Seroprevalence of IgG antibodies to the chlamydia-like microorganism 'Simkania Z' by ELISA

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SUMMARY

The newly described microorganism 'Simkania Z', related to the Chlamydiae, has been shown to be associated with bronchiolitis in infants and community acquired pneumonia in adults. The prevalence of infection in the general population is unknown. A simple ELISA assay for the detection of serum IgG antibodies to 'Simkania Z' was used to determine the prevalence of such antibodies in several population samples in southern Israel (the Negev). The groups tested included 94 medical and nursing students, 100 unselected blood donors, 106 adult members of a Negev kibbutz (communal agricultural settlement), and 45 adult Bedouin, residents of the Negev. IgG antibodies to 'Simkania Z' were found in 55–80 % of these presumably healthy individuals, independently of antibodies to *Chlamydia trachomatis* and *Chlamydia pneumoniae*. The Bedouin had a seropositivity rate of 80 %, while all other groups had rates of between 55 and 64 %. These results indicate that 'Simkania Z' infection is probably common in southern Israel.

INTRODUCTION

Chlamydia trachomatis and *Chlamydia pneumoniae* are both significant pathogens for man. *C. trachomatis* is the etiologic agent of trachoma and inclusion conjunctivitis and widespread genital tract disease in humans [1–3]. *C. pneumoniae* has recently been recognized as a significant respiratory tract pathogen and appears to be involved in the aetiology of chronic cardiovascular disease [4–7].

The microorganism 'Simkania Z' is a recently discovered intracellular bacterium which was first thought to belong to the family *Chlamydiaceae* [8], although more recent evidence indicates that it may belong to a separate family in the order *Chlamydiales* [9]. Its replicative cycle seems to be similar to that of

chlamydia, in that it is characterized by the appearance of electron-dense elementary bodies and dividing reticulate bodies [10]. We have sequenced its 16S ribosomal DNA and compared the sequence to that of organisms with similar morphological and physiological characteristics [8]. While *Chlamydia* species have 95·5 % sequence identity among themselves, 'Simkania Z' has only 83 % with them, and 73 % identity with certain rickettsia such as *Cowdria ruminantium*, *Ehrlichia*, *Anaplasma*, and *Coxiella burnetii*. The known *Chlamydia* species have 73–75 % identity with these rickettsial microorganisms.

We have recently shown that infection with 'Simkania Z' may be associated with bronchiolitis in infants [11] and with community acquired pneumonia in adults [12]. Other potential pathological associations with 'Simkania Z' infection are thus far

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unknown, and the extent of exposure to the organism in the general population is unknown.

In the present study we report the use of an ELISA assay for specific detection of IgG antibodies to 'Simkania Z' and the prevalence of such antibodies in samples of various adult populations residing in southern Israel. We compare these rates to sero-positivity rates to *Chlamydia trachomatis* and *C. pneumoniae*, and for some individual sera, levels of antibodies to 'Simkania Z' with levels of antibodies to *C. trachomatis* and to *C. pneumoniae*.

MATERIALS AND METHODS

Serum samples

Subjects who agreed that their serum samples be used in this study included several laboratory workers (control sera, chosen for their characteristics in the assay system; some were included in each test), 94 healthy medical and nursing students (aged 22–26, of whom half were male) studying at the Faculty of Health Sciences of Ben Gurion University, 106 adult (aged 18–40) members of a Negev kibbutz (communal agricultural settlement), and 45 adult Bedouin, re-siding in the Negev. Also tested were serum samples of 100 unselected voluntary blood donors, for whom no personal or demographic details were available. They were however, at least 18 years of age, relatives or friends of patients requiring transfusions at the Soroka Medical Center, and they denied a history of hepatitis or extensive travel in exotic areas; none were commercial donors.

The 106 members of the Negev kibbutz represent 70 % of the population of those ages residing in the kibbutz. Kibbutz members are considered to be of high socio-economic status; they have extensive medical and other services available to them at all times at no charge. Their sera were obtained in 1989 at a routine screening for blood lipid levels and serosurvey for antibody level to measles virus in the face of an impending outbreak. Of the members aged 18–25, 51 % were male, and of those aged 26–40, 58 % were male.

The Bedouin adults are not necessarily a rep-resentative sample of the Bedouin of the Negev. The serum samples were obtained in 1992 from persons accompanying patients to the hospital. Although some Bedouin live in city apartments, most do not. They are considered to be of lower socio-economic status, although they have access to full medical services. Those living in non-permanent structures usually keep their water in large closed, transportable tanks, while those in permanent structures have running water. Forty percent of the Bedouin aged 18–25 were male, and 44 % of those aged 26–40 were male.

ELISA assay

The ELISA assay for detection of IgG antibodies to *Chlamydia trachomatis* and to *C. pneumoniae* has been described previously [13]. It was shown to have a good positive/negative correlation with microimmuno-fluorescence (MIF), first described by Wang and Grayston [14], and considered to be the gold standard for chlamydial serology. The assay was adapted for the detection of antibodies to 'Simkania Z' by substituting purified elementary and reticulate bodies of 'Simkania Z' for purified chlamydial elementary bodies. The organism was grown in Vero cells as described previously [8] and purified according to the protocol of Caldwell and colleagues for purification of chlamydia [15]. Bands containing elementary and reticulate bodies were pooled before pelleting and resuspension in SPG buffer (0·01 M sodium phosphate, pH 7·2; 0·25 M sucrose, 5 mM L-glutamic acid). Anti-gen was inactivated by treatment with 0·3 % formalin for 1 h at room temperature and stored at −70 °C until use. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Richmond, CA), with bovine serum albumin as standard, after sample treatment with 1 N NaOH to a final concentration of 0·5 N for 10 min. For the assay, antigen was diluted to a concentration of 1·0 μg/ml in 0·05 M carbonate-bicarbonate buffer, pH 9·6 and used to coat the wells of microtitre plates in volumes of 100 μl per well. The plates were incubated for 4 h at room temperature, then overnight at 4 °C, and rinsed four times with phosphate-buffered saline, pH 7·2 (PBS). Each well of the plates was then treated with 100 μl of 2 % deoxycholate monohydrate (Aldrich Chemical Co., Inc., Milwaukee, WI, cat no. 23,839-2) in 5 mM EDTA, preserved with 0·1 % sodium azide, for 1 h at 37 °C. Plates were washed four times with PBS. A blocking solution consisting of 0·5 % gelatin and 5 % bovine serum albumin (BSA) in PBS was added to all wells (200 μl) for 2 h at room temperature. After further rinsing of the plates, serum samples were diluted 1:100 in PBS containing 0·05 % Tween-20 (PBS-T) supplemented with 5 % BSA and incubated for 1 h at room temperature. All serum samples were
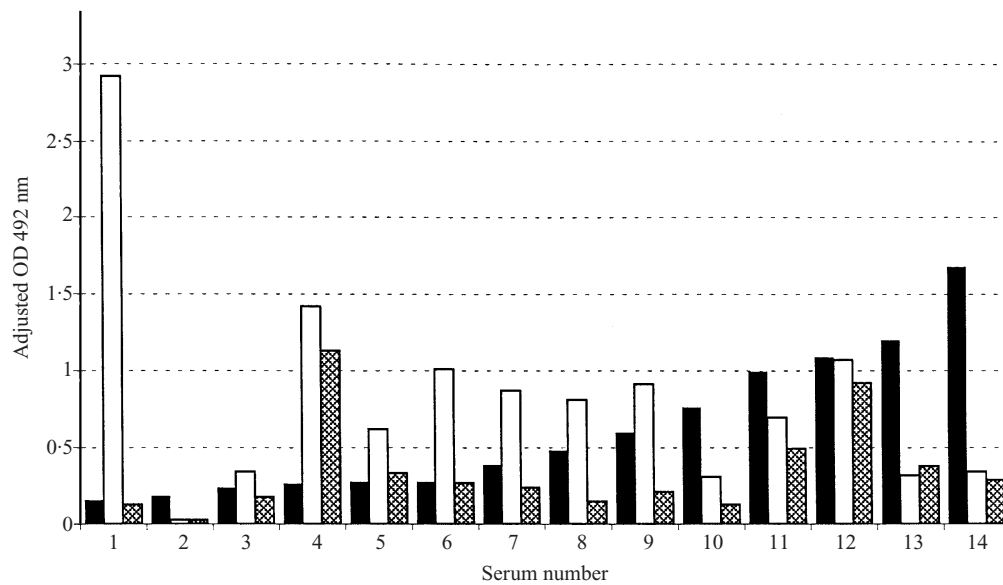
Fig. 1. Levels of antibodies to 'Simkania Z', *Chlamydia trachomatis* and *C. pneumoniae* for 14 healthy medical and nursing students in the urea ELISA, arranged according to increasing adjusted OD 492 nm for 'Simkania Z' antibodies. ■, 'Simkania Z'; □, *C. pneumoniae*; ▨, *C. trachomatis*.

tested in duplicate. Contents of the plate were removed and replaced with 100 μl of 6 M urea (United States Biochemical Co., Cleveland, OH, cat. no. 23036) for 15 min. After four washes with PBS-T, peroxidase conjugated rabbit anti-gamma chain (Dako P-214, Glostrup, Denmark) diluted 1:500 in serum diluent was added and incubated for 0·5 h at room temperature. After four more washes with PBS-T, orthophenylenediamine dihydrochloride substrate was added at a concentration of 0·6 mg/ml in citrate-phosphate buffer, pH 5·0. The reaction was stopped by the addition of 50 μl of 3 N H_2SO_4. Absorbance was read at 492 nm with an Organon Teknika ELISA reader, against a reference wavelength of 650 nm.

Calculation of results and data analysis

Quadruplicate background values (all reagents applied to the wells except for serum diluent in the place of a serum sample) were averaged and subtracted from the average optical density (OD) for each serum sample to give net average absorbance values for each serum sample. Sera were routinely tested in duplicate, and net average absorbance values were normalized with respect to a low positive control serum tested in each experiment, to give an adjusted OD value for the serum. The control serum, tested in 12 separate assays, had an average OD ($\pm$ standard deviation) of 0·619 $\pm$ 0·066. Also included in each experiment was a control negative serum. Since in the chlamydial

ELISA system an adjusted absorbance of 0·400 was shown to give an excellent positive/negative correlation with the MIF test, and since no gold standard exists for 'Simkania Z' serology, we chose to create a grey zone around the 0·400 OD level, and to consider adjusted OD values of < 0·300 as negative, of 0·300–0·500 as borderline, and of > 0·500 as positive.

Statistical analysis

The significance of the differences between proportions of seropositives was calculated by χ^2 or Fisher's exact test as appropriate.

RESULTS

Sera of 94 students and 100 blood donors were tested by the ELISA for IgG antibodies to *C. trachomatis*, *C. pneumoniae*, and to 'Simkania Z'. Figure 1 shows that for 14 representative student sera, levels of IgG antibody to each of the Chlamydia and to 'Simkania Z' were independent. Figure 2 (*a* and *b*) shows the levels of IgG antibodies to each of the three antigens for each of the 100 blood donors; the sera are arranged in order of increasing adjusted OD with respect to 'Simkania Z' antigen. There is clearly no trend for increasing IgG levels to chlamydial antigens with increasing levels of antibodies to 'Simkania Z', nor for lower levels of antibody to chlamydia with lower levels of Z antibody.
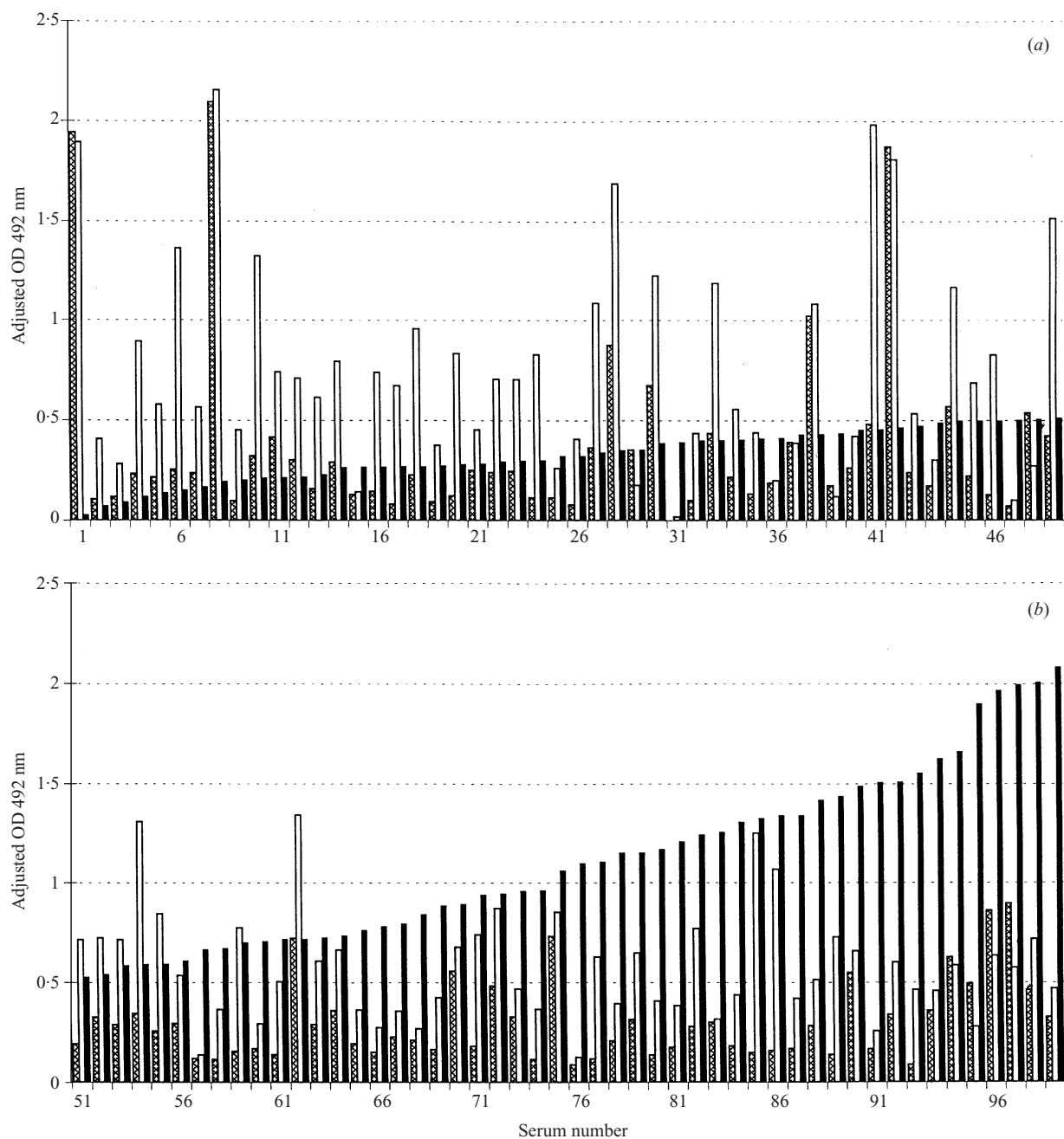
Fig. 2. Urea ELISA levels of antibodies to 'Simkania Z', *Chlamydia trachomatis* and *C. pneumoniae* for 100 blood donors, arranged in order of increasing adjusted OD 492 nm with respect to 'Simkania Z' antibodies. (*a*) Serum samples 1–50. (*b*) Serum samples 51–100. ■, 'Simkania Z'; □, *C. pneumoniae*; ▨, *C. trachomatis*.

The prevalence of past exposure to 'Simkania Z' in these healthy population samples was similar to that to *C. pneumoniae*, but greater than to *C. trachomatis*. Table 1 shows the percent seropositivity to each of the antigens for these population samples and the percent seropositivity for two different age groups among the 106 adult kibbutz members tested (18–25 years and 26–40 years), as well as among the 45 Negev Bedouin tested. Clearly seropositivity to 'Simkania Z' is not

directly related to seropositivity to the two *Chlamydia* species. The seropositivity rates to 'Simkania Z' were higher among the Bedouin than among the other groups, but the difference reached significance ($P < 0.05$) only in comparison to the blood donors. On the other hand, the Bedouin group had significantly less seropositivity to *C. pneumoniae* than the other groups ($P < 0.001$ for each comparison). The Bedouin also had a significantly lower seropositivity rate to *C.*

Table 1. *Seropositivity to 'Simkania Z', C.* pneumoniae *and* C. trachomatis *by the urea ELISA*

| Group | n | Percent seropositive on different antigens* | | |
		'Simkania Z'	C. pneumoniae	C. trachomatis
Students	94	65	60 (n.s.)†§	17 ($P < 0.001$)†§
Blood donors	100	55§	73 ($P < 0.05$)†§	22 ($P < 0.001$)†§
Kibbutz members	106	64	58 (n.s.)†§	17 ($P < 0.001$)†§
Aged 18–25	41	68	41	17
Aged 26–40	65	63	68	17
Bedouin	45	80	27 ($P < 0.001$)†	4 ($P < 0.001$)†
Aged 18–25	20	85	20	0
Aged 26–40	25	76	32	8

* Adjusted OD > 0·500 for 'Simkania Z'; adjusted OD > 0·400 for chlamydia.
† Significance of seropositivity difference compared to 'Simkania Z'; n.s., not significant.
§ Significantly different from the Bedouin seropositivity rate for the same antigen.

trachomatis than the other groups; trachoma was still seen occasionally among the Bedouin as recently as the early 1960s, so that the two seropositives that were found may have had childhood trachoma infection.

DISCUSSION

The ELISA technique used to detect IgG antibodies to 'Simkania Z' was originally designed to differentiate between antibodies to *C. pneumoniae* and antibodies to *C. trachomatis*. We and others have shown by immunoblot [16, 17] extensive cross-reaction between antibodies against the latter two organisms. The basis for the high specificity of the ELISA used in this study is the reduction of cross-reactive lipopolysaccharide epitopes on the antigens by deoxycholate treatment and a wash step with 6 M urea which releases antibodies of low affinity. The levels of antibodies to *C. trachomatis* and *C. pneumoniae*, as detected by the ELISA, are quite independent. Since serologic tests for antibodies to 'Simkania Z' are only now being developed, there is no gold standard by which they can be evaluated. However, by using purified 'Simkania Z' antigen in an assay system previously shown to have very high specificity, in its ability to differentiate between antibodies to *C. trachomatis* and antibodies to *C. pneumoniae* [13], and which is highly reproducible, we believe we have achieved an acceptable assay system for detection of antibodies to 'Simkania Z'.

The results of this study indicate that infection with the newly described microorganism 'Simkania Z' is commonplace, with at least half of the young adult and adult population bearing antibodies to the organism, and a much higher proportion among

Bedouin residents of the Negev. These preliminary results will need to be confirmed in more extensive studies, and if they are, the question of zoonotic infection with 'Simkania Z' or related organisms may arise. Nothing is yet known about such infection in domestic or wild animals.

The possible cross-reactivity of antibodies to known zoonotic organisms, such as *Bartonella* spp., with Simkania antigen is of concern. While there are no published data on the extent of zoonotic *Bartonella* spp. infections in the Negev, or the extent of seropositivity to *Bartonella* spp. in various population groups in the Negev, some local cases of bartonella-associated cat scratch fever have been diagnosed, and the extent of seropositivity and possible cross-reactivities need to be investigated. However, it is unlikely that our ELISA is detecting antibodies to *Bartonella* sp., for example among the Bedouin, since if *Bartonella* sp. seropositivity is widespread, one might expect to find high rates of seropositivity to *C. pneumoniae*, bartonella-chlamydia cross-reactivity having been clearly documented [18]. However, the seropositivity rates to *C. pneumoniae* in our assay system are remarkably low among the Bedouin.

Recently the existence of other chlamydia-like intracellular microorganisms has been reported, especially as uncultivated endosymbionts of amoebae [19, 20]. Some such organisms, including one detected in amoebae isolated from nasal mucosa, have been shown to be geneologically approximately equidistant from 'Simkania Z' and the known Chlamydiae [19, 20]. Antigenic relationships between the newly recognized organisms, if they exist, will need to be explored, as will the possible involvement of amoebae in propagation or spread of the organisms, since

amoebae have been shown to enhance the invasiveness of legionella [21], for example. It is not known whether Simkania can grow in amoebae or whether amoebae can be a vector for the spread of 'Simkania Z' infection.

The ELISA assay used in this study is useful for serosurveys since because of the 6 M urea wash step, it preferentially detects IgG antibodies of high avidity [22]. Its applicability in diagnosis of current infection with 'Simkania Z' needs to be further explored. It may be that in conjunction with testing for IgA, which initially is dimeric [23, 24], and therefore possibly of relatively higher affinity, it can be used for diagnosis of current infection [12]. We are currently exploring the capabilities of various immunoassay systems and testing additional population groups to confirm the results of this study and to determine the age of acquisition of antibodies to 'Simkania Z'.

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