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"Chlamydia pneumoniae and Simkania negevensis

in severe respiratory infection"

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Executive Summary

The overall aim of this research project is to determine the prevalence of infection with *Chlamydia (Chlamydophila) pneumoniae* and *Simkania negevensis* in certain population groups in Lima, Peru, and in the Negev region of Israel; to determine the possible association of these organisms with severe respiratory illness; and to investigate the possibility that drinking water (or waste water) may be a source of infection. During the first year of this project, several laboratory techniques were developed. The serologic dot blot assay is being used to test sera of children with severe respiratory disease in Lima. ELISA and dot-blot testing of sera of pregnant women from various locations in the Negev, showed a high rate of seropositivity to *S. negevensis*, and a lower rate of seropositivity to *Ch. pneumoniae*. The dot blot assay developed for examining water samples for the presence of *Simkania* and amoebae, which may be carriers of *Simkania*, indicated the presence of the organism in association with amoebae in all effluent water samples tested in the Negev and in Lima, Peru, and in about half of the drinking water samples tested from both areas. During the coming year, more serum and water samples will be collected and tested, and the results will be used to formulate an overall picture of the association of the presence of the organisms in the water supply and the extent of seropositivity of the local population. In Lima, more respiratory samples from children hospitalized with severe respiratory tract infections are being collected for testing for the presence of *S. negevensis* and *Ch. pneumoniae*. Thus achievement of the objectives of this project is in sight. If *S. negevensis* and *Ch. pneumoniae* are found to be associated with severe respiratory infection in children in Lima, there will be strong implications for empirical treatment of such infections, as well as for possible water treatment to eliminate or inactivate the organisms. The techniques developed in Beer Sheva are now being used effectively in Lima to achieve the goals of this project.

Section I

A. Research Objectives:

The overall aim of this research project is to determine the prevalence of infection with *Chlamydia pneumoniae* and *Simkania negevensis* in certain population groups in Lima, Peru, and in the Negev region of Israel; to determine the possible association of these organisms with severe respiratory illness; and to investigate the possibility that drinking water (or waste water) may be a source of infection.

Specifically, in this project we set as our goals to:

1. Develop and evaluate several simple assay systems for detection of antibodies in serum samples and antigen in clinical samples or in water samples, including:
 - a. A simple dot blot assay for detection of antibodies to *Chlamydia pneumoniae* and *Simkania negevensis*. This assay was designed for use in remote areas and in laboratories not equipped with an ELISA reader.
 - b. A variation of the dot blot assay in the converse format to be used for the detection of the *C. pneumoniae* and *S. negevensis* in water samples.
 - c. Adaptation of the microimmunofluorescence (MIF) test for detection of antibodies to *S. negevensis*
 - d. Implementation of the direct immunofluorescence assay (DFA) for detection of *S. negevensis* in clinical samples.
2. Examine serum samples from several population groups (and respiratory samples from hospitalized patients) to determine the extent of infection with these organisms.
3. Examine water sources (and waste water) for the presence of the organisms and test for a correlation between their presence and the prevalence of past or present infection in the relevant population groups.

B. Research Accomplishments during the past year:

During the past year, significant further progress has been made, and we are close to attaining all of our research goals.

Development of assay systems for detection of antibody:

Dot blot assay system

We have developed a spot dot assay system for detection of antibodies to *S. negevensis* which is suitable for use in remote areas and requires only an antigen-impregnated membrane, sample tubes, a pipettor for microliter volumes, and developing reagents.

Results are read with the unaided eye. Antigen impregnated and blocked membranes can be stored at room temperature for several months.

Initially the assay was performed using alkali-lysed mixtures of purified *S. negevensis* elementary and reticulate bodies (EB and RB) as antigen. Other types of antigen were also tested:

- a. ELISA antigen, consisting of whole Sn EB and RB particles, formalin-inactivated and deoxycholate-digested;
- b. Microimmunofluorescence (MIF) antigen, consisting of purified *S. negevensis* EB in suspension;
- c. A lipopolysaccharide (LPS) fraction consisting of purified EB and RB particles after lysis and proteinase K digestion of the proteins;

When a small number of sera and controls were assayed employing these antigens, no significant differences were observed when compared with the alkali-lysed antigen, which was easier to prepare and handle. However, further use of the assay with the LPS fraction needs to be investigated to determine the smallest amount of antigen that can be used in a dot-blot assay, since such an assay may show a higher specificity. In Western blot experiments, the pattern of the LPS fraction of *S. negevensis* was totally different from that of *C. trachomatis* L2 (preliminary results).

Forty sera collected from young Peruvian children (up to 5 yrs old) diagnosed with severe acute respiratory disease were tested and 20 (50%) were found to possess IgG antibodies specific for *S. negevensis*. Other sera will be collected in the future and tested on antigen- containing filters which will be supplied from Israel. Figure 1 shows the distribution of titers obtained by the dot-blot assay for IgG and IgA antibodies to *S. negevensis* among these children. It should be noted that it has been determined that for best results, sera tested by the dot-blot assay should be fresh and not hemolytic. When this is the case, children's sera show no background coloring at all, and the titers are very easy to read in the assay.

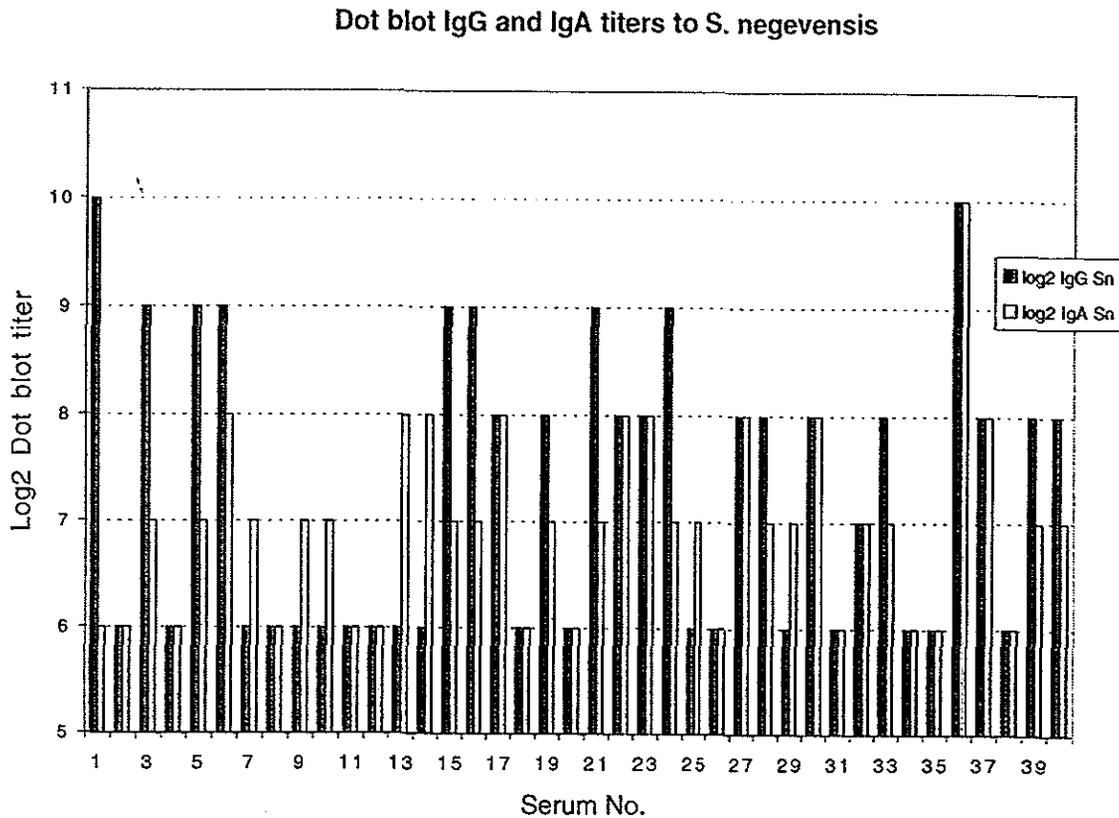


Figure 1. IgG and IgA titers to *S. negevensis* as tested by dot-blot assay for forty sera of children hospitalized in Lima.

ELISA testing of adult sera

In the framework of this project, serum samples from pregnant women living in various localities in the Negev, which are normally discarded after routine serologic tests, are being saved for us by the laboratory of the Health Ministry (in accordance with permission from the local "Helsinki" committee). These sera are being tested for the presence of antibodies to *S. negevensis* and *Ch. pneumoniae* by the ELISA technique. So far 123 such sera have been collected, from women in seven different localities. There seem to be significant differences in seroprevalence of antibodies to *S. negevensis* and to *Ch. pneumoniae* in the various locations—with a tendency for a very high seroprevalence of antibodies to *S. negevensis* in women from Bedouin settlements or cities (78%) and lower in Jewish towns (50%); the corresponding rates of seropositivity to *Ch. pneumoniae* were 31% among Bedouin women and 21% among the Jewish women.

Areas with mixed populations were not taken into account in this brief summary—only towns or cities with exclusively one ethnic group. When water testing will be complete, more extensive analyses will be carried out.

Conclusions and directions for future work with respect to serologic techniques.

ELISA: most specific assay method evaluated to date. However, may miss early IgG antibodies (of lower affinity). IgA cutoff levels may need to be calibrated with respect to an outside assay system for different age groups.

IPA: more time consuming (hands-on time) than ELISA, but seems to be good for IgA detection, especially in young children.

Dot blot: Very simple system. Easy transport of antigen treated membranes; no advanced equipment required for use. Extensive testing showed a measure of cross reactivity with cell lysate antigen currently used. This still needs to be better defined. The dot blot seems to be an ideal test for children's sera.

MIF for antibodies to *S. negevensis*: This assay has been worked out and we are able to prepare slides which can be used for up to three weeks after preparation. The test is not difficult to perform, but does require experience to read accurately and reproducibly, as well as a high quality fluorescence microscope.

2. Techniques for detection of *S. negevensis* and *Acanthamoebae* in water samples

Several techniques were developed or adapted for the purpose of detection of amoebae and *S. negevensis* infected amoebae in water sources. Initially, experiments were carried out to determine whether *S. negevensis* is able to grow in *A. polyphaga*, at least under laboratory conditions.

It was found that after an initial lag period, progeny *S. negevensis* increased exponentially between 7 and 14 days after infection of the amoebae. The amoebae continued to multiply during the infectious process.

After initial experiments to recover *S. negevensis* and *Acanthamoebae* from seeded water samples were not extremely successful, work was begun on development of a dot blot assay for detection of amoebae and *Simkania negevensis*-infected *Acanthamoeba polyphaga* in water supplies. For this purpose hyperimmune antisera were raised by immunization of mice with lysates of *A. polyphaga*. Antibody titers of >1:1000 were obtained (as measured by dot immunoblot).

In reconstruction experiments, techniques were developed for optimization of recovery of amoebae from water samples. Known numbers of amoebae were diluted 1:500 or

1:1000 in tap water, and recovery by filtration and by centrifugation was examined. Centrifugation was found to be more successful than filtration, but still was limited to 16-19% efficiency, which was inadequate. Whether the concentration of the amoebae from the water is carried out by filtration or centrifugation, specific detection of the organisms depends upon successful exposure of antigenic epitopes of interior as well as exterior components, especially since *S. negevensis* also needs to be detected inside the amoebae. After numerous experiments, a three-tiered fixation protocol was adopted: treatment with 30% methanol, 5% hydrogen peroxide for 15 min, followed by 70% ethanol for 5 min, and then by 96% ethanol for 5 min. Efficient blocking was achieved with the use of the polyvinylpyrrolidone solution which we use for the dot blot antibody assay. In the reconstruction experiments, using anti-*S. negevensis* hyperimmune sera we were able to detect $1.6-2.7 \times 10^3$ *S. negevensis*-infected amoebae per spot; using anti-*A. polyphaga* hyperimmune sera we were able to detect 128-256 amoebae per spot. This level of sensitivity was also not satisfactory.

An altered fixation protocol was developed and adopted. With the new protocol, 8-24 infected amoebae were detectable, or 8 uninfected amoebae. This level of sensitivity was far more acceptable, and experiments were begun for detection of *S. negevensis* and acanthamoebae in natural water supplies.

Drinking water samples to be tested were concentrated x 60 and tested. Of 27 samples tested for the presence of amoebae, 15 were positive and 12 negative. Twenty-five of these samples were also tested for the presence of *S. negevensis*. Twelve were positive and 13 were negative. Every sample positive for *S. negevensis* was also positive for amoebae.

When 21 samples of waste water effluents were tested for the presence of *S. negevensis*, all were positive. Seventeen of them were also tested for amoebae, and all were strongly positive.

Water samples in Peru. Eleven samples of drinking water as well as 5 samples of untreated sewage water collected from different parts of Lima. Amoebal antigens were found in 8 samples of drinking water (72%), and *S. negevensis* was found in 6 samples of drinking water. No samples negative for amoebae were found positive for *S. negevensis* antigen. Both antigens were found in abundance in all 5 samples of sewage tested.

It should be noted that additional control experiments need to be carried out in order to make certain that the hyperimmune rabbit sera used for detection of organisms in waste-

water effluents are not detecting some other organism to which the rabbits were naturally exposed. These experiments are now underway.

Conclusions and Significance—Organism detection

S. negevensis was shown to be able to grow in trophozoites of *Acanthamoeba polyphaga* and to be able to persist in amoebal precysts and cysts. (These results have been published in *Applied and Environmental Microbiology*, and a reprint is attached.) Methods were also developed for detection of infected and uninfected amoebae, and these methods were shown to be applicable also in Peru.

Natural residence and replication of *S. negevensis* in free-living amoebae would imply that natural infection with *S. negevensis* may be facilitated by amoebae in a way similar to amoebal facilitation of Legionella transmission. While the apparent involvement of Simkania in respiratory morbidity would seem to indicate probable aerosol transmission, infection may be as common as it is (viz. the high prevalence of antibodies to the organism in the general population and its early age of acquisition) due to early exposure to water or dust bearing Simkania-carrying amoebae. Further studies are necessary to follow survival of *S. negevensis* inside *A. polyphaga* cysts under controlled conditions for encystation. The resistance of such infected cysts to various adverse conditions simulating natural environments is of special interest for understanding possible mechanisms of *S. negevensis* transmission.

The hypothesis that the high prevalence of antibodies to the organism in the general population may be due to early exposure to water or dust bearing Simkania-carrying amoebae will be further tested in the framework of this study by examination of samples of water sources, soil and dust, and correlation of the results with the local prevalence of Simkania infection.

C. Scientific Impact of Collaboration

An understanding of the extent of the presence of Simkania and/or Chlamydia in drinking water in Lima is beginning to be obtained as a result of this research project. As more samples are tested, details of the picture will be filled in, and the implications of our findings will become clearer.

Dr. Jose Delgado is continuing with patient enrollment in Lima, and special measures are being instituted for preservation of patient specimens until they can be tested in the laboratory.

D. Description of Project Impact

The dot-blot assay which we have developed will soon be available for use in underdeveloped regions. The only equipment required for its use is a pipettor, so that it can be used in very simple laboratories. The significance of antigen detection in drinking water will need to be determined, and the implications for possible water treatment considered.

E. Strengthening of Developing Country Institutions

A filtration manifold was obtained which makes possible testing of water samples by the same protocol as that used in Israel. Scientists in Peru were trained by Dr. Kahane during her recent visit there in the performance of the dot-blot serologic assay and the dot-blot assay for detection of *S. negevensis* and other organisms in water samples. The proper storage and treatment of clinical samples was also explored during her visit there. We hope that further exchange visits will be possible during the coming year.

F. Future Work

In Peru, collection of specimens from children hospitalized with severe respiratory illness is continuing. Water samples are also being collected and tested by the dot-blot assay for detection of *Simkania* and amoebae developed in Israel. The project is essentially on schedule in Peru and definitely on schedule in Israel. The work plan has not been revised.

Section II

A. Managerial Issues

No staff changes or site changes are anticipated. In accordance with our progress, Ben Gurion University requested allocation of the full grant budget as originally approved, and this request was approved.

B. Budget

During the past year, because of the security situation, planned trips to Israel were not carried out. Therefore it was agreed that Dr. Simona Kahane would travel to Lima and present the techniques developed in Beer Sheva (see below). For this purpose, \$4,500

were transferred to the Israeli side for travel, from the Peruvian side. This sum was requested because at first it was thought that Dr. Bella Dvoskin would also travel to Peru. Only \$3000. of the amount was used for Dr. Kahane's trip, and it was decided that the remainder would be used toward per diem, insurance, and lodging for our Peruvian colleagues when (hopefully) they will come during the coming year(s) to Beer Sheva.

C. Special Concerns

There are presently no changes in special concerns.

D. Collaboration, Travel, Training, and Publications

At the end of this grant year (February, 2002) Dr. Simona Kahane traveled to Lima and was able to meet and work with Prof. Bob Gilman, Mrs. Pat Sheen, Dr. Jose Delgado, Mrs. Maritza Jimenez Calderon, Ms. Juliana Cordova and Ms. Fanny Azenas. During her stay she had several productive discussions about their work, about the techniques developed by us in Israel, and about our common plans for future studies. The techniques transferred to Lima included both a dot-blot assay for serologic studies and a dot-blot assay for detection of *S. negevensis* and *Acanthamoeba* antigens in water samples. The necessary materials to be used for their demonstration were brought from Israel. Even before her trip, exchange of DNA primers and positive controls took place, by courier mail service, as well as exchange of protocols by email communication, which has been consistently excellent. Additional communication is by fax and telephone.

An article describing the growth of *S. negevensis* in *Acanthamoeba polyphaga* and its survival in amoebal cysts has been published (S. Kahane, B. Dvoskin, M. Mathias, M.G. Friedman. 2001. Infection of *Acanthamoeba polyphaga* with *Simkania negevensis* and *S. negevensis* survival within amoebal cysts. Appl Environ Microbiol 67: 4789-95) and has been enthusiastically received by the scientific community. (We have received a number of very encouraging responses to the publication.) A manuscript describing the association of *S. negevensis* with acute exacerbations of chronic obstructive pulmonary disease has also been accepted for publication (European Journal of Clinical Microbiology and Infectious Disease).

Two students in Israel took part in the development of the dot blot and MIF serologic assays. In Lima, Maritza Jimenez Calderon and Juliana Cordova were trained in the performance of the dot-blot assays by Dr. Simona Kahane. Also, Dr. Kahane was able to offer suggestions for improved collection and storage of nasopharyngeal washes, for optimal detection of Chlamydia and Simkania in the samples to be collected this year.

E. Request for American Embassy Tel Aviv or A.I.D. actions

At this time there are no requests for Embassy or A.I.D. actions.