ISOLATION OF COXIELLA BURNETII BY A CENTRIFUGATION SHELL-VIAL ASSAY FROM TICKS COLLECTED IN CYPRUS: DETECTION BY NESTED POLYMERASE CHAIN REACTION (PCR) AND BY PCR-RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSES

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Abstract. Ticks are the principal vectors and reservoirs of Coxiella burnetii. The identification of isolates is necessary for understanding the clinical diversity of Q fever in different geographic areas. This is the first report of isolation of C. burnetii from ticks by the shell-vial assay and by nested polymerase chain reaction (PCR) assay for the detection of this pathogen in ticks. Of 141 ticks collected in Cyprus (Rhipicephalus sanguineus and Hyalomma spp.), 10% were found to be infected with C. burnetii. Three ticks were positive by hemolymph test, and 11 triturated ticks were positive by nested PCR. Three isolates were obtained by the centrifugation shell-vial technique. Analysis by PCR, then restriction fragment length polymorphism showed that the 3 Cyprus isolates had identical restriction profiles to reference strains Nine Mile and Q212. The methods described are useful in studying the epidemiology and ecology of C. burnetii.

INTRODUCTION

Q fever is a worldwide zoonosis caused by Coxiella burnetii, an obligate intracellular bacterium living in the phagolysosomes of the host cell.1–3 The most important reservoirs of this infection are cattle, sheep, and goats. In these animals, the infection is usually asymptomatic, with no obvious ill effects, although abortion may occur as a result of the infection.1 In nature, C. burnetii has been found primarily in a cycle involving ticks and free-living vertebrates, in particular rodents. Coxiella burnetii is transmitted to domestic animals either by tick bites or through contact with infected excreta.1

Although C. burnetii may be isolated from various tick species, the infection in humans is uncommon because infection takes place after inhalation of contaminated aerosols, consumption of fresh dairy products, and contact with infected animals.1,3 In humans, the infection is presented in 2 forms: acute and chronic. The acute form manifests pneumonia, prolonged fever, granulomatous hepatitis, and, rarely, meningoencephalitis.3 Clinical manifestations of chronic Q fever mainly include endocarditis.1–7 Reports from different countries indicate that epidemiological and clinical features of the disease may vary from one part of the world to another.6,8–15 The reason for this epidemiological and clinical polymorphism of Q fever is not known. However, the role of strain differences cannot be excluded, and thus detection and isolation of C. burnetii strains is of interest.5

In Cyprus, seroepidemiological studies have shown the presence of C. burnetii in both human and animal populations attributable to the close association between rural families and their livestock.16,17 Although these studies provide important information concerning the prevalence of Q fever in the island, the detection, isolation, and subsequent identification and categorization of C. burnetii strains into pathogenic or nonpathogenic is of critical importance in the understanding of the epidemiology, natural history, and potential threat to human health by these bacteria. Large-scale tick studies are difficult to carry out because classic techniques are time- and material-consuming; they also require viable ticks for hemolymph testing or ticks frozen at −80°C for shell-vial centrifugation.16–20 We have applied the nested polymerase chain reaction (PCR) assay to triturated ticks collected from Cyprus for the sensitive and specific detection of C. burnetii. Also, to facilitate the isolation of C. burnetii from ticks, we used the centrifugation shell-vial assay.

MATERIALS AND METHODS

Ticks. Thirty ticks were collected from goats and 111 from sheep from different areas of Cyprus (Table 1). The animals belonged to people with high immunoglobulin G titer against C. burnetii by the indirect immunofluorescent antibody test (IFA). All ticks were adults and were identified according to the usual taxonomic keys21–23 as Rhipicephalus sanguineus (100 ticks) and Hyalomma spp (41 ticks).

Hemolymph test and isolation of C. burnetii by the shell-vial method. The living ticks were disinfected and subjected individually to a hemolymph test by cutting off a leg under sterile conditions.26 A further drop of hemolymph was mixed with 500 µL of Earle’s minimum essential medium (MEM) containing 4% fetal calf serum, 2 mM L-glutamine, and inoculated into shell vials. All collected ticks were disinfected, triturated in 1 mL of MEM, and inoculated into duplicate shell vials. After inoculation, the shell vials, which contained monolayers of Vero cells, were centrifuged at 700 × g for 1 hr at 22°C. The ticks not tested by the hemolymph test were triturated in 1 mL of MEM, and inoculated into duplicate shell vials. After inoculation, the shell vials, which contained monolayers of Vero cells, were centrifuged at 700 × g for 1 hr at 22°C. The inoculum was then removed, and 1 mL of growth medium was added to the cells. The shell vials were incubated in a 5% CO₂ incubator at 37°C.

At the same time, 2 shell vials were inoculated with MEM and used as negative controls. The isolation of C. burnetii from ticks was carried out in a biosafety cabinet used only for shell-vial infection. The cell monolayers in the shell vials were examined for C. burnetii by the IFA technique on Day...
6, and if the first test was negative, again on Day 12. For the detection of C. burnetii, human sera collected by our laboratory (which displayed a high titer of immunofluorescent antibody to C. burnetii of >1/40,000), at a dilution of 1/100, and fluorescein-conjugated goat antiserum to human immunoglobulin G (dilution, 1/2,000) (Kallestad, Austin, TX) were used. Specificity was evaluated by simultaneous staining of uninfected cell monolayers and inoculated cultures negative to C. burnetii human sera. Infected Vero cells were inoculated into 25-cm² flasks containing a Vero monolayer. Establishment of the C. burnetii isolates was performed through serial passages on Vero cells.

**Extraction of DNA.** Fifty microliters of triturated ticks or 200 μL of infected cell suspension were used for the DNA extraction. The sample was incubated in the presence of 400 ng/mL proteinase K (stock 20 mg/mL in H₂O) overnight at 56°C. Subsequently, proteinase K was inactivated by boiling for 10 min, and the solution was centrifuged at 2,000 rpm for 5 min at 22°C. The supernatant was kept at −20°C.

**Amplification by PCR.** For direct detection of C. burnetii in triturated ticks, a nested PCR assay was performed by the primers Hfrag1/Hfrag2 in the first PCR and the primers HF1/HF2 for the nested PCR. The specificity of these primers was confirmed after testing 42 different bacteria isolates by nested PCR. To perform DNA amplification of the isolates from Cyprus and compare them with the reference strains Nine Mile and Q212, the genomic primers CB1/CB2 were used. The specificity of these 2 primers were tested in PCR with purified DNA from 25 other bacteria species. The PCR amplification was carried out in 100-μL volumes with 10 μL of boiled sample, 1 μM of each primer, 200 μM of each deoxynucleotidetide 2.0 mM MgCl₂, and 0.5 U of Taq polymerase (GIBCO BRL Life Technologies, Gaithersburg, MD). Four controls, 2 negative (distilled water and uninfected ticks or Vero cells) and 2 positive (Nine Mile and Q212 strains), were included in each test. The PCR amplification was performed in a DNA thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer, Norwalk, CT). The cycling conditions were performed as described previously. The PCR product was separated on 2% agarose gel and visualized under ultraviolet light.

**Restriction endonuclease digestion.** The specificity of the amplification was evaluated by restriction analysis of the PCR products. The CB1/CB2 products from infected cells were digested with the enzymes TaqI and AluI (New England Biolabs, Beverly, MA), as previously described. The restriction fragments were examined by electrophoresis on 3% low-melting-point agarose gel (GIBCO). The patterns obtained were compared with those obtained with the reference strains Nine Mile and Q212.

### RESULTS

**Isolation of C. burnetii.** Three of the 5 ticks examined were hemolymph positive. From the tick hemolymph, 1 Vero cell culture was positive by IFA test for C. burnetii on Day 12 after infection. Two cell cultures from the 141 examined triturated ticks were positive by IFA test after incubation for 14 days at 37°C. The subcultures derived from the initial shell vials were considered heavily infected after 15 to 21 days by IFA test, Gimenez staining, and PCR (Table 1). At this point, the voluminous vacuolar formations in the cell cytoplasm, due to the cytopathic effect of C. burnetii, were prominent.

**Nested PCR detection.** The PCR methods that used primers CB1/CB2 were not sensitive enough to detect C. burnetii in triturated ticks. Direct detection of C. burnetii was achieved in 11 of 142 triturated ticks by nested PCR. The product obtained had a size of 183 bp (Figure 1). Amplification products of the predicted size were not generated from the negative controls.

**Polymerase chain reaction-restriction fragment length polymorphism identification.** A PCR was performed on infected cells of the 3 Cyprus isolates and the C. burnetii reference strains Nine Mile and Q212 by the genomic primers CB1/CB2. The length of the C. burnetii genome target amplified was 257 bp. The assay specificity had been previously evaluated by restricted digestion of the PCR products. The amplified fragments obtained (by use of the primers CB1/CB2) from our samples and the reference strains Nine Mile and Q212 were subsequently digested with AluI (Figure 2) and TaqI (Figure 3). The generated fragments were identical in size in the 3 Cyprus isolates and were characterized by the same restriction profiles as the reference strains Nine Mile and Q212.

### DISCUSSION

Coxiella burnetii is an obligate intracellular parasite. In ticks, it can grow to very high titers, remains viable during their entire life, and can be transmitted transovarially to a new generation. In the enzootic cycle, ticks and vertebrates such as rodents are important components. Every tick species that preys up a susceptible host in an area endemic for Q fever can potentially harbor and spread C. burnetii. Ticks seem to play a central role in maintaining the viability of this pathogen in the environment. In nature, C. burnetii is transmitted to domestic animals, either by tick bites or indirectly through contact with infected excreta.

**Coxiella burnetii** was initially isolated from guinea pigs in embryonated chicken eggs, and later in cell cultures
A nested PCR approach has been applied recently for the highly sensitive and specific direct detection of *C. burnetii* in clinical samples, collected from animals and humans and using primers that are based on conserved plasmid sequences. A less hazardous technique for the isolation of *C. burnetii* was proposed by Raoult and others, who suggested a simplified shell-vial culture system. This method was then successfully applied to valves, arterial prostheses, bone, skin biopsy, bone marrow, and blood for the isolation of *C. burnetii* from patients. The shell-vial culture method has also been used in the isolation of rickettsiae from ticks, but to our knowledge, it has never been used to isolate *C. burnetii* from these arthropods.

This communication confirms the presence of *C. burnetii* in *Rhipicephalus sanguineus* and *Hyalomma* spp. *Coxiella burnetii* was isolated from tick hemolymph (1 isolate) and triturated infected ticks (2 isolates) by the shell-vial technique. The technique proved to be faster than any other isolation method and was applied to tick samples with excellent results.

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**Figure 1.** Direct detection of *Coxiella burnetii* in triturated ticks by nested polymerase chain reaction. The 183-bp amplification products are from 11 triturated ticks and 1 reference strain. Lane A, negative control (uninfected tick). Lanes B–F, 5 triturated ticks. Lane G, molecular size markers (PUC19/Sau3AI/TaqI). Lanes H–M, 6 triturated ticks. Lane N, strain Nine Mile. Lane O, negative control (distilled water).

**Figure 2.** Restriction endonuclease profile analysis of the 257-bp amplification products of the 3 Cyprus *Coxiella burnetii* isolates and the 2 *C. burnetii* reference strains, digested with *AluI*. Lanes A–C, three Cyprus *Coxiella burnetii* isolates. Lane D, negative control (uninfected cells). Lane E, strain Nine Mile. Lane F, molecular size markers (φX174 cleaved with *HindIII*). Lane G, strain Q 212.

**Figure 3.** Restriction endonuclease profile analysis of the 257-bp amplification products of the 3 Cyprus *Coxiella burnetii* isolates and of the 2 *C. burnetii* reference strains, digested with *TaqI*. Lane A, molecular size markers (φX174 cleaved with *HaeIII*). Lanes B–D, 3 Cyprus *Coxiella burnetii* isolates. Lane E, reference strain Nine Mile. Lane F, reference strain Q212. Lane G, negative control (uninfected Vero cells).
In the present study, *C. burnetii* was detected in the same 3 of 141 tick samples by both nested PCR and shell-vial technique. Thus, the nested PCR assay provides a rapid, sensitive, and specific detection of the pathogen in tick samples. It also offers the possibility of testing a large number of ticks collected in the field (after fixation in alcohol or formaldehyde), where culturing of the pathogen is not always possible.

Although the genome of *C. burnetii* is still thought to be highly conserved, previous studies showed that *C. burnetii* isolates could be differentiated by pulsed gel electrophoresis, plasmid DNA content, or both. The technique of PCR-restriction fragment length polymorphism (RFLP) is useful in detecting and identifying *C. burnetii* in early shell-vial cultures and in certain clinical specimens (heart valves). The restriction enzymes used for the identification of *C. burnetii* after PCR amplification, with the primers CB1-CB2, are *AluI* and *TaqI*. No heterogeneity has been reported with other *C. burnetii* isolates after these enzymes were used.

We believe that the shell-vial technique is efficient in the isolation of *C. burnetii* from ticks and is a good alternative to animal or egg inoculations. The method is especially adapted to large isolations that may allow further genetic and physiological studies.

The shell-vial assay, the nested PCR technique, and the PCR-RFLP analysis proved useful tools in this study for the detection, isolation, and identification of *C. burnetii* in ticks. The 3 isolates of *C. burnetii* from ticks collected in Cyprus had the same profile as that of Nine Mile and Q212 *C. burnetii* reference strains. The reference strain Nine Mile was isolated from ticks in the United States and has the same profile with isolates from patients with Q fever in France and Greece. More samples from ticks, human, and animal populations are needed, however, to isolate, identify, and compare the isolates with the reference strains for a better knowledge of the epidemiology of Q fever in Cyprus.

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REFERENCES


