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IDENTIFICATION OF SPOTTED FEVER GROUP RICKETTSIAE USING POLYMERASE CHAIN REACTION AND RESTRICTION-ENDONUCLEASE LENGTH POLYMORPHISM ANALYSIS

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Summary. - In order to facilitate the isolation and identification of spotted fever group (SFG) rickettsiae from their tick vectors, we used the centrifugation shell vial technique or traditional isolation procedures and genotypic identification using the restriction fragment length polymorphism analysis of polymerase chain amplified fragments. The presence of *Rickettsia conorii* both in *Rhipicephalus sanguineus* ticks collected in southern France, and in *Rhipicephalus simus* and *Haemaphysalis leachi* from Zimbabwe was demonstrated. This procedure seems to be of particular interest for studying the epidemiology and ecology of SFG rickettsiae.

Key words: spotted fever group rickettsiae; isolation; identification; polymerase chain reaction

Introduction

The isolation and the subsequent precise identification of spotted fever group (SFG) rickettsiae from their tick vectors, is of critical importance in the understanding of the epidemiology, natural history, and potential thread to human health of these bacteria. The isolation of SFG rickettsiae has previously been carried out by the inoculation of infected tick tissues into laboratory animals (including mice, rabbit, guinea pig, and meadow voles) (Burgdorfer *et al.,* 1979), embryonated hen eggs (Cox, 1942), and cultured cells (Philip and Casper, 1981). Direct isolation of rickettsiae from whole blood and tick hemolymph has been reported (Wike and Burgdorfer, 1972). All these procedures are somewhat time-consuming and not always successful. Further, identification of the isolate by serological techniques is complicated by significant cross-reactivity between currently recognized SFG rickettsiae. Serologic

cross-testing of the different SFG rickettsiae with specially prepared mouse antisera can distinguish 12 or 13 serological patterns (serotypes) (Philip *et al.*, 1978). A new SFG isolate must be tested with the complete panel of antisera, representing all serotypes, in order to be definitively identified. Likewise, sera of naive, experimentally infected animals must be tested with the complete panel of antigens, and weak or double seroconversion can occur (Cacciapuoti *et al.*, 1985) leading to ambiguous results. Few monoclonal antibodies are currently available, most of which are not species-specific, and a combination of several monoclonal antibodies must be used in order to identify a new isolate (Anacker *et al.*, 1987; McDade *et al.*, 1988). The SDS-polyacrylamide gel electrophoresis of the rickettsial proteins has been used to compare different species and to identify new isolates (Péter *et al.*, 1985). The analysis of antigenic proteins is commonly achieved by western immunoblotting of protein profiles with a panel of polyclonal or monoclonal antisera. These techniques, however, require large quantities of protein, and purification steps may result in deletion or modification of some epitopes.

In order to facilitate the isolation and identification of SFG rickettsiae, we isolated SFG rickettsiae by the centrifugation shell vial and traditional techniques and used genotypic identification using the restriction fragment length polymorphism analysis of polymerase chain reaction amplified genomic fragments (Regnery *et al.*, 1991).

Materials and Methods

Isolation procedure. The centrifugation shell vial isolation procedure has been previously reported (Péter *et al.*, 1990). Briefly, ticks were disinfected by immersion in iodated alcohol and washed twice in sterile distilled water, before haemolymph testing (Burgdorfer, 1970). Isolation from positive ticks was attempted one or two days later with the ticks being maintained in a sterile tube. A drop of haemolymph was placed in 300 μl of brain heart infusion broth which was divided into two shell vials containing human embryonic lung fibroblasts grown on 12-mm round cover slips. The shell vials were centrifuged at 700 x g for 1 hr at 37 °C and the supernatant discarded. Fresh medium (minimum essential medium supplemented with 5 % foetal calf serum) was added and the shell vials were incubated in a 5 % CO_2 incubator at 37 °C. After 7 days the cover slip from one vial was stained with an indirect fluorescent-antibody test (IFA) inside the shell vial with hyperimmune rabbit serum to *Rickettsia conorii*. Final establishment of the strains was made through serial subcultures in human embryonic lung fibroblasts, and finally the strains were adapted to Vero cells. Isolates A and C from Zimbabwe were obtained from *Rhipicephalus simus* and *Haemaphysalis leachi* respectively by previously described methods (Kelly and Mason, 1990).

DNA preparation. Culture medium was removed from the flasks, infected Vero cell monolayers were washed three times with PBS and harvested using sterile glass beads, and resuspended in sterile distilled water. An aliquot of this preparation was boiled for 10 min, and used for further DNA amplification.

PCR assay. Ten microliters of boiled samples were PCR-amplified according to the protocols supplied with the GeneAmp DNA amplification reagent kit (Perkin Elmer Cetus, Norwalk, U.S.A.) using 35 cycles of denaturation, annealing and extension as previously described (Regnery *et al.*, 1991). PCR amplifications always included negative control containing no template DNA, control material from non-infected Vero cells, and *Rickettsia conorii* Moroccan

strain DNA as a positive control. Two sets of primers were used, one of them delineating a 381 base pair region of the citrate syntetase gene from *Rickettsia prowazekii* (Wood *et al.*, 1987), the other a 532 base pair region of the 190-kD antigen gene from *Rickettsia rickettsii* (Anderson *et al.*, 1990). These primers have been described previously (Regnery et al., 1991). Successful amplifications of DNA were verified by rapid agarose electrophoresis. PCR products were digested using *Alu*I and *Rsa*I restriction-endonucleases according to the protocols of the supplier, and the digested products were separated on 8 % polyacrylamide vertical gels. The identification of strains was achieved by comparing the PCR/RFLP patterns with those previously defined for the typhus-group and SFG rickettsiae (Regnery *et al.*, 1991).

Results

From 540 *Rhipicephalus sanguineus* ticks collected in Marseille, 52 SFG rickettsiae isolates were made using the centrifugation shell vial technique

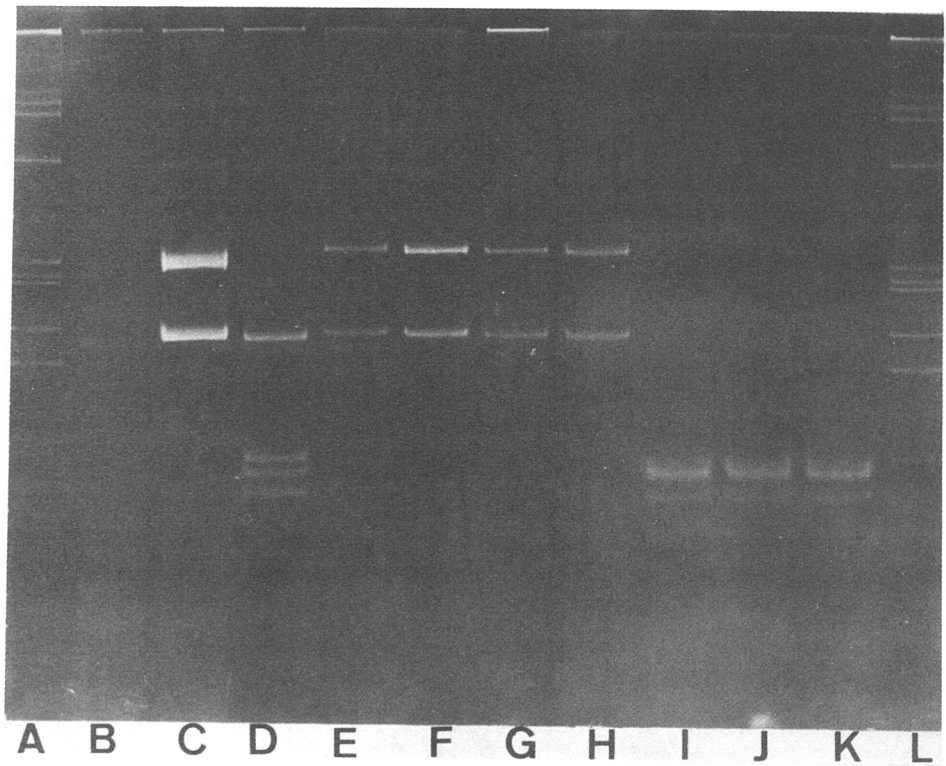

Fig. 1

Patterns obtained after migration on polyacrylamide gel of PCR-amplified DNA
DNA samples were amplified using a pair of primers delineating a 381 base pair region of the citrate synthetase gene of *R. prowazekii*. Lanes C, F, I, - *Phi* X 174 RF DNA cleaved with *Hinf*I; lanes A, E, - *R. conorii* Moroccan strain; lanes B, G, - isolate A from Zimbabwe; lanes D, H, - isolate C from Zimbabwe; lanes A, B, D, - digestion with *Alu*I; lanes E, G, H - digestion with *Rsa*I.

(Péter *et al.*, 1990). The overall contamination rate was of 6 %, and SFG rickettsiae have been isolated from about 88 % of *Rhipicephalus sanguineus* ticks positive by the haemolymph test with immunofluorescence staining (Péter *et al.*, 1990).

Eight of the 52 SFG rickettsiae isolated from *Rhipicephalus sanguineus* in France, and 2 Zimbabwean isolates grew on Vero cells, stained with Gimenez staining (Gimenez, 1964) and fluoresced when tested by immunofluorescence assay with sera from Mediterranean spotted fever patients. They were all amplified by the two sets of primers. Qualitative and quantitative comparisons between these 10 isolates DNA profiles and those of SFG prototypes identified them as *Rickettsia conorii*, the aetiologic agent of Mediterranean spotted fever (Raoult and Walker, 1989) (Fig. 1).

Discussion

The isolation and subsequent precise identification of spotted fever group rickettsiae recovered from their tick vectors has been hampered by several factors. Isolation procedures using inoculation into laboratory animals or embryonated eggs are somewhat time and material consuming, and some of the SFG rickettsiae were not easily isolated in animals (Burgdorfer *et al.*, 1979). Contamination can be troublesome when tissue culture is used for isolation. Disinfection of tick with iodated alcohol has proven to be effective in preventing contamination, without having any side effects on rickettsial viability.

The use of the centrifugation shell vial technique clearly enhances the isolation rate because of the increased infectivity of centrifuged samples (Weiss and Dressler, 1960) and the small number of cells relative to inoculum size. The technique has proved to be faster than other isolation methods, and has been successfully applied to blood samples from patients suffering from Mediterranean spotted fever (Espejo-Arenas and Raoult, 1989; Marrero and Raoult, 1989).

The PCR/RFLP analysis technique, recently developed by Regnery *et al.* (1991) was successfully used for identification of the new isolates. However, in this report DNA was not extracted and purified, but released from infected cells by boiling. The technique is simple, rapid, and circumvents the theoretical and technical difficulties associated with serological methods, with protein analysis methods, or with previously described nucleic acid methods. For DNA amplification, two sets of primers were used. The first one encodes a region of the citrate synthetase gene of *R. prowazekii* and has been proved to prime with both typhus group and with SFG rickettsiae. The second encodes a region of the 190 kD antigen gene of *R. rickettsii*, and has been shown to prime with SFG but not with typhus group rickettsiae. Restriction enzyme digestion of this region of the gene enables the production of DNA profiles characteristic of SFG species (Regnery *et al.*, 1991). The advantages of this technique include

the absence of animal manipulation, the absence of rickettsial purification from their cellular hosts, the absence of DNA purification, and the nonambiguous nature of the results.

The preliminary results reported here show that a combination of the centrifugation shell vial technique for isolation and of PCR/RFLP analysis for identification is a suitable procedure for the rapid isolation and accurate identification of SFG rickettsiae. Further studies using these techniques are in progress to extend present data and to obtain an accurate knowledge of the distribution of SFG species in the different tick species encountered in South-East France and other parts of the world.

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