Evaluation of the High-Density Agglutination Test for Coxiella burnetii Antibodies in Animals

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The usefulness of the high-density particle agglutination (HDPA) test as a potential tool for the detection of anti-Coxiella burnetii antibodies in animal sera was studied by using 619 cow, 589 dog, and 150 cat serum samples and antisera from rabbits, guinea pigs, and mice. The sensitivity and specificity of the test versus those of the reference microimmunofluorescence test were determined at two different threshold titer values. At the cutoff value of 1:16, the sensitivities of the HDPA test for cow, dog, and cat sera were 94.3, 95, and 91.3%, respectively, and the specificities were 95.5, 95.3, and 91.3%, respectively. At the cutoff value of 1:32, the sensitivities were 86.7, 88.3, and 82.6%, respectively, and the specificities were 99, 99.2, and 98.4%, respectively. For the group of immune laboratory animals all samples from rabbits, guinea pigs, and mice were positive by the HDPA test. The erythrocyte-sensitizing substance from phase II C. burnetii was found to contain protein and carbohydrate, and both fractions are immunoreactive. The study results show that the HDPA test is a useful tool in the epizootiological survey of Coxiella infection in animals.

Coxiella burnetii, the etiological agent of Q fever, is an obligate intracellular parasite which has an extremely broad host range. The organism has been isolated from different domestic and wild animal species (12). By shedding the agent into the environment these animals serve as major sources for human infection. While in humans the infection usually results in a variety of diseases, e.g., acute, flu-like illness, pneumonia, and chronic endocarditis and hepatitis, in animals in most cases it causes no apparent symptoms. The diagnosis, therefore, is based on isolation and serology.

Various serological tests have been used for the diagnosis of C. burnetii infection in animals (12). However, the broad-host range nature of the agent and the difficulty in its propagation have caused a lot of problems in identification by conventional methods. Therefore, for mass screening and surveillance of animals for C. burnetii infection a simple test which is readily applicable to different animal species and which does not require large quantities of antigen is desirable. The passive agglutination tests using one or another kind of soluble antigens to coat erythrocytes or inorganic man-made carriers have considerable value in this respect. The erythrocyte-sensitizing substance (ESS), first described by Chang, has been shown to be a good antigen and has been used widely in indirect hemagglutination (IHA) (1, 2, 16) and latex agglutination (4, 6, 7) tests for the serodiagnosis of rickettsial infections. This soluble antigen is used. In this paper, for convenience, the ESS without treatment with chloroform will be called ESS1, whereas chloroform-treated ESS will be called ESS2.

Enzymatic and periodate treatment of ESS. Antisera. Four lots of antiserum against C. burnetii were raised in four rabbits each, with Freund adjuvant. Two of the antisera were treated enzymatically by the addition of proteinase K to make a final concentration of 200 μg/ml and were incubated at 55°C for 1 h. The periodate treatment of ESS was carried out as described previously (14). Protein quantitation of ESS and whole-cell suspensions was performed by the method of Lowry et al. (13). The ESS used to coat HDP for the seroepizootiological survey of animals was prepared without the chloroform treatment step because we found that such treatment results in some loss of ESS and is unnecessary when purified C. burnetii antigen is used. In this study, for convenience, the ESS without treatment with chloroform will be called ESS1, whereas chloroform-treated ESS will be called ESS2.

Materials and Methods

Antigen preparation. The ESS from purified phase II C. burnetii was prepared as described previously (14). Protein quantitation of ESS and whole-cell suspensions was performed by the method of Lowry et al. (13). The ESS used to coat HDP for the seroepizootiological survey of animals was prepared without the chloroform treatment step because we found that such treatment results in some loss of ESS and is unnecessary when purified C. burnetii antigen is used. In this study, for convenience, the ESS without treatment with chloroform will be called ESS1, whereas chloroform-treated ESS will be called ESS2.

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titers of ESS samples, HDP-C. burnetii reagents were prepared by sensitizing HDP with the same volume of antigens in the same condition. The micro-IF test was performed with two conjugates, one of which was specific for immunoglobulin G (IgG) and the other of which was specific for IgM. Samples with micro-IF titers of 1:16 or higher were referred to as positive.

**SDS-PAGE and Immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (11) by using a discontinuous system with 5% stacking and 15% resolving gels. Samples of whole-cell antigen and ESS were mixed with the same volume of sample buffer containing 0.0625 M Tris (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.001% bromphenol blue and were boiled at 100°C for 5 min. Electrophoresis was run at 20 mA of constant current with Tris-glycine buffer of sample buffer containing 0.0625 M Tris (pH 8.3) plus 0.1% SDS buffer until the dye migrated to the bottom of the gels, which were used for staining or immunoblotting.

Immunoblotting was done on a polyvinylidene difluoride (PVDF) membrane as described elsewhere (20). Electrophoresis transfer was performed for 60 min at 200 mA on a semidyry electroblotter. The PVDF membrane was blocked for 1 h at room temperature in blocking buffer consisting of 0.1% Tween 20 in Tris-glycine (pH 8.3) plus 0.1% SDS buffer until the dye migrated to the bottom of the gels. The specificity of the HDPA test versus that of the micro-IF test was shown in Table 2. At the threshold value of 1:16, the HDPA test was in agreement with the micro-IF test for 99 cow, 57 dog, and 21 cat sera. Thus, the specificities of the HDPA test for these animals were 94.3, 95, and 91.3%, respectively. The predictive values for a positive result were 81.1, 69.5, and 65.6%, respectively. At the threshold value of 1:32, the specificities were 86.7, 88.3, and 82.6%, respectively and the predictive values were 94.8, 93, and 90.5%, respectively.

**Detection of C. burnetii antibodies in rabbit, guinea pig, and mouse antisera by HDPA test.** Since there have been reports that the IHA test using ESS failed to demonstrate specific rickettsial antibodies in the sera of common laboratory animals (1, 3, 16), we attempted to determine whether the HDPA test could detect C. burnetii antibodies in immune rabbit, guinea pig, and mouse sera. In our study all four rabbit, four guinea pig, and six mouse antisera were positive by the HDPA test, with titers ranging from 1:64 to 1:2,048 (micro-IF test phase II...
titers ranged from 1:128 to 1:2,048). No significant changes in titers were observed when ESS1 and ESS2 were used as the antigens to sensitize HDP.

**Immunochromatographic characteristics of ESS.** The protein amounts in the whole-cell suspension, ESS1, and ESS2, as measured by the method of Lowry et al., were 3.5, 1.25, and 1.2 mg per ml, respectively. The silver-staining profiles of untreated and treated ESS are shown in Fig. 1A. No differences in staining pattern between ESS1 and ESS2 were observed. Both untreated ESS samples (lanes 4 and 7) show smearing patterns and contain no clearly distinguished bands except for one faintly stained band of roughly 19 kDa and a heavily stained band at the bottom of the gel. In the proteinase K-treated lanes (lanes 5 and 8), as expected, only the band at the bottom of the gel remained. This fast-migrating band is characteristic of the phase II LPS migration pattern (5). This band was removed in lanes of NaIO4-treated ESS samples (lanes 6 and 9), which exhibit a faintly stained pattern in the upper part of the gel and the apparent 19-kDa protein band.

Figure 1B shows the immunoblot profiles of ESS and whole-cell antigens which served as controls. Striking similarities are seen in the immunoblot and silver-staining patterns. In lanes of untreated ESS samples (lanes 4 and 7) both a smeared upper band and a fast-migrating band at the bottom of the gel react strongly with phase II antibodies. The fact that these two bands belong to the protein and carbohydrate fractions of ESS was clearly demonstrated in the proteinase K- and NaIO4-treated lanes (lanes 5 and 8 and lanes 6 and 9), in which the upper and lower parts, respectively, of the gel were removed by enzymatic and periodate treatments. The contribution of each of these two moieties in the HDPA test was determined by a comparison of HDPA titers for two antisera, one each from a rabbit and a guinea pig. Table 3 shows the HDPA titers obtained with reagents prepared from treated and dialyzed ESS samples in comparison with those obtained with reagents prepared from untreated ESS. Treatments with proteinase K and NaIO4 both resulted in a decrease in titers, with the former treatment having a greater effect. Meanwhile, no agglutination was seen when the reagent was prepared from NaIO4-treated and undialyzed ESS samples.

**DISCUSSION**

The most important advantages of agglutination tests are their simplicity and applicability to sera from different sources. A previous study showed that the HDPA test developed by us is a promising tool for the rapid serodiagnosis of Q fever in humans (14). In this study the applicability of the HDPA test to sera from different animal species was evaluated by using 619 cow, 589 dog, and 150 cat serum samples and antisera from rabbits, guinea pigs, and mice. Our results show that the HDPA test also paralleled the reference micro-IF test in the detection of C. burnetii antibodies in animal sera.

For a limited number of samples from different animal species, the results of the HDPA test were in good agreement with those of the micro-IF test with regard to specificity and sensitivity. In a previous report we established the titer of 1:32 as the cutoff value for human sera. In this study the specificity and sensitivity of the HDPA test were evaluated at two different threshold values. At the threshold value of 1:16 the HDPA test detected C. burnetii antibodies in more than 91% of all micro-IF test-reactive samples. The specificity of the test at this low cutoff value is still good, ranging from 91.3% in cat sera to 95.5% in cow sera. The predictive values for a positive result, however, were relatively low, especially for dog and cat sera (69.5 and 65.6%, respectively). Raising the cutoff value to 1:32 will make a positive result more reliable (the predictive values were >90%), but at the cost of a decrease in sensitivity. Therefore, in a serological survey either the lower or higher cutoff value should be chosen depending on the sample population and the research requirement, which may emphasize the sensitivity or specificity of the test. The specificity of the HDPA test was a bit lower for cat sera, and one sample gave a nonspecific reaction at a dilution of up to 1:64. This lower specificity may be explained by the limited number of samples tested and by the fact that many cat serum samples used in the study were hemolyzed, thus making the test more difficult to interpret.

So far, agglutination tests using ESS have been used mainly for serodiagnosis in humans (1, 2, 4, 6, 7, 14, 16). There were only a few reports about their application to animals. For some unclear reasons, the IHA test, although highly sensitive for the detection of specific antibodies in human patients, in many cases failed to detect antibodies in the sera of immunized and infected rabbits, rats, and guinea pigs. In a study by Chang et al. (3) the IHA test results were not consistent for infected rabbits and guinea pigs. Shirai et al. (16) found that the IHA test using glutaraldehyde-fixed sheep erythrocytes generally failed to detect specific antibodies in immune mice, rabbits, and guinea pigs. Anacker et al. (1), using fresh erythrocytes and modified ESS from *Rickettsia rickettsii*, were able to demonstrate specific antibodies in all infected rabbits and guinea pigs but in only three of five groups of pooled mouse sera, with strong prozones observed in every case. In our study, however, the HDPA test was able to detect *C. burnetii* antibodies in all four rabbit, four guinea pig, and six mouse antisera. This apparent advantage of the HDPA test over the IHA test may be

**TABLE 3. Effect of enzyme and periodate treatment on HDPA titer**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>HDPA titer of antiserum sample from:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rabbita</td>
</tr>
<tr>
<td>Untreated ESS</td>
<td>2,048</td>
</tr>
<tr>
<td>ESS-proteinase K</td>
<td>128</td>
</tr>
<tr>
<td>ESS-periodate</td>
<td>512</td>
</tr>
</tbody>
</table>

a Titers are shown as dilution factors (e.g., a titer of 2,048 corresponds to a 1:2,048 dilution).

b Titer by micro-IF test, 2,048.

c Titer by micro-IF test, 1,024.
explained by the nature of carriers. Unlike erythrocytes, HDP are inorganic, biologically inert particles and so are not interfered with by components of animal sera. It has also been recognized that the functional groups coating the particle surface have great binding capability and can effectively sensitize different antigens, resulting in a sensitivity to antibody that is greater than that of similarly used erythrocytes (18).

In an agglutination test using one or another kind of sensitized particles as the reagents, the most important factor determining the test sensitivity is the preparation of a soluble antigen which contains mainly serologically reactive components capable of sensitizing the carriers. The ESS has been shown to be a useful antigen in various passive agglutination tests. Earlier studies with other bacterial antigens have revealed that exposure to heat and high pH increases their efficiency of binding to erythrocytes (15). Also, the ESS from *Rickettsia typhi* was detected in biologic fluid from infected guinea pigs and so could be used as a marker for antigen diagnosis (9). The increasing importance of ESS has encouraged researchers to investigate its chemical and biological natures. In some previous studies the ESS from *R. rickettsii, R. typhi* (16), *Rickettsia prowazekii*, and *Rickettsia conorii* (15) was found to be a protein-carbohydrate mixture. The ESS from phase II *C. burnetii* also contained protein, as determined by chemical quantitation. The chemical quantitation of carbohydrate was not carried out due to the lack of an adequate amount of antigen. However, silver staining of untreated and treated ESS samples reveals that ESS from phase II *C. burnetii* contains both protein and carbohydrate components (Fig. 1A). The results of both chemical analysis and silver staining of ESS and whole-cell antigens indicate that hot alkali treatment, while retaining rickettsial LPS material, removed and altered a large percentage of protein, which was seen as multiple bands in the control whole-cell lanes. This was further confirmed by the fact that hardly any protein bands could be seen in the less sensitive Coomassie brilliant blue-stained SDS-PAGE gels which employed the same amount of ESS per lane (data not shown).

Since the ESS from *R. rickettsii* has been suggested to be a carbohydrate and/or LPS-like material (9), in this study the chemical nature of serologically reactive components of ESS from *C. burnetii* was determined by immunoblot assay and HDPA test with both untreated and treated ESS samples. The immunoblotting profiles of different antigens shown in Fig. 1B were strikingly similar to their silver-staining patterns. This similarity indicates that both protein and carbohydrate moieties in ESS are immunoreactive. However, the contribution of each moiety in the HDPA test is difficult to determine since it is known that the ESS components have different binding capacities (1, 8). In a study by Osterman and Eisemann (15) it was found that the serological activity of ESS from *R. prowazekii* and *R. conorii* was unaffected by trypsin but that both antigenicity and erythrocyte-binding capacity were significantly reduced after exposure to sodium metaperiodate, which suggests an important role for the carbohydrate moiety. However, the authors found that the role of carbohydrate was complex since periodate oxidation also affected the binding of ESS to erythrocytes as well as the interaction of ESS with antibody. In our study portions of ESS samples were dialyzed to remove the chemicals and adjust the pH, which is an important factor in the HDPA test. The interference of NaIO₄ in the test reactivity was clearly shown by the fact that undialyzed periodate-treated ESS rendered the HDPA test nonreactive with both antisera, while the dialyzed sample just reduced HDPA titers. The greater effect of proteinase K on the HDPA antibodies in animals. Unlike erythrocytes, HDP are inorganic, biologically inert particles and so are not interfered with by components of animal sera. It has also been recognized that the functional groups coating the particle surface have great binding capability and can effectively sensitize different antigens, resulting in a sensitivity to antibody that is greater than that of similarly used erythrocytes (18).

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