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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 this project, several laboratory techniques were developed. Of these, 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 dot blot assay developed for examining water samples for the presence of Simkania and amoebae, which may be carriers of Simkania, has been used during the past year to test water samples in both Israel and Peru. In Lima, more respiratory samples from children hospitalized with severe respiratory tract infections were collected for testing for the presence of *S. negevensis* and *C. pneumoniae* by various methods. The dot blot assay for detection of Simkania in water samples has been adapted for detection of the microorganism in clinical respiratory samples, and it is now being evaluated in comparison to genome amplification methods. The techniques developed in Beer Sheva are being used in Lima to achieve the goals of this project. 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.

Section I: Technical Progress

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) or membrane immunoassay 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, looking for a possible correlation between their presence and the prevalence of past or present infection in the relevant population groups.

B) Research Accomplishments

1.0 Development of assay systems for detection of antibody:

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 impregnated membranes; no advanced equipment required for use. For the optimization of the assay, different types of antigen were tested: alkali-lysed purified *S. negevensis*; "ELISA antigen" (whole *S. negevensis* particles, formaldehyde-treated and deoxycholate-digested); "MIF" antigen (suspension of whole EB particles) and LPS (semi-purified fraction of lipopolysaccharide). No significant differences were observed when the various types of antigen were compared. The alkali-lysed antigen was less expensive and easier to handle. Hyperimmune rabbit or murine polyclonal sera to *S. negevensis* showed no cross-reaction to *C. trachomatis* L2 or *C. pneumoniae* antigens when used in the dot-blot assay. When the assay was tested using human sera, the sera tested needed to be "fresh" and not hemolytic. Although more experiments need to be done to establish sensitivity and specificity of the assay, dot blot may be used as an easy assay for primary serologic screening of population groups. Suspected positive samples may be further analyzed using the ELISA assay.

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.

2.0 Detection of microorganisms.

2.1 Detection of antigen in water samples by MEIA

2.1.1. Protocol for MEIA

Cellulose nitrate filter membranes (S&S 0.45 micron or Pall-Supor-800, 0.8 micron) were used for filtration of control or tested water samples. Samples were mixed, and any coarse particles if present were removed by drainage through a viscose polypropylene sheet. A sample volume of 100-500 microliters of reclaimed waste water or 500 ml of drinking water was filtered (using an S&S 96-place manifold system with a 0.45 micron membrane sheet or a 47 mm diameter Pall Supor filter, respectively).

For each test, the following control samples were used: *S. negevensis* -infected Vero cell lysates (stored frozen in 50% fetal calf serum) or lysates of *A. polyphaga* persistently infected with *S. negevensis*. Two-fold serial dilutions of these controls in distilled water (200 microliter volumes) were filtered in duplicate through the manifold as calibration standards (see Figure 1). Filters were processed as follows: they were frozen for 10 min at –70 °C and incubated for 30 min at room temperature in 0.1 % saponin in distilled water with vigorous

shaking. The solution was removed and the filter frozen again at –70 °C. The filter was then fixed for 15 min in a solution consisting of 30% methanol and 5% hydrogen peroxide; then 5 min in 70% ethanol; and 5 min in 96 % ethanol. When the filter was almost dry, it was incubated for 45 min in a blocking solution containing 0.3% polyvinyl pyrollidone (PVP) dissolved in TBS-Tween buffer containing 10 mM Tris pH 7.6, 150 mM NaCl, and 0.05 % Tween-20, to which normal swine immunoglobulins (DAKO X-0906) diluted 1:5000 were added. The addition of swine immunoglobulins into the blocking buffer reduced background due to non-specific binding of rabbit or mouse immunoglobulins or of labeled second antibodies to biologic material on the filters.

For detection of the organisms trapped on the filters in the MEIA, the filters prepared as described above were incubated for 45 min at room temperature (RT) in rabbit anti-*S. negevensis* polyclonal hyperimmune serum diluted 1:20,000 in blocking solution. After washing three times for five min with TBS-Tween, the filters were incubated with affinity purified horse-radish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories, Inc., 111-035-003) diluted at 1:20,000 in blocking solution. After further washing, they were incubated in diaminobenzidine (DAB) substrate solution. In each assay a duplicate control filter was prepared in parallel and processed as above omitting the serum incubation step. Only signals stronger than those obtained on the control filter were considered positive.

The same procedure was employed for detection of amoebal antigens using mouse polyclonal anti-*A. polyphaga* (at 1:5000) and HRP-conjugated , affinity purified anti-mouse antibodies (Jackson, 115-035-003), diluted 1:2000.

2.1.2. Evaluation of the sensitivity and specificity of the MEIA. The sensitivity of the dot immunoblot (MEIA) protocol was evaluated by determination of the number of infectious center-forming particles of S. negevensis or of the number of amoebae present in the highest dilution of control material giving a positive signal (121 IFU of *S. negevensis* and 468 infected amoebae). The sensitivity and specificity of the MEIA assay are demonstrated in Figures 1 and 2 (pages 6 and 7).

2.1.3. Use of the MEIA assay. Both *S. negevensis* and amoebal antigens were detected in the majority of drinking water samples taken on several occasions from specific sources in Beer Sheva and in three satellite communities near Beer Sheva (28/35 for *S. negevensis* and 24/35 for amoebae). In contrast, drinking water from Eilat, which is obtained by desalination (by reverse osmosis) has no traces of either *S. negevensis* or amoebal antigens.

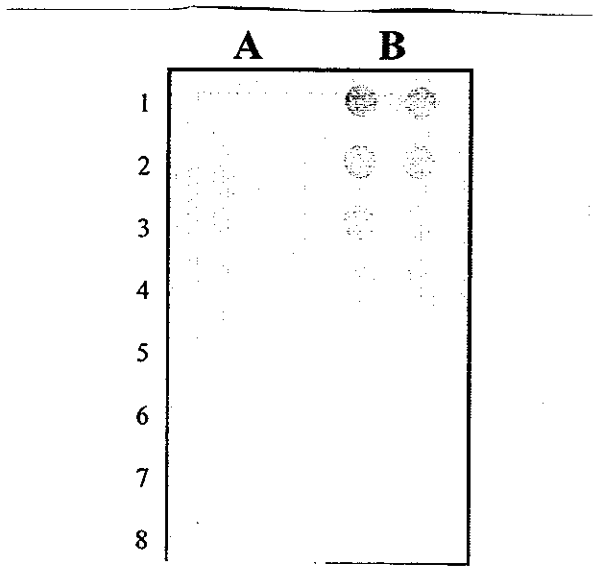

Figure 1. Specificity of MEIA assay with respect to other bacteria commonly found in the respiratory tract. The MEIA assay was performed as described in Materials and Methods. Column A: 100 microliters of the following bacteria were filtered in duplicate: *B. (Moraxella) catarrhalis*; *Proteus vulgaris*; *Staphylococcus aureus*; *Escherichia coli*; *S. epidermitis*; *Klebsiella pneumoniae*; *Haemophilus influenzae*; and *Streptococcus pneumoniae*. Column B: 100 microliters of *S. negevensis* suspension in two-fold dilutions.

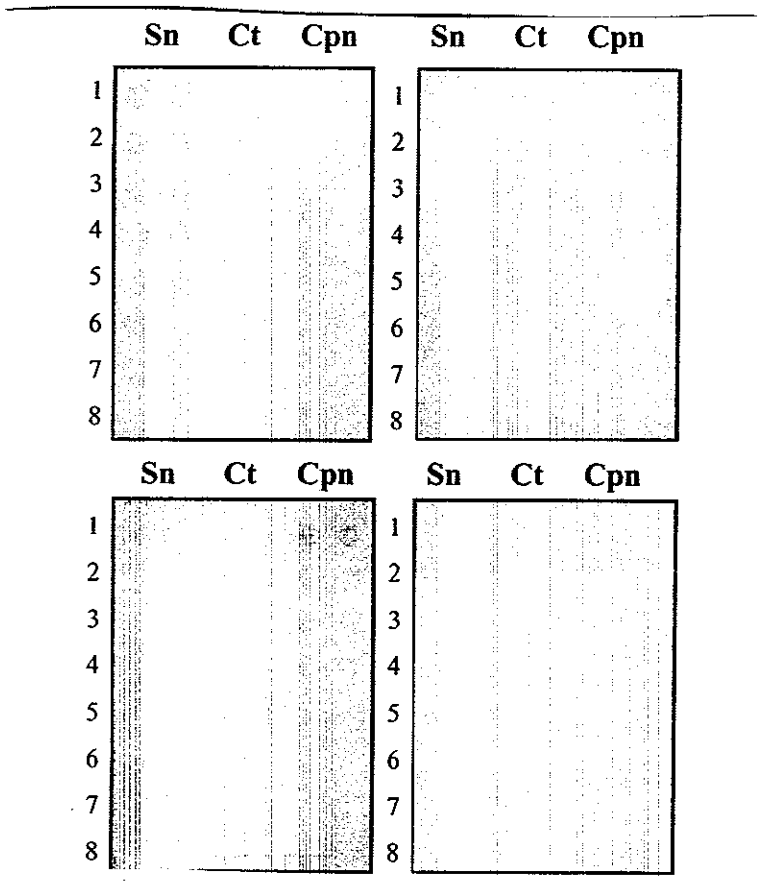

Figure 2. Specificity of MEIA assay with respect to members of *Chlamydiaceae*. Duplicate samples of 100 microliter volumes of two-fold dilutions of bacterial suspensions were applied to each of the membranes, as indicated. Sn: *S. negevensis*; Ct: *C. trachomatis* L2; Cpn: *C. pneumoniae*. Starting concentrations were: Sn, 4 x

10^4 per dot; Ct, 2.5 x 10^4 per dot; Cpn, 2.5 x 10^4 per dot. Upper left panel: rabbit anti-Sn serum used at 1:20,000; upper right panel, rabbit anti-Ct serum used at 1:20,000; lower left panel, rabbit anti-Cpn serum at 1:20,000; lower right panel, serum diluent only, with second antibody.

Reclaimed waste water is routinely examined by the Israel Ministry of Health laboratories before being approved for use in agriculture. Forty samples of such water obtained during a 1.5 month interval from various locations in the Negev were tested for the presence of *S. negevensis* and amoebal antigens by MEIA. The majority of samples (39/40 = 97.5%) were shown to contain *S. negevensis* antigens. No samples were found to contain antigens of *S. negevensis* in the absence of amoebal antigens.

2.2. Detection of *S. negevensis* or amoebal DNA by PCR. The sensitivity of our original PCR assay specific for *S. negevensis* was improved by using a nested PCR assay.

2.2.1. Protocols for DNA preparation and amplification. DNA from drinking water samples was prepared as follows: a volume of 500 ml water sample was drained through a cellulose acetate filter (Sartorius 12342-47-N, 5 micrometer); the filter was incubated with Page's saline buffer for 1 hr; the solution containing the biological material was centrifuged for 20 min at 1000 rpm to sediment amoebae; and the precipitate was used to prepare DNA with the QIAamp kit (Qiagen). Assays for the detection of the *S. negevensis* genome by PCR were performed using two sets of nested primers:

The first was primers ccF (CTT CGG GTT GTA AAG CAC TTT CGC) and ccR (CCC CGT CAA TTC TTT TGA GTT T) recognizing conserved chlamydial 16S rDNA sequences (433-457 and 933-945, respectively) and amplifying a 512 bp fragment, followed by nested ZpF and ZpR primers specific for *Simkania negevensis* Z (as previously described (15)), amplifying a 405 bp fragment.

The second set of nested primers were AF and BR amplifying a 1099 bp fragment within the 23S rDNA of *S. negevensis* containing the LSU intron (3) and IntD and IntR nested primers amplifying a 338 bp fragment within this intron. If the DNA tested does not contain the intron, a 441 bp fragment is obtained with the AF and BR primers, and no sequence is amplified by the IntF/IntR primer pair.

The use of the nested primers allowed increased sensitivity of the PCR assay when it was necessary; however, when DNA was abundant, primer sets AF/BR or IntF/IntR or ZpF/ZpR could also be used alone. PCR assays for the presence of amoebal sequences were carried out with the primers AmP1 and Amp2 described by Lai *et al.* (Mol. Cell Probes **8**:81-9) using the cycling program described below for *S. negevensis*.

2.2.2. Results obtained with the new PCR protocols. To confirm the finding of *S. negevensis* antigen in water samples, DNA prepared from the same volume of water used in the MEIA assay was also tested by PCR. All samples containing *S. negevensis* or Simkania-like antigens detected by MEIA that were tested for the presence of *S. negevensis* DNA contained such DNA as detected by PCR. Representative experiments are shown in Figures 3 and 4.

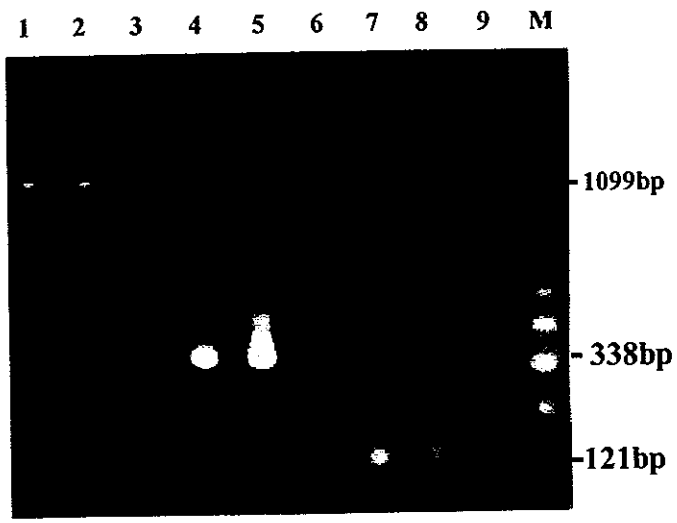

Figure 3. Amplicons detected by PCR assay of DNA prepared from water samples (lanes 2,5 and 8) as compared with DNA from purified cell-culture grown *S. negevensis*. (lanes 1, 4, and 7) using the following primer pairs: AF/BR (lanes 1 and 2), intF/intR (lanes 4 and 5), and amp1/amp2 (lanes 7 and 8). M: marker, 50-2000 bp ladder (Bio-Rad).

Figure 3 also demonstrates the presence of amoebal DNA in both control and test samples (121 bp amplicon). Figure 4 shows the amplification of *S. negevensis* DNA using the ccF/ccR and ZpF/ZpR nested primers for a number of DNA samples obtained from water. It should be noted that the nested PCR proved to be more sensitive for the detection of *S. negevensis* DNA in water samples than the standard PCR previously described (JID **177**:1425-9 and JID **178**:1553).

In Peru, 96 water sample sediments were tested by PCR for the presence *of S. negevensis* DNA. One was positive. However, these and more samples will be tested by the improved PCR assay (and by the MEIA), and it is expected that more will be positive.

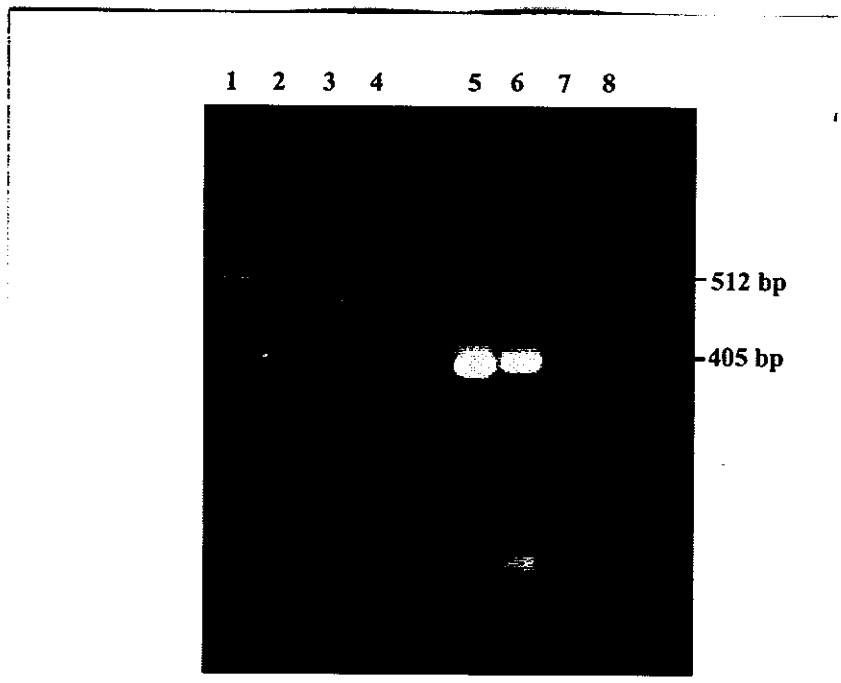

Figure 4. Nested PCR for detection of *S. negevensis* sequences in DNA prepared from water samples. Primers used: lanes 1-4, ccF/ccR; lanes 5-8 ZpF/ZpR (nested primers). Lanes 1 and 5: positive control; lanes 2-3 and 6-7: DNA prepared from two water samples; lanes 4 and 8, negative controls (no DNA).

3.0 Isolation and cultivation of Simkania or Simkania-like microorganisms from water. The procedure involves filtration of 500 ml of water sample through cellulose acetate filters (5 micron pore size), elution of protozoa containing Simkania from the filter and cultivation in cell culture in the presence of elevated concentrations of antibiotics. Out of eight attempts at isolation, six were successful and two were contaminated beyond recovery. The isolates are currently being studied in comparison to the *S. negevensis* laboratory strain.

4.0. Recent serologic studies. Sera obtained from a number of groups of healthy pregnant women (n=192) living in the same areas from which water samples were obtained for this study, showed an average prevalence of antibodies to *S. negevensis* of 75% (range: 50% to 92% for the different groups, unpublished results), as assayed by the ELISA assay (Epidemiol. Infect. **122**:117-23). The serum samples were from Jewish and Bedouin women living in rural and urban locations. The seropositivity ranges are consistent with published data (review article attached and *ibid.*).

In Peru, 127 serum samples have been collected from patients in Dos del Mayo Hospital and 91 from patients in the Hospital del Nino. These will be tested by more than one of our serologic tests for IgG and IgA antibodies to *S. negevensis*.

5.0 Summary of project accomplishments and implications. *S. negevensis* was initially isolated as a contaminant of cell cultures, and its natural hosts were unknown. With the help of techniques developed in this study, the presence of Simkania or Simkania-like bacteria in drinking water and in reclaimed waste water was demonstrated. Further studies are needed to understand the significance of the presence of *S. negevensis* or *S. negevensis*-like microorganisms in water. Aspects of such studies could include determination of the possible correlation between a high seroprevalence of antibodies to *S. negevensis* in healthy population samples and the quality of drinking water or agricultural irrigation water used in the same area. Thus, further research will enable conclusions about the environmental, epidemiological and health care implications of the common existence of Simkaniae in various water sources.

C) Scientific Impact of Collaboration:

Collaborating scientists in Peru have run the dot blot assay on all water specimens . This transfer of techniques occurred after Dr. Simone Kahane's visit to Peru. The results will be part of a forthcoming joint publication on water. The new nested PCR technique and primers has just been transferred and works well on positive and negative controls. In the final phase of the project they will use this technique to test their water and nasopharyngeal samples.

D) Description of Project Impact

If confirmed, the findings of this study will be reported to the National Institute of Health laboratories so that they can incorporate testing for *S. negevensis* in their water testing regimen.

E) Strengthening of Developing Country Institutions

This grant has mad possible acquisition of an oximeter for the study in children in the Dos de Mayo hospital. It also has provided a strong collaboration between the Pediatric Department of the Dos de Mayo hospital and the AB PRISMA project.

F) Future Work:

The project is on schedule. Efforts in Peru will be directed to running the acute and convalescent serology in patients as well as in age-matched well children. In addition, they will run the nested PCR for *Simkania negevensis* in both water and nasopharyngeal specimens.

Section II: Project Management and Cooperation

A) Managerial Issues

No staff changes are anticipated at this time. With respect to site changes, because of the continued Palestinian terrorist threats, it has not been feasible for young Peruvian scientists to be sent to Israel for training. Prof. Gilman has now come to Israel (see below), and Prof. Friedman will plan to visit Peru for consultations. Exchange of experimental protocols is being carried out efficiently by email correspondence.

We recently requested a transfer of funds between different budget lines of the Peruvian side of the budget, in accordance with their needs. They had increased need for funds in the "Salaries line" for several reasons, which were detailed in a letter to Mr. Boaz Ayalon of the US. Embassy in Tel Aviv. Some materials were bought more cheaply by other grants from the USA, and equipment was obtained from other grants. The changes were approved and implemented.

B) Special Concerns

There are presently no changes in special concerns

C) Collaboration, Training, Travel and Publications:

1) In Peru, the project is headed and coordinated by PhDc - Patty Sheen - The major investigator in the project is Ms. Maritza Calderone MS who is assisted by Ms Fanny Arenas and Ms Juliana Cordova. Specimen collection for water was supervised by Ms. Lilia Cabrera RN. - Specimen collection from patients was performed by Dr. Ninosko Donaires and is now completed. Collaboration with Dr. Nora Espirato at the Hospital Dos de Mayo enabled these specimens to be collected from the pediatric ward.

2) At the time of the last semi-annual report, Dr. Simona Kahane had just traveled to the USA to meet with Dr. Gilman in Baltimore, review progress, plans, and new protocols and give him new reagents for our colleagues in Peru. Dr. Gilman from the Peru group visited Israel to meet with Dr. Maureen Friedman and Dr. Simone Kahane to coordinate the final phases of the project and discuss joint manuscripts. Ms Arenas was trained at the JHU over July and August at the Institute of Tropical Health -and successfully completed the certificate course . This was training was in part funded from the Peru - Israel CDR -. Using the internet the Israel side of the grant has transferred the most recent set of primers to Peru as well as the new water membrane technique for the detection of Simkania. In addition, Israel will transfer new membrane technology to Peru as part of the visit of Dr. Gilman .

3) Since the last semi-annual report, we have published a review article entitled: "Infections with the chlamydia-like microorganism *Simkania negevensis*, a possible emerging pathogen" in Microbes and Infection, Vol 5, pages 1013-1021 (2003). Reprints are attached to this report. In that article some serologic results that were not previously formally published were reported on. We have also submitted for publication an article describing the detection of *S. negevensis* in water supplies, as detailed in this semi-annual report.

4) The Peruvians have been transferred the latest technology using nested PCR and have demonstrated that they are obtaining positive and negative controls similar to that of Israel. They also have used the dot blot and demonstrated a significant portion of stored water had Simkania antigen present whereas tap water and clean well water were all negative for this pathogen. During the next six months, we hope to confirm by PCR, results obtained in use of the adapted MEIA assay to detect *S. negevensis* in clinical nasal wash samples. (In order to reduce non-specific signals, NPW samples were centrifuged (13,000 rpm for 30'). The precipitate was resuspended to the original volume in distilled water and a 100 microliter sample was filtered in duplicate for the MEIA assay. Of 99 samples, 34 gave a specific positive antigen signal.) In Peru, additional serologic tests will be carried out by the dot blot assay, and additional water samples will be tested for the presence of *S. negevensis* and acanthamoebae by MEIA and by the improved nested PCR assay. The 58 properly collected and stored NPW samples will be tested by the improved PCR for *S. negevensis* sequences, and if the volume is sufficient, by the improved MEIA as well.

D) Summary of Requests for CDR Program Actions:

See "Managerial Issues", above.