequence analysis revealed that the Greek CCHF virus strain had high sequence identity with other Balkan CCHF virus strains.

Keywords: Crimean–Congo haemorrhagic fever, cytokines, Greece, PCR, quantitative real-time PCR
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Introduction

Crimean–Congo haemorrhagic fever (CCHF) is a severe viral human disease with fatality rates up to 30% [1]. It is the most widely disseminated tick-borne viral disease, with a geographical distribution following that of *Hyalomma* spp. ticks, the main vectors of CCHF virus (CCHFV). With the 48° latitude as the northern geographical limit, the disease is present from eastern China to Russia, the Balkans, Central Asia, and Africa. Besides the tick bite, an additional mode of transmission is direct contact of broken skin or mucous membranes with blood or tissues of infected livestock or CCHF patients. Risk groups include shepherds, farmers, soldiers, veterinarians, and healthcare workers. CCHF is a significant public health threat because of the associated high fatality rate, the absence of an FDA-approved vaccine, and the potential for human-to-human transmission and nosocomial outbreaks.

CCHF is endemic in the Balkan peninsula [2–7]. Since 2002, when the disease was first detected in Turkey, 2508 confirmed CCHF cases have been reported in that country, involving 133 deaths, mainly in middle and eastern Anatolia [8]. CCHF cases had not been reported in Greece prior to 2008. However, a CCHFV strain (strain AP92) was isolated in 1975 from *Rhipicephalus bursa* ticks collected from goats in Vergina village, northern Greece [9]. Antibodies against CCHFV were detected in four of 64 residents of the prefecture where strain AP92 was isolated; however, none of them recalled any illness resembling CCHF [10]. A serosurvey revealed that 1% of a human population had antibodies to CCHFV [11]. As no CCHF case had been reported in Greece, it was suggested that the human antibodies were against strain AP92, which seems to be moderately pathogenic to humans, and thus a good candidate for vaccine studies. Genetically, strain AP92 differs by more than 20% at the nucleotide level in the S RNA segment from all other known CCHFV strains [3,5]. Here we present the laboratory findings on the first clinical CCHF case in Greece with a fatal outcome.

The Case

On 21 June 2008, a 46-year-old woman was admitted to the Alexandroupoli General University Hospital, with high fever (39.5°C), headache, chills, malaise, nausea, vomiting and abdominal pain following a tick bite 4 days before [12]. The patient was engaged in agricultural activities in a rural area, 2 km north of Komotini, a city in Rhodope prefecture, in north-eastern Greece. The city is located at an altitude of

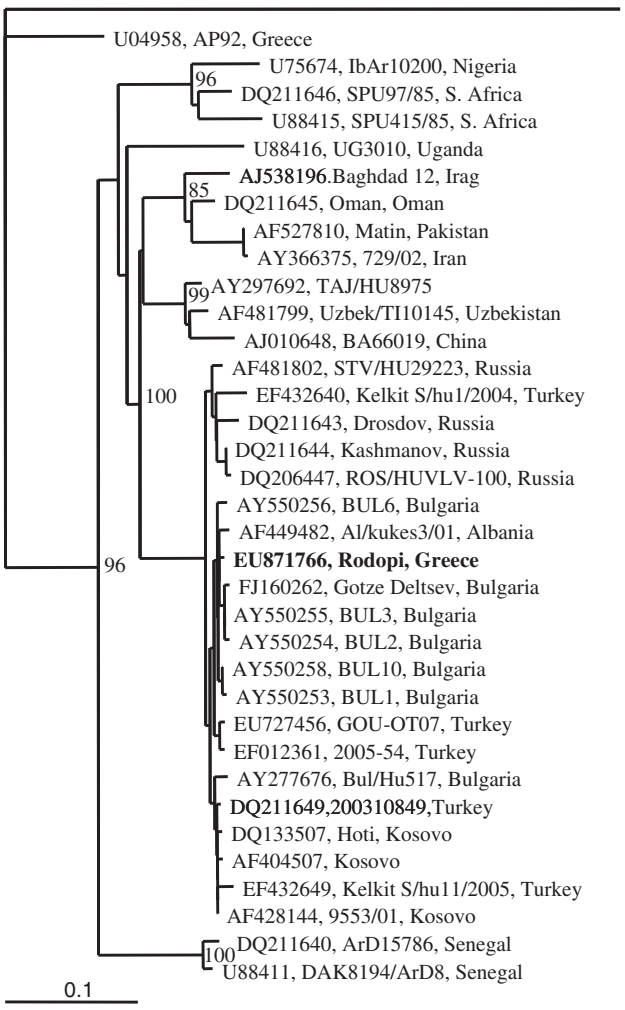

FIG. 1. Crimean–Congo haemorrhagic fever virus S RNA-based phylogenetic tree. Dugbe virus was used as the outgroup. The numbers at the nodes indicate percentage bootstrap replicates of 100; values below 60% are not shown. Horizontal distances are proportional to the nucleotide differences. The scale bar indicates 10% nucleotide sequence divergence. Sequences in the tree are indicated by GenBank accession number, strain name, and country. The Greek strain of the present study is given in bold.

32–38 m, close to the feet of the Rhodope mountains (latitude 41°7′22″ N, longitude 25°23′47″ E), and 18 km south of the Greek–Bulgarian border (Fig. 1). The patient did not report any travel abroad. Physical examination revealed mild sensitivity during palpation of the right hypochondrium, and a red papule at the site of tick bite was observed. Laboratory examination on admission showed leukocytes at 5620/μL (reference range 4500–11 000/μL), with 81.4% neutrophils, a haematocrit of 33.5%, platelets at 158×10^9/L, an aspartate transaminase (AST) level of 139 IU/L (reference range <31 IU/L), an alanine transaminase (ALT) level of 46 IU/L (reference range <34 IU/L), a γ-glutamyl-transpeptidase level of 57 IU/L (reference range 9–38 IU/L), a lactate dehydrogenase (LDH) level of 701 IU/L (reference range 25–248 IU/L), an activated partial thromboplastin time (APTT) of 64.3 s (reference range 24–35 s), an International Normalized Ratio of 1.77 (reference range 0.68–1.17), and D-dimers above 10 000 ng/mL. A few hours after admission, blood re-examination revealed that the platelet level was 100×10^9/L, the APTT was 82.6 s, and the fibrinogen level was 227 mg/dL.

A chest X-ray showed a right lower lobe linear atelectasy and bilateral pleural effusion. Echocardiography revealed mild hepatomegaly without focal damage, gall bladder dilatation, and the presence of pericholecystic oedema, as well as fluid collection in the Morrison and Douglas spaces. A computed tomography scan of the abdomen revealed severe ascites under pressure in the whole peritoneum, and liver enlargement. The blood supply was decreased in the arterial phase of the examination. Remarkable oedema surrounding the whole portal vein was seen.

Supportive treatment was initiated, combined with cephalosporins and metronidazole; in addition, doxycycline was administered, as rickettsiosis was included in the differential diagnosis. CCHF was not included in the initial differential diagnosis.

On the next day, APTT could not be determined, D-dimers were above 10 000 ng/mL, the AST level was 293 U/L, the ALT level was 95 U/L, and the LDH level was 1661 U/L. The patient developed disseminated intravascular coagulation, and fresh frozen plasma was given.

On 23 June, the patient was afebrile and complained of severe rachialgia. A petechial rash on the extremities was seen, and she suffered heavy haemorrhage from the genital tract, and diarrhoea, which became haemorrhagic.

Large haematomas were seen at the injection and venipuncture sites. Increased levels of transaminases (AST level of 3962 U/L, and ALT level of 1545 U/L) and LDH (8085 U/L), and an undefinable APTT were detected. Serological examination for hepatitis A, B and C was negative. On the same day, oliguria, mental disturbance and severe lactic acidosis were established. Bone marrow aspiration, which was performed to exclude haematological malignancy, revealed haemophagocytosis of neutrophils and erythrocytes by histiocytes. No blastic infiltration was seen. The patient's condition deteriorated rapidly, and she was transferred to the intensive-care unit, where she was intubated and given inotrops, fresh frozen plasma, erythrocytes, corticosteroids, and antithrombin III.

Convulsions were present and were attributed to a probable intracranial haemorrhage. On 25 June (seventh day of the disease), the patient died with multiple organ failure. An autopsy was not performed (it was not approved by the patient's family).

Laboratory Diagnosis

Stored serum and blood specimens taken at the time of admission (third day of the disease) were sent to Aristotle University of Thessaloniki after the death of the patient. Viral RNA was extracted by using a Viral RNA extraction kit (Qiagen, Hilden, Germany). A nested RT-PCR that amplifies a 240-bp fragment of the S RNA genome segment of CCHFV [13] was performed, and gave a positive result; sequencing of the product of the nested PCR confirmed the result (accession number EU871766). An additional 452-bp fragment of the M RNA segment was amplified and sequenced (accession number FJ643480). The sequences of the S and M segments were aligned with the corresponding segments retrieved from the GenBank database, using CLUSTAL W, and phylogenetic analysis was performed using the DNAdist, Neighbor and Consence programs of the PHYLIP software. Analyses showed that the causative CCHFV strain clusters in the European/Turkish clade, together with other Balkan strains (Figs 1 and 2). In the S segment, the Rodopi strain presents the highest identity with the Balkan strains, differing from the Russian strains by 3.1–4.4%, whereas the M segment is genetically closer to that in the Russian strains than in the Balkan strains (genetic distances of 4.6% and 7.4% respectively). A quantitative real-time PCR [14] revealed a CCHFV load of 2.97×10^8 copies/mL. No CCHFV antibodies were detected.

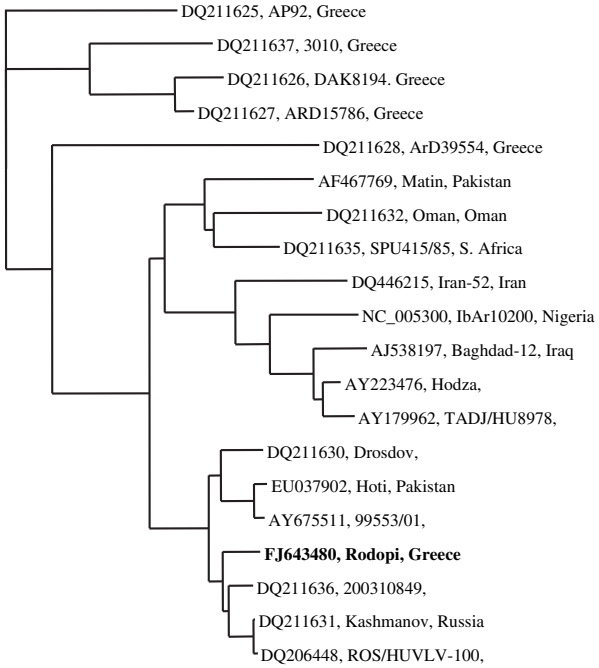

FIG. 2. Crimean–Congo haemorrhagic fever virus (CCHFV) M RNA-based phylogenetic tree. The AP92 CCHFV strain was used as the outgroup. The numbers at the nodes indicate percentage bootstrap replicates of 100; values below 60% are not shown. Horizontal distances are proportional to the nucleotide differences. The scale bar indicates 10% nucleotide sequence divergence. Sequences in the tree are indicated by GenBank accession number, strain name, and country. The Greek strain of the present study is given in bold.

To investigate the patient's innate immune response, the following details concerning cytokines, chemokines and growth factors were obtained by using the BioPlex Human Cytokine 27-plex panel in a Bio-plex suspension array system (Bio-Rad Laboratories): interleukin (IL)-1b, IL-1 receptor antagonist, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor, granulocyte colony-stimulating factor, granulocyte–macrophage colony-stimulating factor, interferon-γ, interferon-inducible protein-10, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α, macrophage inflammatory protein-1β, platelet-derived growth factor-bb, RANTES, tumour necrosis factor-α (TNF-α), and vascular endothelial growth factor. A strong innate immune response was observed, with increased expression of IL-6 (361.7 pg/mL), IL-8 (480.6 pg/mL), IL-9 (52.2 pg/mL), IL-15 (63.1 pg/mL), IL-1 receptor antagonist (41.1 pg/mL), TNF-α (53.7 pg/mL), RANTES (65.5 pg/mL), eotaxin (25.2 pg/mL), interferon-γ (69.5 pg/mL), MCP-1 (382.5 pg/mL), and vascular endothe-

lial growth factor (30.1 pg/mL). The levels of the remaining factors were below 10 pg/mL, and in healthy (control) individuals all respective factors were below 10 pg/mL.

Discussion

Although the Balkan peninsula has been known to be a CCHF-endemic region since the early 1950s, no CCHF case was reported in Greece prior to 2008. In the early spring of 2008, a cluster of CCHF cases was reported in Bulgaria, very close to the Greek–Bulgarian border [15]. Additionally, in the same year, many CCHF cases were observed in Turkey, where, during January to June of 2008, 688 confirmed cases were reported, 5.96% of them fatal [8]. We cannot exclude the possibility that unrecognized CCHF cases may have occurred in previous years in Greece.

The patient had no underlying disease; the presentation of severe illness and the rapid progression to death seem to have been associated with a massive inflammatory response and a catastrophic immune cascade. Increased levels of IL-6 and TNF-α have been previously associated with severe or fatal CCHF in humans [16,17]. Significantly high levels of MCP-1 were detected in our patient, suggesting that MCP-1 plays an important role in the course and, probably, in the outcome of the disease. In addition, high levels of IL-8, one of the major mediators of the inflammatory response and a major chemotaxis inducer, were detected. Haemorrhages occurred on the fifth day of the disease. Haematuria, melena, gingival bleeding and bleeding from the vagina may commence on days 4 and 5 of the illness [18]. Among the sites of bleeding, that from the vagina accounts for 10%, and the most common haemorrhagic manifestation is epistaxis [18].

Thrombocytopenia and prolonged APTT and International Normalized Ratio were among the first altered parameters. Criteria predicting a fatal outcome, during the first 5 days of the disease, include the following: leukocytes $\geq 10 \times 10^9$/L, platelets $\leq 20 \times 10^9$/L, AST $\geq$200 U/L or ALT $\geq$150 U/L, APTT $\geq$60 s, and fibrinogen $\leq$110 mg/dL [19]. Recently the criterion of leukocyte counts was excluded, and the level of transaminases was modified to AST $\geq$700 IU/L and ALT $\geq$ 900IU/L [20]. Our patient fulfilled all four modified criteria. Recently, it was shown that a high CCHFV load is predictive of fatal outcome [7,14,21]. The viral load of the patient on the third day of the disease was 2.97×10^8 copies/mL. In comparison, viral load of 3.45×10^9 copies/mL was observed on the sixth day of illness in a fatal case in Albania, whereas other primary cases who survived had viral loads ranging from 3.9×10^3 to 8.6×10^7 copies/mL, and all secondary cases had viral loads below 2.8×10^4 copies/mL [14]. It could be suggested that a viral load greater than 1×10^8 copies/mL is associated with fatal outcome.

CCHFV IgM antibodies are detectable as early as day 4 of illness in survivors [22]. In the present case, the available serum specimen was taken on the third day of illness; it was therefore not expected to detect CCHFV-specific antibodies. In addition, an antibody response is rarely demonstrable in fatal cases, the detection of antibodies being generally a favourable sign [22].

The remarkable haemophagocytosis seen in our patient is a phenomenon that has also been reported by other researchers; it was suggested to play a role in the development of pancytopenia in CCHF [23–25].

The patient did not report any travel abroad. She was working in a tobacco plantation, and she had livestock very close to her house. Dissemination of infected ticks, and hence spread of CCHFV, is possible via the livestock trade or movements of livestock or wild animals infested with infected ticks. It was not possible to determine the exact origin and the conditions that influenced the emergence of CCHF in Greece.

No secondary infections have been observed, probably because haemorrhages started when the patient was already hospitalized and all general precautions and protective measures had been taken. The situation would have been worse if haemorrhagic manifestations had started before hospitalization, when household members might have come into contact with blood or excreta of the patient.

According to mathematical modelling of CCHFV transmission, if the first infected person happens to die before he or she has a chance to infect anyone else, an epidemic will not occur [26]. Immediately after establishment of the laboratory diagnosis, control measures were taken, coordinated by the Hellenic Centre for Disease Control and Prevention [27].

In general, CCHF outbreaks have developed on a background of favourable climatic factors and environmental changes that are beneficial for the survival of large numbers of *Hyalomma* ticks and of the hosts of both their immature and adult stages [28]. In the northern hemisphere, *Hyalomma marginatum marginatum* is activated by the rising temperature in the spring. The mean temperature in Greece in April 2008 was high (18.1°C), and rainfall was increased (67 mm) in comparison with the dry April of 2007 (8 mm).

CCHFV has been detected in a variety of ixodid ticks, and *H. marginatum marginatum* is considered to be the main vector. The Greek CCHFV strain AP92 was isolated from *R. bursa* ticks [9]. It was suggested that the great genetic difference of that strain is related to the different genus of the tick vector. However, in Turkey, CCHFV of the European/

Turkish clade was detected in *H. marginatum marginatum* and in *R. bursa*, with almost identical nucleotide sequences [29]. Although it is difficult to incriminate an arthropod as an actual vector, it seems that *R. bursa* plays an important role in the CCHFV life cycle. Both tick species are abundant in northern Greece, with *H. marginatum marginatum* and *R. bursa* representing 12.8% and 19.8% of ticks, respectively [30]. It cannot be predicted whether additional CCHF cases will occur in the future in Greece. However, taking into account that vectors are present in Greece and endemic foci are present in neighbouring countries, and the emergence of the disease in this country, it can be suggested that the risk of more cases occurring in the future is high.

Clinicians should include CCHF in the differential diagnosis of febrile syndromes accompanied by thrombocytopenia, especially in regions neighbouring endemic countries and in individuals returning from an endemic region. Detailed knowledge of the medical history is very helpful. Effective surveillance and reporting are necessary for the control of CCHF, and require the collaboration of multidisciplinary experts.

Transparency Declaration

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References

1. Schmaljohn CS, Nichol ST. Bunyaviridae. In: Knipe DM, Howley PM, eds. *Fields virology*, 5th edn, Vol. 2. Philadelphia, PA: Lippincott, Williams and Wilkins, 2007; 1741–1789.
2. Gligic A, Stamatovic L, Stojanovic R *et al.* The first isolation of Crimean hemorrhagic fever virus in Yugoslavia. *Vojnosanit Pregl* 1977; 34: 318–321.
3. Papa A, Bino S, Llagami A *et al.* Crimean-Congo hemorrhagic fever in Albania, 2001. *Eur J Clin Microbiol Infect Dis* 2002; 21: 603–606.
4. Papa A, Bozovic B, Pavlidou V *et al.* Genetic detection and isolation of Crimean-Congo hemorrhagic fever virus, Kosovo, Yugoslavia. *Emerg Infect Dis* 2002; 8: 852–854.
5. Papa A, Christova I, Papadimitriou E *et al.* Crimean-Congo hemorrhagic fever in Bulgaria. *Emerg Infect Dis* 2004; 10: 1465–1467.
6. Drosten C, Minnak D, Emmerich P *et al.* Crimean-Congo hemorrhagic fever in Kosovo. *J Clin Microbiol* 2002; 40: 1122–1123.
7. Duh D, Saksida A, Petrovec M *et al.* Viral load as predictor of Crimean-Congo hemorrhagic fever outcome. *Emerg Infect Dis* 2007; 13: 1769–1772.
8. Yilmaz GR, Buzgan T, Torunoglu MA, *et al.* A preliminary report on Crimean-Congo haemorrhagic fever in Turkey, March–June 2008. *Euro Surveill* 2008; 13: pii: 18953.
9. Papadopoulos O, Koptopoulos G. Crimean-Congo hemorrhagic fever (CCHF) in Greece: isolation of the virus from *Rhipicephalus bursa* ticks and a preliminary serological survey. *Zentbl Bakteriol Hyg* 1980; 1 (suppl 9): 189–193.
10. Antoniadis A, Casals J. Serological evidence of human infection with Congo-Crimean hemorrhagic fever virus in Greece. *Am J Trop Med Hyg* 1982; 31: 1066–1067.
11. Antoniadis A, Alexiou-Daniel S, Malissiovas N *et al.* Seroepidemiological survey for antibodies to arboviruses in Greece. *Arch Virol* 1990; 1: 277–285.
12. Papa A, Maltezou HC, Tsiodras S, *et al.* A case of Crimean-Congo haemorrhagic fever in Greece, June 2008. *Euro Surveill* 2008; 13: pii: 18952.
13. Schwarz TF, Nsanze H, Longson M *et al.* Polymerase chain reaction for diagnosis and identification of distinct variants of Crimean-Congo hemorrhagic fever virus in the United Arab Emirates. *Am J Trop Med Hyg* 1996; 55: 190–196.
14. Papa A, Drosten C, Bino S *et al.* Viral load and Crimean-Congo hemorrhagic fever. *Emerg Infect Dis* 2007; 13: 805–806.
15. Kunchev A, Kojouharova M. Probable cases of Crimean-Congo haemorrhagic fever in Bulgaria: a preliminary report. *Euro Surveill* 2008; 13: pii: 18845.
16. Papa A, Bino S, Velo E *et al.* Cytokine levels in Crimean-Congo hemorrhagic fever. *J Clin Virol* 2006; 36: 272–276.
17. Ergonul O, Tuncbilek S, Baykam N *et al.* Evaluation of serum levels of interleukin (IL)-6, IL-10, and tumor necrosis factor-alpha in patients with Crimean-Congo hemorrhagic fever. *J Infect Dis* 2006; 193: 941–944.
18. Ergonul O. Crimean-Congo haemorrhagic fever. *Lancet Infect Dis* 2006; 6: 203–214.
19. Swanepoel R, Shepherd AJ, Leman PA *et al.* Epidemiologic and clinical features of Crimean-Congo hemorrhagic fever in southern Africa. *Am J Trop Med Hyg* 1987; 36: 120–132.
20. Ergonul O, Celikbas A, Baykam N *et al.* Analysis of risk-factors among patients with Crimean-Congo haemorrhagic fever virus infection: severity criteria revisited. *Clin Microbiol Infect* 2006; 12: 551–554.
21. Wölfel R, Paweska JT, Petersen N *et al.* Virus detection and monitoring of viral load in Crimean-Congo hemorrhagic fever virus patients. *Emerg Infect Dis* 2007; 13: 1097–1100.
22. Burt FJ, Leman PA, Abbott JC *et al.* Serodiagnosis of Crimean-Congo haemorrhagic fever. *Epidemiol Infect* 1994; 113: 551–562.
23. Karti SS, Odabasi Z, Korten V *et al.* Crimean-Congo hemorrhagic fever in Turkey. *Emerg Infect Dis* 2004; 10: 1379–1384.
24. Cagatay A, Kapmaz M, Karadeniz A *et al.* Haemophagocytosis in a patient with Crimean Congo haemorrhagic fever. *J Med Microbiol* 2007; 56: 1126–1128.
25. Tasdelen Fisgin N, Fisgin T, Tanyel E. Crimean-Congo hemorrhagic fever: five patients with hemophagocytic syndrome. *Am J Hematol* 2008; 83: 73–76.
26. Cooper BS. Mathematical modelling of CCHF transmission. In: Ergonul O, Whitehouse CA, eds. *Crimean Congo hemorrhagic fever. A global perspective*. Dordrecht: Springer, 2007; 187–203.
27. Maltezou HC, Papa A, Tsiodras S, *et al.* Crimean-Congo hemorrhagic fever in Greece: a public health perspective. *Int J Infect Dis* 2009; Jan 18 [Epub ahead of print].
28. Hoogstraal H. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. *J Med Entomol* 1979; 15: 307–417.
29. Tonbak S, Aktas M, Altay K *et al.* Crimean-Congo hemorrhagic fever virus: genetic analysis and tick survey in Turkey. *J Clin Microbiol* 2006; 44: 4120–4124.
30. Pavlidou V, Gerou S, Kahrimanidou M *et al.* Ticks infesting domestic animals in Northern Greece. *Exp Appl Acarol* 2008; 45: 195–198.