Isolation by a Sensitive Centrifugation Cell Culture System of 52 Strains of Spotted Fever Group Rickettsiae from Ticks Collected in France

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Received 9 November 1989/Accepted 23 January 1990

Boutonneuse fever caused by Rickettsia conorii is transmitted mainly by the brown dog tick, Rhipicephalus sanguineus. We collected 540 ticks in Marseille, and tried to isolate as many strains of rickettsia as possible. Ticks were evaluated for the presence of rickettsia by the hemolymph test and by a new culture system, the centrifugation-shell vial technique. We avoided contamination in the culture system. Prior to ticks being submitted to the hemolymph test, they were disinfected. Only 5.6% (27 of 478) of the cultures were contaminated. A drop of hemolymph from each of 478 R. sanguineus ticks was cultured in two shell vials, and another drop was stained by the Gimenez method or indirect immunofluorescence. Since Gimenez staining in our hands was not satisfactory, comparison of the hemolymph test and culture is based on the results of indirect immunofluorescence. Thus, 50 of 369 (13.5%) examined ticks were hemolymph test positive, and 44 (11.9%) cultures were positive. After disinfection, another pool of 62 ticks was examined by the hemolymph test. The ticks were kept individually in a sterile environment. A few days later, the hemolymph of these ticks was collected again and cultured. The contamination rate was not significantly higher (6.4%) than in the above-described conditions. It allowed us to isolate eight more strains. Thus, we recommend screening ticks with the hemolymph test and culturing only the hemolymph test-positive ticks.

MATERIALS AND METHODS

Ticks. A total of 503 adult R. sanguineus ticks were collected from houses and dogs in Marseille. Nine Rhipicephalus turanicus and 28 Dermacentor marginatus ticks were collected in southeastern France. Prior to performance of the HT, pools of 10 to 20 ticks were disinfected by immersion for 10 min in iodated alcohol (12 ml of tincture of iodine and 6 ml of ethylene glycol per liter of 70% ethanol). The ticks were then rinsed twice for 5 min in distilled water and allowed to dry on sterile filter paper in petri dishes.

Cells. Human embryonic lung fibroblasts were grown in minimal essential medium with 10% fetal calf serum. Shell vials (3.7 ml; Sterilin, Feltham, England) with 12-mm round cover slips were seeded with 1 ml of medium containing 50,000 cells and incubated in a 5% CO₂ incubator for 3 days to obtain a confluent monolayer.

HT and inoculation. The first group of 478 R. sanguineus ticks were disinfected and subjected individually to an HT by cutting of a leg under a laminar flow hood. The hemolymph drop was deposited in 300 μl of brain heart infusion broth, and this mixture was placed into two shell vials. The shell vials were centrifuged at 700 × g for 1 h at 37°C. The inoculum was discarded, and fresh medium (minimal essential medium supplemented with 5% fetal calf serum) was added. The vials were placed in a 5% CO₂ incubator at 37°C. A second HT was performed with another leg of each tick. The droplet was placed on a microscope slide and allowed to dry until it was stained. A second group of 62 ticks (25 R. sanguineus, 9 R. turanicus, and 28 D. marginatus) were subjected to HT. Then each tick was maintained individually in a sterile test tube with a cotton

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TABLE 1. Comparison between HT by immunofluorescence and the shell vial culture of 369 ticks

<table>
<thead>
<tr>
<th>No. of ticks</th>
<th>Result* with:</th>
<th>HT</th>
<th>IFA on dissected tick tissuesa</th>
<th>Shell vial culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>ND</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>315</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* a: +, Positive; -, negative; ND, not done.
b: Confirmed by dissection in cases of discrepant results.
c: Two were contaminated.

Confirmation of discrepant results. We considered a tick to be positive or negative when the HT and culture results were in accordance. When discrepant results were observed between the HT and culture system, living ticks were dissected for preparation of multiple tissue smears with the hypodermis, salivary glands, and Malpighian tubules. The smears were stained by an IFA as described above.

RESULTS

The initial use of Gimenez staining proved unsatisfactory; thus, the remaining 369 tick hemolymph samples were examined by an IFA. Inoculation of 478 tick hemolymph samples into shell vials yielded only 27 contaminated cell cultures (5.6%). The comparison between the HT and culture system is based on the results from only the 369 ticks whose hemolymph was stained by IFA. A total of 50 ticks were found infected with rickettsiae by the HT; 44 cultures were positive (Table 1). We observed that cultures inoculated with material from unfed female ticks frequently showed few scattered cells infected with rickettsiae (Fig. 1a). In contrast, many foci of rickettsia-infected cells (Fig. 1b) or cell cultures packed with rickettsiae (Fig. 1c) were regularly observed with the hemolymph of engorged females. Cultures varied from being strongly positive to containing fewer than 10 organisms. The 14 discrepant results were as follows: 10 positive HT yielded negative cultures for SFG rickettsia, and four positive cultures were observed from HT-negative ticks. The tissue smears from 11 of these ticks confirmed the results of eight positive and three negative HT. Thus, six cultures gave false-negative results, and two were contaminated (they were weakly positive cultures). Three cultures gave false-positive results. Furthermore, one false-negative HT was found as tissue smears of one tick revealed a typical rickettsial infection. Two ticks were dead and could not be dissected (Table 1).

In part 2 of this study, only 4 of the 62 cultures (6.4%) were positive.
were contaminated when the ticks were first examined by the HT and subsequently cultured. Of these cultures, we isolated eight strains of SFG rickettsiae from the three tick species (R. sanguineus, R. turanicus, and D. marginatus).

DISCUSSION
The shell vial culture has been successfully used for diagnosis of viral diseases (7, 11). Recently, Marrero and Raoult (9) and Espejo-Arenas and Raoult (5) demonstrated in a small series of cases that it could be easily adapted to the diagnosis of boutonneuse fever. Indeed, blood from febrile patients in whom rickettsiosis is suspected may be inoculated into this cell culture system. Results were available within 72 hr, and strains were isolated and established in the laboratory for future investigation. In the overall study of SFG rickettsiae in Marseille, we tried to isolate strains of SFG rickettsia from R. sanguineus. So far, the isolation process required inoculation of infected ticks into embryonated hen eggs, sensitive animals (guinea pigs or meadow voles), or Vero cells. Shell vial culture was evaluated as a new isolation procedure which had never been applied to rickettsial isolation from ticks.

The time for recovery of rickettsiae from ticks in the shell vial assay is longer than from human or guinea pig blood (7 days from hemolymph and only 2 days from blood).

The comparison of the HT and shell vial culture was based on results obtained with IFA staining. Indeed, in our hands, the Gimenez method gave poorly stained rickettsia which did not allow a correct evaluation of both systems. The shell vial culture gave excellent results in detecting 80% of the positive ticks. When compared with Vero cell culture as reported in the literature, this system is much more efficient: Anderson et al. (1) isolated five SFG rickettsiae from 25 IFA HT-positive ticks, Philip and Casper (10) isolated 120 rickettsiae from 177 such ticks, and Magnarella et al. (8) isolated 4 rickettsiae from 14 such ticks. The explanation may be the increased infectivity of centrifuged samples (15, 17). In one instance, we isolated rickettsiae from an HT-negative tick.

We were unable to compare the shell vial assay to animal inoculation because inoculation of 540 animals was cost prohibitive. Moreover, some of the SFG rickettsiae are not easily isolated in animals (3). The HT is more sensitive and less time-consuming than the shell vial culture as a screening test. This was the reason that in part 2 of this study, ticks were screened by a stained HT with IFA, followed 1 or 2 days later by the culture of a second drop of hemolymph. Since the contamination rate remained low, we recommend this procedure and that only the hemolymph from rickettsia-infected ticks be cultured in the shell vials.

Shell vial culture is definitely very efficient in the isolation of rickettsiae from ticks and is without any doubt a good alternative to the animal inoculations. Its sensitivity seems to be better than that of the usual Vero cell culture, and it does not require specific equipment. It is specially adapted to large scale isolation campaigns in tick population and makes it possible to isolate both pathogenic and nonpathogenic rickettsiae.

ACKNOWLEDGMENTS
We gratefully thank G. Vestris, V. Galicher, M. Enea, and M. C. Lafforge for excellent technical assistance.
This work was supported by a grant from the Centre National de la Recherche Scientifique (convention 86208).

LITERATURE CITED