Serological Cross-Reactivity between Different *Chlamydia*-Like Organisms[▽]

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The serological cross-reactivity between different recently described *Chlamydia*-related organisms was determined. Mouse sera exhibited a strong reactivity against autologous antigen and closely related heterologous antigen but no cross-reactivity with distantly related species. These results are important to better interpret serological studies and assess the pathogenic role of these obligate intracellular bacteria.

New *Chlamydia*-related species have been isolated and assigned to the *Chlamydiales* order (8), including *Parachlamydia acanthamoebae* (1), *Simkania negevensis* (16), *Neochlamydia hartmannellae* (14), *Waddlia chondrophila* (22), *Protochlamydia amoebophila* (3), *Criblamydia sequanensis* (23), and *Rhabdochlamydia crassificans* (5). Serological studies have suggested that some of these intracellular bacteria may cause respiratory tract infections in humans. *P. acanthamoebae* antibody reactivity was documented in patients with community-acquired pneumonia (2, 21) and aspiration pneumonia (11). Enzyme-linked immunosorbent assay suggested a possible role of *Simkania negevensis* in acute respiratory infections (20), in exacerbation of chronic obstructive pulmonary disease (19) and in community-acquired pneumonia in children (6). Since immunofluorescence is the gold standard method for the diagnosis of infection due to *Chlamydiaceae* (7) and *Chlamydia*-like organisms (4), we used this technique to define the level of cross-reactivity between the different bacterial species. The molecular weights of immunogenic proteins were also determined by Western blotting. Moreover, we inferred a serological differentiation index (SDI) phylogenetic tree (9, 24) and compared its topology to that of the 16S rRNA phylogenetic tree.

Chlamydia-like organisms grown using amoebal coculture (12, 13) and purified on sucrose barrier were used as antigens to immunize mice. *Acanthamoeba castellanii* (ATCC 30010) was used to grow *P. acanthamoebae* strain BN9 (ATCC VR-1476), *P. acanthamoebae* strain Hall's coccus, *P. amoebophila* strain UWE25 (ATCC PRA-7), *S. negevensis* (ATCC VR-1471), *W. chondrophila* (ATCC 1470), *C. sequanensis* (CRIB-18), and *R. crassificans* (CRIB-01). *Hartmannella vermiformis* strain BL was used to grow *N. hartmannellae* (ATCC 50802). Purified bacteria were injected intravenously to 6-week-old BALB/c mice (two mice per antigen). After four immunizations, blood was collected, and sera were obtained by centrifugation at $5,200 \times g$ for 5 min at 4°C. Twofold-diluted sera were tested by immunofluorescence for antibody reactivity against purified antigens. Fluorescein isothiocyanate-coupled anti-mouse immunoglobulin antibody (Bio-Rad, Rheinach, Switzerland) was

used as a secondary antibody. Phosphate-buffered saline–milk and uninfected axenic *A. castellanii* (ATCC 30010) were used as negative controls. The IgG/IgM Micro-IF test kit (ANILab-systems, Vantaa, Finland) was used to test the antibody reactivity of mice sera against *Chlamydia trachomatis*, *Chlamydia psittaci*, and *Chlamydophila pneumoniae* antigens.

Mice responded adequately to injected antigens. Very high titers of 1/32,768 and 1/16,384 were obtained after immunization with *W. chondrophila* and *P. acanthamoebae* strain Hall's coccus, respectively. Similar results were obtained for each duplicate sera, with titers varying by less than one dilution. Sera elicited against any of all *Chlamydia*-like organisms tested did not exhibit cross-reactivity against amoeba. The serological cross-reactivity (SDI) between two bacteria, expressed as the number of twofold dilution steps separating homologous and heterologous titers, were calculated as described previously (9). Table 1 shows antibody titers and SDIs obtained from reciprocal cross-reactions of mouse antisera with different *Chlamydia*-like organisms, as determined by immunofluorescence. Significant cross-reactivity was observed between closely related species, for instance, between the *P. amoebophila* and *P. acanthamoebae* strains. In contrast, no or little cross-reactivity was detected between distantly related *Chlamydia*-like organisms. No cross-reactivity against *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* was detected when we tested sera elicited with any *Chlamydia*-like organisms, except *P. acanthamoebae* strain Hall's coccus that reacted with *C. pneumoniae* (titer of 1/128). A tree inferred from SDIs was built by using the neighbor-joining option of the PHYLIP software (10). 16S rRNA sequences were used to infer another neighbor-joining tree (see Fig. 1A for the GenBank accession numbers), using Kimura-corrected p-distance and the complete deletion option of the MEGA software (18). Both SDI and 16S rRNA-based trees were rooted using *W. chondrophila* as an outgroup. The phylogenetic tree based on the SDI was congruent with the tree inferred from 16S rRNA encoding gene sequences (Fig. 1A). This suggests that cross-reactivity between members of the *Chlamydiales* order is proportional to the level of relatedness between each species, i.e., to the likelihood of common epitopes.

To determine the molecular weight of immunogenic proteins of *Chlamydia*-like organisms, Western blots were performed as described previously (11). The membrane was first incubated with the mouse serum diluted 1/64. After washing steps, the membrane was further incubated with a 1/500 dilu-

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TABLE 1. Antibody titers and SDIs obtained from reciprocal cross-reactions of mouse antisera with different *Chlamydia*-like organisms, as determined by immunofluorescence

Species studied	Antibody titer (SDI)							
	<i>P. acanthamoebae</i> strain BN9	<i>P. acanthamoebae</i> strain Hall's coccus	<i>P. amoebophila</i>	<i>N. hartmannellae</i>	<i>R. crassificans</i>	<i>S. negevensis</i>	<i>C. sequanensis</i>	<i>W. chondrophila</i>
<i>P. acanthamoebae</i> strain BN9	1,024 (0)	1,024 (4)	128 (1)	32 (10)	<32 (10)	<32 (11)	<32 (17)	<32 (17)
<i>P. acanthamoebae</i> strain Hall's coccus	1,024 (4)	16,384 (0)	512 (3)	64 (12)	<32 (12)	32 (12)	<32 (19)	64 (19)
<i>P. amoebophila</i>	1,024 (1)	1,024 (3)	256 (0)	32 (7)	64 (3)	<32 (5)	128 (9)	64 (10)
<i>N. hartmannellae</i>	128 (10)	128 (12)	128 (7)	2,048 (0)	<32 (11)	<32 (12)	<32 (18)	<32 (18)
<i>R. crassificans</i>	32 (10)	64 (12)	128 (3)	<32 (11)	256 (0)	64 (3)	256 (8)	64 (10)
<i>S. negevensis</i>	<32 (11)	64 (12)	256 (5)	<32 (12)	256 (3)	512 (0)	512 (8)	128 (12)
<i>C. sequanensis</i>	32 (17)	64 (19)	128 (9)	<32 (18)	128 (8)	128 (8)	32,768 (0)	256 (13)
<i>W. chondrophila</i>	<32 (17)	<32 (19)	128 (10)	<32 (18)	128 (10)	32 (12)	512 (13)	32,768 (0)

tion of a peroxidase-labeled goat anti-mouse immunoglobulin G (Bio-Rad).

P. amoebophila had immunogenic proteins of approximately 130, 70, 43, and 30 kDa. For *P. acanthamoebae* strain BN9, a protein of approximately 70 kDa exhibited the strongest immunogenicity. For *N. hartmannellae*, proteins of approximately 89, 70, 53, and 45 kDa exhibited the strongest immunogenicity, whereas proteins of *W. chondrophila* of about 70, 53, 45, and 30

FIG. 1. (A) Congruence of the phylogenetic tree based on the 16S rRNA encoding gene (left panel) and the phyletic tree based on the SDI (right panel). Both trees were built by using the neighbor-joining method and were outrooted with *Waddlia chondrophila*. The 16S rRNA sequences were downloaded from GenBank. The accession numbers are as follows: *Waddlia chondrophila*, AF042496; *Rhabdochlamydia crassificans*, AY928092; *Simkania negevensis*, U68460; *Neochlamydia hartmannellae*, AF177275; *Protochlamydia amoebophila*, BX908798; *Parachlamydia acanthamoebae* strain Hall's coccus, AF366365; and *Parachlamydia acanthamoebae* strain BN9, Y07556. (B) Western blot pattern of each *Chlamydia*-like organisms. Each serum elicited in mice (two sera raised against each bacteria) was tested against its autologous antigen. All sera were tested at a 1/64 dilution. Lanes: A and B, *Parachlamydia acanthamoebae* strain BN9; C and D, *Parachlamydia acanthamoebae* strain Hall's coccus; E and F, *Protochlamydia amoebophila*; G and H, *Neochlamydia hartmannellae*; I and J, *Waddlia chondrophila*; K and L, *Simkania negevensis*; M and N, *Rhabdochlamydia crassificans*. The molecular sizes reported on the left of the figure were determined by using the Kaleidoscope prestained marker (Bio-Rad).

kDa exhibited the highest immunogenicity. For *S. negevensis*, a protein of approximately 30 kDa exhibited the strongest immunogenicity, although having immunogenic proteins of approximately 130, 35, and 20 kDa. *R. crassificans* possess immunogenic proteins of 130, 89, 43, and 25 kDa (Fig. 1B). Earlier, Western blot has been used to confirm immunofluorescence results in two studies investigating the role of *Chlamydia*-like organisms in pneumonia (11, 25). The first study used as a positive control a rabbit serum elicited with *P. acanthamoebae* that exhibited a strong reactivity against proteins of about 70, 40, and 28 kDa (11). Here, we also identified a 70-kDa parachlamydial immunogenic protein. In the second study, a 64-kDa protein exhibited the strongest immunogenicity (4, 25).

Since serology was not discriminative at the species level for *Parachlamydiaceae*, previous serological studies suggesting a role of *Parachlamydia* as a new agent of pneumonia (2, 11, 21) should be interpreted as a hint for a role of either *Parachlamydia* or *Parachlamydia*-related species in respiratory tract infections. Conversely, serological studies aiming at defining the pathogenicity of *Simkania* spp. showed a high seroprevalence. The absence of cross-reactivity between *Simkania* spp., *Parachlamydia* spp. (Table 1), and all three *Chlamydiaceae* tested in our study support the idea that humans are frequently exposed to *Simkania* spp. or cross-reacting related species. Whether *Simkania* spp. cause respiratory tract diseases remains to be demonstrated since Kumar et al. (17) and Johnsen et al. (15) did not confirm early studies by Lieberman et al. (19, 20). Discrepant results might be due to some cross-reactivity of the enzyme-linked immunosorbent assay used by Lieberman et al. with *Chlamydia*-like organisms such as *Rhabdochlamydia* that exhibit common immunogenic proteins.

In conclusion, we have described the level of cross-reactivity and the molecular weight of immunogenic proteins of *Chlamydia*-like organisms. Understanding the pathogenic potential of *Chlamydia*-like organisms in humans and animals should be reconsidered using these findings.

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