Detection of *Simkania negevensis* in cell culture by using a monoclonal antibody

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SUMMARY

In the present study, a monoclonal antibody (mAb), D5-14, raised in our laboratory against *Chlamydia trachomatis* LGV2 serotype, stained *Simkania negevensis* inclusions in *S. negevensis*-infected cells by using the immunofluorescence test. D5-14 mAb, reacting in immunoblot with an approximately 64-66-kDa protein of *C. trachomatis* LGV2 serotype, recognized a protein with the same molecular mass when tested with *S. negevensis* elementary bodies.

KEY WORDS: Simkania negevensis, *Chlamydia trachomatis*, Monoclonal antibody.

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Symkania negevensis, belonging to the family *Simkaniaceae* in the order *Chlamydiales*, is associated with bronchiolitis in infants (Kahane *et al*., 1998), community-acquired pneumonia in adults (Lieberman *et al*., 1997) and acute exacerbation of chronic obstructive pulmonary disease (COPD) (Lieberman *et al*., 2002).

S. negevensis is sensitive to tetracyclines and macrolides, similar to chlamydiae; a quinolone resistance is present in *S. negevensis* (Casson and Greub, 2006) whereas no tetracycline resistance has been shown in this bacterium to date, unlike the presence of many *C. suis* tetracycline-resistant strains in animal chlamydial infections (Di Francesco *et al.*, 2008).

Few data on the immune response in *S. negevensis* infection can be found in the literature, while several studies on chlamydial immune response have been published (Meoni *et al.*, 2009). Although only few reports on the prevalence of *S. negevensis* in human infections are present, owing to the unavailability of commercial diagnostic tests, a high seroprevalence of antibodies to *S. negevensis* in healthy adults from different part of the world has been reported (Friedman *et al*., 1999; Friedman *et al*., 2000; Friedman *et al*., 2006).

Evidence of infection with *S. negevensis* has been supported by culture, polymerase chain reaction (PCR) and in-house serological tests (Friedman *et al*., 2006). Culture needs polyclonal hyper-immune animal sera raised against *S. negevensis* and is generally performed by using monolayers of various cell lines of human or simian origin, such as HeLa cells or Vero cells (Kahane *et al*., 1993; Kahane *et al*., 1998). Additional cell culture model systems have been tested showing the organism was able to infect epithelial cells originating in the respiratory, genital, or gastrointestinal tract, in addition to macrophages (Kahane *et al*., 2007a).

The microorganism can replicate in free-living amoebae and persists for long periods of time in amoebal cysts (Kahane *et al*., 2001). Various amoebae may harbor *S. negevensis* thus representing important reservoirs and vectors of this microorganism (Michel *et al.*, 2005; Kahane *et al.*, 2007b).

Since monoclonal antibodies (mAbs) to *S. negevensis* are not commercially available, we

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tested 20 mAbs, raised in our laboratory against *Chlamydia trachomatis* LGV2 serotype, against *S. negevensis* infected cells. The mAb D5-14 was the only one which stained *S. negevensis* inclusions in *S. negevensis*-infected cells by using the immunofluorescence test. D5-14 mAb reacted also against *C. pneumoniae* and *C. psittaci*-infected cells.

S. negevensis and *C. trachomatis* LGV2 serotype were grown in monolayer cultures of LLC-MK2 cells (a continuos cell line prepared from Rhesus monkey kidney). Murine mAbs directed against *C. trachomatis* LGV2 serotype were produced as previously described (Cevenini *et al.*, 1987).

In order to evaluate the time of appearance of positive immunofluorescence in infected cell cultures, D5-14 mAb was tested by an indirect immunofluorescence assay in which *S. negevensis* or *C. trachomatis* infected LLC-MK2 cells were used as antigen. At various time intervals after infection with *S. negevensis* or *C. trachomatis*, the cells were fixed with methanol. Uninfected LLC-MK2 cells were used as control. Bound mAb was detected with fluorescein-conjugated anti-mouse immunoglobulins (Dako, Copenhagen, Denmark).

For immunoblotting identification of the protein recognized by D5-14 mAb, *S. negevensis* elementary bodies (EBs) were purified by sucrose density-gradient (Fukushi & Hirai, 1988). *C. trachomatis* LGV2 serotype EBs were purified in the same way. The sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli (1970) was followed using a 4-12% (w/v) precast gel (Life Technologies). The molecular weight marker (Invitrogen) was used as a molecular mass marker. After protein electrophoretic transfer from gels into nitrocellulose sheets (Comanducci *et al.*, 1994), the nitrocellulose paper was stained with Ponceau-S (0.1% in 1% acetic acid) to ensure the transfer of proteins. The strips were then blocked with 3% albumin in sodium phosphate, 0.15M NaCl, 0.05% (v/v) Tween 20 (PBST). The nitrocellulose paper was then cut into strips which were incubated with the mAb overnight at room temperature under gentle agitation. The strips were washed 3 times with PBST, followed by incubation for 2 h at room temperature with peroxidase-labelled rabbit anti-mouse immunoglobulins (Dako) diluted 1:1000 in PBST. The strips, washed 3 times, were then transferred to distilled water and the immunoblots developed by using 4-chloro-naphtol (Bio-Rad) and then rinsed with distilled water.

In order to characterize the immunoglobulin subclass of D5-14 mAb, the IsoQuick Strips for mouse monoclonal isotyping (Sigma-Aldrich, St. Louis, MO USA) was used.

The D5-14 mAb, reacting in immunoblot with a 64-66-kDa protein of *C. trachomatis* (LGV2 serotype), recognized a protein with the same molecular mass when tested with *S. negevensis* EBs (Figure 1).

The results of the immunofluorescence test of *S. negevensis*-infected cell cultures at various times of infection revealed that D5-14 mAb, reacting positively as early as 8 h post-infection with

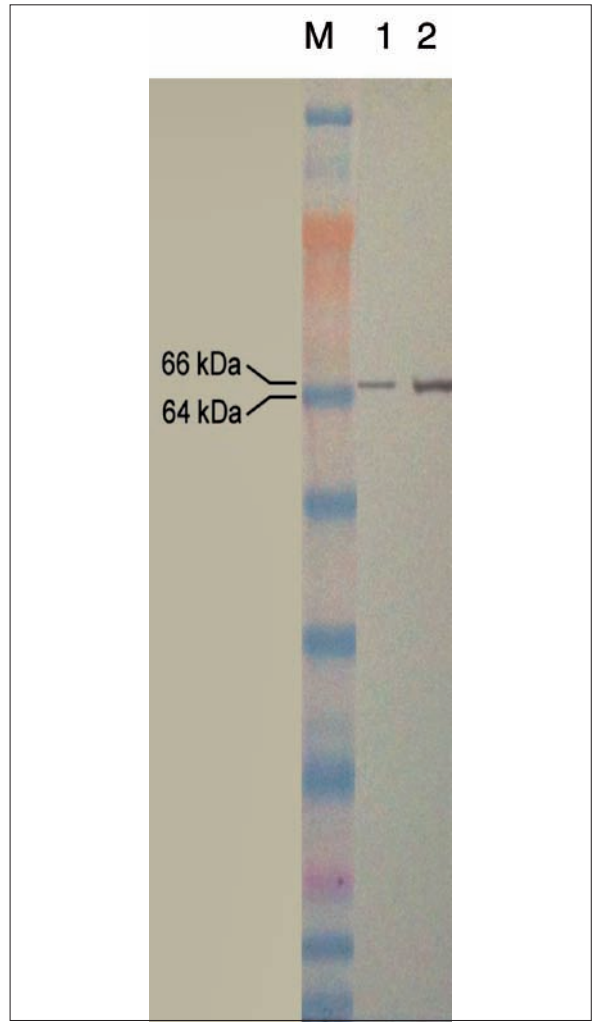

FIGURE 1 - *Western blot analysis of mAbD5-14 with C. trachomatis (lane 1) and Simkania negevensis (lane 2).*

C. trachomatis inclusions, reacted 16 h post-infection with *S. negevensis* inclusions.
In addition, the intensity of the fluorescence and the dimension of the detected inclusions of *C. trachomatis* and *S. negevensis*, peaked at 48 h and 72 h p.i., respectively (Figure 2).
IgG1 K chain was the isotype class identified in D5-14 mAb.
S. negevensis from clinical samples can be isolated in cell line monolayers and polyclonal hyperimmune animal sera are used for its detection. The D5-14 mAb described in the present study was able to detect *S. negevensis* in cell monolayers by immunofluorescence as early as 16 h p.i. with a more intense reactivity 72 h p.i., in comparison to the reactivity against *C. trachomatis* LGV2 serotype detectable as early as 8 h p.i. with a high intensity of fluorescence 48 h p.i.
In immunoblot D5-14 mAb recognized a protein very close to the 64 kDa protein of the molecular weight marker in both *C. trachomatis* and *S. negevensis* EBs. Previous studies performed on antigens and the membrane structure of *S. negevensis* showed a polypeptide pattern significantly different from that of other *Chlamydiales* members. Monoclonal antibodies against chlamydial OMP1 and OMP2 proteins did not react with any corresponding polypeptides of *S. negevensis*, while reactivity with polyclonal, monospecific antibodies to the 60 kDa chlamydial heat shock pro-

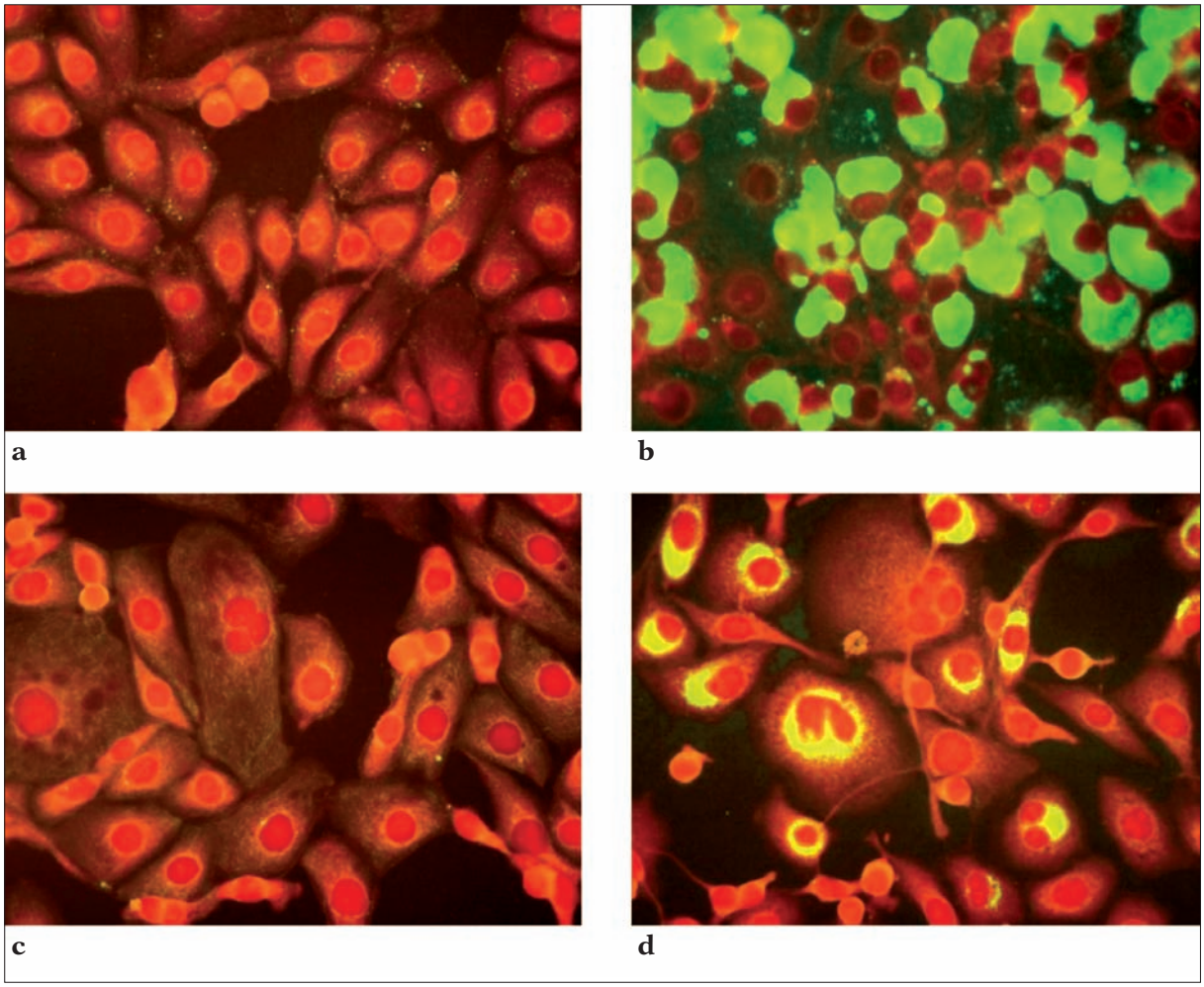

FIGURE 2 - *Chlamydia trachomatis inclusions detected by monoclonal antibody D5-14 after 8 h (a) and 48 h (b) post infection; Simkania negevensis inclusions detected by monoclonal antibody D5-14 after 16 h (c) and 72 h (d) post infection.*

tein was observed (Kahane *et al*., 1993). Additional immunoblot analysis of *S. negevensis* using hyperimmune murine sera (Friedman *et al*., 2003) or microimmunofluorescence–positive human sera (Yamaguchi *et al*., 2005) showed a strong immunoreactivity to a 37-42 kDa set of three bands and a 64 kDa antigen. Our results demonstrate the reactivity of a monoclonal antibody against a protein showing the same molecular weight of the protein recognized as a major target of the humoral immune response to *S. negevensis*. Therefore, D5-14 mAb could be used, as a confirmatory test, in the diagnosis of *S. negevensis* infection by the immunofluorescence test.

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