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STUDY OF CROSS-REACTION BETWEEN *COXIELLA BURNETII* AND *LEGIONELLA PNEUMOPHILA* USING INDIRECT IMMUNOFLUORESCENCE ASSAY AND IMMUNOBLOTTING

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Summary. – Parients with Q fever and legionellosis may present identical clinical symptom. Differentiation of these diseases is made by serology, mainly the indirect immunofluorescence assay (IFA). Using IFA the authors tested 154 Q fever positive sera from 55 patients with acute Q fever and 28 patients with chronic Q fever for *Legionella pneumophila* antibodies and 57 sera from 57 patients with legionellosis for *Coxiella burnetii* antibodies. Of the 211 sera tested, four sera from different patients had antibodies to both *C. burnetii* and *L. pneumophila.* Using cross-adsorption studies and protein immunoblotting, no cross-reaction between *C. burnetii* and *L. pneumophila* antibodies could be identified. The moderate antibody titers against *L. pneumophila* in two Q fever patients and vice versa for one legionellosis patient are consistent with the incidence of seroprevalence in healthy blood donors and were not due to cross-reactivity. One patient was identified with concurrent Q fever and legionellosis.

Key words: Coxiella burnetii; Q fever; Legionella pneumophila; cross-reaction; indirect immunofluorescence assay; immunoblotting

Introduction

C. burnetii and *L. pneumophila* are the causative agents of Q fever and legionellosis, respectively. Both may present clinically identical symptoms, mainly pneumonia, fever in immunocompromised patients (Heard *et al.*, 1985; Meyer, 1981) or endocarditis (Mc Cabe *et al.*, 1984). It is of great importance to differentiate between these two diseases as their antibiotic sensitivities are different. The standard treatment of legionellosis with erythromycin is ineffective in Q fever (Spicer *et al.*, 1981). Tetracyclines are currently recommended in

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treatment of Q fever but are unreliable in legionellosis. Laboratory diagnosis of legionellosis and Q fever is most commonly performed using IFA. A recent publication has reported a case of Q fever pneumonia in which the diagnosis was complicated by the presence of cross-reacting antibodies to *L. pneumophila* serogroup IV (Dwyer and Gibbons, 1988). Another publication has reported a dual infection with *C. burnetii* and *L. pneumophila* (Domaradzki *et al.*, 1984). The aim of our study was to find patients showing positive serologic responses to *C. burnetii* and *L. pneumophila* in order to determine whether cross-reacting antibodies were present.

Materials and Methods

C. burnetii phase II antigen. C. burnetii strain Nine Mile was cultivated in mouse fibroblasts L929. The cells were grown in antibiotic free MEM-EARLE medium with 2 mmol/l L-glutamin and 4 % heat inactivated foetal calf serum (Seromed Biochrom KG). The harvested infected cells were centrifuged at 700 × g for 10 min; the supernatant was centrifuged at 10 000 × g for 10 min and pellet containing bacteria was resuspended in 0.5 % formaldehyde. The pelleted infected cells were resuspended in 10 ml of distilled water and disrupted with 1 ml of 5 % trypsin (Seromed Biochrom KG) at 37 °C for 1 hr. Then the released bacteria were purified by differential centrifugation through a solution of sucrose-phosphate-glutamin pH 7.2 (SPG; 7.64 % sucrose, 0.049 % potassium dihydrogenophosphate, 0.124 % dipotassium hydrogenophosphate, 0.072 % L-glutamin; all from Sigma) and 7 % renografin (Squibb). Three cycles of low speed-high speed centrifugation resulted in bacteria free of host material, which were suspended in 0.1 % formaldehyde.

C. burnetii phase I antigen. We inoculated mice with *C. burnetii* phase II strain Nine Mile; ten days later, we recovered the spleens of the rodents which contained *C. burnetii* phase I. Each spleen was grinded in 7.5 ml MEM-EARLE medium and inoculated into three 75 cm^2 culture flasks containing L929 cell monolayer (2.5 ml/flask). The infected cells were then harvested and the bacteria purified by the same methods as described above. The *C. burnetii* suspension (phase I and II) used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were concentrated to obtain a protein concentration of 2 mg/ml (Lowry *et al.*, 1951).

L. pneumophila. The bacteria used for SDS-PAGE and immunoblotting were grown on buffered charcoal yeast extract agar (BCYE, Biomérieux). The colonies were suspended in distilled water and centrifuged at 10 000 × g for 10 min. The cell pellet was washed three times with distilled water. Then the bacterial suspension was centrifuged at 700 × g for 10 min to clarify the pellet. The bacteria were killed with 0.5 % formaldehyde. The bacterial suspension was concentrated to obtain a protein concentration of 1.5 mg/ml. The strains were *L. pneumophila* serogroup I (Philadelphia 1 strain) and serogroup VI (Chicago 2 strain). The antigens used for IFA were formalin fixed antigens prepared from bacteria cultivated in embryonated hen's yolk sac (Taylor *et al.*, 1981). Ten serogroups were used: serogroup I - Philadelphia 1 strain, serogroup II - Togus 1 strain, serogroup III - Bloomington 2 strain, serogroup IV - Los Angeles 2 strain, serogroup V - Dallas 1E strain, serogroup VI - Chicago 2 strain, serogroup VII - Chicago 8 strain, serogroup VIII - Concorde 3 strain, serogroup IX - In 23 G1 C2 strain and serogroup X - Leiden 1 strain.

IFA. C. burnetii phase I and II antigens were placed on slides, air dried and fixed in cold acetone for 15 min. The sera were diluted in phosphate-buffered-saline (PBS) with 3 % non fat dry milk. After serum distribution, the slides were incubated for 30 min at 27 °C in a moist chamber. The slides were then washed three times for 10 min in PBS. The conjugated antisera diluted in PBS-3 % non fat dry milk with 0.025 % Evans Blue were added and allowed to react 30 min in a moist chamber at 37 °C. The slides were washed three times for 10 min in PBS and once for 10 min

in distilled water. The slides were dried and mounted with buffered glycerol (Fluoprep Biomérieux). The slides were observed with an epifluorescent microscope with a 40 × F objective. Positive sera screening was performed with a serum dilution 1:25 and using a goat anti-human globulin fluorescein conjugate (Fluoline H, Biomérieux) with a dilution 1:400. The antigens were *C. burnetii* phase II. Sera with titers higher than 1:25 were tested to determine IgG, IgM and IgA levels for both phase I and II antigens. An important step is to remove IgG prior to testing for IgM and IgA. This is necessary not only because of rheumatoid factor, often present in Q fever endocarditis and causing false positive IgM, but also because of very high levels of IgG which can saturate antigen sites and cause false negative results for both IgM and IgA (Raoult *et al.*, 1988). IgM and IgA titers were determined using a goat anti-human IgG fluorescein conjugate (Fluoline G, Biomérieux) diluted to 1:200, a goat anti-human IgM fluorescein conjugate (Fluoline M, Biomérieux) diluted to 1:200. *L. pneumophila* positive sera screening was performed with a serum dilution 1:16, using the following antigens: *L. pneumophila* serogroup I antigen, *L. pneumophila* serogroups II, III, IV polyvalent antigen, *L. pneumophila* serogroups V, VI polyvalent antigen and *L. pneumophila* serogroups VII, VIII, IX, X polyvalent antigen, and an anti-human globulin fluorescein conjugate diluted to 1:400 (Fluoline H, Biomérieux). For each positive serum, we determined antibody levels against each serogroup. The technique was identical to the *C. burnetii* IFA. However, the main difference was that the type of immunoglobulin was not determined.

Serum selection. For Q fever positive sera, IFA was used to determine titers of IgG, IgM and IgA. There were 154 sera from the Centre National de Référence des Rickettsioses: 99 sera from 72 patients with acute Q fever and 55 sera from 28 patients with chronic Q fever. The 72 patients had clinical manifestations compatible with acute Q fever as well as antibody levels: IgG, IgM and IgA phase II titers were significantly higher than IgG, IgM and IgA phase I titers, with IgG phase II level $\geq$ 200 and IgM phase II level $\geq$ 50. The 28 patients had clinical manifestations compatible with chronic Q fever, and their antibody levels were compatible with this diagnosis (Peacock *et al.*, 1983; Raoult *et al.*, 1988): IgG phase I titers $\geq$ IgG phase II titers and IgA phase I titers $\geq$ IgA phase II titers with IgG phase I level $\geq$ 800 and IgA phase I level $\geq$ 25. For legionellosis positive sera, IFA was used to determine antibody levels. There were 57 sera from 57 patients with legionellosis which were provided by the Centre National de Référence des Légionelloses. The patients were clinically compatibles with legionellosis as well as antibody levels (Bornstein *et al.*, 1986): for *L. pneumophila,* a presumptive diagnosis can be established with a single titer of 1:256 and a confirmed diagnosis with seroconversion and minimum level of 1:128 for the late serum.

Serum adsorption. The serum was diluted to 1:20 in PBS and 1 ml aliquot was mixed with centrifuged deposit from a bacterial suspension and shaken for 3 hr at room temperature. This mixture was centrifuged at 10 000 ×g for 10 min and the supernatant was retained and treated again in the same manner. Then, the supernatant was tested against homologous and heterologous antigens.

SDS-PAGE and immunoblotting. The acrylamide concentration of stacking and separating gels were 3.2 % and 12 % respectively. The bacterial suspensions were dissolved in buffer described by Laemmli (1970). After 6 min boiling, 20 ml aliquots were added to each well: *C. burnetii* and *L. pneumophila* protein concentrations in each well were 1 mg/ml and 0.5 mg/ml respectively. The gels were electrophoresed at 20 mA for 4 hr. Then the gels were electroblotted to nitrocellulose papers at 50 V for 4 hr. The blots were blocked overnight at room temperature with Tris buffered saline (TBS; 0.02 % merthiolate (Sigma), 2.33 % Tris (Sigma) and 29.25 % NaCl) with 5 % non fat dry milk. The blots were washed with distilled water. The sera being tested were diluted to 1:100 in TBS-3 % non fat dry milk. The blots were then incubated overnight at room temperature with diluted sera. After incubation, the blots were washed in TBS three times for 10 min. After washing, the blots were incubated with biotin diluted to 1:1000 in TBS-3 % non fat dry milk for 1 hr at room temperature. After incubation, the blots were washed as described above. The blots were then incubated with avidin diluted to 1:2000 according to an idential scheme. After removal of avidin by washing, the bands were visualized by incubation with a freshly prepared solution containing a-chloronaphtol, methanol, TBS and H_2O_2. The reaction was terminated by washing with distilled water.

Identification of sera positive for both C. burnetii and L. pneumophila. The 57 legionellosis positive sera were tested against *C. burnetii* and the 154 Q fever positive sera were tested against *L.*

pneumophila. The sera with double positivity were adsorbed with *C. burnetii* and *L. pneumophila* antigens. Native and adsorbed sera were subjected to protein immunoblotting.

Statistical test. We used the χ^2 test to compare the prevalences of anti-*C. burnetii* and -*L. pneumophila* antibodies in the population of patients with legionellosis or Q fever to the prevalences of anti-*C. burnetii* and -*L. pneumophila* antibodies in an healthy population.

Results

Out of the 211 sera studied, four sera showed antibodies at a diagnosis level to both *C. burnetii* and *L. pneumophila* (Table 1). Sera No. 1 and 2 came from different patients diagnosed as having legionellosis. Serum No. 1 presented antibody level against *L. pneumophila* serogroup VI of 1:1024, and IgG *C. burnetii* phase II titer of 1:200. Serum No. 2 presented antibody titer against *L. pneumophila* serogroup VI of 1:2048 and IgG *C. burnetii* phase II titer of 1:200 and IgM phase II titer of 1:100. Sera No. 3 and 4 were from two patients diagnosed as suffering from acute Q fever and Q fever endocarditis, respectively. Serum No. 3 presented IgG phase II and IgM phase II titers of 1:400 and 1:50, respectively with antibody level against *L. pneumophila* serogroup I of 1:128. Serum No. 4 presented phase II titers of 1:1600 (IgG), 1:50 (IgM) and 1:100 (IgA), and phase I titers of 1:1600 (IgG), 1:50 (IgM) and 1:100 (IgA), with antibody level against *L. pneumophila* serogroup VI of 1:512.

Serum adsorption data. The four sera were adsorbed with *C. burnetii* phase II antigen as previously described; antibody levels to *L. pneumophila* were identical, whereas antibodies against *C. burnetii* disappeared. Serum No. 4 was adsorbed with *C. burnetii* phase I antigen: antibody titer to *L. pneumophila* was identical, whereas antibodies against *C. burnetii* disappeared. The four sera were adsorbed with *L. pneumophila* antigens: *L. pneumophila* serogroup VI for sera No. 1, 2 and 4 and *L. pneumophila* serogroup I for serum No. 3. Antibodies against *L. pneumophila* disappeared but antibody levels to *C. burnetii* were unchanged. Thus, after adsorption with *C. burnetii* or *L. pneumophila* antigens, heterologous antibodies did not disappear.

Table 1. Antibody titers of sera with antibodies to both C. burnetii and L. pneumophila

Serum No.	*C. burnetii*						*L. pneumophila*	
	antigen phase II			antigen phase I				
	IgG	IgM	IgA	IgG	IgM	IgA		serogroup
1	200	0	0	0	0	0	1024	VI
2	200	100	0	0	0	0	2048	VI
3	400	50	0	0	0	0	120	I
4	1600	50	100	1600	225	100	512	VI

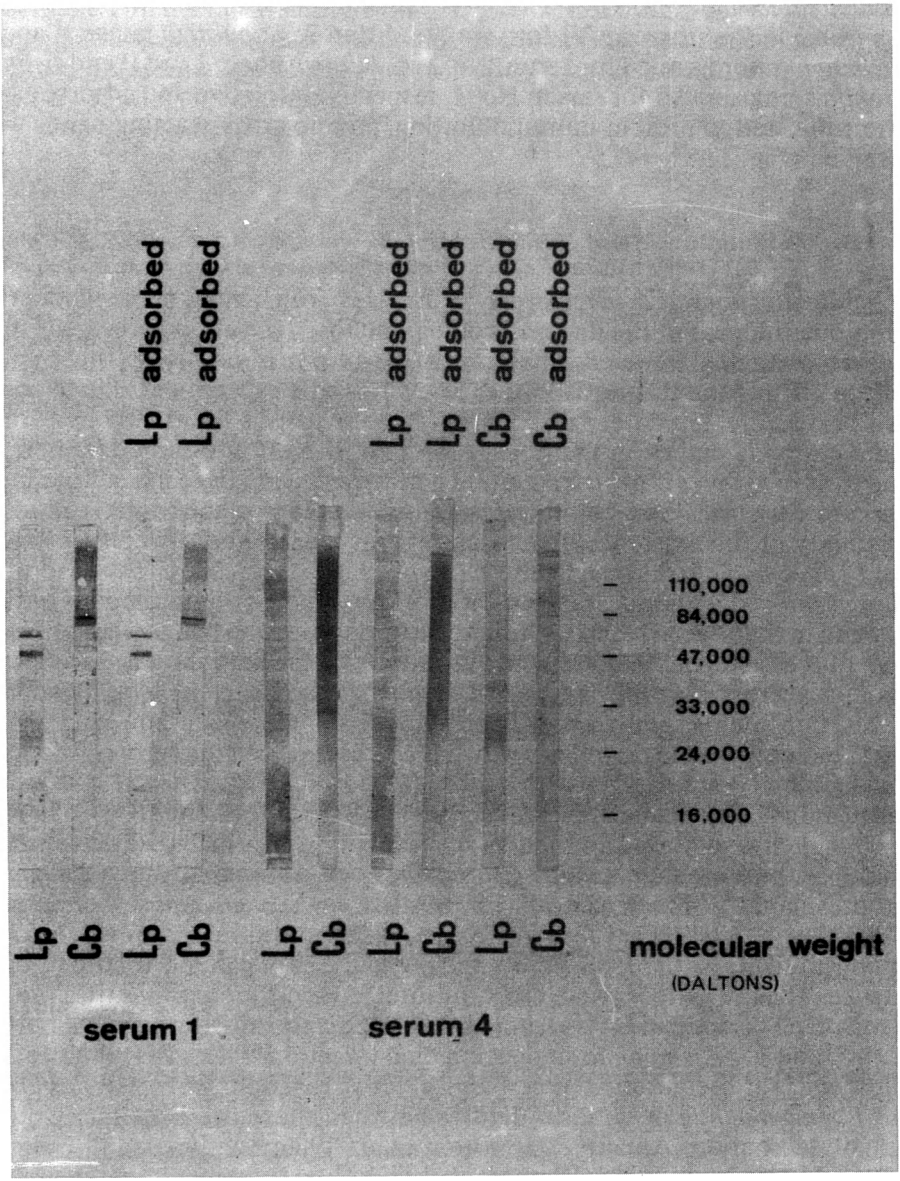

Fig. 1

Western blots of *C. burnetii* and *L. pneumophila* carried out with serum No. 1 and No. 2 before and after adsorption

L: *L. pneumophila;* Cb: *C. burnetii;* Lp adsorbed: serum adsorbed with *L. pneumophila;* Cb adsorbed: serum adsorbed with *C. burnetii*

Immunoblotting data. The bacterial suspension were *C. burnetii* phase II and *L. pneumophila* serogroup VI for sera No. 1 and 2, *C. burnetii* phase II and *L. pneumophila* serogroup I for serum No. 3, *C. burnetii* phase I and II and *L. pneumophila* serogroup VI for serum No. 4, respectively. Native and adsorbed sera were subjected to protein immunoblotting, and no cross-reacting bands were observed (Fig. 1).

Discussion

Legionellosis and Q fever are two diseases which may present identical symptomatology. For the diagnosis of legionellosis, the cultivation is the most specific method. However, isolation of *Legionella* is not easy: the positive culture affirms the diagnosis, but the negative one can not exclude legionellosis. Therefore, laboratory diagnosis of legionellosis is frequently performed using antibody detection methods, especially IFA. In the same manner, the diagnosis of Q fever is most frequently performed using IFA, the culture being reserved for specialized laboratories. Thus, it is important to determine the possibility of the existence of serological cross-reactions between *C. burnetii* and *L. pneumophila* that can be the cause of a false diagnosis. Cross-adsorption studies showed a disappearance of homologous but not heterologous antibodies and in the Western blot technique, no cross-reacting bands were observed in all four cases. The adsorption and immunoblotting data show the absence of serological cross-reactions between *C. burnetii* and *L. pneumophila.* In support of this, our findings show a prevalence of anti-*C. burnetii* antibodies (3.5 %) in the 57 patients with legionellosis which is consistent with the seroprevalence in healthy blood donors (2.7 % with a titer $\geq$ 128; χ^2=0.8) (Raoult *et al.*, 1987). The same conclusions can be drawn for serum No. 3; the prevalence (1 %) of anti-*L. pneumophila* serogroup I antibodies at a level of 1:128 is in agreement with the results of the Bornstein's study (Bornstein *et al.*, 1986) that showed a similar seroprevalence in blood donors (1 % positive to *L. pneumophila* serogroup I with a titer of 1:64 to 1: 128; χ^2=0.1). No significant differences were noticed among populations of blood donors and populations of patients for the seroprevalence. These 3 patients were probably infected at different times with the two agents. One of our patients (serum No. 4) had diagnostic titers against both *C. burnetii* and *L. pneumophila* suggesting dual infection. Thus, the results of our study confirm the absence of serological cross-reactions between *C. burnetii* and *L. pneumophila* despite similarities between these two bacteria involving lipopolysaccharide (Amano *et al.*, Otten *et al.*, 1986; Schramek and Galanos, 1984; Schramek *et al.*, 1982; Wong *et al.*, 1979). Our study shows that in patients with suspected legionellosis or Q fever, laboratory confirmation of these infections is possible using IFA which has been shown to have a good specificity. However, it must be born in mind that patients suffering from legionellosis can have antibodies against *C. burnetii* at a moderate titer and vice versa. This is in

agreement with the fact that there is a seroprevalence of anti-*C. burnetii* antibodies and anti-*L. pneumophila* antibodies in the normal population. In conclusion, the differentiation of legionellosis and Q fever should not be compromised by serological methods.

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