Prevalence of Antibodies to Spotted Fever Group Rickettsiae along the Eastern Coast of the Adriatic Sea

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A seroepidemiological survey in coastal Croatia detected antibodies reactive with Rickettsia conorii in 4.2% of sera by immunofluorescence assay and in 5.0% of sera by enzyme immunoassay. Western immunoblotting demonstrated antibodies to the 120-kDa surface protein in all 20 positive serum samples examined and to rickettsial lipopolysaccharide in 3 of these serum samples. Humans in this area are clearly being exposed to spotted fever rickettsiae.

Mediterranean spotted fever, a tick-transmitted rickettsiosis endemic in southern Europe, Africa, and Asia (24), has seldom been reported in the Yugoslavian literature (25). The incidence of Mediterranean spotted fever appears to be increasing in the Mediterranean basin (11), including the Adriatic coast of Croatia (15). A spotted fever group (SFG) rickettsiosis was first described for this area by Tartaglia in 1935 (22). According to serosurveys in the Mediterranean basin, the prevalence of antibodies to SFG rickettsiae is as high as 73.5% by the indirect immunofluorescence assay (IFA) (8). In 1984, antibodies to SFG rickettsiae were demonstrated by complement fixation in the sera of 5% of 730 healthy persons from eight islands in North Dalmatia (15), and in 1990 the IFA showed a substantial seroprevalence among rural and urban populations (5).

The area of investigation consists of a typical stony karst region, covered with dense shrubs and bushes of Mediterranean type in places altered by human changes. The vegetation and microclimatic factors provide a splendid habitat for small mammals, reptiles, and a large population of ticks (4, 12, 13).

Sera collected from 477 healthy subjects living in the Adriatic coastal region including Istra (n = 42), Zadar (n = 57), Split (n = 103), and Dubrovnik (n = 275) were tested by IFA, enzyme immunoassay (EIA), and Western immunoblot assay (WB) to detect antibodies reactive with Rickettsia conorii antigens.

R. conorii (Moroccan and Malish-7 strains) isolates obtained from the American Type Culture Collection were cultivated in confluent Vero cells, which were applied to microscope slides, air dried, and fixed with 95% ethanol (14). Sera were serially diluted in phosphate-buffered saline (PBS) containing 3% nonfat dry milk, incubated on the antigen slides in a moist chamber for 30 min at 37°C, and washed three times with PBS. Fluorescein isothiocyanate-conjugated goat anti-human immunoglobulin G (IgG) (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted 1:100 was incubated on the slides for 30 min at room temperature and washed in PBS.

For microtiter EIA, wells of polystyrene U-bottom micro-
titer plates were coated overnight with 100 μl of R. conorii antigens diluted 1:25 in 0.1 M sodium carbonate buffer (pH 9.6). The wells were then washed three times with 0.25 ml of PBS with 0.05% Tween 20 and covered with 100 μl of PBS plus 3% nonfat dry milk for 1 h at 37°C. After the plates were washed, 100 μl of test or control serum diluted 1:200 in PBS was added. The plates were reincubated and washed, and 100 μl of conjugate (alkaline phosphatase-labeled goat anti-human IgG) diluted 1:1,000 in PBS was added to each well. After 1 h at 37°C, the wash was repeated, and p-nitrophenyl phosphate substrate (1 mg/ml in 10% diethanolamine buffer, pH 9.8) was added. Following incubation for 45 min at 37°C, the reaction was stopped with 3 M NaOH (50 μl per well), and optical density (OD) was measured with a microplate reader (Bio-Tek Instruments, Winooski, Vt.) at a wavelength of 405 nm. For each specimen, the net OD was calculated. The mean and standard deviation of net ODs from 37 negative controls were 0.06 ± 0.03. Any net OD of more than 3 standard deviations above the mean negative value (0.17) was regarded as positive.

For the WB assay, purified Malish-7 strain rickettsiae were dissolved in final sample buffer, and the proteins were separated by electrophoresis in a 7% sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose paper (10). Nonspecific binding sites were blocked with PBS containing 5% nonfat dry milk. Each human serum sample was diluted at 1:200 in PBS-3% milk and incubated with the nitrocellulose strips for 1 h with gentle shaking at room temperature. Alkaline phosphatase-conjugated goat anti-human immunoglobulins (anti-IgG plus anti-IgM; Bio-Rad Laboratories, Richmond, Calif.) diluted 1:100 in PBS-3% milk were incubated with the strips for 1 h. After three 10-min washes in PBS, the bound enzyme was detected with 4 ng of fast red salt per ml-2 ng of napthol AS-MX phosphate per ml in 50 mM Tris, pH 8, and the strips were washed in distilled water for 10 min to stop the reaction.

Of 477 serum samples tested by IFA, 20 (4.2%) contained antibodies to R. conorii at a titer of ≥64 (Table 1). Two serum samples from Dubrovnik reacted with the Malish-7 strain at the cutoff titer of 64 and did not react with the Moroccan strain. Similarly, one serum sample each from Istra and Zadar reacted at a titer of 64 with only the Moroccan strain. By EIA, 24 (5.0%) were positive for IgG.
TABLE 1. Antibodies to different *R. conorii* strains in sera from inhabitants living along the Adriatic coastal regions of Croatia tested by IFA and EIA

<table>
<thead>
<tr>
<th>Region</th>
<th>No. of serum samples tested</th>
<th>No. positive (%) by assay with strain:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Morocan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IFA</td>
</tr>
<tr>
<td>Istra</td>
<td>42</td>
<td>1 (2.4)</td>
</tr>
<tr>
<td>Zadar</td>
<td>57</td>
<td>5 (8.8)</td>
</tr>
<tr>
<td>Split</td>
<td>103</td>
<td>14 (13.6)</td>
</tr>
<tr>
<td>Dubrovnik</td>
<td>275</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>477</td>
<td>20 (4.2)</td>
</tr>
</tbody>
</table>

antibodies. Three serum samples reacted only with the Malish-7 strain. Twenty-one serum samples were positive by both IFA and EIA, whereas one serum sample was positive only by IFA and two serum samples were positive only by EIA. The WB assay demonstrated that all 20 serum samples containing IFA and/or EIA antibodies that were examined reacted with the 120-kDa protein antigen, three serum samples reacted with the rickettsial lipopolysaccharide bands at molecular weights of 20 to 50 kDa, and one serum sample reacted with the 60-kDa antigen. A pool of 37 negative serum samples from Vermont, where there are no SFG rickettsioses, did not react with any rickettsial antigens by WB. A pool of five convalescent serum samples from Spanish Mediterranean spotted fever patients used as the positive WB control reacted with the 120-kDa antigen.

In Croatia and many other parts of the world, SFG rickettsiosis is seldom considered or diagnosed, in part because of the difficulty of the clinical diagnosis of SFG rickettsioses and in part because of the frequent lack of availability of specific laboratory diagnostic tests. Thus, recognition of a persistent, endemic SFG rickettsiosis can be delayed for years even when a substantial number of cases are occurring (21).

Serologic surveys have been employed to estimate the importance of various infectious diseases. This approach offers the possibility of greater sensitivity and specificity than does reviewing medical records or questioning persons regarding a past history of SFG rickettsiosis. Serologic surveys in the area considered to be endemic for boutonneuse fever have included Portugal (1), Spain (6, 8, 20), France including Corsica (16, 17), Italy including Sicily (23), Israel (19), Egypt (3), the Central African Republic (7), Ivory Coast (18), and Zimbabwe (9). Problems with serosurveys include the selection of an assay that will detect persistent antibodies and selection of a cutoff value that provides useful sensitivity and specificity. The use of the three most specific techniques available (IFA, EIA, and WB) yielded a high degree of agreement. It appears that SFG rickettsiae are endemic in this area. Inhabitants and visitors, either military personnel or tourists, are at risk of infection. Although this investigation has documented the presence of antibodies to SFG rickettsiae in a healthy population more convincingly than previous reports, the question of what SFG rickettsial species stimulated the antibodies remains unanswered. The possibilities include the diverse strains of *R. conorii* (24), the Israeli SFG rickettsia, *R. akari*, *R. sibirica*, *R. slovaca*, *R. helvetica*, and the two recently identified novel SFG rickettsial serotypes from *Rhipicephalus turanicus* ticks (2). Isolation and identification of rickettsiae from patients will provide definitive etiologic data, and recovery and identification of rickettsiae from ticks and mites will suggest the possibilities for the other rickettsial species that might be involved. Ultimately, serologic specificity will be achieved only when an assay of antibodies to species-specific rickettsial epitopes becomes available.

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