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Development of Specific Assays for Diagnosis of *Chlamydia trachomatis* and TWAR (*C. pneumoniae*) Infections

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TABLE OF CONTENTS

	Page
1. Cover Page	1
2. Table of Contents	2
3. Executive Summary	3
4. Research Objectives	4
5. Methods and Results	
Development of serologic tests	5
Results of testing sera from Peru	11
Development of agent detection techniques	12
Results of agent detection	14
6. Impact, Relevance, and Technology Transfer	15
7. Project Activities	16
8. Project Productivity	16
9. Future Work	17
10. Literature Cited	17
11. Appendix A (Translation of informed consent forms)	20
12. Appendix B (Article in press describing urea ELISA)	22
13. Figures 1-13B	29-43

EXECUTIVE SUMMARY:

The purpose of this project was to develop, together with scientists in Peru, simple laboratory techniques for the detection and differentiation of infections due to *Chlamydia trachomatis* and *Chlamydia pneumoniae*, and to begin to apply these techniques in Lima and surrounding areas. These two chlamydial species are known to be responsible for a variety of pathologies in the United States and in Europe, including, sexually transmitted, ocular and respiratory tract diseases, although the extent of their prevalence in less developed areas is largely unknown. In the framework of the project two different types of serologic assays capable of differentiating between antibodies to the two organisms were developed, and one of these was adopted and used by Maritza Calderone (Peru) and Israeli scientists to test some 1400 sera originating in Israel and in Peru for antibodies to the two organisms. Subsequently, Maritza trained other Peruvian scientists and technicians in the use of the assay, with control sera and antigen brought back from Israel. Many of the serum and clinical specimens used either for the development of the techniques or for prevalence estimation were collected in the framework of the project. Advanced techniques were also developed for sensitive detection of the organisms in clinical specimens. Important contributions were made in this field by Dr. Guillermo Madico, visiting the Israeli laboratories, and further refinements were subsequently made in Peru by him and scientists whom he trained. We found serologic evidence that *C. pneumoniae* infection is extremely common in the population groups tested, ranging to 80 and even 100% in certain groups. *C. trachomatis* infection was found to be quite common in certain groups, while rare in others. Although we did not succeed in bringing the antigen detection system to a very simple format, it is capable of detecting chlamydia in easily obtainable urine specimens, and there is now no doubt of the necessity for such detection systems. The very full collaboration achieved in this project demonstrates the capabilities of the young Peruvian scientists involved and has led to an awareness of the apparently strong contribution of chlamydial infection to morbidity in this developing region.

RESEARCH OBJECTIVES:

The primary objective of this research project was to develop techniques for differentiation of infection with *Chlamydia trachomatis* from infection with *Chlamydia pneumoniae*. This involved development of serologic assays able to identify the presence of antibodies against each of these two species, confirmation of the validity (sensitivity and specificity) of these assays by comparison to "gold standard" methodologies, and development of direct detection techniques capable of detecting the organisms in appropriate clinical specimens.

Both of these organisms are notorious pathogens for man; however, very little is known about their prevalence in most population groups in South America. In this research project we aimed to develop serologic techniques to detect past and present infection in several different population groups, both in Israel and in Peru. We also hoped to develop accurate and rapid organism detection techniques for use with a variety of clinical specimens.

The significance of infection with *C. pneumoniae* is only now beginning to be appreciated. Besides causing simple and more serious respiratory tract infection [1-2], it has now been shown to be a risk factor for coronary heart disease [3] and is strongly suspected of being involved in development of atheromas and athersclerosis [4-6]; furthermore, it is believed to be involved in development of pulmonary hyperreactivity and asthma [7-9]. Only now are studies beginning to be carried out with respect to the effect of early treatment on the development of associated diseases--such studies could not be carried out before the development of simple tests to ascertain the presence of the organism and its eradication. Thus the diagnosis of infection is important both to determine the clinical entities in which the organism is involved as well as the effect of treatment on the resolution of the clinical problem. It has been known for sometime that infection with *C. pneumoniae* is quite common in Europe and the United States. Its prevalence in Peru and other South American countries has rarely been investigated, and this project, first and foremost, was aimed at developing simple tools with which the extent of infection with Chlamydia species could be determined in various population groups in Lima and surrounding areas.

Infection with *C. trachomatis* is today associated almost exclusively with sexually transmitted disease, at least in North America and Europe. As with *C. pneumoniae*, relatively little is known about the prevalence of infection with *C. trachomatis* in South America. It is also not known to what extent the presence of antibodies to *C. trachomatis* actually reflects sexually transmitted disease in these settings, or whether some seropositivity may be due to ocular disease. Although it is thought that there is little trachoma in S. America, recent reports indicate that ocular chlamydial infection may occur without classical signs of trachoma [10-12].

The development of simple but accurate serologic tests for antibodies to *C. pneumoniae* has been an elusive goal. Cross reactions between proteins of different Chlamydia species is not limited to a few proteins, but rather genus-specific epitopes may be found on many polypeptides as well as on lipopolysaccharide (LPS) moieties on chlamydial elementary bodies[13-15]. Efforts to develop serologic tests based on recombinant proteins or synthetic peptides have not as yet met with great success. In fact even microimmunofluorescence (MIF) serology is not considered to have an excellent correlation with other methods of determining infection with *C. pneumoniae* [16-17], but for want of alternatives, it is considered the golden standard by which other serologic tests must be measured.

The physician, faced with decisions on how best to treat patients with community acquired pneumonia (CAP), must take into consideration the possibility that *C. pneumoniae* may be an etiologic agent [2, 18-20]. Isolation of *C. pneumoniae* from sputum or nasopharyngeal secretions is not routinely carried out. Therefore the physician

would like to be able to request a serologic test for acute infection, preferably with a good possibility of diagnosis already in the first serum sample. Most laboratories do not carry out MIF, which requires great technical expertise for proper evaluation. ELISA assays are considered to be more objective, and therefore find wider application in clinical laboratories.

Development of specific serologic assays helps pinpoint population foci of infection, but it is generally agreed that diagnosis of chlamydial infection in the specific individual is often best done by detection of the infectious agent. To this end we wished to develop a very sensitive molecular assay PCR--polymerase chain reaction) for detection of *C. trachomatis* in swab or urine samples, and for detection of *C. pneumoniae* in throat swabs or nasal washes. The ultimate objective was development of an assay system that would be simple to implement even without advanced equipment.

METHODS AND RESULTS

1. Development of serologic tests:

1.1 Serum samples

1.1.1 Israel

a. CAP patients. Paired serum specimens were collected from 308 patients hospitalized with community acquired pneumonia (CAP) in Soroka Medical Center (SMC). The etiological diagnosis for CAP was based on positive blood cultures and/or significant changes in antibody titers to *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis*, respiratory viruses (adenovirus, respiratory syncytial virus, parainfluenza viruses 1 and 3, influenza viruses A and B), *Coxiella burnetti, Mycoplasma pneumoniae, Chlamydia pneumoniae*, and *Legionella* sp.[21]. Serology for *C. pneumoniae* infections was performed by microimmunofluorescence (MIF) in three different laboratories, and such infection was diagnosed in 62 of the patients [18].

b. Women with suspected PID or vaginitis/cervicitis. Serum samples were collected from 157 women with suspected pelvic inflammatory disease (PID) and 94 women with cervicitis attending the SMC gynecologic emergency service or neighborhood gynecologic clinics, after informed consent forms were signed. The women were aged from 17 to 55. Seventy-two percent were born in Israel (of whom 91 percent were Jewish and 9% were Bedouin), 11% were born in states of the former USSR or Eastern Europe, 12% were born in North Africa or Iraq, 2% were from North or South America or Western Europe, and 2% were from Ethiopia or India. These percentages do not reflect the ethnic makeup of the Negev population, and no conclusions of prevalence of seropositivity in groups of different ethnic origins can be drawn from them. The purpose of collecting these samples was to obtain sera with a higher than average prevalence of antibodies to *C. trachomatis* for use in development of the serologic tests. A translation into English of the informed consent form is attached as Appendix A. The Hebrew version of the informed consent form was approved by the IRB of the Soroka Medical Center.

c. Healthy students. Serum samples from 61 healthy medical and nursing students were available for testing. Demographic data were not available for these samples.

1.1.2 Peru

a. Women living in a high altitude region attending a gynecology clinic in Junin (n=58, average age: 33; median age: 33; range 20-55)

b. Children hospitalized with pneumonia (aged 0-10 yrs, n=88, most with two serum samples)

c. San Juan poor in Lima, living in the Pampas de San Juan de Miraflores (n=84, ages 1-55 years).

d. Women bringing children for medical attention in El Alto, Bolivia (n=84).

e. Children in Bolivia: Capillary blood samples (n=142, age range: 3 months to 4 yrs).

1.2 Automasking ELISA for specific detection of antibodies to *C. pneumoniae* and *C. trachomatis*.

a. Antigen. Chlamydial antigen used in the automasking ELISA consisted of elementary bodies prepared from *C. pneumoniae* TW-183, originally obtained from the Washington Research Foundation and *C. trachomatis* L_2/434/Bu, originally obtained from Dr. R. Cevenini, Dept. of Microbiology, University of Bologna, Italy. They were adapted to grow in HeLa 229 cells, and were harvested and purified as described by Caldwell et al. [22]. Purified elementary bodies (EB) were suspended in SPG buffer (0.01 M sodium phosphate [pH 7.2], 0.25 M sucrose, 5mM L-glutamic acid), inactivated by treatment with 0.3% formalin, and stored at -70°C until use. Concentrations of EB protein were determined by the Bio-Rad protein assay (Bio-Rad, Richmond, California), with bovine serum albumin as standard, after sample treatment with 1N NaOH to a final concentration of 0.5 N for 10 min.

b. Sera tested. Group A: Sera of 154 consecutive CAP patients in Israel. Group B: sera of an additional 31 CAP patients diagnosed by MIF serology has having acute C. pneumoniae infections.

c. Masking antibodies. Masking antibodies were produced in New Zealand white rabbits by hyperimmunization with *C. pneumoniae* or with *C. trachomatis* elementary bodies as described by Caldwell [23], using two rabbits for each antigen. An immunoglobulin fraction was prepared from the pooled sera by ammonium sulfate precipitation followed by extensive dialysis against saline [24], and the immunoglobulin fractions were brought to a volume equal to that of the serum from which they originated. Different batches of masking antibody were produced from the same serum pools, under identical conditions, and these consistently gave comparable results when tested in one test with the same control sera.

d. ELISA procedure. For the ELISA procedure, each serum was tested in four wells, two of which had been treated with masking antibodies in addition to antigen, and two of which had received masking antibody diluent alone in addition to antigen. The masking antibodies corresponded to the antigen applied to the plate--i.e. antibodies against *C. pneumoniae* on plates coated with *C. pneumoniae* antigen. Purified elementary bodies of *C. pneumoniae*, the ELISA antigen, were diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6, to the desired concentration, and 0.1 ml volumes were distributed to all the wells of Corning Easy Wash microtiter plates (Bibby Sterilin Ltd., Stone, UK). After 4 hours at room temperature, the plates were held at 4°C. overnight. Then plates were emptied and washed four times with phosphate buffered saline, pH 7.3 (PBS). After a 1 hr treatment with 2% sodium deoxycholate in 5mM EDTA in PBS and washing of the plates, wells to be masked were treated with a dilution of 1:40 of *C. pneumoniae* hyperimmune rabbit IgG fraction (in PBST supplemented with 5% bovine serum albumin--serum diluent) for 2 hr at room temperature, while the other half received masking antibody diluent alone. Plates were washed in PBS containing 0.05% Tween-20 (PBST), and human sera to be tested were diluted 1:100 for IgG and 1: 30 (final dilution) for IgA in serum diluent, supplemented with 2% normal rabbit serum. Prior to

testing for specific IgA, sera were depleted of their IgG content by incubation with Protein G and filtration in Mini Rapi-Sep-M vials (Integrated Diagnostics, Inc., Baltimore MD, USA). After incubation for 1 hr at room temperature, plates were washed with PBST, and peroxidase conjugated rabbit antihuman IgG (Dako P-214) or rabbit antihuman IgA (Dako P-216) was added to all wells at a dilution of 1:1000 or 1:250, respectively, and incubated for 1/2 hr at room temperature. After further washing in PBST, the chromogenic substrate OPD at a concentration of 0.6 mg/ml in phosphate citrate buffer, pH 5.0 was added. The reaction was stopped after 15 min. with 2 M sulfuric acid, and the absorbance was read at 490 nm.

e. Calculation of percent masking. Calculation of percent masking for IgG and for IgA for each serum was carried out by averaging duplicate wells and subtracting the appropriate background values. Then the difference between OD results for masked and unmasked wells was divided by the value for the unmasked wells and multiplied by 100%.

Data analysis: Kappa agreement. The K value for agreement between MIF and AM-ELISA results beyond agreement due to chance was calculated as the number of observed cases in agreement less the number of expected agreements, divided by the total number of cases less the number of expected agreements, times 100%. A kappa value of 75% or more represents excellent agreement, and values of 40-74% indicate fair to good agreement [25]. The predictive value of a positive test, or positive predictive value is the proportion of true positives among the total positive results given by the test being developed. The negative predictive value is the proportion of true negatives among the total number of negative results given by the test.

This ELISA technique was optimized with several control sera, and it was used to analyze paired serum samples from 154 CAP patients.
Optimization of ELISA assay: Various concentrations of chlamydial antigen were tested for their ability to discriminate between positive and negative sera, and 1 μg/ml was chosen as a concentration that gave reasonably high OD values for positive sera at a serum dilution of 1:100 and low values for negative sera, and was not wasteful of antigen. It was the concentration used in all further tests. Deoxycholate was used at a concentration of 2% since this concentration gave better reduction of background than lower or higher concentrations. Application of the hyperimmune rabbit immunoglobulins at a dilution of 1:40 was found to result in greater masking than either more concentrated or more dilute solutions. Supplementation of the test serum diluent with 2% normal rabbit serum was found to result in minimal non-specific binding of human sera to the coated microtiter plates.

Reproducibility. The reproducibility of percent masking for a given serum tested on different days is shown in Table 1.

Table 1. Reproducibility of masking for a given serum tested on different days.

Antibody isotype	No. of tests	Mean % masking ± SD*	Coefficient of variation
IgG	11	60.2 ± 3.0	5%
IgA	11	50.4 ± 5.7	7%

*SD: Standard deviation

Effectiveness of automasking for determination of species-specificity of antibodies in human sera. The effectiveness of automasking for determination of species-specificity of antibodies in human sera was shown by testing all sera in parallel on *C. trachomatis* antigen (also at 1 μg/ml) masked by immunoglobulins of rabbits hyperimmunized to *C. trachomatis* (L2). Table 2 shows the results of such testing for paired sera of eight patients with *C. pneumoniae* related CAP. The table shows that even when OD levels appeared to be significant on *C. trachomatis* antigen, the masking level was consistently lower than that on the *C. pneumoniae* antigen system. The exceptions were patients who

were shown to be highly seropositive to *C. trachomatis* by MIF using *C. trachomatis* antigen (for example Patient 5 in the table).

AM-ELISA criteria for implication of a *C. pneumoniae* etiology in CAP patients. Optical density at 490 nm and masking results were obtained for IgG and IgA antibodies to *C. pneumoniae* for 154 consecutive CAP patients with paired sera and various etiologic diagnoses, among whom 25 were diagnosed by MIF serology as having acute *C. pneumoniae* infections (Group A sera). In this ELISA, the degree of binding of human antibodies to chlamydial antigen was expressed as OD, while the degree of specificity of that binding was expressed as percent masking generated by the rabbit hyperimmune immunoglobulin. We wished to include IgA results in our criteria and not just IgG for indication of recent or current infection since it has been shown [25] that in 25% of patients with *C. pneumoniae* pneumonia, an IgA response alone was seen. We were unable to develop a reliable masking ELISA for IgM antibodies specific for *C. pneumoniae*. We could not rely on rises in antibody titer for diagnosis since we wished to limit the testing to one serum dilution for IgG and one for IgA. Upon examining our data we found several striking correlations which enabled us to establish the following criteria for association of *C. pneumoniae* with a case of CAP.

1. An IgA O.D. of 1.100 or more and 40% IgA masking, and/or,
2. An IgA O.D. of ≥1.400, and/or
3. A drop of 0.600 OD units or more achieved by the masking for IgG.

The last criterion could be calculated as a function of both OD and masking by multiplying the OD without masking by the percent masking.

Table 2. Comparison of optical density (OD) levels and percent masking for paired sera of eight CAP patients, when tested for IgG and IgA on *Chlamydia pneumoniae* and on *Chlamydia trachomatis* antigens in the AM-ELISA.

	IgG				IgA			
	Chlamydia pneumoniae		*Chlamydia trachomatis*		*Chlamydia pneumoniae*		*Chlamydia trachomatis*	
Patient	OD	% masking	OD	% masking	OD	% masking	OD	% masking
1-A	0.73	38	0.52	22	1.58	43	0.81	7
1-B	1.48	26	1.30	15	2.05	33	1.20	0
2-A	0.88	66	0.26	19	1.34	38	0.90	13
2-B	1.15	52	0.59	28	1.80	35	1.31	11
3-A	0.95	46	0.63	11	1.81	56	0.69	17
3-B	1.14	41	0.90	17	2.09	51	0.91	16
4-A	1.10	65	0.43	21	1.43	53	0.68	18
4-B	1.15	63	0.50	26	1.50	45	0.77	13
5-A*	0.64	65	0.62	68	0.77	47	0.78	26
5-B*	1.03	65	0.80	62	0.33	74	0.33	47
6-A	1.34	46	0.78	17	2.04	37	0.83	6
6-B	1.19	49	0.69	22	1.96	44	0.91	13
7-A	0.72	54	0.31	25	1.63	48	0.47	3
7-B	0.77	60	0.38	34	1.70	41	0.46	2
8-A	0.45	51	0.17	22	1.37	43	0.52	0
8-B	0.52	50	0.23	24	1.58	51	0.52	8

*This patient had an IgG titer of 1:256 by MIF on both *C. pneumoniae* and *C. trachomatis* antigens. He had a MIF IgA titer of 64 on *C. pneumoniae* antigen, but <16 on *C. trachomatis* antigen.

Evaluation of the ELISA. The criteria determined were applied to the sera comprising group B, and the results are shown in Table 3. For comparison's sake, the summary statistics for Group A, compared to MIF were: sensitivity, 83%; specificity, 86%; positive predictive value, 74%; negative predictive value, 92%.

Table 3. Comparison of results by ELISA and by MIF for patient group B:

MIF	AM-ELISA		
	Positive	Negative	Total
Positive	20	6	26
Negative	7	59	66
Total	27	65	92

Sensitivity: 20/26 = 77%
Specificity: 59/66 = 89%
Positive predictive value:74%
Negative predictive value: 91%
Total agreement: 79/92 = 86%
Kappa: Agreement over chance: 74%

The effectiveness of each of the criteria in identifying positive patients in group B is shown in Table 4. The total number (39) is greater than the number of positive patients in the group (20) since some patients were positive by more than one criterion. Also shown in Table 4, for each criterion, is the number of patients positive by that criterion in the first serum sample, in the second, or in both.

Table 4. Effectiveness of each of the criteria for identification of patients positive for acute *Chlamydia pneumoniae* infection in group B.

	Criteria	No. true positive			No. false positive
		Both sera	1st serum only	2nd serum only	
1	IgA O.D.≥ 1.400	15	3	6	3*
2	IgA O.D.≥ 1.100 and ≥ 40% masking	14	1	2	2*
3	IgG net red'n ≥ 0.600 O.D.	9	–	3	3

* including one patient who was false positive by two criteria.

Thus, of the total of 20 patients identified as being positive by the ELISA, 75% were identified by the first criterion alone, 70% by the second, and 45% by the third alone. Of the 15 identified by the first criterion, 9 (60%) were identified already in the first serum sample; for the second criterion, 12 (86%), and for the third, 6 (67%). Overall, 80% of those correctly identified as positive were identified in the first serum sample by at least one criterion; 50% by at least two, and 5% (one patient) by all three criteria. It may be noted that a similar number of these positive patients were identified by MIF criteria as positive already in the first serum sample.

This automasking ELISA system showed promise. However, because the use of immunoglobulins derived from polyclonal rabbit sera as a critical component of the assay introduces a factor which may be difficult to control in the long run, and because of the large number of wells (8) needed for testing each serum sample in the assay if the serum sample is to be tested on both antigens, we decided to try to design a simpler assay system.

1.3 Urea ELISA for detection of IgG antibodies to *C. trachomatis* and *C. pneumoniae.*

Evaluation of this assay method was done in comparison with a commercial MIF test with respect to IgG. (This assay method is now being published after efforts to patent it resulted in the determination that it was probably not patentable. Proofs of the article in press are attached as Appendix B--"A simple ELISA capable of distinguishing between IgG antibodies to *Chlamydia trachomatis* and *Chlamydia pneumoniae*".) The urea ELISA was also used to detect chlamydia-specific IgA antibodies; these results have not yet been published.

1.3.1 The microimmunofluorescence test (MIF)--See Appendix B.

1.3.2 The ELISA assay procedure and calculation of results--See Appendix B.

1.3.4 Optimization and evaluation of the ELISA urea assay--See Appendix B.

1.3.5 Application of the assay--Some data appears in Appendix B. Other unpublished data follows.

a.ELISA screening of sera of CAP patients. The ability of the urea ELISA to discriminate between antibodies to *C. trachomatis* and to *C. pneumoniae* in patients with severe respiratory infection was investigated. For this purpose the ELISA seropositivity for the two chlamydial antigens in paired sera of 15 consecutive CAP patients was compared. In **Figure 1** the patients are arranged in descending order of their maximal *C. pneumoniae* antibody level. The figure shows the level of absorbance at 492 nm for each of the sera on the two antigens. Two sera were available and are represented from each patient. None of the patients whose sera are represented in Figure 3 were determined to have *C. pneumoniae* infections by MIF (MIF IgG titer of ≥516 or four-fold rise in IgG titer, or IgM titer of ≥16 [18]). Although some patients have rather high levels of IgG antibodies against *C. pneumoniae*, there do not seem to be any drastic changes between paired serum samples. On the other hand the third patient seems to have been seropositive to *C. pneumoniae*, but to have undergone seroconversion to *C. trachomatis* between the drawing of her two serum samples. (This was confirmed by MIF testing of the sera.) Apparently as a result of this infection, her antibody level to *C. pneumoniae* also rose. The figure shows that there is some tendency to see slightly elevated antibody levels to *C. trachomatis* in conjunction with high levels of antibody to *C. pneumoniae* among the CAP patients.

b. ELISA screening of sera of healthy students. For comparison purposes, a similar plot was drawn for 61 healthy students with a single serum sample each, and the results are shown in **Figure 2**. Among the healthy students, the tendency for slightly elevated *C. trachomatis* antibody levels was not found, and the demarcation of seropositive and seronegative to each of the chlamydial antigens was quite clear. We concluded that for patients with severe respiratory infections, if antibodies to both antigens are measured, there should be no problem in identifying seropositivity, although one may want to modify somewhat the criteria for positivity cutoff level. For example one might question whether patients 1, 2, and 5 are actually *C. trachomatis* seropositive. However, for seropositivity screening in healthy populations, such adjustments are probably unnecessary.

c. ELISA screening of sera of 253 women attending the gynecologic emergency room of SMC or neighborhood gynecologic clinics, presenting with symptoms of cervicitis/vaginitis or suspected PID (sPID). The sera were tested for antibodies to *C. trachomatis* and *C. pneumoniae* by the urea ELISA. Table 5 shows that seropositivity rates to *C. pneumoniae* did not vary geatly by age group, and were rather similar for both vaginitis and sPID patients. Seropositivity rates to *C. trachomatis* tended to increase with age in vaginitis patients, while the youngest sPID patients were for the most part seronegative to *C. trachomatis*. After age 20, the rates leveled off at about 20%.

Table 5. Seropositivity to *C. trachomatis* and *C. pneumoniae* in patients with cervicitis/vaginitis or suspected pevic inflammatory disease (sPID), by age group.

Age group (yrs)	Diagnosis	n	Seropositivity (IgG)	
			C. trachomatis	*C. pneumoniae*
17-20	vaginitis	36	5 (14%)	19 (53%)
17-20	sPID	19	1 (5%)	8 (42%)
21-30	vaginitis	31	4 (10%)	19 (61%)
21-30	sPID	56	12 (21%)	29 (52%)
31-40	vaginitis	14	5 (36%)	7 (50%)
31-40	sPID	61	13 (21%)	33 (54%)
41-55	vaginitis	13	6 (46%)	10 (77%)
41-52	sPID	21	3 (14%)	12 (57%)

2. Results of testing of sera from Peru

Table 6 shows the overall seropositivity to *C. trachomatis* and *C. pneumoniae* in the various groups of sera collected in the vicinity of Lima, Peru, and neighboring Bolivia. It will be recalled (Tables 2 and 3 of Appendix B) that an adjusted OD 492 cutoff of 0.4 was determined to give good correlation with a MIF cutoff titer of 16. What is immediately apparent is that seropositivity to *C. trachomatis* is extensive, except among children hospitalized with pneumonia in Lima. Seropositivity to *C. pneumoniae*, on the other hand, is extremely prevalent among all the groups examined.

Table 6. Seropositivity to *C. pneumoniae* and *C. trachomatis* by urea ELISA in several South American population samples.

Group	n	*C. pneumoniae* positive (%)	*C. trachomatis* positive (%)
High altitude women	58	48 (83%)	25 (43%)
Bolivian women	82	57 (70%)	31 (38%)
Children with pneumonia			
Up to 2 yrs old	42	5 (12%)	0 (0%)
2-3 yrs old	22	9 (41%)	0 (0%)
4-10 yrs old	25	12 (48%)	1 (4%)
San Juan poor			
1-4 yrs	18	9 (50%)	4 (22%)
5-10 yrs	19	10 (53%)	7 (37%)
11-20 yrs	16	11 (69%)	6 (38%)
21-29 yrs	19	12 (63%)	8 (42%)
30-55 yrs	11	11 (100%)	8 (73%)

2.1 Women of a high altitude region attending a gynecology clinic.
Among these women, seropositivity to *C. trachomatis* reached 43% and to *C. pneumoniae*, 83%. **Figure 3** shows that only 3 of the *C. trachomatis* IgG seropositive women had *C. trachomatis*-specific serum IgA (~12% of IgG seropositives). These women were aged between 20 and 55. On the other hand, 21 women had IgA antibody to *C. pneumoniae* (**Figure 4**), that is, 44% of the IgG seropositives. **Figure 5** shows that there was no particular correlation between levels of IgG antibody to *C. trachomatis*

and *C. pneumoniae*. In Figures 3-5 the sera are arranged in order of increasing age of the women from whom they were drawn.

2.2 Women bringing children for medical attention in El Alto, Bolivia.
In figures 6-8, the sera are arranged in order of increasing IgG antibody level to *C. pneumoniae*, as ages were unavailable for many of the subjects. **Figure 6** shows that there is no correlation between IgG antibody levels to *C. trachomatis* and *C. pneumoniae*, while **Figure 7** shows that IgA antibody to *C. trachomatis* was more common in this group than it was in the high altitude women, reaching 35% of the IgG seropositives. IgA to *C. pneumoniae* was found in 54% of the IgG seropositives (**Figure 8**), somewhat higher than the rate among the high altitude women.

2.3 Children hospitalized with pneumonia in Lima, Peru.
A convalescent serum sample was available from 71 of the 88 children from whom sera were obtained in the study. The children ranged in age from newborns to 10 years of age, but were for the most part, less than 4 years of age. Only one child (age 9) was borderline positive for *C. trachomatis* IgG. The others were all seronegative. IgG levels to *C. pneumoniae* over 1.0 adjusted O.D. were found in 15 children (**Figure 9**). IgA levels of >0.5 adjusted O.D. were found in 13 children, but only in children above the age of 2.5 years (**Figure 10**). In 8 of these children, IgG levels were also high (>1.0 adjusted O.D.). From the two graphs it can be seen that *C. pneumoniae* infection occurred already at one year of age, and that it was quite common among these children with pneumonia at 2 and 3 years of age.

Interestingly, in 11 of the 13 children with IgA levels >0.5 adjusted O.D. units, the level in the second serum sample was lower than that in the first. This may be due to the fact that when serum IgA initially appears in response to an infection, it is largely dimeric, but it becomes predominantly monomeric during the coming weeks. Dimeric IgA would be expected to bind antigen more strongly under the conditions of our assay system.

2.4 San Juan poor.
The subjects from whom serum samples were obtained in the peri-urban pueblo Joven (shanty town) the Pampas de San Juan de Miraflores, located in the Cono Sur of Lima, were aged 1-55 years. Their seropositivity to the two chlamydia species is shown in **Figure 11**, by age group. Two observations stand out. One is the high degree of seropositivity to *C. trachomatis*, and the second, the very early acquisition of infection with *C. pneumoniae*, with seropositivity reaching 100% in the oldest age group.

Examination of **Figures 12A and 12B**, showing IgG and IgA adjusted OD levels by increasing age for children aged 1-12 (Fig. 12A) and for subjects aged 13-55 (Fig. 12B), reveals that although the number of *C. trachomatis* seropositives is rather high, the levels of OD reached are for the most part not impressive. The OD levels to *C. pneumoniae* are more impressive (**Figures 13A and 13B**). An obvious question is whether exposure to some other chlamydia species is common, perhaps some *C. psittaci* strains prevalent among the animals that live together with the people and around their shack-like homes. Another possible explanation for the high rate of *C. trachomatis* seropositivity in children is that some trachoma-like serovars circulate in this population, although typical trachoma is not seen (but note [10-12]). A third possibility is that intrapartum infection of newborns with *C. trachomatis* is common and has been for some years.

Only further research can answer these questions, but clearly, exposure to chlamydial organisms is extremely common in this population. One may note that Carballal et al. reported high rates of (respiratory) infection with *C. trachomatis* in children of low socioeconomic status in Argentina, and they postulate possible horizontal transmission [27].

In summary, our data indicate that infection with chlamydial organisms is extremely common in Lima and its environs. The ELISA technique which we developed seems to

distinguish quite well between both IgG and IgA antibodies to *C. trachomatis* and to *C. pneumoniae*.

3. Development of agent detection techniques

Even before the start of this project it was determined that there was little purpose in trying to develop chlamydial antigen detection techniques, since these have been for the most part unsuccessful in spite of tremendous effort and resources invested. It was decided that techniques to be developed should be based on genome detection, preferably by polymerase chain reaction (PCR).

3.1 Clinical samples

3.1.1 Israel

a. Throat swabs from 308 CAP patients (see description for serum samples, page 5).

b. Cervical swabs from women attending gynecologic clinics in Beer Sheva (see p. 5)

3.1.2. Peru

a. Urine and cervical swab samples from high altitude women attending a gynecology clinic in Junin (n=63 for each sample type, see description of serum samples, p. 5).

b. Urine samples, boiled, concentrated, and centrifuged, from children hospitalized in Lima with pneumonia (n=55, see serum samples) and nasal lavage fluid from these children (n=25).

c. Cervical swab samples (n=54) and urine samples (n=56) from women attending a family planning clinic in Loayza Hospital (n=54, age range: 14-41).

d. Urine samples from young adults in Bolivia (n=91).

e. Nasopharyngeal swabs from children in Bolivia (n=139, see also capillary blood samples) and urine samples from these children (n=52).

3.2 Detection techniques

3.2.1 Detection by culture and enzyme immunoassay kit.

Throat swabs from the CAP patients were not cultured for chlamydia since they were not taken with swabs suitable for chlamydial culture, nor were they transported and stored in chlamydia transport medium. All cervical swabs from women attending the gynecologic clinic were cultured in shell vials on McCoy cells in Eagle's MEM supplemented with 10% FCS and cycloheximide. Vials were centrifuged for one hr at 38°C. and incubated for 72 hr at 37° C. Coverslips were fixed with methanol, and staining for inclusion bodies was done with glycerine/iodine. Some swabs were tested also by commercial enzyme immunoassay in parallel with culture.

3.2.2 PCR

The rationale behind the choice of primers for the PCR technique developed was that a DNA sequence should be amplified from the Chlamydial genome of *C. trachomatis*, *C. pneumoniae* or *C. psittaci*, corresponding to some 1000 base pairs of the 60 Kda cysteine rich protein common to these chlamydia species. Because there are differences in the

amplified sequences, however, in the different species, probes could be developed which could confirm exactly which species was found in the specimen tested. In practice, conditions were optimized which resulted in the ability to detect one infectious organism, at least in the case of *C. trachomatis* (which may correspond to 300 physical chlamydia particles). This type of PCR can never be as sensitive as one which employs primers for detection of sequences in the chlamydial plasmid or chlamydial ribosomal DNA, since the latter two sequences are present in many copies in each bacterium. However, it was thought that the loss in sensitivity would not be critical and that the advantage of being able to use one set of primers for all the organisms would override the loss in sensitivity. As will be seen below, this turned out not to be the case, although other factors apparently contributed to the problems as well.

DNA was prepared from all of the above samples for PCR amplification. Two different methods of preparing DNA were tested: one was with a commercial kit, QIAamp Blood Kit (QIAGEN Inc., Chatsworth, CA), and one was by means of Chelex-based extraction, as described by Walsh et al. [28].

A reconstruction experiment was performed in which cells containing a known number of infectious units of *C. trachomatis* L-2 were added to urine, and DNA was extracted (by both techniques) from total urine, and from the pellet and the supernatant of a 5' microfuge centrifugation . Chlamydia were detected in the urine pellet by both extraction techniques, but not in the whole urine extract. Figure?

A reconstruction experiment was also performed with endocervical swabs in which swabs were soaked in chlamydia-infected cells containing ~7,200 infectious particles or a suspension of chlamydial elementary bodies (~7,200 infectious units). DNA from the swab extract was then extracted by both techniques, QIAamp and Chelex. The chelex extraction gave a stronger signal than the QIAamp extraction, whether it was the original suspension, or pellet or supernatant that was being tested.

A third reconstruction experiment was carried out with nasopharyngeal swabs. In this experiment, dilutions were performed, and one infectious unit per reaction tube was detectable. (The corresponding number of physical particles is unknown, but may have been about 300.)

The last result was considered quite promising, and it was decided to continue most extractions with Chelex, and for urine to use only the pellet from urine samples. An experiment was carried out to test whether ethanol precipitation of DNA extracted by Chelex would be helpful in raising the sensitivity of the assay, but it was found not to improve detection, and inhibition controls showed that some inhibition was taking place. Therefore it was not used in further experiments.

4. Results of agent detection.

4.1 Culture and antigen detection

Five cervical swabs of women with vaginitis were culture positive, one of which was also antigen positive by the Clearview Chlamydia antigen detection kit. None of the specimens from women with suspected PID was culture positive. No culture was attempted from any of the Peruvian samples since no material likely to be viable was transported from Peru into Israel.

4.2 PCR detection

All specimens listed above from Peru were extracted with Chelex. Amplification was performed on 30 urine samples from Bolivian women. All were negative.

DNA was extracted with QIAamp from 128 cervical swab specimens from Beer Sheva. Fifty-four of these samples were amplified, including 5 that had been positive by culture. None were positive by PCR.

Testing of a sampling of the specimens for the presence of human DNA with primers for interferon- receptor indicated that DNA was present in the sample specimens.

It was decided that further efforts at improvement of the PCR would be carried out in Peru, as no funds for laboratory personnel were available to continue the work in Israel past the third year of the grant. The DNA specimens are still available, and it is hoped that at some point we will be able to test them with a very efficient (and inexpensive!) PCR assay.

In Peru, we have now started using the PCR which utilizes the the plasmid DNA for the detection of Chlamydia. This increased our yield of results between three and four-fold.

IMPACT, RELEVANCE AND TECHNOLOGY TRANSFER

This grant has enabled estimation of prevalence rates of chlamydial infections in groups at high risk for such infections of the respiratory and genital tracts, as well as in the general population of both countries. The prevalence of chlamydial infections turned out to be quite high in Peru, and therefore such infections must be of significant public health concern.

The grant has also had a significant impact on both the Israeli and Peruvian laboratories and their workers. On a personal level, I think that we in Israel have discovered that the ability to use sophisticated equipment and develop sophisticated techniques is not limited to residents of the "developed" world. I personally (MGF) was impressed by the quality of research that could be carried out under difficult laboratory conditions, and am convinced that investment in quality equipment for such laboratories is justified.

The Israeli side was able to develop two ELISA format serologic assays capable of distinguishing between antibodies to *C. trachomatis* and antibodies to *C. pneumoniae*. One of these assays is now being published and is in routine use in our research laboratories. The Peruvian side has also established the assay in their laboratory. The PCR techniques for detection of *C. trachomatis* and *C. pneumoniae* originated in the Israeli laboratory, but were refined and applied in both the Israeli and Peruvian laboratories. Unfortunately, at the time of the expiry of this grant, the PCR assays are not yet developed as completely as we would have liked. However, the results obtained with the serologic assays and the perhaps more preliminary results obtained with the PCR assay indicate very clearly that infection with *C. trachomatis* and *C. pneumoniae* are of great public health significance in the areas sampled in Peru. Whether testing for these organisms will now become more routine may depend on availability of funds, but they can clearly no longer be ignored, and larger scale testing for them is certainly warranted.

This grant permitted Peru to develop two technologies. We were able to train our personnel in both ELISA and PCR. The PCR equipment and the -70°C freezer were essential to the development of our laboratory. We have now used these instruments to develop our laboratory in the PCR detection of chlamydia, but also for *Trichomonas vaginalis* and Gonnorhea. In addition we have begun work on the PCR for tuberculosis and toxoplasmosis. Dr. Guillermo Madico is now entered in a Ph.D. program with Dr. Tom Quinn and Dr. Charlotte Gaydos on the detection of Chlamydia in urine. None of this would have occurred without the assistence of the CDR project. In summary, not only was the program able to produce important scientific results, but also resulted in the Peru section having a significant input into building its institution.

Equipment: This grant was used to buy either all or part of a microscope, a -70°C freezer, and a thermocycler. It also allowed us to buy the reagents necessary to complete both ELISA and PCR assays.

PROJECT ACTIVITIES

In the framework of this project several meetings were held among the senior investigators, and very fruitful exchange visits were made. Dr. Batia Sarov met with Dr. Gilman in Atlanta, Georgia, during the Annual Meeting of the American Society for Tropical Medicine in December, 1993, and Dr. Friedman met with Dr. Gilman in New York in July, 1994. Although neither of these trips was paid for by this research budget, the purpose of the meetings was to discuss the ongoing study and plans for the future. A two-day meeting between Dr. Gilman and the Beer Sheva investigators took place in Beer Sheva in December, 1994, coinciding with Dr. Gilman's trip to Cairo. During this meeting we reviewed with the progress made in development of the serologic and PCR assays, as well as the progress made in the collection of genital and respiratory samples in Peru.

Maritza Calderone spent close to three months in our laboratory in Beer Sheva, between October, 1995, and January, 1996. She brought with her serum samples from children with respiratory infections, from control children, and from women with gynecologic problems to be tested with our ELISA, as well as nasal washes, urine samples, and genital swabs to be tested for the presence of chlamydia by PCR. During her stay she learned the ELISA technique as well as preparation of all the reagents and tested the sera which she brought with her from Peru. She brought back to Peru sufficient chlamydial antigen for an extended period of time until we will supply more or they will be able to produce their own. Her trip and stay were covered by the grant.

Dr. Guillermo Madico, M.D., spent five weeks in our laboratories in March-April, 1996. He brought with him additional serum and urine samples and while in Beer Sheva refined the PCR technique so that he was able to harvest DNA from very dilute samples. Upon his return to Lima he trained Maritza in the refined technique, which she can now perform routinely. His trip and stay were covered by the grant.

Prof. Maureen Friedman visited the laboratories in Peru during June, 1996. She was impressed with the ability of our Peruvian collaborators to accomplish scientifically sophisticated techniques in much poorer physical conditions than are usually expected in diagnostic laboratories. This is a tribute not only to the ingenuity of the laboratory personnel and students, but also to their intense determination to succeed. Her trip was covered in part by the grant.

Publications: Initially, our data was not submitted for publication so long as we believed there was a possibility of submitting a patent application for the urea ELISA technique. When that turned out to be highly unlikely, an article describing the urea ELISA was submitted for publication and is now in press. A copy is attached as Appendix B. Other publications are planned and will acknowledge the support of this grant.

Communication: Since we began using Email for communication, it has been rather simple, and matters arising are efficiently dealt with.

PROJECT PRODUCTIVITY

This project was extremely productive; however, our original goals were ambitious, to say the least, and we did not manage to fulfill all of them to the extent that we had hoped. We did not succeed, in the time frame of the grant, in bringing the PCR detection test to the level of applicability and ease of performance that we had wished to because of the

design of the primers as well as an initial problem with inhibitors in the swabs. We also have had to learn the best way to handle urine samples and still are involved in this process. We still have not gotten the sensitivity of urine to equal that of vaginal specimens in testing women for STD. On the other hand, the urea ELISA developed in Israel is now being used to screen many additional serum samples from various population groups for antibodies to *Chlamydia trachomatis* and *Chlamydia pneumoniae*.

FUTURE WORK

This project is leading to further work, both in Peru and in Israel. It is now very clear that good and simple Chlamydia detection techniques are needed since chlamydial infections are so widespread and have a strong tendency to cause chronic disease. Furthermore, the urea ELISA should be developed for detection of *C. psittaci* strains as well in order to determine the degree of exposure to these organisms, especially in certain population groups.

This work has demonstrated the importance of *C. pneumoniae* as an agent of respiratory disease in children in Peru. Respiratory disease is now the major cause of infant mortality in Peru. We hope in the future to define the frequency of this disease and obtain a rapid diagnostic so that children with this condition can be treated promptly.

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Appendix A

DUMMY FORM

Proposed Informed Consent for high risk groups treated in the medical facilities (gynecology clinics and wards) (Translation of Hebrew form approved by IRB)

Dear Mr./Mrs.

We are asking you to volunteer to participate in a study on Chlamydia trachomatis.

A small portion of the blood and the smear test taken from you for routine medical examination are needed for a medical research project which aims to develop new and better tests to detect infection with a treatable, sexually transmitted agent named Chlamydia. This agent, if not properly diagnosed and treated may cause complications. Your illness will be diagnosed according to routine and accepted techniques and you will be treated during the acute phase and during the follow-up period according to the established treatment in our institute. The additional specific tests which will be developed in the framework of this study will result in the availability of more sensitive, rapid, and specific tests.

Part of the clinical information gathered will be used for the purpose of validating the new tests. All information which is gathered will be kept under strict confidence in your medical files at the Clinic, and will be protected in accordance with the normal guidelines regarding medical confidentiality. The information will be available only to members of the medical staff treating you and the Clinic, and also to the research team.

Only statistical results of research findings will be used in future publications in professional medical journals. Your personal data will be kept in the strictest confidence and there will be no way of identifying you. Your name will be known only to those members of the medical staff responsible for your treatment. If you have any additional questions, the clinic's staff will be most willing to assist you.

You are, of course, entitled to refuse participation in the study. Your acceptance or refusal will not affect in any way your treatment at the Clinic. A decision to change your mind and to refuse participation, during the course of the study, will be honored.

You are requested to provide your signature, thereby granting us your consent to be included in the study.

We thank you for your cooperation.

Dr. _____

_____

Appendix A

CONSENT FORM

I hereby declare that I received a detailed explanation (both in writing, and orally) of the purposes of the study and my participation in it. It was made explicit that I am free to withdraw my participation in this study, and that I will be able at any time to change my mind and refuse to participate, without this act disrupting my medical treatment, or affecting the quality of my medical treatment in the present or future. I hereby give my consent to be included in the study on chlamydia trachomatis as detailed here.

Name of Patient: _____ Signature: _____

Date: _____

I (signed below) hereby declare that I presented this consent form to the patient and answered all of his or her questions.

Name of Doctor: _____ Signature of Doctor: _____

Date: _____

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UNCORRECTED PROOFS

Serodiagnosis and Immunotherapy in Infectious Disease 00 (1997) 000–000

Serodiagnosis & Immunotherapy IN INFECTIOUS DISEASE

NB corrections on p. 1 p. 3 p. 4 p. 5

A simple ELISA capable of distinguishing between IgG antibodies to *Chlamydia trachomatis* and *Chlamydia pneumoniae*

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Abstract

Simple assays which reliably distinguish between past infection with *Chlamydia pneumoniae* and with *Chlamydia trachomatis* are needed. We developed an enzyme-linked immunosorbent assay (ELISA) for this purpose by reducing antigen cross reactive lipopolysaccharide epitopes with deoxycholate treatment and releasing antibodies of low affinity with a 6 M urea wash step. Paired serum samples from 212 patients with non-*C. pneumoniae*-associated community acquired pneumonia and single serum samples from 61 healthy students, 61 women with gynaecological complaints, and 100 blood donors were tested in the assay system. Excellent positive/negative correlation with the microimmunofluorescence (MIF) test was shown. This urea ELISA assay may be useful in assessment of past infection with these organisms, especially in epidemiological studies. © 1997 Elsevier Science B.V.

Keywords: Chlamydia serology; ELISA; *Chlamydia trachomatis*; *Chlamydia pneumoniae*; Antibody avidity; Seroepidemiology

1. Introduction

Chlamydia trachomatis and *Chlamydia pneumoniae* are both significant pathogens for man. *C. trachomatis* is the etiologic agent of trachoma and inclusion conjunctivitis and of genital tract disease in humans [1,2]. *C. pneumoniae* has recently been recognized as a significant respiratory tract pathogen, responsible for clinical syndromes ranging from mild upper respiratory tract infection to pneumonia [3,4]. It also seems to be involved in the etiology of cardiovascular disease [5–7]. We and others have shown by immunoblot [8,9] extensive cross reaction between antibodies against *C. pneumoniae* and antibodies against *C. trachomatis*, attributable to the presence of genus-specific epitopes. The only assay that reliably distinguishes between antibodies to *C. trachomatis* and antibodies to *C. pneumoniae*, and the 'gold standard' for chlamydial serology, is the microimmunofluorescence test (MIF), first described by Wang and Grayston [10] and extensively used for *C. pneumoniae* serology [11,12]. However, this test is cumbersome, and results are very much dependent upon the expertise of the reader. Therefore it is not widely employed in routine laboratory practice. The prevalence of past infection and the age of acquisition of infection with *C. trachomatis* and *C. pneumoniae* is currently unknown for many population groups. For epidemiological surveys of prevalence of chlamydial disease, a simple serologic assay which can distinguish between antibodies to the two agents is necessary. We have developed such an assay and here describe it.

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2. Materials and methods

2.1. Serum samples

Four groups of serum samples were used in this study: Group 1 consisted of paired serum samples drawn one month apart from 212 adults hospitalized with community acquired pneumonia (CAP). They were participating in a study of the etiologic agents of CAP in the Negev region of Israel. Details of the study have been reported elsewhere [13]. For the present study of past infection with *Chlamydia* species, only sera of patients whose illness was not associated with current *C. pneumoniae* infection (as determined by MIF) were used; the paired samples were helpful in assessment of the reliability of the ELISA assay. Group 2 consisted of serum samples of 61 healthy medical and nursing students. Group 3 consisted of serum samples of 61 women with vaginitis or suspected pelvic inflammatory disease attending the Soroka emergency gynaecologic clinic. These women were among patients included in a study aimed at development of simple laboratory techniques for differentiation of infections caused by *C. pneumoniae* from those caused by *C. trachomatis*. Extensive testing for etiologic agents was not carried out on these patients, but cervical swabs were obtained which were tested by culture and by direct enzyme immunoassay for the presence of *C. trachomatis*. Sera were tested by IPAzyme Chlamydia (Savyon Diagnostics, Ashdod, Israel) for the presence of antibodies to *C. trachomatis*. If any of these tests were positive, the patient and the physician were notified, and the patient was advised to report for follow-up testing. Medical treatment of the patients was without intervention by the investigators. Group 4 consisted of serum samples from 100 blood donors. No demographic or other identifying details were available for these samples. In addition serum samples of five laboratory workers were used in calibration of the assay; two of them were included as positive and negative controls in each test.

2.2. The MIF test

The MIF used as the reference standard for determination of the presence of IgG antibodies to *C. pneumoniae* or *C. trachomatis* was a commercial kit (IO International, London, UK), used for published Chlamydia serologic studies [14–16] before it was commercially available. *C. trachomatis* antigen was a pool of serovars D–K, and *C. pneumoniae* antigen was strain IOL-207. The kit was used according to the manufacturer's instructions, with sera being tested at a dilution of 1:16 for screening purposes, or titrated in two-fold dilutions as required. Results were read independently by two investigators (SI and MGF). End point titers were read as the last positive serum dilution. Positive results were even bright apple green fluorescence of elementary bodies. Discrepant readings were usually resolved by repeat testing. Sera which could not be characterized as clearly positive or negative were not included in the analyses of sensitivity and specificity of the ELISA (see results).

2.3. ELISA assay

2.3.1. Antigen preparation

Chlamydia strains: The chlamydia strains used were *C. pneumoniae* TW-183, originally obtained from the Washington Research Foundation and *C. trachomatis* L_2/434/Bu, originally obtained from Dr R. Cevenini, Department of Microbiology, University of Bologna, Italy. They were adapted to grow in HeLa 229 cells, and were harvested and purified as described by Caldwell et al. [17]. Purified elementary bodies were suspended in SPG buffer (0.01 M sodium phosphate (pH 7.2), 0.25 M sucrose, 5 mM L-glutamic acid) and stored at −70°C until use. Concentrations of elementary body protein were determined by the Bio-Rad protein assay (Bio-Rad, Richmond, CA), with bovine serum albumin (BSA) as standard, after sample treatment with 1 N NaOH to a final concentration of 0.5 N for 10 min.

2.3.2. Assay procedure

For the assay, purified elementary bodies were diluted to a concentration of 1.0 μg/ml in 0.05 M carbonate–bicarbonate buffer, pH 9.6. Each well of a microtiter plate (Corning Costar, Cambridge, MA) was coated with 100 μl of either *C. trachomatis* or *C. pneumoniae*. The plates were incubated for 4 h at room temperature (RT), then overnight at 4°C and rinsed four times with phosphate buffered saline (PBS), pH 7.2. Each well of the plates was then treated with 100 μl of 2% deoxycholate monohydrate (Aldrich Chemical, 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% BSA in PBS was added to all wells (200 μl) for 2 h at RT. 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 RT. All serum samples were tested in duplicate wells for each of the chlamydial antigens.

Contents of the plate were removed and replaced with 100 μl of 6 M urea (US Biochemical, Cleveland, OH) which remained on the plates for 15 min. After four washes with PBS-T, peroxidase conjugated rabbit anti-gamma chain (Dako P-214, Glostrup, Denmark) diluted 1:1000 in serum diluent was added and incubated for 0.5 h at RT. After four more washes with

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PBS-T, Orthophenylenediamine dihydrochloride (OPD) substrate was added at a concentration of 0.6 mg/ml in citrate-phosphate buffer, pH 5.0. The reaction was stopped after 14 min 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.

2.3.3. Calculation of results

Quadruplicate background values (all reagents applied to the wells except serum diluent instead of a serum sample) were averaged and subtracted from the average OD for each serum sample to give net average absorbance values for each serum sample. After final conditions for the assay were established, sera were routinely tested in duplicate on each antigen and net average absorbance values were normalized with respect to a positive control serum tested in each experiment. An adjusted absorbance of greater than 0.400 was considered positive for each serum tested. All these calculations were carried out independently for values obtained on *C. trachomatis* antigen and on *C. pneumoniae* antigen.

2.4. Data analysis

Sensitivity and specificity of the ELISA compared with MIF was calculated for each of the chlamydial antigens, and the kappa value of agreement beyond chance was calculated as the number of observed cases in agreement less the number of expected agreements, divided by the total number of cases less the number of expected agreements, times 100%. A kappa value of 75% or more represents excellent agreement, and values of 40–74% indicate fair to good agreement [18].

3. Results

3.1. Urea dissociation of antibodies from solid phase antigen

In optimizing the assay, various concentrations of urea were tested on several control sera. The MIF titers of the sera are shown in Table 1. The results of a representative experiment are shown in Fig. 1; in this experiment four different concentrations of urea were tested on five sera. Each urea concentration was tested on quadruplicate samples of the serum on *C. trachomatis* (Fig. 1A) and on *C. pneumoniae* (Fig. 1B). It is clear that increasing concentrations of urea did not result in increasing antibody dissociation, but that a plateau was reached at which the positive sera remained clearly positive on their appropriate antigen, but clearly negative on the antigen to which they were negative by MIF.

Table 1
MIF titers of five sera shown in Fig. 1

Serum no.	MIF titer	
	C. trachomatis	*C. pneumoniae*
1	256	64
2	64	<16
3	<16	128
4	<16	128
5	<16	<16

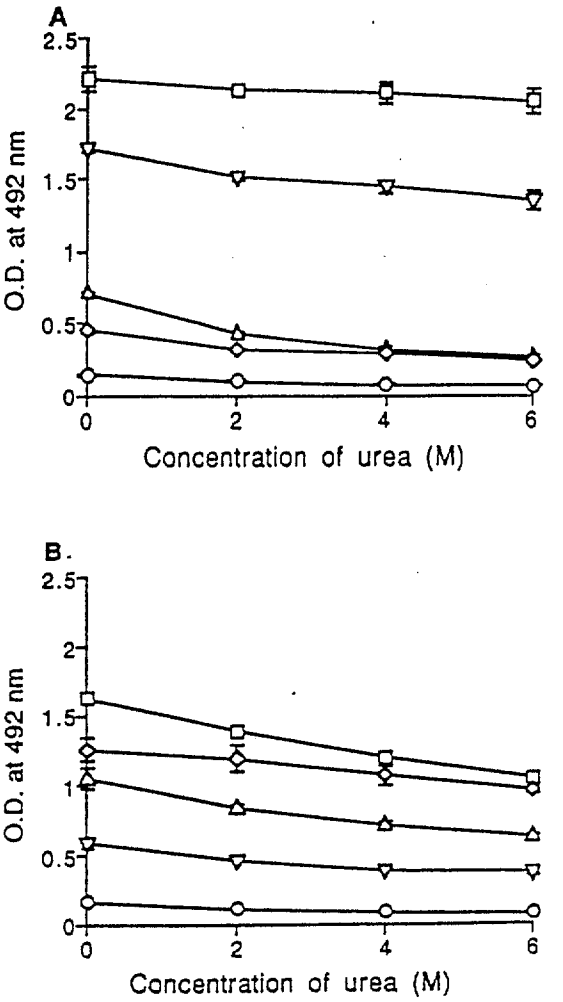

Fig. 1. Influence of increasing concentrations of urea on net antibody binding of five sera to chlamydial antigen in the ELISA. (A) binding to *C. trachomatis* L-2 antigen; (B) binding to *C. pneumoniae* TW-183 antigen. Bars mark standard deviation for quadruplicate determinations. Squares, downward pointing triangles, upward pointing triangles, diamonds, and circles represent sera 1, 2, 3, 4 and 5 in Fig. 1, respectively. Sera were tested at a dilution of 1:100.

3.2. Reproducibility of results

The inter- and intra-assay reproducibility of OD values obtained with the urea ELISA was examined in

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Table 2
Correlation of *Chlamydia trachomatis* and *Chlamydia pneumoniae* MIF and ELISA results for sera of 61 healthy students

	C. trachomatis antigen					*C. pneumoniae* antigen			
	MIF+	MIF−	MIFi[a]	Total		MIF+	MIF−	MIFi	Total
ELISA (*C. trachomatis*)+	2	0		2	ELISA (*C. pneumoniae*)+	31	2		33
ELISA (*C. trachomatis*)−	0	57		57	ELISA (*C. pneumoniae*)−	1	23		24
Total	2	57	2	61	Total	32	25	4	61

ELISA positivity was an adjusted absorbance of greater than 0.400 OD at 492 nm. Calculations of sensitivity, specificity and agreement take into account only those sera which were clearly MIF+ or MIF− at a dilution of 1:16.
Sensitivity of ELISA, 100%; sensitivity of ELISA, 97%; X specificity of ELISA, 100%; specificity of ELISA, 92%; overall agreement, 100%; overall agreement, 95%; kappa for agreement beyond chance, 100%; kappa for agreement beyond chance, 89%.
[a] MIFi, MIF indeterminate.

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several ways. First, the mean and standard deviation (S.D.) of the values for the positive control serum tested in ten different experiments (interassay variation) was 1.72 ± 0.14 on *C. trachomatis* antigen and 0.96 ± 0.10 when tested on *C. pneumoniae* antigen. The control negative serum tested in 10 different experiments had a mean OD + S.D. of 0.05 ± 0.02 on *C. trachomatis* and 0.08 ± 0.03 on *C. pneumoniae* antigen. Second, Fig. 1 demonstrates the low S.D. for quadruplicate determinations of five sera. The error bars are practically not visible because the S.D. was so small, ranging, in the case of 6 M urea, from 0.002 to 0.088 on *C. trachomatis* antigen and from 0.003 to 0.030 on *C. pneumoniae* antigen (intra-assay variation). Finally, the reliability of the assay was assessed by testing sequential serum samples from 212 patients with community acquired pneumonia not associated with *C. pneumoniae* infection. Each serum pair was tested together in the same assay. With respect to *C. trachomatis*, 179 pairs (84.4%) had adjusted OD values within 0.1 OD units of each other; another 22 (10.4%) had values within 0.2 OD units of each other; and 11 (5.5%) had OD values differing from each other by more than 0.2 OD. Of the last group, six were rising values and five were falling. With respect to *C. pneumoniae* antigen, 150 (70.7%) of the serum pairs had adjusted OD values within 0.1 OD of each other; another 39 (18.4%) had OD values within 0.2 OD of each other; and 23 (10.8%) had OD values different by more than 0.2 OD. Of the last group, 15 were rising values and eight were falling values. It is likely that at least some of these were caused by exposure to *C. pneumoniae*, even though the exposures were not strong enough to give MIF values indicative of current infection [13,19].

3.3. Correlation between MIF and ELISA

The sensitivity and specificity of the ELISA test were determined by examining the correlation between ELISA IgG seropositivity to one or the other chlamydial antigen and MIF positivity for antibodies against the corresponding chlamydial antigen. This was done for sera of 61 healthy medical and nursing students (Table 2), and for sera of 61 women with genital complaints (Table 3). In order to simplify the presentation, we limited the analyses to sera which were clearly positive or negative in the MIF at a dilution of 1:16. Because we were not certain that the correlations would be similar for healthy and potentially currently infected persons, since with the ELISA we were looking at an antibody population with higher avidity than with the MIF, we analyzed the two groups separately. Indeed, while the correlations were very good for both groups (94–100% sensitivity, 90–100% specificity, and 93–100% overall agreement), they were somewhat better for the healthy students than for the women with genital complaints.

3.4. Application of the ELISA

The ELISA assay was used to test serum samples of 100 blood donors. At a cut-off value of 0.4 adjusted OD, 20% were seropositive to *C. trachomatis*, and 67% to *C. pneumoniae*. These values are somewhat higher than those of the students represented in Table 2, where the seropositivity rates were 3 and 58% respectively.

4. Discussion

In this report we describe an immunoassay in the ELISA format which is capable of distinguishing between IgG antibodies to *C. pneumoniae* and IgG antibodies to *C. trachomatis*, with excellent correlation of seropositivity to the two agents with the MIF test. The basis of the specificity of this assay is twofold: first, the treatment of the chlamydial antigen attached to the ELISA plate with deoxycholate to remove crossreactive LPS antigens, and second the removal of antibodies of low avidity by treatment with 6 M urea. The deoxycholate treatment was suggested previously by Ladany et al. in the case of *C. pneumoniae* [20]. However, in

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Table 3
Correlation of *Chlamydia trachomatis* and *Chlamydia pneumoniae* MIF and ELISA results for sera of 61 women with genital complaints

	C. trachomatis antigen					*C. pneumoniae* antigen			
	MIF+	MIF−	MIFi[a]	Total		MIF+	MIF−	MIFi	Total
ELISA (*C. trachomatis*)+	29	1		30	ELISA (*C. pneumoniae*)+	33	2		35
ELISA (*C. trachomatis*)−	2	18		20	ELISA (*C. pneumoniae*)−	2	18		20
Total	31	19	11	61	Total	35	20	6	61

ELISA positivity was an adjusted absorbance >0.400 OD at 492 nm. Calculations of sensitivity, specificity and agreement take into account only those sera which were clearly MIF+ or MIF− at a dilution of 1:16
Sensitivity of ELISA, 94%; sensitivity of ELISA, 94%; specificity of ELISA, 95%; specificity of ELISA, 90%; overall agreement, 94%; overall agreement, 93%; kappa for agreement beyond chance, 88%; kappa for agreement beyond chance, 84%.
[a] MIFi, MIF indeterminate.

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that publication no direct comparisons were made, for the sera tested, on both chlamydial antigens. We chose to work with 2% deoxycholate after preliminary experiments showed this concentration to be most effective in reducing crossreactivity without significantly reducing antigenicity (data not shown). Agents such as 6–8 M urea, varying concentrations of thiocyanate ion, and guanidine hydrochloride have increasingly been used to determine the avidity of serum (and mucosal) antibodies to various antigens, including *E. coli* polysaccharide and Diphtheria toxin [21] and to differentiate between recent and past infection [22] with various infectious agents [23]. We found that a 15 min wash with 6 M urea allowed differentiation of antibodies directed against *C. trachomatis* and *C. pneumoniae*. In contrast, when increasing concentrations of thiocyanate ions were tested, the amount of antibody bound to antigen steadily decreased, until at 4 M sodium thiocyanate, almost all sera were negative (data not shown). This phenomenon is actually well known, and when thiocyanate is used for determination of antibody avidity, it is on the basis of reaching 50% of the no-treatment binding value [21,23–25]. When increasing concentrations of urea were tested (Fig. 1), non specific binding of negative sera was reduced and a plateau appeared to be reached in the binding of positive sera to the antigen. This implied that it may be possible to achieve reliable and reproducible results using a single concentration of urea. We chose to use 6 M urea and compared results obtained in the urea ELISA with results obtained by MIF for 61 healthy subjects and for 61 women with gynaecological complaints. The reason for including the second group was to increase the number of *C. trachomatis* positive sera tested, since seropositivity rates to *C. trachomatis* in the general population in our area are rather low ([8] and unpublished data). The results of the comparison, shown in Tables 2 and 3, indicate that the technique shows promise for seroepidemiologic studies of past infection with the two chlamydial species. The somewhat lower sensitivity of the ELISA compared with the MIF in the case of the women with gynaecologic complaints may be due to the presence of antibodies of low avidity, which are removed by the urea treatment in the ELISA, but can be detected in the MIF.

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The *C. trachomatis* seropositivity rate for the group of 100 blood donors (20%) is similar to that reported by Ben-Yaakov et al. [26] in our region, using MIF, although they found significant variation over a 3 year period. The *C. pneumoniae* seropositivity rate (67%) is somewhat higher than the rates which they report for adults aged 19–50 (4455%), but similar to their rates for older persons (66%).

44

Both inter- and intra-assay reproducibility of the ELISA was shown to be good. The close correlation of the adjusted OD levels for paired sera of adults with CAP (not associated with *C. pneumoniae* infection) indicates that the assay gives reproducible results not only for individual sera tested many times, but also for sequential sera of the same person. This will be an important consideration in future experiments designed to explore the potential of the technique in serologic diagnosis.

The adjusted OD cutoff value which we used in the ELISA to determine seropositivity (0.400) is relatively high compared with cutoff points used in ELISA assays with viral or peptide bacterial antigens. However, for a whole-organism bacterial system this is not unreasonable, similar cutoff points having been previously described [27,28]. The exposure of individuals to gram negative bacterial antigens is varied and sometimes heavy; moreover, many bacteria have proteins with common functions, and structures related to some extent. As noted, the cutoff of 0.4 OD units gave an excellent positive/negative correlation with MIF titers ≥ 216 (Tables 2 and 3).

≥16

It should be noted that the *C. trachomatis* antigen used in the MIF test was a mixture of serovars D–K, associated with genital tract infection, while the antigen used in the ELISA was the L2 serovar, shown in the past to be broadly cross-reactive within the species *C. trachomatis* [29] and most easily grown in cell culture.

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This antigen reflected well the seropositivity to genital serovars (viz the correlation with MIF, Table 3) as well as the seropositivity due to infection with trachoma-associated serovars (A–C) (unpublished data). The *C. pneumoniae* MIF antigen was IOL-207, while the ELISA antigen was TW183. These two strains have been shown to be antigenically closely related [30]. We do not know to what extent they are predominant in our area, but the high rates of seropositivity found (Tables 2 and 3, [8], and unpublished data) indicate that it is likely that our local strains are closely related to them.

There have recently been reports questioning the specificity of the MIF test [31,32], but no other serologic assay has been shown to be superior to it, and while there are conflicting views as to the utility of immunoblotting [8,9], immunoblotting is not a practical alternative for large scale studies. Thus, for now at least, MIF remains the 'gold standard'. The ELISA described here, if its efficiency will be confirmed in more extensive studies, will make possible the non-subjective examination of large numbers of sera for antibodies to the two chlamydia species. Knowledge of the prevalence of infection and age of acquisition of antibodies in various population groups is useful for the pinpointing of such infection. The possible potential of the assay as a diagnostic tool needs to be closely examined, including its ability to detect specific IgA antibodies. Experiments in this direction are currently underway in our laboratory.

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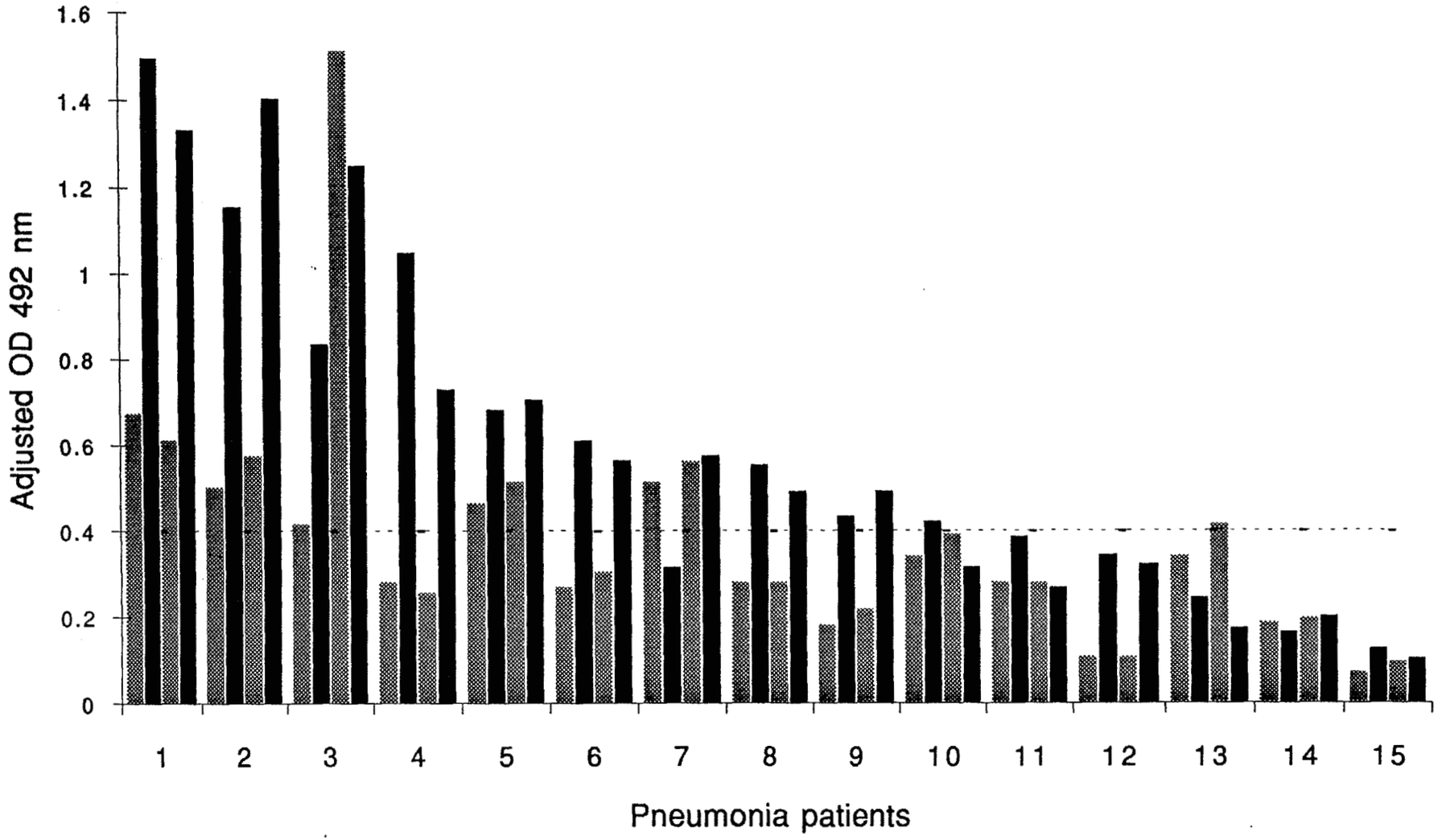

Figure 1

Figure 1. Binding of paired sera from 15 adults hospitalized with CAP to chlamydial antigens in the ELISA. The two sera of each patient were drawn approximately one month apart. Grey bars: *C. trachomatis antigen*. Black bars: *C. pneumoniae* antigen.

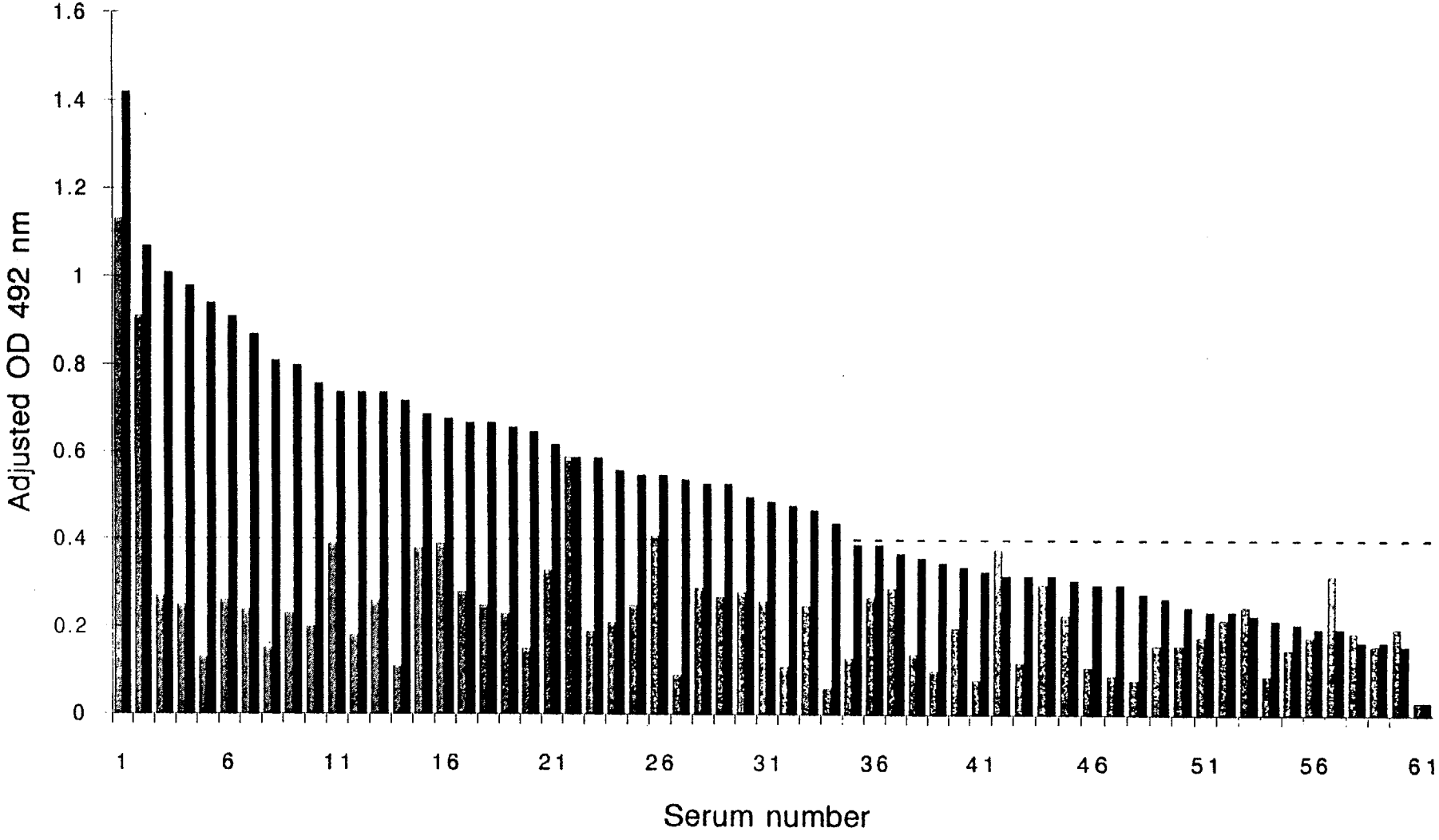

Figure 2

Figure 2. Binding of 61 sera of healthy students to chlamydial antigens in the ELISA . Grey bars: *C. trachomatis antigen*. Black bars: *C. pneumoniae* antigen.

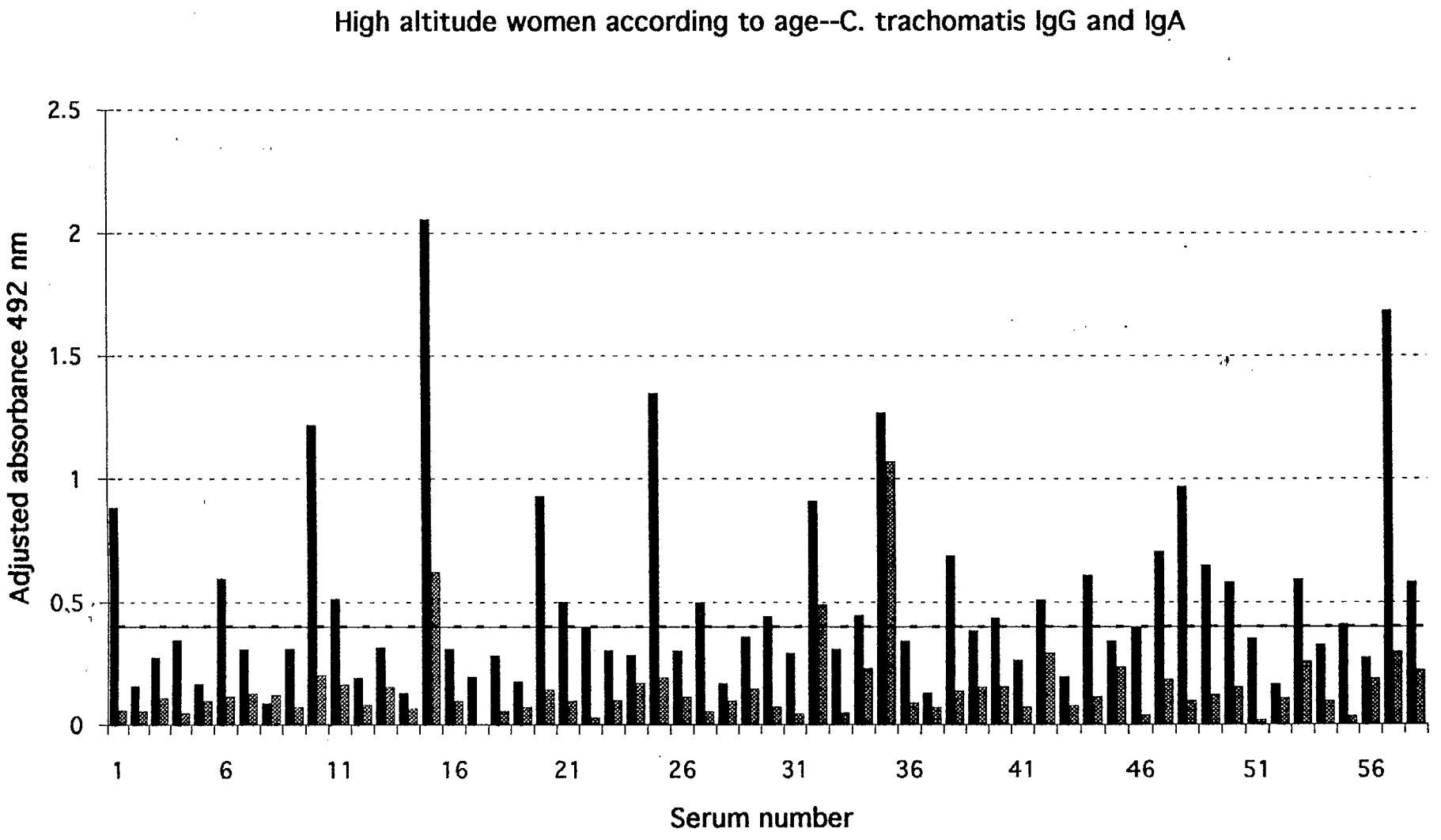

Figure 3. *C. trachomatis* specific IgG and IgA levels in 58 women from a high altitude region in Peru, as measured by the urea ELISA. The ELISA results are arranged in order of increasing age of the subjects. An adjusted OD level >0.4 is considered positive, although values between 0.4 and 0.5 may be considered borderline positive. Black bars: IgG; grey bars: IgA.

Figure 3

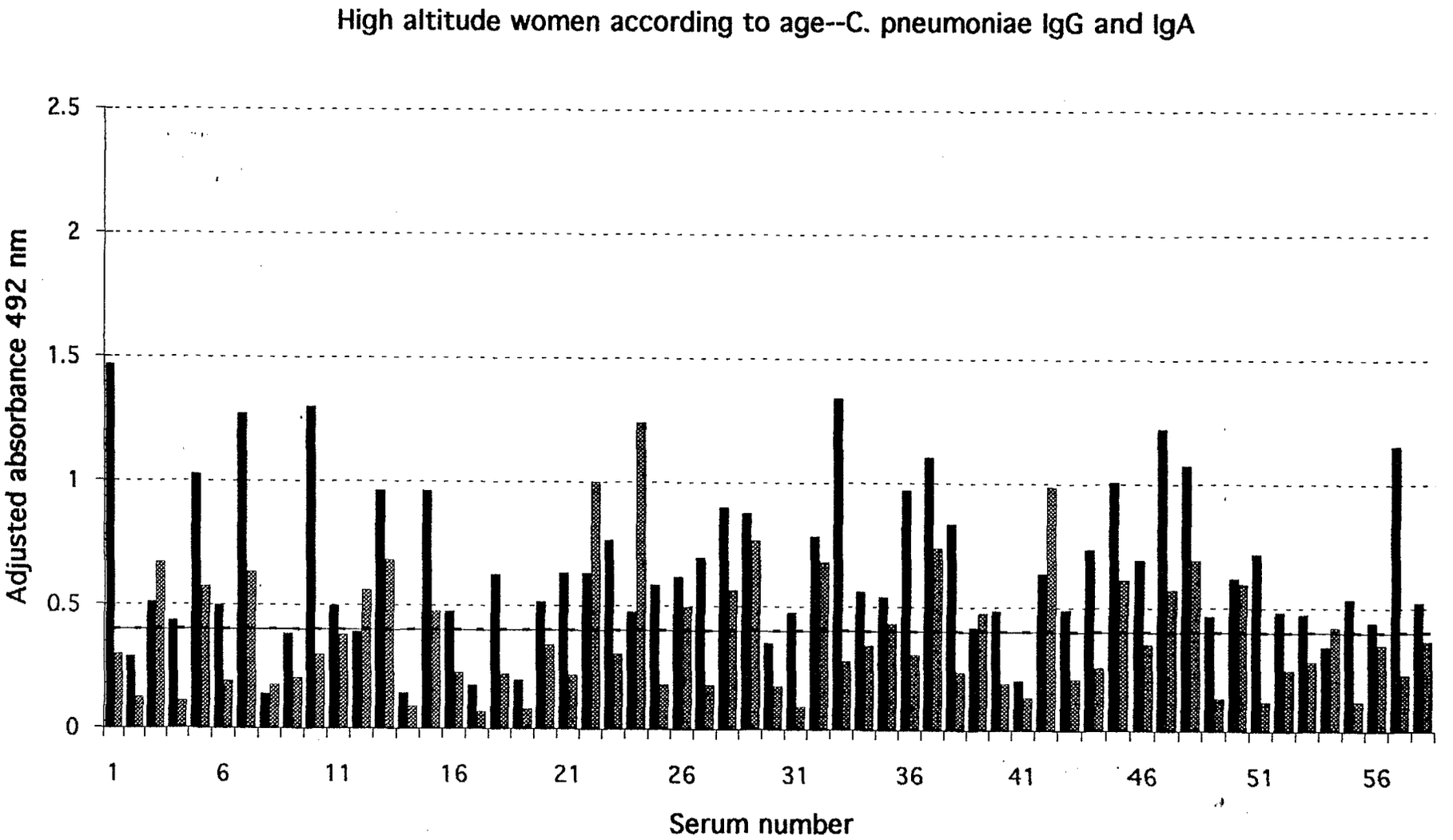

Figure 4. *C. pneumoniae* specific IgG and IgA levels in 58 women from a high altitude region in Peru, as measured by the urea ELISA. The ELISA results are arranged in order of increasing age of the subjects. An adjusted OD level >0.4 is considered positive, although values between 0.4 and 0.5 may be considered borderline positive. Black bars: IgG; grey bars: IgA.

Figure 4

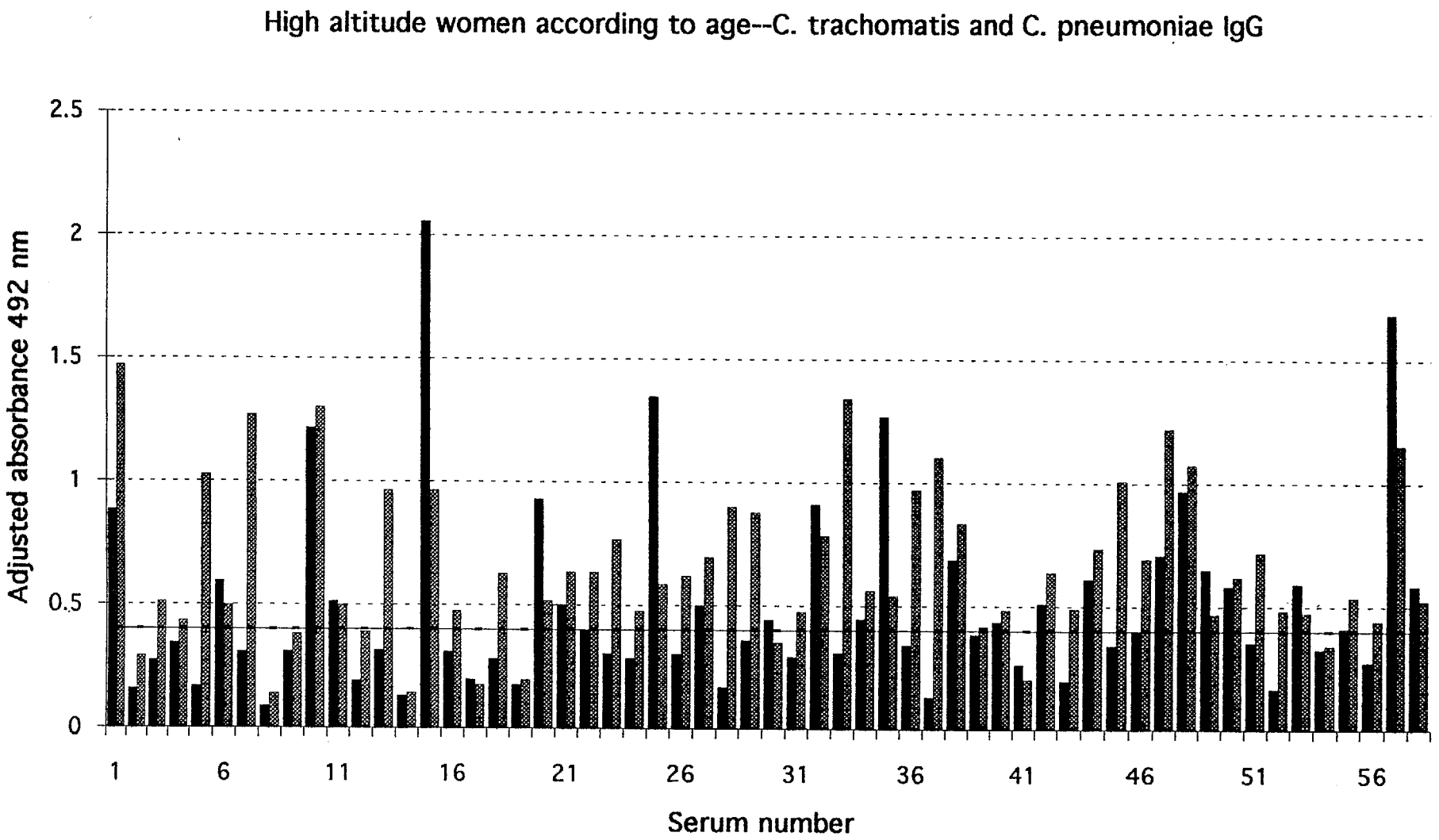

Figure 5

Figure 5. IgG antibody levels to *C. trachomatis* (black bars) and to *C. pneumoniae* (grey bars) in individual sera of 58 women from a high altitude region in Peru, as measured by the urea ELISA. The ELISA results are arranged in order of increasing age of the subjects. An adjusted OD level >0.4 is considered positive, although values between 0.4 and 0.5 may be considered borderline positive.

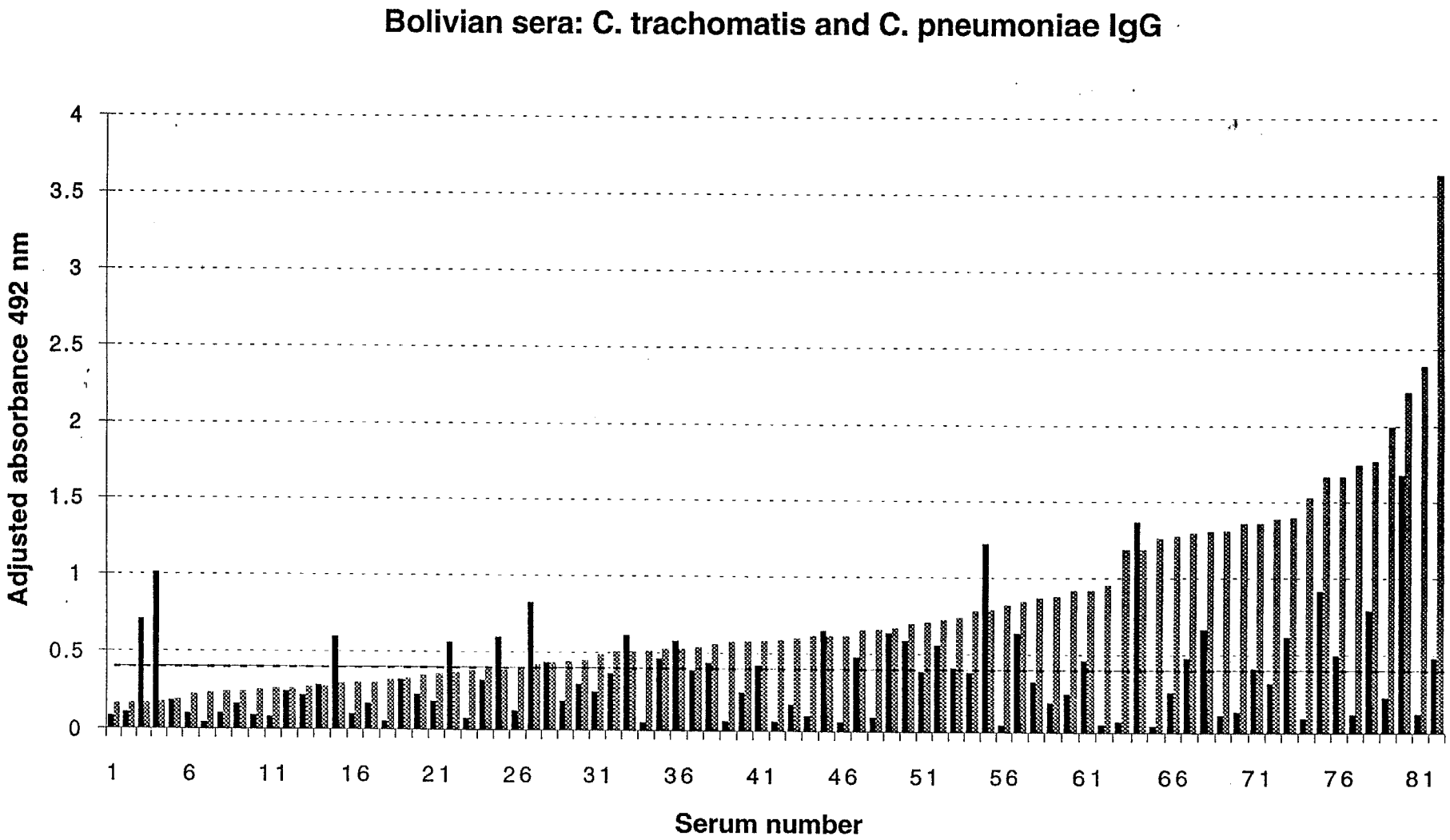

Figure 6

Figure 6. IgG antibody levels to *C. trachomatis* (black bars) and to *C. pneumoniae* (grey bars) in sera of 82 Bolivian women as measured by the urea ELISA. The sera are arranged in order of increasing IgG levels to *C. pneumoniae*. An adjusted OD level >0.4 is considered positive, although values between 0.4 and 0.5 may be considered borderline positive.

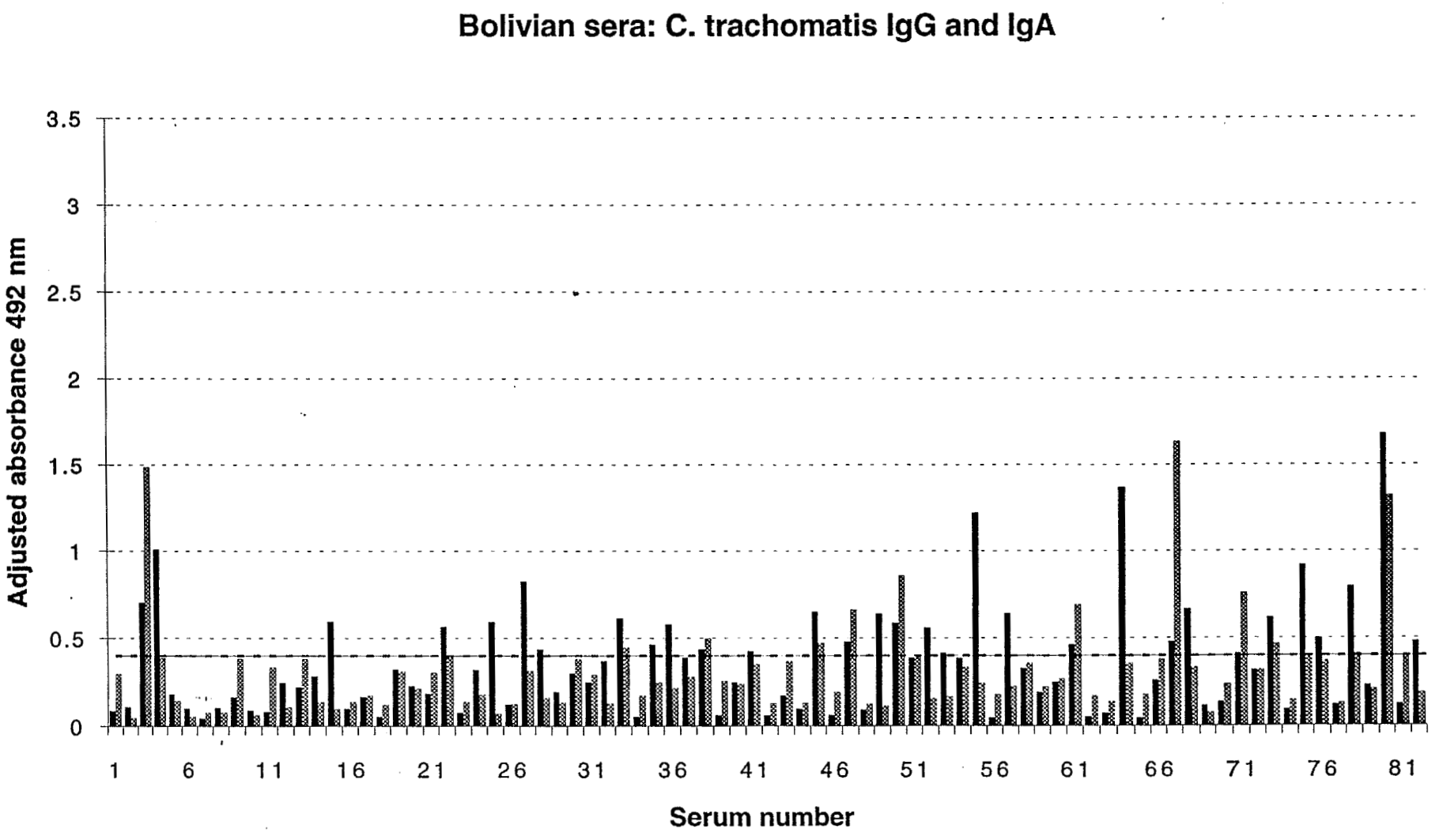

Figure 7

Figure 7. *C. trachomatis* specific IgG and IgA levels in 82 Bolivian women, as measured by the urea ELISA. An adjusted OD level >0.4 is considered positive, although values between 0.4 and 0.5 may be considered borderline positive. Black bars: IgG; grey bars: IgA.

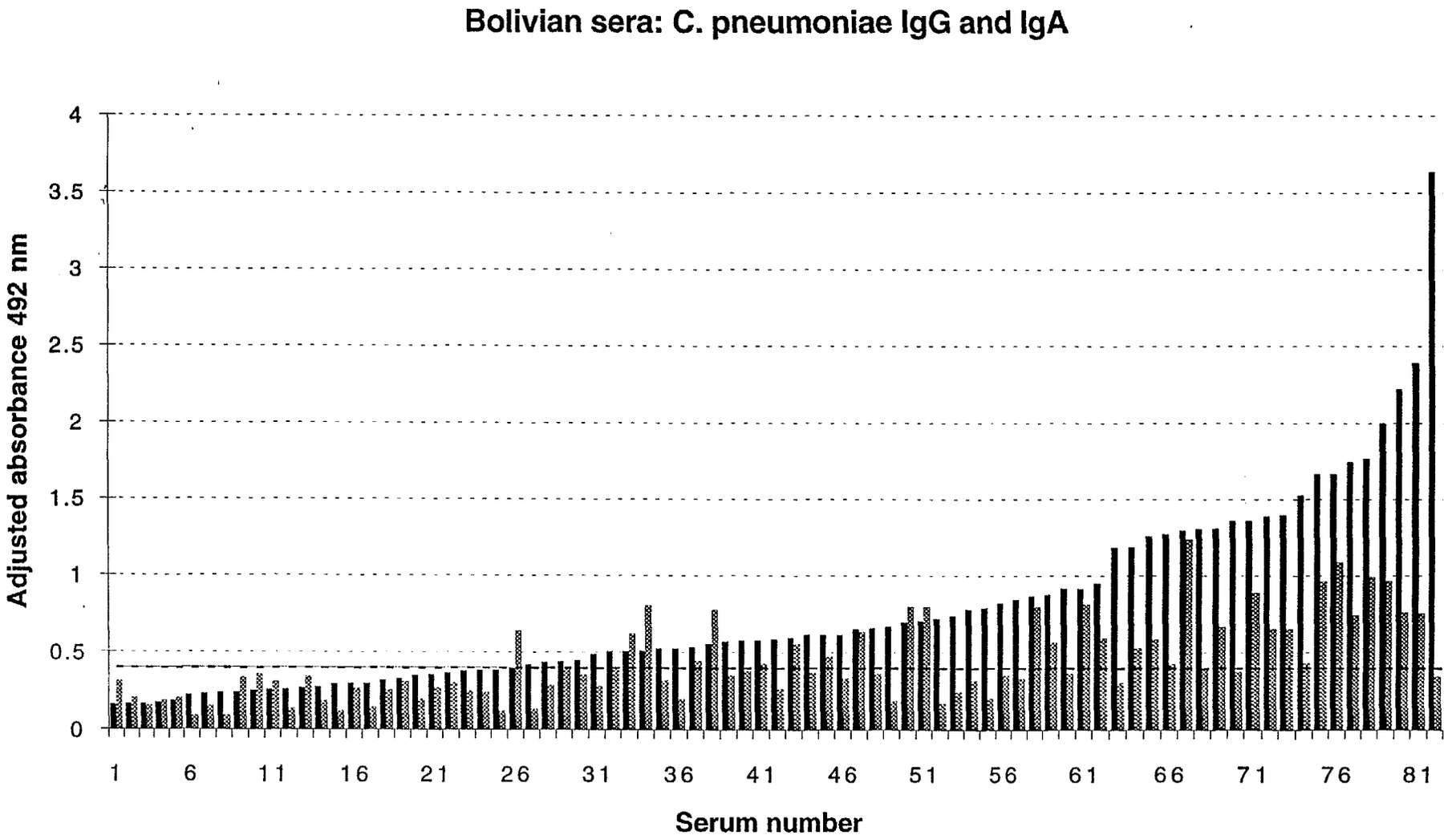

Figure 8

Figure 8. *C. pneumoniae* specific IgG and IgA levels in 82 Bolivian women as measured by the urea ELISA. The sera are arranged in order of increasing IgG levels to *C. pneumoniae*. An adjusted OD level >0.4 is considered positive, although values between 0.4 and 0.5 may be considered borderline positive. Black bars: IgG; grey bars: IgA.

Figure 9

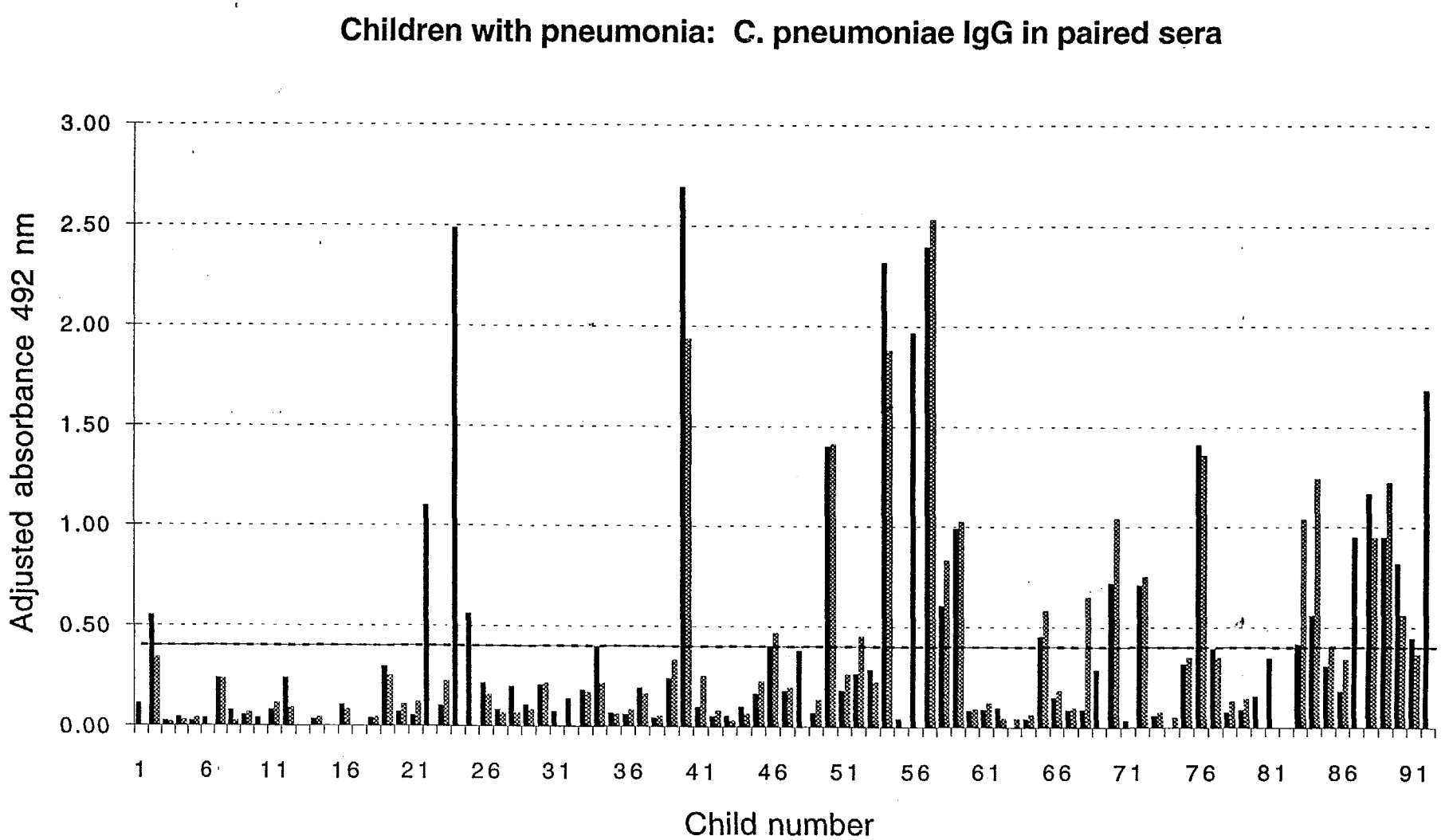

Figure 9. IgG levels to *C. pneumoniae* in paired sera of children hospitalized with pneumonia in Lima, as determined by the urea ELISA (Not all children have two serum samples.) The representation is in order of increasing age of the children. Subjects 1-20 are up to 1 year old, subjects 21-45 are up to 2 years old, subjects 46-66 are 2 to 4 years old, subjects 67-84 are 4-7 years old, and subjects 85-92 are 7-10 years old.

Figure 10

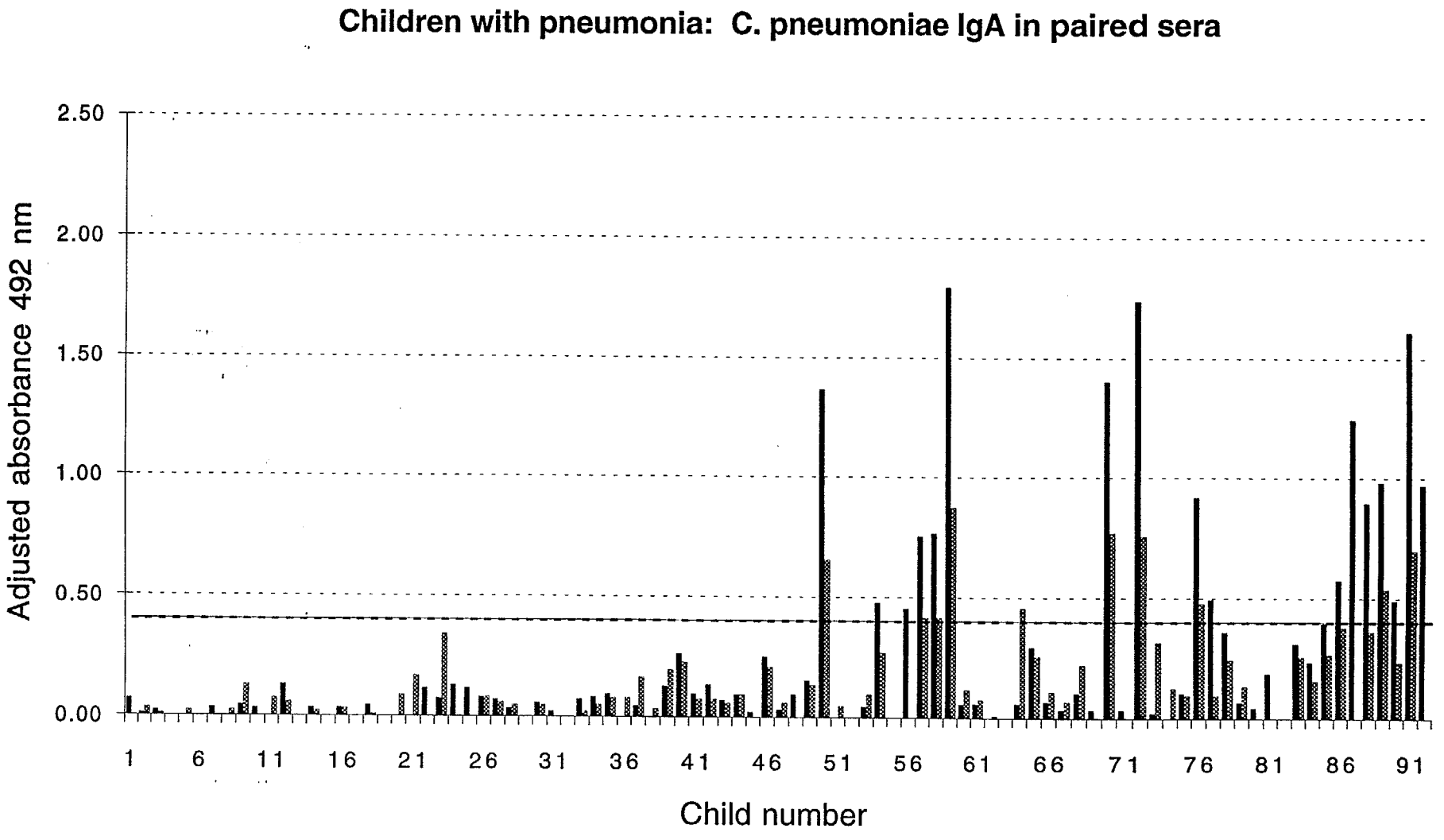

Figure 10. IgA levels to *C. pneumoniae* in paired sera of children hospitalized with pneumonia in Lima, as determined by the urea ELISA (Not all children have two serum samples.) The representation is in order of increasing age of the children. Subjects 1-20 are up to 1 year old, subjects 21-45 are up to 2 years old, subjects 46-66 are 2 to 4 years old, subjects 67-84 are 4-7 years old, and subjects 85-92 are 7-10 years old. Black bars: first serum sample; grey bars: second serum sample

Figure 11

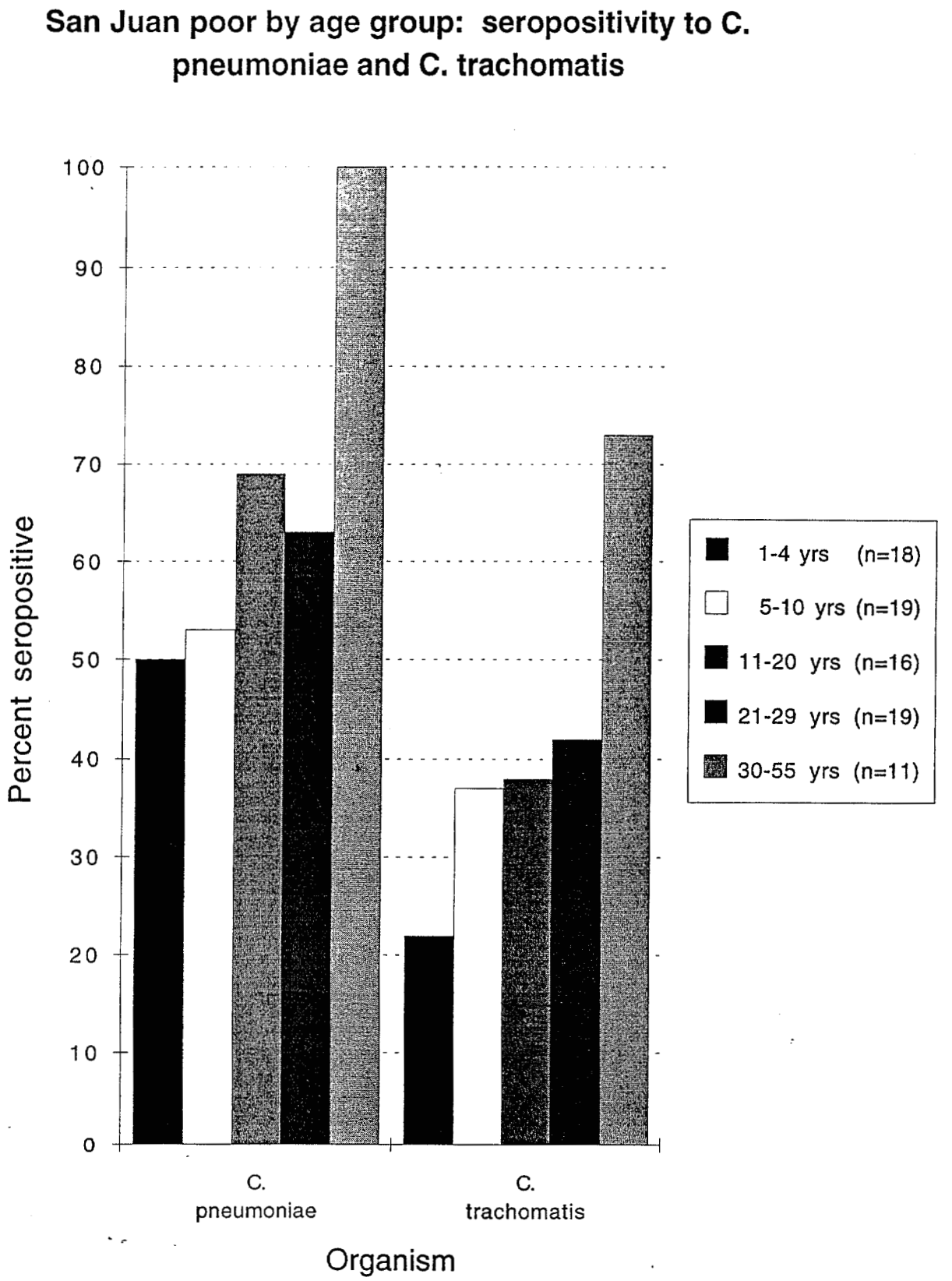

Figure 11. Seropositivity to *C. pneumoniae* and *C. trachomatis* by the urea ELISA in 83 residents of San Juan de Miraflores, by age group.

A.

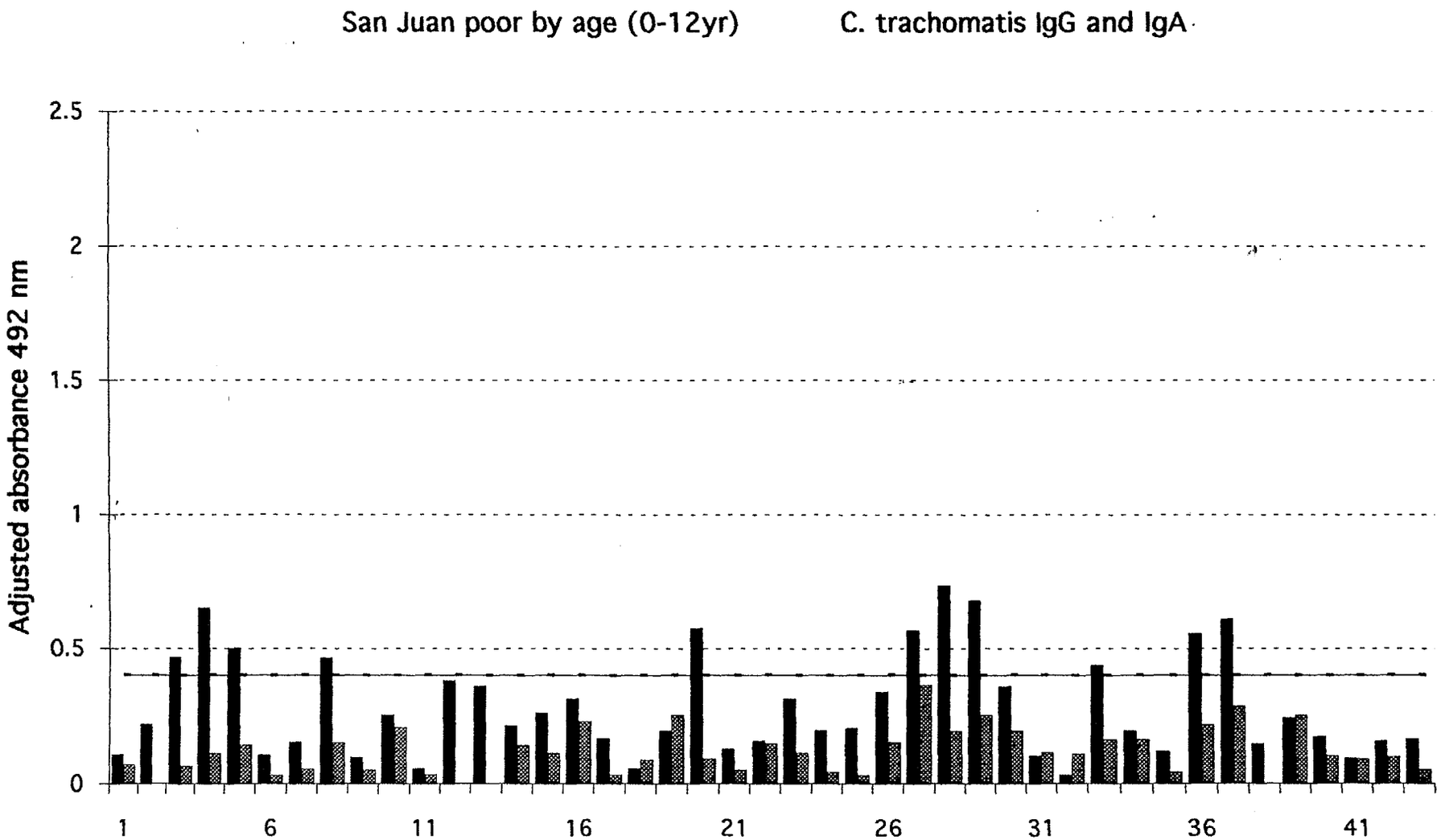

Figure 12. IgG (black bars) and IgA (grey bars) levels to *C. trachomatis* in sera of residents of San Juan de Miraflores, as determined by the urea ELISA. The sera are arranged in order of increasing age. A: children aged 1-12; B: subjects aged 13-55.

Figure 12A

B.

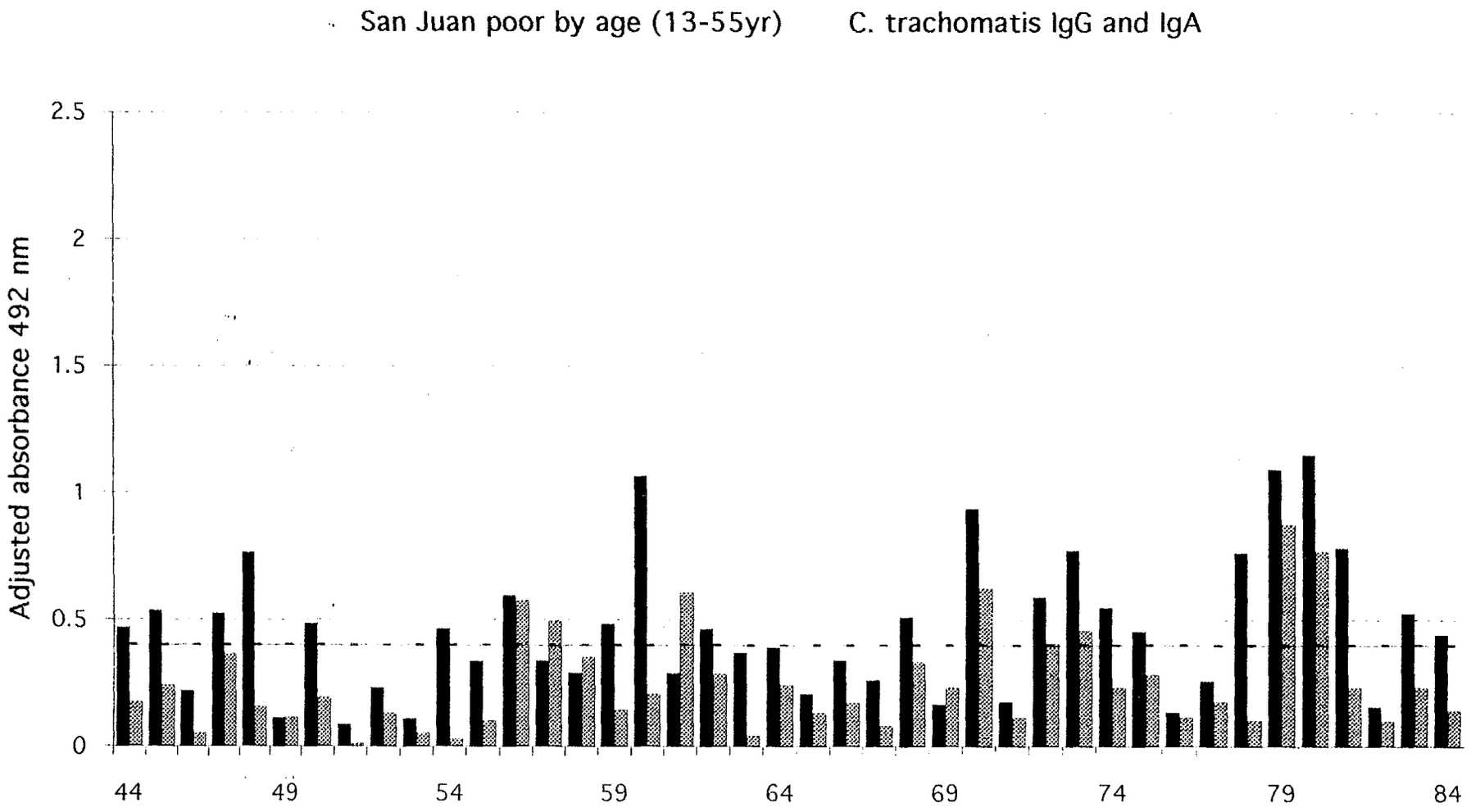

Fig. 12 B

Figure 12 B

A.

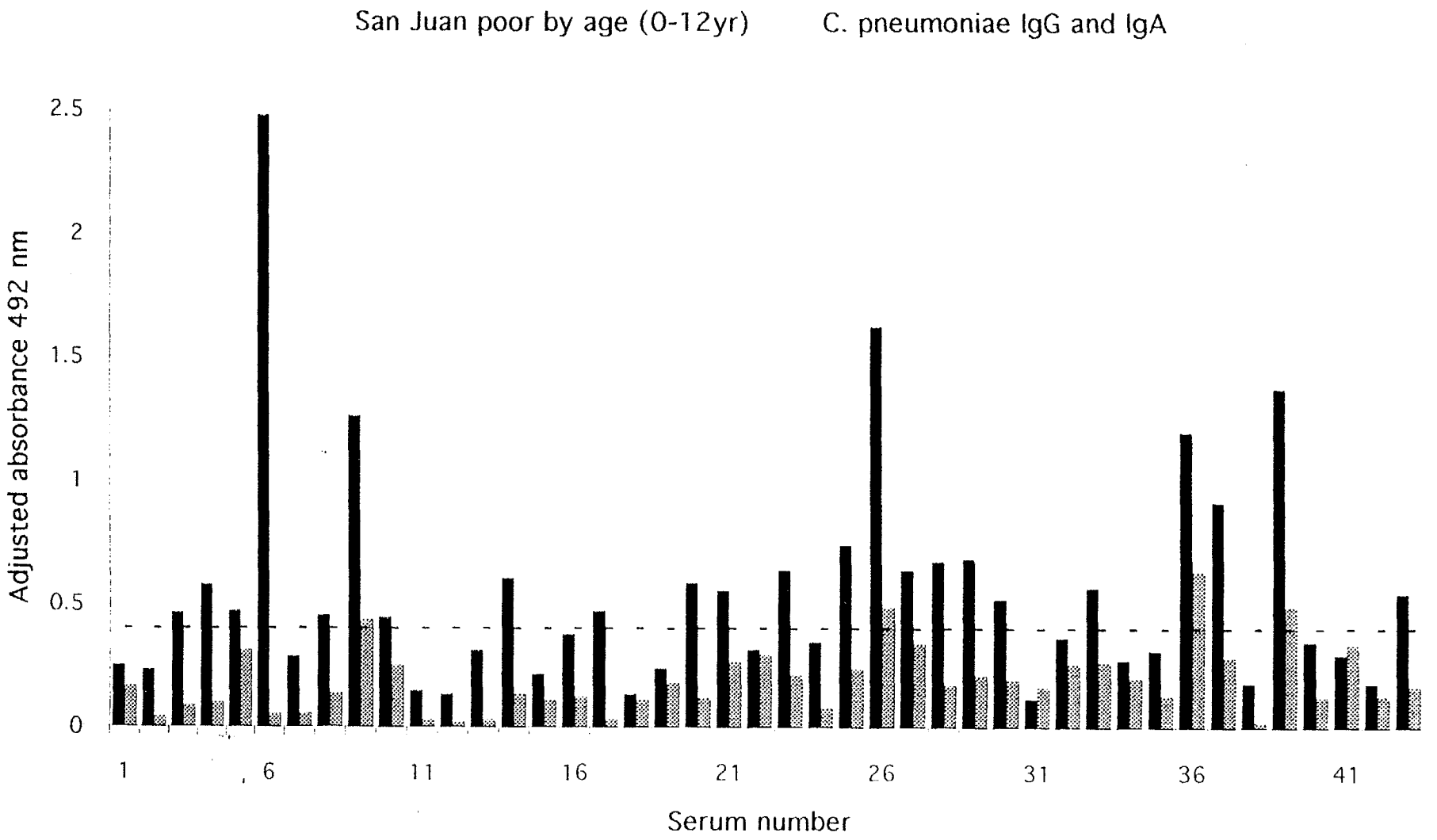

Figure 13. IgG (black bars) and IgA (grey bars) levels to *C. pneumoniae* in sera of residents of San Juan de Miraflores, as determined by the urea ELISA. The sera are arranged in order of increasing age. A: children aged 1-12; B: subjects aged 13-55.

42

Figure 13A

B.

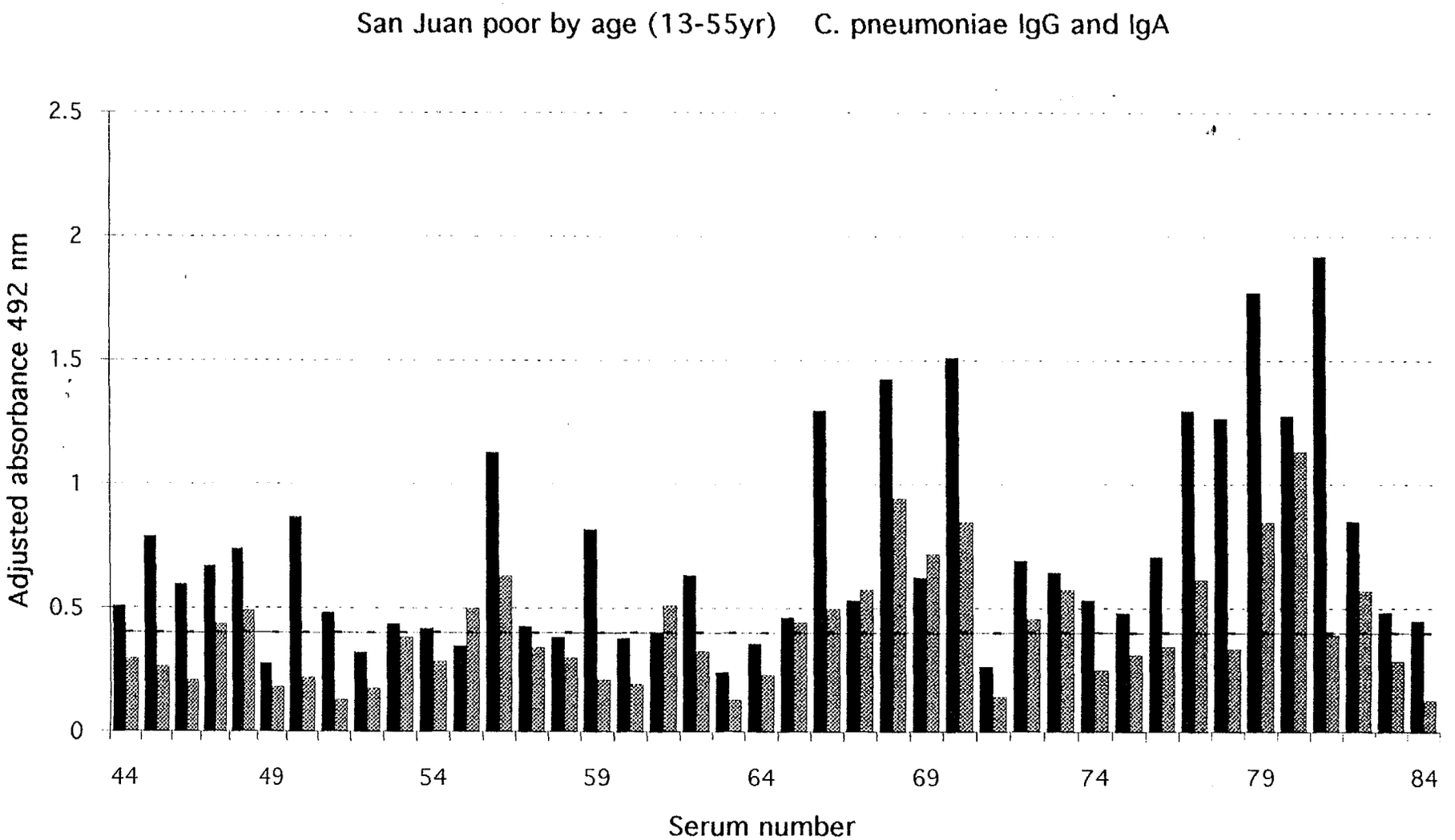

Figure 13 B

Figure 13 B