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Rapid Method for Detection of *Coxiella burnetii* Antibodies Using High-Density Particle Agglutination

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A high-density particle agglutination test, using erythrocyte-sensitizing substance from phase II *Coxiella burnetii* adsorbed to high-density composite particles, was developed for rapid serodiagnosis of Q fever. The test was compared with the microimmunofluorescence test for sensitivity and specificity by using 3,036 human serum samples collected in Gifu Prefecture, Japan. An excellent agreement was found between the two tests for the acute-phase group and paired serum samples, but some discordant results were observed in the single-sample group. The sensitivity and specificity of the high-density particle agglutination test were both 100% in the former group and 81.6 and 99.9%, respectively, in the latter group. The test is a very promising tool for routine serodiagnosis of Q fever because of its simplicity, sensitivity, and specificity.

Q fever, a zoonosis of worldwide distribution, is caused by the rickettsia *Coxiella burnetii*. This organism is found in urine, feces, milk, and birth products of infected animals (7, 10). Humans become infected through inhalation of contaminated aerosols or ingestion of raw milk. Because of the nonspecific initial clinical signs, such as fever, pneumonia, headache, myalgia, cough, and weakness, Q fever is rarely diagnosed clinically.

Since isolation and identification of the causative agent are time-consuming, hazardous, and expensive, the diagnosis of Q fever is usually based on the results of serological tests. So far, a number of serological methods have been used for detecting *C. burnetii* antibodies. However, they are still far from satisfactory. The complement fixation test, although specific, is cumbersome and lacks sensitivity (9, 23, 34). The microagglutination test is simple and sensitive and can detect an early antibody response to *C. burnetii* (17), but it has the disadvantage of requiring large amounts of antigen. The microimmunofluorescence (micro-IF) test has considerable value in serodiagnosis of Q fever and is the test of choice. However, this test is time-consuming and requires skilled technicians and expensive equipment. Of the newer methods, the enzyme-linked immunosorbent assay (ELISA) has been reported by some authors as being more sensitive than other tests used (9, 24, 31), but its application in Q fever diagnosis is still limited.

In Japan, a high-density particle agglutination (HDPA) test has recently been used by some investigators for rapid serodiagnosis of human infections (26, 28, 30). Studies by these authors have shown that the test is very specific and sensitive. The present study describes a rapid, specific, and sensitive HDPA test for detection of antibodies to *C. burnetii* in human sera.

MATERIALS AND METHODS

Carrier particles. High-density composite particles (18) (HDP; Tokuyama Soda Co., Tokyo, Japan) were used as the carrier. These particles have a silica core surrounded by a red dye layer which in turn is covered by a second silica layer. The particle surface is covered with functional groups designed to adsorb antigens. The density of the particles is approximately 2.0 g/cm^3, and their diameter is 1.8 μm.

Antigen preparation. The antigen used to coat the HDP was the erythrocyte-sensitizing substance (ESS) prepared as described by Chang (5) and adapted to *C. burnetii*. Phase II *C. burnetii* Nine Mile cultivated in BGM cell cultures was purified as described previously (16). The purified *C. burnetii* cells were suspended in phosphate-buffered saline (PBS; 1 mg/ml), adjusted to 0.2 N with saturated NaOH solution, and boiled for 30 min. Then the suspension was dialyzed overnight at 4°C against Chang buffer (NaCl, 6.8 g; Na_2HPO_4, 1.05 g; KH_2PO_4, 0.81 g; distilled H_2O, 1,000 ml; pH 6.8), mixed with an equal volume of chloroform, and shaken for 20 min. The mixture was then centrifuged at 400 × *g* for 20 min to remove chloroform from the ESS suspension.

Preparation of antigen-coated HDP. For preparation of *C. burnetii* antigen-coated HDP, the ESS, which was diluted in PBS (1/60 M) to an optimum concentration, was mixed with the same volume of 0.5% HDP suspension in PBS (1/60 M; pH 7.2) and incubated for 2 h at room temperature with frequent shaking. Then the HDP were washed twice and suspended in PBS (1/60 M) containing 10% stabilizing buffer (0.01% bovine serum albumin, 1% dextran, 1% sodium glutamate, 0.5% glycine, and 0.1% sodium azide) to make a final HDP concentration of 0.5%.

The optimum volume of antigen varied from 60 to 150 μl/1.0 ml of HDP-*C. burnetii* reagent. This was determined by box titration with positive sera of known micro-IF titers.

Sera. A total of 3,036 serum samples collected in Gifu Prefecture, Japan, was used in the study. Of these, 3,000 samples were collected between September and December 1995 from 1,740 patients of Gifu University Medical Faculty Hospital, 20 acute-phase single samples were from laboratory-confirmed Q fever patients (29), and 10 follow-up samples were collected from two Q fever patients in 1995 and 1996. Demographic characteristics (e.g., age and gender) and clinical information on most patients were obtained from case reports submitted with serum samples. All sera were stored at −20°C before testing for *C. burnetii* antibodies by micro-IF and HDPA tests.

HDPA test. For screening, the sera, which were inactivated at 56°C for 30 min, were first diluted 1:16 in buffer (PBS [1/60 M] with stabilizing buffer) in a 96-well plate, and only reactive specimens were further titrated at serial twofold dilutions. To each well, which contains 20 μl of serum dilution, 20 μl of antigen-coated HDP was added. The plates were then mixed with a mixer, incubated at room temperature for 30 min, and read macroscopically. A positive agglutination reaction is indicated by a thin even layer of particles covering the entire bottom of the wells, whereas a negative reaction is indicated by the formation of a small red button in the center of the well bottom.

Micro-IF test. The micro-IF test was performed on all specimens by the technique described by Philip et al. (25) and adapted to the *C. burnetii* antigen (phase II, strain Nine Mile) as described elsewhere (16). The serum samples were screened at a dilution of 1:16. The conjugate was fluorescein-conjugated goat affinity-purified antibody to human immunoglobulins (immunoglobulin G [IgG], IgA, and IgM)-2MG (Organon Teknika N.V. Cappel Products) (micro-IF/Ig). Positive samples were then diluted in twofold steps to 1:4,096, and subsequent immunoassays were performed with goat anti-human IgM(μ) (Tago, Inc., Burlingame, Calif.) (micro-IF/IgM), IgG (Fc) (Organon Teknika N.V. Cappel Products) (micro-IF/IgG), and polyvalent conjugate (IgG, IgA, and IgM). A positive control and negative control were run with each test. Titers of 1:16 or higher were considered positive.

In this report the micro-IF results are considered to be true results which

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TABLE 1. Agreement of the HDPA test with micro-IF/Ig test results

HDPA titer	No. of samples at indicated micro-IF/Ig titer									Total no.
	Nonreactive (<16)	Reactive								
		16	32	64	128	256	512	1,024	≥2,048	
<16	2,815	1								2,816
16	20	13	2							35
32	1	9	19	6	1					36
64	1[a]	2	7	29	18	6	4	1		68
128			7	9	10	7	2	3		38
256				4	5	2	9	1		21
512				1	2	2	8	2		15
1,024							1	2	1	4
≥2,048							1	1	1	3
Total	2,837	25	35	49	36	17	25	10	2	3,036

[a] This sample was nonreactive by the micro-IF/Ig test but had a micro-IF/IgM titer of 1:16 and therefore was scored positive.

indicate the presence or absence of antibodies to *C. burnetii*. The sensitivity and specificity of the HDPA test were determined by comparison with the micro-IF test values.

Statistical analyses. The correlation coefficient was calculated by the assumed-mean method. The sensitivity, specificity, and predictive values of positive and negative test results were calculated according to standard methods.

RESULTS

The results of micro-IF and HDPA tests for all 3,036 serum samples are shown in Table 1. Overall, 200 samples were positive by the micro-IF test, including 1 sample reactive only by the micro-IF/IgM test. The screening by HDPA at the dilution of 1:16 revealed, in total, 220 reactive samples.

Threshold value of the HDPA test. To determine the threshold value of the HDPA test, all examined serum samples were grouped according to their micro-IF/Ig titers into positive (≥16) and negative (<16) categories (Fig. 1).

At the HDPA titer of 1:16, more than half of the samples were negative by the micro-IF test. At the HDPA titer of 1:32, 35 samples were positive and only 1 sample was negative by the micro-IF test. At the HDPA titer of 1:64, 68 samples were positive. One sample was negative by the micro-IF/Ig test but had a micro-IF/IgM titer of 1:16 and so was scored positive. At the HDPA titer of ≥1:128, all samples were positive by the micro-IF test. We therefore determined the titer of 1:32 to be the threshold value of reactivity for the HDPA test.

Sensitivity. The HDPA and micro-IF tests results were in agreement for 183 micro-IF/Ig-reactive serum samples (Table 1). One sample, which had an HDPA titer of 1:64, was negative by the micro-IF/Ig test but had a micro-IF/IgM titer of 1:16. Sixteen micro-IF/Ig-reactive samples were negative by the HDPA test.

The results of the HDPA and micro-IF tests were in agreement for all 113 paired and follow-up serum samples from 34 patients (Table 2). All 20 acute-phase serum samples were positive by the HDPA test. Of 87 micro-IF-reactive single serum samples, 71 were positive by the HDPA test. Thus, the sensitivity of HDPA test results for all positive sera was 91.96%, that for paired and acute-phase sera was 100%, and that for single sera was 81.6%. The predictive values were 99.46, 100, and 98.6%, respectively.

Specificity. The HDPA test results were in agreement with the micro-IF results for 1,121 single and 1,714 paired nonreactive serum samples (Table 2). Only one single sample with an HDPA titer of 1:32 was negative by the micro-IF test. Thus, the specificity of the HDPA test for single serum samples was 99.9%, and the predictive value of a negative result was 98.6%. For paired and follow-up sera, the specificity and the predictive value of a negative result were 100%.

At a dilution of 1:4, *C. burnetii* antigen-coated HDP gave no reaction with all control sera, which included sera from guinea pigs immunized with *Rickettsia rickettsii*, *R. prowazekii*, *R. tsutsugamushi*, *R. conorii*, and *R. typhi* and sera from psittacosis patients (micro-IF titers ranked from 1:512 to 1:4,096). These sera were also negative by the micro-IF test.

Agreement of HDPA and micro-IF tests in titers. For 183 samples reactive by both tests (Table 1), HDPA titers did not

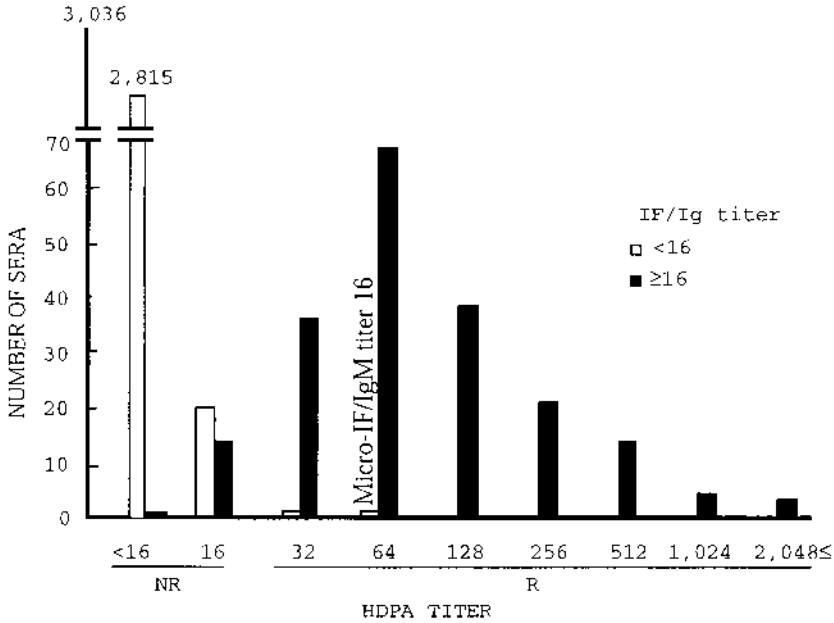

FIG. 1. Determination of threshold values of reactivity for the HDPA test. NR, nonreactive; R, reactive.

TABLE 2. Sensitivity and specificity of HDPA test versus micro-IF test results

Population of sera	Sensitivity		Specificity		Predictive value of result:			
					Positive[a]		Negative[b]	
	No. of samples[c]	%	No. of samples[d]	%	No. of samples	%	No. of samples	%
Single[e]	71/87	81.6	1,121/1,122	99.9	71/72	98.6	1,121/1,137	98.6
Acute	20/20	100			20/20	100		
Paired	113/113	100	1,714/1,714	100	113/113	100	1,714/1,714	100

[a] True positive/(true positive + false positive).
[b] True negative/(true negative + false negative).
[c] HDPA positive/micro-IF reactive.
[d] HDPA negative/micro-IF negative.
[e] Included acute sera.

always parallel micro-IF titers. Some HDPA titers were lower than micro-IF titers; others were higher. However, the relatively high correlation coefficient ($r = 0.788$; $P < 0.01$) showed that titer agreement of the two tests was meaningful. The correlation was higher in the group of acute-phase serum samples ($r = 0.91$; $P < 0.01$). In this group, 16 of 20 samples had an HDPA titer higher than or equal to the micro-IF/Ig titer. Of 34 positive cases, in which two or more consecutive serum samples were obtained, 13 were diagnosed as having active Q fever by both methods (by an at least fourfold rise in antibody titer) (Table 3). Four other cases had a fourfold rise in antibody titer only by the micro-IF/Ig test, whereas another case had a fourfold rise in titer only by the HDPA test. In the 16 remaining cases no significant seroconversions by both tests were observed, though the antibody titers remained high (≥128).

DISCUSSION

The use of the HDPA test for detection of specific antibodies to infectious agents in humans has recently been reported (26, 28, 30). The present paper is the first to describe the use of this test for detection of specific antibodies to *C. burnetii*. For a new test to be confirmed as a useful tool for routine diagnosis, all its aspects should be evaluated in detail. In our study, the HDPA test was evaluated by comparison with the micro-IF test, the best currently available serological test, for some main criteria. Our results showed that the two tests were in good agreement for specificity and sensitivity.

The HDPA test has several advantages over other conventional methods. The test is technically simple to perform in small local laboratories or a physician's office. It requires neither elaborate equipment nor skilled technicians, as the micro-IF test does. Test results are obtained within 1 h, whereas other conventional methods require from 4 h to overnight. Like the indirect hemagglutination test, the HDPA test offers economy of reagents, but antigen-coated HDP are much more stable than sensitized erythrocytes. Preliminary observations showed that the former were still active after preservation for 10 months at 4°C.

The HDPA test is highly specific. If micro-IF test results are considered to be the reference value, the HDPA test gave a nonspecific reaction for only 1 of 2,836 negative serum samples tested. Antisera to other rickettsiae, if inactivated by heating at 56°C for 30 min, did not agglutinate *C. burnetii* antigen-coated particles even at the dilution of 1:4 (native antisera gave cross-reactions at dilutions up to 1:16). The high specificity of the HDPA test in distinguishing anti-*C. burnetii* antibodies from those to other rickettsiae was supported by the fact that *C. burnetii* is immunologically distinct from other rickettsiae (10). The attempts by some authors in searching for cross-reacting epitopes between *C. burnetii*, *Francisella tularensis*, and *R. rickettsii* were futile (32, 33). In fact, all current serological tests for Q fever (complement fixation, the immunofluorescent-antibody assay, microagglutination, indirect hemagglutination, and ELISA) are highly specific, and no cross-reactions between *C. burnetii* and any bacterial or viral microorganisms were

TABLE 3. Result of paired and follow-up samples from 13 patients

Patient	Bleeding date (mo/day/yr)	Antibody titer measured by:			
		HDPA	Micro-IF/Ig	Micro-IF/IgG	Micro-IF/IgM
1	9/18/95	64	128	128	64
	10/27/95	128	256	256	64
	11/29/95	1,024	1,024	512	128
2	9/18/95	128	128	128	64
	10/17/95	256	256	256	64
	11/28/95	512	1,024	512	<16
3	10/27/95	64	512	512	128
	11/17/95	512	2,048	1,024	64
4	10/2/95	64	64	16	64
	11/14/95	128	128	256	16
	11/27/95	512	512	1,024	<16
5	9/18/95	128	128	32	128
	10/2/95	256	1,024	1,024	64
	11/14/95	1,024	2,048	2,048	16
6	10/11/95	32	32	16	32
	10/30/95	128	128	64	64
	11/14/95	512	512	256	32
7	11/7/95	32	16	16	16
	11/23/95	128	128	64	32
8	9/18/95	64	32	16	32
	10/16/95	512	128	128	64
9	9/18/95	64	64	32	128
	10/12/95	128	128	64	64
	11/13/95	256	512	128	64
10	10/17/95	64	64	64	16
	11/20/95	256	256	128	16
11	11/7/95	32	32	16	32
	11/24/95	256	128	64	64
12	5/14/95[a]	16	<16	<16	<16
	5/19/95	128	32	16	64
	6/30/95	512	1,024	1,024	64
	8/24/95	256	1,024	1,024	32
	2/23/96	32	128	256	<16
13	2/29/96[b]	32	32	32	32
	3/8/96	64	64	64	32
	3/15/96	64	128	256	16
	3/26/96	128	256	256	<16
	4/5/96	128	256	256	<16

[a] This sample was collected on the fifth day after onset.
[b] This sample was collected 3 weeks after onset.

observed (21, 24). Our study with the HDPA test, though still limited, also confirmed this specificity. Rheumatoid factor seems to have no effect on antigen-coated HDP, since all five rheumatoid factor-positive serum samples (titers of 1:50 to 1:2,500) were negative by the HDPA test.

The high sensitivity of the HDPA test in detecting specific antibodies in human sera has been reported by several authors (26, 28, 30). Satoh et al. (26) and Shitara et al. (28) showed that this test is more sensitive than complement fixation and particle agglutination tests in diagnosis of mycoplasma pneumonia. Tomiyama and Lee (30) found that the sensitivity of the HDPA test was superior to that of the immunofluorescent-antibody test in detecting hantavirus antibodies. The authors explained this superiority by the excellent capability of HDP in sensitizing protein and lipid antigens on their surfaces. In our study, 16 micro-IF/Ig-reactive serum samples were negative by the HDPA test (Table 1), which showed that the HDPA test is less sensitive than the micro-IF test. However, the sensitivity of the HDPA test in acute cases was equal to that of the micro-IF test. Hechemy and Rubin (15) and De La Fuente et al. (6) in their studies with latex tests also found that higher test sensitivity was observed in the samples collected from Rocky Mountain and Mediterranean spotted fever patients during the early days after onset. In our study, four patients showed a fourfold rise in antibody titer only by the micro-IF test. Although the time of onset of disease in these cases was not known, this indicates that specimens collected earlier during the acute phase of the disease may be necessary to demonstrate a fourfold rise in titer by the HDPA test.

The advantages of agglutination tests in the early diagnosis of infections were well documented (4, 14, 15, 17). In this respect, however, our investigation had some limitations. Because Q fever is still not diagnosed routinely in Japan, we could not obtain serum samples from patients during the first days after onset. The course of disease in most patients was also unknown. With such limitations, we examined the micro-IF/IgM and IgG titers and used them as reference value to evaluate the newly developed HDPA test. It is well-known that the presence of anti-*C. burnetii* IgM antibody to phase II in the serum indicates an active or recent infection (7, 23). In our study, the sensitivity of the HDPA test was higher in micro-IF/IgM-positive samples, which was most clearly shown in the group of acute-phase sera. This fact suggests that the HDPA test is useful for detection of an early antibody response in patients. In one patient (patient 12 in Table 3), specimens from whom were available from the day of onset, a titer of 1:16 was obtained by the HDPA test on the fifth day (the day the patient was diagnosed as having Q fever by PCR [data not shown]), and 5 days later the HDPA titer had risen to 1:128. In this case the HDPA test detected anti-*C. burnetii* antibodies earlier than micro-IF test, but the HDPA titer dropped faster than micro-IF titer. In some cases, from which two or more follow-up samples were obtained (patients 2, 3, 4, 5, and 13 in Table 3), the kinetics of the HDPA test was similar to that of the micro-IF/Ig test but different from that of the micro-IF/IgM test. This fact indicates that the HDPA test, although more efficient in detection of specific IgM antibody, can measure the IgG antibody level in the sera as well.

Although a good correlation was found between the HDPA and micro-IF tests in acute-phase and paired serum samples, discrepant results were observed in the group of single serum samples. In this group, 16 micro-IF/Ig-reactive samples were negative by the HDPA test (Table 2). All these samples had low micro-IF/Ig titers and were negative by the micro-IF/IgM test, so they probably were collected from cases of past infection. This suggests that the application of the HDPA test in seroepidemiological investigations is limited. The discrepancies between the two tests results may be explained by the suggestion by Hechemy and Rubin that some rickettsial antibodies, not detected by agglutination tests using ESS as the antigen, are directed against a heat-labile, species-specific protein found in the whole organism, which is used in the micro-IF test (15). This heat-labile protein is lost during treatment by boiling *C. burnetii* suspensions with NaOH. Moreover, Hechemy et al. (12) found that the ESS from *R. rickettsii* plays a minor role in the micro-IF test, and the micro-IF test measures antibodies to at least some antigens separate and distinct from ESS. In a study by Bakemeier (2), the absorption of rabbit anti-*C. burnetii* phase I plus II serum samples with either phase I or phase II whole-cell antigens did not significantly reduce the titers of the IHA test which used soluble antigens from either phase I or phase II.

In humans, host response to *C. burnetii* is characterized by an early formation of anti-phase II antibodies, which are elevated and largely dominate in primary acute Q fever, and a later rise in anti-phase I antibodies, which are present at high titers only in the chronic form of the disease (3, 8, 11, 17, 22–24, 34). This explains why the use of phase II *C. burnetii* antigens is essential for any serological test in the diagnosis of acute Q fever. Most current serological tests for Q fever use the intact whole cell or chemical-treated particulate antigens. The use of soluble antigens prepared by ultrasonication of both phase I and phase II was first reported by Bakemeier (2). ESS, although used widely for the diagnosis of various rickettsial infections (1, 5, 6, 14, 15, 27), has not been applied to *C. burnetii*. Our study showed that ESS from phase II *C. burnetii* is also a good antigen for serodiagnosis of Q fever. Studies by Anacker et al. (1), Murphy et al. (19), Osterman and Eisemann (20), and Hechemy et al. (12) showed that ESS is a complex mixture which contains more than one antigenic component. Enzymatic and chemical treatments of ESS suggested an important role of the carbohydrate and/or LPS-like moiety but at the same time could not preclude the role of the protein moiety in the agglutination reaction (13, 20). To our knowledge, no information on the nature of the ESS from *C. burnetii* is available to date. At present, the chemical analysis of the ESS from *C. burnetii* and the investigation of different ESS fractions role in the agglutination reaction are under way.

The results of this preliminary evaluation of the HDPA test for detection of antibodies to *C. burnetii* are very encouraging. Although more research is still needed, our HDPA test is a promising tool for rapid serodiagnosis of Q fever. Because of its simplicity, specificity, and sensitivity, we recommend its use in hospitals and local laboratories where the IF test is not available.

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