

ANNUAL REPORT

Covering March 2002-February 2003

Submitted to the U.S. Agency for International Development; Bureau for Economic
Growth, Agriculture and Trade

"Chlamydia pneumoniae and Simkania negevensis

in severe respiratory infection"

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PRISMA (Products in Information Systems, Health,
Medicine, and Agriculture)
Lima, Peru

Project No. C19-033

Grant No. TA-MOU-99-C19-033

USAID Grant Project Officer: Mr. William H. Crane

Project duration: March 2000-February 2004

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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*, with a lower rate of seropositivity to *C. pneumoniae*. The specificity of the dot blot assay developed for examining water samples for the presence of *Simkania* and amoebae, which may be carriers of *Simkania*, was improved during the past year. The modified protocol will be used during the coming year to test more water samples in both Israel and Peru. Together with the results of more serum sample testing, 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 *C. pneumoniae* by various methods. If *S. negevensis* and *C. 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 being used 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:

1. Development of assay systems for detection of antibody:

Dot blot assay system

The dot blot assay system for detection of antibodies to *S. negevensis* 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. In its final format, the dot blot antibody assay system uses an antigen-impregnated filter paper (8x 96 dots, corresponding to microtiter plate

format) in which the antigen consists of an alkali-lysed mixture of purified *S. negevensis* elementary and reticulate bodies (EB and RB). Because of its homogeneity on the filter, we preferred the use of this antigen preparation to the use of our standard ELISA antigen (consisting of whole Sn EB and RB particles, formalin-inactivated and deoxycholate-digested) or the microimmunofluorescence (MIF) antigen (consisting of purified *S. negevensis* EB in suspension).

The LPS (lipopolysaccharide) antigen (consisting of purified EB and RB particles after lysis and proteinase K digestion of the proteins) turned out to be too expensive for routine use. We were unable to significantly reduce the amount used per spot, and the growth of the quantities of bacteria required for extraction of the needed LPS was quite costly.

The dot blot assay has not yet been extensively applied for testing of children's sera in Lima, but will be during the coming year.

Following is a brief review of serologic techniques developed and/or used in the framework of this study:

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

The dot immunoblot method for detection of *S. negevensis* and *A. polyphaga* in samples of reclaimed water and drinking water was refined and thoroughly tested for specificity as described below. Because the results indicated a possibility of cross-reactions, modifications were implemented which resulted in greater specificity.

Antigen was prepared from a panel of 8 bacteria (from ATCC standard cultures) and tested for reactivity with the anti-Simkania antibodies in the dot blot assay. These bacteria included: *B. catarrhalis*, *P. vulgaris*, *Staphylococcus aureus*, *Escherichia coli*, *Staphylococcus epidermitis*, *Klebsiella pneumoniae*, *H. emophilus influenzae*, and *Streptococcus. Pneumoniae*. The results indicated some binding of rabbit immunoglobulins or binding of the labeled second antibodies to biologic material on the filter. The addition of normal swine immunoglobulins into the blocking buffer and serum dilution buffer reduced this background. Furthermore the use of affinity-purified goat anti-rabbit HRP-conjugated second antibodies increased the specificity of the test and eliminated the possibility that antibodies in the enzyme conjugate would bind to unknown microbial antigens in the water samples being tested.

Possible cross-reactivity of anti-Simkania antibodies with *C. pneumoniae* or *C. trachomatis*: With a serum dilution of 1:20,000 no cross-reactivity could be detected. The use of this serum dilution also made the assay system very economical.

In addition, all tests are now done \pm anti-Simkania serum. Any signal that appears at the same intensity without addition of serum as with serum is considered false positive.

With the modified procedure, 2000 infectious center forming units of *S. negevensis* could be detected in water samples. For drinking water, 500 ml samples are filtered through Super 800 47 mm 0.8 micron filters (Gelman Laboratories). Such filters can then be cut into four and tested for both *S. negevensis* and Acanthamoebae, with and without specific serum. For testing of waste water effluents or reclaimed water, very turbid samples are first filtered through viscose-polypropylene fiber "cloth" and 200 - 400 microliters are applied to the filter manifold (microtiter plate format).

Detection of amoebal antigens in water samples

1. *A. polyphaga* persistently infected with *S. negevensis* or uninfected, as two-fold or four-fold dilutions in distilled water, serve as positive controls in each experiment.
2. Filtration, processing of the filter, and blocking are as described for *S. negevensis* antigens.
3. The protocol uses mouse anti-*A. polyphaga* serum diluted 1:5000 in blocker as above and Jackson peroxidase-conjugated, affinity purified goat anti-mouse (115-035-003) diluted 1:2000.
4. All other details of the protocol are as used for the detection of *S. negevensis*, with and without anti-amoebal serum.

Of note with respect to the sensitivity of the dot-blot antigen detection method for water samples:

1. Non-specific background can be due to sewage debris on the filter or unknown bacteria cross-reacting. We have tried several types of pre-filtration to reduce the problem of debris, and so far the fiber cloth seems to be best.
2. We have managed to increase the specificity of the dot-blot antigen detection method, at the expense of some sensitivity; however, at this stage in the research, high specificity is probably more important than high sensitivity. In the future, when the importance of the presence of the organism in water supplies will be better understood, more sensitive techniques will be able to be developed, especially when the genome of the organism will have been sequenced.

Survival of organisms in aqueous environments

Several parameters of the survival of amoebae, *S. negevensis* and Chlamydia in aqueous environments were investigated by reconstruction experiments.

- a. Antigen detection over time in water. *S. negevensis* and *C. trachomatis* L2 were mixed with sterilized purified water samples, and the presence of antigen and infectivity were followed for 7 days at room temperature. In both cases antigen could be detected after 7 days almost as efficiently as at the beginning of the experiment.
- b. Infectivity: *C. trachomatis* infectivity in water was lost within 8 hours. *S. negevensis* infectivity could be found even 7 days post infection (8-15%). *S. negevensis* -infected *A. polyphaga* survived for 7 days and even significantly increased in number.
- c. Sensitivity to routine chlorination. *S. negevensis* infectivity was shown to be completely resistant to chlorination as performed routinely by health authorities.

3. Detection of *S. negevensis* in clinical samples

Use of the PCR assay for detection of *S. negevensis* in clinical samples, that had been developed in Beer Sheva, has not been very successful in Lima. There are several possible sources of difficulty, which include the technique used to obtain the sample, the storage of the sample until processing for DNA extraction, or the storage of the DNA extract, the extraction procedure, and less than optimal sensitivity of the PCR protocol. We are presently trying to determine the source of the problem in order to correct it and are considering the possibility of sending some respiratory samples from this winter season for isolation studies in Israel.

In parallel, we are continuing to test and develop the use of the dot blot antigen detection procedure for application to clinical samples. If it will be successful, it would be a simpler and cheaper method of detection than the PCR procedure.

Conclusions and Significance—Organism detection

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. The resistance of infected amoebal cysts to various adverse conditions simulating natural environments is of special interest for understanding possible mechanisms of *S. negevensis* transmission. Our findings that *S. negevensis* is resistant to routine chlorination procedures and can survive in simple aqueous environments, within or without amoebae, may have great significance for its mode of 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.

Patient enrollment is continuing 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

We have again benefited from the Israel transfer of technology to Peru. We have used the new Amebic cysts sent to us to demonstrate that the PCR reaction was specific and also functioning. We also tested out Dr. Montenegro's new PCR primers for *S. negevensis* which initially looked promising but after more extensive testing were found to be lacking specificity. In addition we strengthened the Pediatric service of Dos de Mayo hospital purchasing a pulse oximeter for the project. Finally we have trained Indira Martinez – in the PCR technique used for Simkania –

Water samples are now being collected to be used for the new dot blot technique provided to us the last month. In addition bronchiolitis samples will continue to be collected over the next winter.

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. In Israel, more water samples are being tested, and it is anticipated that more serum samples of healthy expectant women will be tested as well. Also, adaptation of the dot-blot antigen detection method for use with nasopharyngeal wash specimens is to be finalized. The project is essentially on schedule in Peru and is on schedule in Israel. The work plan has not been revised.

Section II

A. Managerial Issues

No staff changes or site changes are anticipated.

B. Budget

During the past year, because of the security situation, planned trips to Israel were not carried out. Dr. Kahane was able to take advantage of a trip to the United States to make a detour to meet with Prof. Gilman in Baltimore on the 4th of March, 2003, to discuss progress in the project and transfer some reagents needed by our colleagues in Lima. To make this meeting possible, she used some travel funds (\$700) from the grant. Prof. Gilman then returned to Peru with the reagents and protocols that were discussed.

We may submit a request for adjustment of some of the budget lines on the Peruvian side, as the breakdown of expenses has been somewhat different than originally anticipated.

C. Special Concerns

There are presently no changes in special concerns.

D. Collaboration, Travel, Training, and Publications

As mentioned above, Dr. Kahane traveled to the U.S, and there met with Prof. Gilman. During that meeting it was agreed that the serologic data from ELISA assays for antibodies to Simkania (samples from previous study) be prepared for publication. The problems with *S. negevensis* detection referred to above were discussed, as well as possible methods for solving or getting around them. It is hoped that during the coming year at least one of the young Peruvian scientists will be able to come to Beer Sheva to practice the various techniques in this laboratory. Perhaps the outlook is better now for peaceful times. In any case, it is anticipated that Prof. Friedman will travel to Peru—if relatively early in the year, then for purposes of aiding in the solution of problems with techniques. If these problems are relatively easily solved without her, then she will travel later for purposes of summarizing and analyzing data obtained in the project, with the members of the Peruvian team.

A review article on *S. negevensis* infections has been submitted for publication and is now in final stages of revision.

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.