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AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: 8-24-88

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18

FROM: AID/SCI, ~~Victoria Ose~~

SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. 5.224
Final Report

Attachment

FINAL PROGRESS REPORT

"IMMUNODIAGNOSIS AND SEROEPIDEMIOLOGY OF TUBERCULOSIS"

A RESEARCH PROJECT

USAID/PSTC PROGRAM

GRANT No.936-5542-G-00-5043-00

SUBMITTED BY

SANIT MAKONKAWKEYOON, Ph.D.

PROJECT LEADER

DEPARTMENT OF CLINICAL IMMUNOLOGY

FACULTY OF ASSOCIATED MEDICAL SCIENCES

CHIANG MAI UNIVERSITY

CHIANG MAI, THAILAND.

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PROJECT PROFILE

Country	: Thailand
Grant Number	: 936-5542-G-00-5043-00
Program	: Program of Science and Technology Cooperation
Project Title	: Immunodiagnosis and Seroepidemiology of Tuberculosis
Project Leader	: Sanit Makonkawkeyoon, Ph.D.
Organization	: Department of Clinical Immunology Faculty of Associated Medical Sciences Chiang Mai University Chiang Mai, Thailand
Co-investigators	: Sichon Songsiri, M.S. Sakchai Dettrairat, M.S. Luksana Makonkawkeyoon, M.S. Busara Pongthong, M.D., M.PH.
Project Consultant	: None
Authorized Officer	: Sanit Makonkawkeyoon, Ph.D.
Total Project Budget	: US$ 102,180
Project Duration	: April 1985-April 1988
Reporting Period	: Final

1. BACKGROUND/INTRODUCTIONS :

Tuberculosis is one of the oldest and most widespread of the life-threatening infectious diseases still affecting the human population throughout the world. The estimated prevalence is about 15-20 million of active cases, with an annual incidence of about 10 million and at least 3 million deaths each year. Although the problem of tuberculosis in developed countries has been decreasing since the turn of this century, there is a very low decline in developing countries. The World Health Organization (WHO) reported that pulmonary tuberculosis was still widespread in the developing world with an annual incidence of 200-350 cases per 100,000 inhabitants in some parts of Africa, Asia and Oceania.

The diagnosis of tuberculosis is therefore of importance both to the individual patients and to the community. There is no simple diagnostic test for tuberculosis. Clinical and radiological signs and bacterial staining by smear are notoriously nonspecific. Culture techniques, although providing a definitive diagnosis, are expensive and time-consuming; and more seriously, they vary considerably in their reliability from laboratory to laboratory. There is no doubt that tuberculosis is often misdiagnosed; many cases are not recognized until a postmortem examination is performed, and cases of renal, meningitis, bone and intestinal tuberculosis are often only diagnosed late in the course of the disease.

Extensive studies have established beyond doubt that *M.tuberculosis* induces humoral immune response in the infected

host. Until the present time, no investigator has developed a completely reliable test for serodiagnosis of tuberculosis. Many researchers have shown that there is a high degree of sharing of antigens between the various mycobacterial species and that some of the antigens are also shared with other bacterial genera.

A serologic test that would indicate active tuberculosis with reasonable precision would be of great value in the numerous conditions in which tuberculosis is part of a differential diagnosis, and in the diagnosis of diseases of internal organs that are difficult to assess, such as those of the spine or the gut. A serologic test would also save the time required for culture of specimens containing too few tubercle bacilli to yield a positive direct smear examination. The development of an effective serologic test to detect antibody in preclinical or clinical tuberculosis would also greatly aid the effectiveness of tuberculosis control programs. Such an assay could both facilitate early diagnosis of the diseases and could decrease incidence of the disease by early detection in multibacillary individuals. We have tried to isolate a species specific antigen from M.tuberculosis and tried to develop a specific serologic test for tuberculosis.

2. OBJECTIVES:

The overall objectives of this research project are :

(1) To obtain species specific antigens or haptens from M.tuberculosis.

(2) To develop an enzyme linked immunosorbent assay by using the species specific antigens or haptens for serodiagnosis of tuberculosis

(3) To develop a simple and practical method of ELISA for field study and seroepidemiology of tuberculosis.

3. MATERIALS AND METHODS :

PART I. ISOLATION AND PURIFICATION OF SPECIES SPECIFIC ANTIGEN FROM M.TUBERCULOSIS H37Rv.

1.) LIPOPOLYSACCHARIDE EXTRACTION AND PURIFICATION

METHOD 1. 90% Phenol-Water Extraction, and Sonication.

METHOD 2. 90% Phenol-Water Extraction, Sonication, Sephadex G-25, G-100 and Thin Layer Chromatography.

METHOD 3. Sonication, 90% Phenol-Water Extraction, Sephadex G-25, G-100 and Thin Layer Chromatography.

2.) POLYSACCHARIDE EXTRACTION AND PURIFICATION

METHOD 1. Sulfosalicylic-Ether Extraction and Sonication

METHOD 2. Sulfosalicylic-Ether Extration

3.) OLIGOSACCHARIDE EXTRACTION AND PURIFICATION

METHOD 1. Ethanol Extraction, Chloroform-Methanol Extraction and Chromatography on Dowex Column

4.) GLYCOPEPTIDOLIPIDS/LIPOOLIGOSACCHARIDE EXTRACTION AND PURIFICATION

METHOD 1. Chloroform-Methanol Extraction and Thin Layer Chromatography

METHOD 2. Chloroform-Methanol Extraction, DEAE-Cellulose Acetate Column and Thin Layer Chromatography

5.) ISOLATION AND PURIFICATION OF CELL WALL POLYSACCHARIDE

METHOD 1. Trichloroacetic Ethyl-Ether Extractions and Sephadex G-75, G-25 Chromatography

6.) ISOLATION AND PURIFICATION OF LIPID FROM M.TUBERCULOSIS

METHOD 1. Pyridine, Methanol and Chloroform Extraction

7.) SELECTED METHODS FOR ISOLATION AND PURIFICATION OF ANTIGEN FROM M.TUBERCULOSIS

A. Preparation of Crude Sonication M.tuberculosis Antigen (CTB-Ag).

M.tuberculosis H37Rv was cultured in Sauton's medium for 6 weeks and the bacilli were killed by autoclave. They were washed 3 times with distilled water and resuspended in phosphate buffered-saline containing 0.4% sodium azide to give 25% w/v (wet weight). This suspension was sonicated in an ice bath 3 time, at 300 watts for 15 min, using Braun-Sonic Model 1510, transducer diameter 2 cm. (Braun, Melsungen, U.S.A.). The sonicate was centrifuged at 15,000 G for 20 min. and the supernatant was stored at 4 ^{o}C as the source of sonicated M.tuberculosis H37Rv antigen (CTB-Ag). The optimal antigen dilution, after determined by titration, was 1:400.

B. Preparation of Total Lipid Antigen from M.tuberculosis (TL-Ag).

Mycobacterial lipid was obtained from M.tuberculosis H37Rv (TMC#102). The organism was grown in Sauton's medium for 6 weeks. The well-grown cultures were autoclaved and washed with distilled water. One gram of dried bacilli was extracted with 40 ml chloroform-methanol (2:1) mixture by stirring at 50 ^{o}C 18 hours. After centrifugation at 3000 G 15 min, the residue was re-extracted with half volume of chloroform-methanol mixture. Pooled supernatants (TL antigen) were stored at 4 ^{o}C. The total lipid was titrated to find out optimal dilution for ELISA test.

C. Preparation of X-Antigen (X-Ag).

After M.tuberculosis H37Rv was grown in Sauton's media for 6 weeks, the organisms killed by autoclave and washed 3 times with distilled water. One gram of dried bacilli was extracted with 40 ml chloroform-methanol (2:1) mixture as the same procedure for total lipid antigen. The chloroform-methanol extracted materials was dried under nitrogen gas, then dissolved with chloroform. The lipid materials in chloroform was passed through a Florisil column, then stepwise elution with 2-5% methanol in chloroform. Each eluate was tested with 22 different rabbit sera against various different species of mycobacterium. Eluate reacted only with rabbit anti-M.tuberculosis H37Rv was pooled (we called X-antigen).

PART II. DEVELOPMENT OF APPROPRIATE ELISA FOR SERODIAGNOSIS OF TUBERCULOSIS

1. ELISA Procedure for TL Antigen.

An ethanol solution of optimal dilution (1:200) of TL antigen (50 microliters) was added to the wells of microtiter plate (Dynatech, Alexandria VA, U.S.A), and the solvent was evaporated in 70 ^{o}C hot air oven for 40 min. Fifty microliters of tested serum (diluted 1:400 with 0.1% Tween 20, 0.02% sodium azide in phosphate buffered saline, PBSTS) was added, and the plates were incubated at room-temperature for 1 hour. Wells were washed 3 times in phosphate buffered saline (PBS). Peroxidase-conjugated anti-human immunoglobulin (G+A+M) antibody (Cappel, Laboratories, Cochraville, PA, U.S.A.) (50 ul), diluted with 0.1% Tween 20 in phosphate buffered saline (PBST), was added into each well and incubated at room-temperature for 30 min. After removing unreacted conjugate with PBS, 50 microliters of substrate solution (O-phenylenediamine [0.4 mg/ml], 30% hydrogen peroxide [0.5 ul/ml] in citrate-phosphate buffer [pH 5.0], SUBS.) was added, plates were developed in the dark for 30 min, and the reaction was stopped by addition of 50 microliters of 2.5 N sulfuric acid. The optical density was measured with microplate reader at 490 nm. (Biotek. U.S.A.). Diagram of the procedure was show in figure 1A.

2. Amplified ELISA for Purified X-Antigen.

The optimal dilution (1:200) of X-Ag was diluted from stock with absolute ethanol. Fifty microliters was distributed into 96

well flat-bottomed microtiter plates (Dynatech). The plates were dried at 70 ^{o}C for 30 min. Before use, the plates were wetted with PBS. Fifty microliters of human tested serum, diluted 1:400 in PBSTS, was added to each precoated well and incubated for 60 min. at room temperature. The plates were then washed, 50 microliters of 1:1000 of rabbit anti-human IgG + IgA + IgM (Amplifier) were added, and the preparations were incubated at room temperature for 30 min. The plates were washed with PBS, 50 microliters of a 1:5000 dilution of goat anti-rabbit IgG+IgA+IgM conjugated to horseradish peroxidase (Cappel) in PBST was added to each well, and the preparations were incubated for 30 min at room temperature. The plates then were washed, 50 microliters of substrate mixture were added to each well, and the preparations were incubated in the dark at room temperature for 30 min. The color reaction was stopped with 50 microliters of 2.5 N sulfuric acid. The optical density was read with microtiter plate reader (Biotek, U.S.A.). A diagram of the procedure is shown in figure 1B.

3. <u>ELISA Procedure for CTB Antigen</u>.

The CTB antigen was diluted in coating buffer (Carbonate-bicarbonate buffer pH 9.6) to optimal dilution (1:1600, previous titration). One hundred microliters of diluted antigen was dispensed into each well of the microtiter plate and incubated for 2 hours. All of the following steps were the same as ELISA with TL antigen excepted that reactants volume were 80 microliters instread of 50 microliters.

PART III. DEVELOPMENT OF AN ELISA FOR FIELD STUDY OF TUBERCULOSIS

1. Nature of Specimens.

Two groups of samples were collected for this study. The first group consisted of various tuberculosis patients from Tuberculosis Control Center Region 10, Chiang Mai. The second group composed of sera samples from 2 villages in Lampoon province (16 miles south of Chiang Mai).

A total of 1,735 samples of sera and filter paper preserved blood were obtained from 406 tuberculosis patients, 220 specimens from 2 villages of Lampoon province. Sixteen samples from patients infected with atypical mycobacteria, 69 specimens from various types of leprosy patients and 199 from professional blood donors. Filter paper preserved blood samples were taken from most of the samples except from atypical mycobacterial infected and leprosy patients.

The nature, classifications, and types of specimens are shown in Tables 3,4 and 5 in the section of results.

2. Collection, Separation and Storage of Sera.

Blood was drawn from donors by venipuncture using vacutainer tube. Serum was separated from blood clot by centrifugation. All sera were stored in glass vials and kept at -20 °C until used.

3. Filter Paper for Preservation of Antibody Activity in Whole Blood.

3.1 Preservation of Blood by Filter Paper.

One free drop of blood from a capillary tube (approximate 25 ul) was dropped on a 1.0x1.5 cm filter paper (Whatman No.1). After drying at room temperature, the bloodly filter paper strip was kept in a plastic bag at 4-8 ^{o}C for various periods of time.

3.2 Elution of Preserved Blood.

Blood preserved filter paper strip was cut at the margin of the blood stain. The bloodly filter paper was extracted by submerging in 1 ml of 0.05% Tween 20 and 0.02% sodium azide in PBS by shaking at 56 ^{o}C for 1 hour. The extracted solution was tested for antibody to mycobacterium species.

PART IV. ISOLATION AND IDENTIFICATION OF M.TUBERCULOSIS FROM PATIENTS

1. Smear and Stain

Sputum was collected from various groups of TB patients at the Tuberculosis Control Center Region 10, Chiang Mai. Sputum specimens were smeared on a microscopic slide and stained by Ziehl-Neelsen technic for acid fast bacilli and Auramine O staining technic for auramine fluorescent bacilli.

2. Cultivation and Identification of M.tuberculosis.

Sputum was treated with 5% oxalic acid for 25 minutes to decontaminate non-acid fast bacteria. The treated sputum was inoculated onto 3 Lowenstein-Jensen Slants. Cultures were incubated at 37 ^{o}C and observed for growth at weeks 1 and 4.

Cultures showing typical colonies and no sign of contamination were subcultured on Lowenstein-Jensen slant and Lowenstein-Jensen deep for further biochemical identification. After 2 weeks of incubation, cultures showing maximum growth were tested for Niacin accumulation, Nitrate reduction and semiquantitative and heat-stable catalase production. Cultures showing confused biochemical reactions were subcultured and tested once again.

PART V. PRODUCTION OF RABBIT HYPERIMMUNE SERA TO VARIOUS SPECIES OF MYCOBACTERIA.

1. Mass Culture for Antigen Preparation.

M.tuberculosis H37Rv and other 28 mycobacterium species were cultured for mass antigen preparation. All mycobacterium species were cultured at 37 °C for 2-6 weeks in Sauton's liquid media, except *M.marinum* at 30 °C. Cultures were done in 500 ml culture flask containing 250 ml culture media. Cutures showing maximum growth were autoclaved and harvested by centrifugation. One culture flask yielded approximately 2-4 gm of bacilli (wet weight). The bacilli were washed twice with normal saline solution (NSS) and resuspended in NSS to get a suspension containing 60 mg bacilli/ml (wet weight). This suspension was sonicated for 5 minutes, 4 times in an ice bath by a Braun-Sonic 1510 with an effect of 100 W. The sonicated material was divided into 2 portions; one was mixed with an equal volume of incomplete Freund's adjuvant and stored at -20 °C until used for immunization of rabbits. The other portion was centrifuged at 8,000 g for 30 min, supernatant fluid was collected and kept at -20 °C until used

for ELISA assay.

Antigen preparation of 35 mycobacterium species or strains were produced for immunization of two rabbit per one mycobacterium species. The following antigens of mycobacterium species were prepared for rabbit immunization:

1. M.avium TMC#706
2. M.avium serotype 2, NIH#724
3. M.bovis BCG TMC#1020
4. M.bovis NIHJ#1608
5. M.chelonei abscessus NIHJ#1609 ATCC#19977
6. M.chelonei chelonei NIHJ#1611 ATCC#19237
7. M.flavescense
8. M.flavescense NIHJ#1614 ATCC#14474
9. M.fortuitum NIHJ#1615 ATCC#19542
10. M.fortuitum TMC#1529
11. M.fortuitum
12. M.gastri NIHJ#1616 ATCC#15754
13. M.gordonae TMC#1324
14. M.intracellulare TMC#1403
15. M.intracellulare NIHJ#1618 ATCC#15980
16. M.kansasii TMC#1204
17. M.malmoense ATCC#22620
18. M.marinum TMC#1218
19. M.nonchromogenicum NIHJ#1622 ATCC#19530
20. M.phlei NIHJ#1624 ATCC#11758
21. M.phlei TMC#1548
22. M.scrofulaceum
23. M.scrofulaceum NIHM#1626 ATCC#19981
24. M.smegmatis TMC#1546
25. M.smegmatis NIHJ#1628 ATCC#14468
26. M.tuberculosis TMC#102

27. M.tuberculosis H37Rv
28. M.terrae
29. M.terrae NIHJ#1603
30. M.triviale
31. M.vaccae TMC#1526
32. M.ulcerans NIHJ#1636
33. M.xenopi NIHJ#1638
34. M.simiae NIHJ#1627
35. M.szulgai ATCC#10831
36. M.novum ATCC#19621

2. Production of Rabbit Hyperimmune Sera against Mycobacteria.

Two New Zealand white rabbits were used for immunization with each antigen emulsion. A single intramuscular injection of 0.5 ml antigen emulsion at the hind leg was gently done. Rabbits were further immunized with 0.1 ml of antigen emulsion intravenously at the neck region every 14 days, e.g. on days 14, 28, 42 and at monthly intervals thereafter. Bleeding was done every 7 days, e.g. on days 0, 7, 14, 21 and so forth. Sera were separated and stored at -20 ^{o}C for antibody assay.

Antibodies were assayed by two techniques, indirect ELISA and double immunodiffusion (Ouchterlony). For the indirect ELISA assay, a flat bottom 96 wells microtiter plate was coated with 200 ul of a 1/3200 dilution of mycobacterial antigens. After overnight incubation at 4 ^{o}C, excess antigens were washed out. A 180 ul of various dilutions, e.g. 1/640, 1/1280, 1/2560 and 1/5120, of rabbit anti-mycobacterium serum was added, incubated, washed, a 1/1000 dilution of peroxidase-conjugated goat anti-rabbit immunoglobulins (Cappel) was added. After washing, ABTS/H_2O_2 chromogen/substrate was added to react for 30 min at room temperature. All color reactions were read at 405 nm in an ELISA reader (Microplate Autoreader EL 309, Bio-Tek Instruments, U.S.A.).

In double immunodiffusion technique, agar gel is poured evenly onto glass plates and holes or well cut into the solidified gel. Antigens of various mycobacteria are placed in the center wells while various dilutions of the corresponding rabbit anti-mycobacteria serum in the peripheral wells. The reacting gel is covered in a moist chamber and allow to stand in a refrigerator. Any sample positive for immune precipitin line will be further test by ELISA.

4. RESULTS

PART I. ISOLATION AND PURIFICATION OF SPECIES SPECIFIC ANTIGEN FROM M.TUBERCULOSIS H37Rv.

1. LIPOPOLYSACCHARIDE EXTRACTION AND PURIFICATION

Extraction and purification of lipopolysaccharide by phenol-water extraction then further purified by gel filtration resulting no fraction is good enough for species-specific antigen of *M.tuberculosis* (see first and second progress reports).

2. POLYSACCHARIDE EXTRACTION AND PURIFICATION

Extraction and purification of polysaccharide by sulfosalicylic acid and ether did not yield any fraction good enough to be the species-specific antigen of *M.tuberculosis* (see first progress report).

3. OLIGOSACCHARIDE EXTRACTION AND PURIFICATION

Extraction and purification of oligosaccharide from *M.tuberculosis* by ethanol and chloroform-methanol mixture did not yield any species-specific antigen when evaluated by ELISA using pooled human TB serum, pooled human leprosy serum and rabbit anti-*M.tuberculosis* serum (see first progress report).

4. GLYCOPEPTIDOLIPID EXTRACTION AND PURIFICATION

Glycopeptidolipid and lipooligosaccharide extraction and purification from *M.tuberculosis* by chloroform-methanol extraction and thin layer chromatography or by chloroform-methanol extraction, DEAE-cellulose acetate column and thin layer chromatography in chloroform-methanol. Species-specific antigen of *M.tuberculosis* is still not able to obatin by these techniques (see first progress report).

5. ISOLATION AND PURIFICATION OF CELL WALL POLYSACCHARIDE

Cell wall polysaccharide of *M.tuberculosis* was extracted with trichloroacetic acid (TCA), ethyl ether then passing through Sephadex G-75 and G-25. There is no fraction showing species-specific antigen (see first progress report).

6. ISOLATION AND PURIFICATION OF LIPID

M.tuberculosis was extracted with pyridine, methanol and chloroform then chromatographed on TLC. Materials in some spots gave very encouraging results. However, when they were further evaluated with individual leprosy serum, non-specificity was observed (see second and third progress reports).

7. SELECTED METHODS FOR ISOLATION AND PURIFICATION OF ANTIGEN FROM M.TUBERCULOSIS

A. Crude Sonicated M.tuberculosis Antigen (CTB-Ag).

This type of antigen was prepared for ELISA evaluation of tuberculous patients' sera. It is the crude antigen of M.tuberculosis.

B. Total Lipid Antigen from M.tuberculosis (TL-Ag).

This lipid antigen was prepared for ELISA evaluation of TB patients' sera against lipid antigen of M.tuberculosis.

C. X-Antigen from M.tuberculosis (X-Ag).

At least many hundred grams of M.tuberculosis H37Rv were used for trial of many isolation and purification methods for species-specific antigen of M.tuberculosis. Finally, a species-specific antigen of M.tuberculosis could be obtained by extracting with chloroform-methanol and passing through Silicic/ Celite or Florisil column. The specificity of X-Ag was evaluated by rabbit hyperimmune sera against various different species of mycobacterium as shown in tables 1 and 2 and figure 2.

PART II. DEVELOPMENT OF APPROPRIATE ELISA FOR SERODIAGNOSIS OF TUBERCULOSIS

After many modification of ELISA techniques have been tried, we ended up using one step ELISA antibody assay for CTB-Ag and TL-Ag and amplified ELISA antibody assay for X-Ag. Both techniques are summarized schematically as in figures 1A and 1B respectively.

PART III. DEVELOPMENT OF AN ELISA FOR FIELD STUDY OF TUBERCULOSIS

1. Nature of Specimens. Sera samples were collected from many different types of subjects. The detail of nature, types and classification of specimens were shown in tables 3, 4 and 5.

2. Collection and Stroage of Sera. All sera specimens were kept at -70 ^{o}C until use for ELISA test.

3. Filter Paper for Preservation of Antibody Activity in Whole Blood. Whole blood was preserved on filter paper Whatman No.1 for 140 days at 4-8 ^{o}C in a refrigerator. After blood was extracted by PBS containing Tween 20. The extracted solution was assayed for antibody against antigens of *M.tuberculosis*. Antibody activity in whole blood is generally preserved by filter paper as shown in figures 3, 4 and 5.

PART IV. ISOLATION AND IDENTIFICATION OF M.TUBERCULOSIS FROM PATIENTS

All suspected tuberculous patients will be collected for sputum. After sputum was decontaminated and concentrated, it was smeared and stained for acid-fast bacilli. The other portion of the decontaminated and concentrated sputum was cultured for mycobacterium. *M.tuberculosis* was identified by colonial morphology, Niacin accumulation, Nitrate reduction, semiquantitative and heat-stable catalase production

All sera specimens were classified by acid-fast stain, cultivation and identification of M.tuberculosis, group or type and history of subjects (see tables 3,4 and 5).

PART V. PRODUCTION OF RABBIT HYPERIMMUNE SERA TO VARIOUS SPECIES OF MYCOBACTERIA

Rabbit hyperimmune sera were produced against 36 different species or strains of mycobacteria. When antibody titer react 1:100 in ELISA test with O.D. value more than 1.500. Blood was taken from immunized rabbit, serum collected and stored at -20 °C. The antibody response in each rabbit against each mycobacterium species or strain is shown in figures 6-14.

PART VI. SEROLOGICAL EVALUATION OF M.TUBERCULOSIS SPECIFIC ANTIGEN

The sensitivity and specificity of M.tuberculosis specific antigen for serological evaluation of tuberculosis infection were evaluated by sera from high prevalent T.B. cases village and low prevalent T.B. cases village, tuberculous patients, leprosy patients, atypical mycobacterial infected patients and normal blood from blood bank. Results of serological evaluation by different types of antigens and sera were shown in figures 15-44.

5. DISCUSSION AND CONCLUSION

From our research investigation, a species specific antigen or hapten was isolated from M.tuberculosis H37Rv. This species-specific antigen or hapten was highly specific for M.tuberculosis by reacting only with rabbit hyperimmune sera against M.tuberculosis, but did not react with rabbit hyperimmune sera

against other mycobacterial species or strains. When this antigen or hapten was characterised by thin layer chromatography using diisobutylketone : acetic acid : water = 80:50:7 and chloroform : methanol : water = 50:49:1 as developing agents, its Rf value was different from the Rf value of phenolic glycolipid-I (PGL-I) of M. leprae.

When this species-specific antigen or hapten, crude sonicated and crude lipid extract (total lipid) antigens from M. tuberculosis were evaluated by sera from different groups of subjects, this species-specific antigen or hapten showed high specificity. This species-specific antigen is now being considered for use as a routine laboratory diagnosis of tuberculosis at the "Tuberculosis Control Center Region 10" Chiang Mai.

We tried to study the possibility of serodiagnosis of tuberculosis in villages by developing a filter paper preserved blood technique for antibody preservation and an eluation technique for eluting antibody from filter paper preserved blood. Our preserve and eluating techniques worked well in our hand. We also tried to develop a spot test for preliminary serodiagnosis of tuberculosis. The species-specific antigen was spotted on a cellulose acetate paper, dried, tested serum added, washed, rabbit anti-human globulin added, washed. Goat anti-rabbit globulin conjugated with peroxidase was added, washed, substrate added. Positive result yielded pink color while negative test gave colorless. Our results seem promising to use this technique for field study.

6. PUBLICATIONS OR MANUSCRIPTS RESULTING FROM THIS WORK.

1. Songsiri, S., Makonkawkeyoon, L., Dettrairat, S. and Makonkawkeyoon, S. (1988) Amplified enzyme linked immunosorbent assay. Journal Medical Technologists Association of Thailand (in press).
2. Makonkawkeyoon, S., Makonkawkeyoon, L., Songsiri, S. and Dettrairat, S. Evidence for species-specific lipid antigen from *Mycobacterium tuberculosis*. American Review of Respiratory Disease (submitted for publication).
3. Pongtong, B., Makonkawkeyoon, S., Songsiri, S. and Niraruth, V. (1988) Comparative study between culture method and ELISA technique in the diagnosis of tuberculous meningitis. Thai Health Journal (in press).
4. Niraruth, V., Pongtong, B., Makonkawkeyoon S., Songsiri, S. and Dettrairat S. Comparison of bacterial culture and enzyme linked immunosorbent assay for the diagnosis of pulmonary tuberculosis. Thai Journal of Tuberculosis and Chest Diseases (submitted for publication).
5. Pontong, B., Ukarasevi, P., Makonkawkeyoon, S. and Songsiri, S. Evaluation of seroepidemiology of pulmonary tuberculosis by enzyme linked immunosorbent assay. Thai Journal of Tuberculosis and Chest Diseases (submitted for publication).

6. Pongtong, B., Makonkawkeyoon, S., Songsiri, S. and Niraruth, V. Comparison of bacterial culture and enzyme linked immunosorbent assay for the diagnosis of tuberculous meningitis. Thai Journal of Tuberculosis and Chest Diseases (submitted for publication).

7. Makonkawkeyoon, S., Songsiri, S., Dettrairat, S. and Makonkawkeyoon, L. Evaluation of species-specific antigen from Mycobacterium tuberculosis for serodiagnosis of tuberculosis (manuscript in preparation).

8. Makonkawkeyoon, S. and Kasinrerk, W. Interleukin 1 and interleukin 2 production in tuberculous patients (manuscript in preparation).

Table 1.Test for specificity of eluates from florisil column by ELISA

Fraction No.	Rabbit Hyperimmune Sera to Mycobacterium			
	M.tuberculosis	*M.avium*	*M.bovis*(BCG)	*M.paratuberculosis*
1% Methanol in chloroform				
9	2.061	2.411	2.474	2.671
11	2.049	0.039	0.024	0.033
13	2.056	0.000	0.003	0.014
15	2.070	0.016	0.019	0.068
17	2.110	0.002	0.013	0.081
19	2.033	0.022	0.018	0.051
21	1.918	0.014	0.011	0.009
23	1.932	0.027	0.005	0.055
25	2.000	0.023	0.039	0.063
5% Methanol in chloroform				
41	2.076	0.211	0.364	0.313
43	2.669	0.385	2.673	2.435
45	1.539	0.232	0.255	0.795
47	1.091	0.341	0.260	0.601
49	1.586	0.693	0.194	1.093
51	0.943	0.440	0.226	0.879
53	1.104	0.652	0.372	0.956
55	1.084	0.708	0.314	0.995
57	1.069	0.843	0.339	0.905
59	0.920	0.687	0.310	0.924
61	0.929	0.662	0.344	0.736
63	1.444	1.044	0.375	0.841
65	1.066	0.926	0.453	0.836
67	1.054	0.997	0.400	1.097
69	1.180	0.921	0.355	1.037
71	1.225	0.887	0.423	1.171
73	1.690	1.752	1.256	1.254
75	2.051	2.003	1.404	1.589
77	1.359	1.210	0.608	1.071
79	2.245	1.811	0.797	1.346

Table 2. Test for specificity of pooled fractions from florisil column

Rabbit antiserum to	Blank*	crude lipid from *M.tuberculosis*	pooled fraction 11-25
M.avium TMC 706	0.000	2.088	0.019
M.avium NIHJ 724	0.057	1.253	0.185
M.bovis (BCG) TMC 1030	0.234	1.897	0.424
M.bovis NIHJ 1608	0.000	0.038	0.000
M.chelonei	0.000	1.208	0.000
M.flavescense	0.017	2.195	0.055
M.flavescense 1614	0.000	1.478	0.000
M.fortuitum ATCC 6841	0.000	1.448	0.000
M.fortuitum NIHJ 1615	0.000	1.032	0.000
M.gastri NIHJ 1616	0.000	1.833	0.236
M.gordonae TMC 1324	0.000	0.654	0.000
M.intracellulare	0.000	0.013	0.000
M.intracellulare TMC 1403	0.000	1.450	0.540
M.kansasii TMC 1204	0.000	1.213	0.000
M.marinum	0.000	1.545	0.000
M.nonchromogenicum NIHJ 1622	0.000	0.347	0.000
M.phlei	0.000	0.694	0.000
M.phlei NIHJ 1624	0.000	1.665	0.000
M.scrofulaceum	0.000	0.807	0.000
M.smegmatis TMC 1546	0.000	0.645	0.000
M.smegmatis NIHJ 1628	0.000	1.380	0.062
M.terrae	0.000	1.708	0.000
M.triviale	0.000	0.173	0.000
M.tuberculosis H37Rv TMC 102	0.000	1.599	2.319
M.vaccae TMC 1526	0.000	0.036	0.000
M.bovis (BCG)	0.131	1.536	0.000
M.duvalii	0.318	2.012	0.000
M.paratuberculosis (bovine)	0.033	2.182	0.046

TABLE 3 CLASSIFICATION OF SAMPLES.

SAMPLE GROUP	NUMBER OF SAMPLE
NORMAL FROM BLOOD BANK	199
PEOPLE IN VILLAGES	220
TUBERCULOSIS PATIENTS	406
ATYPICAL MYCOBACTERIAL INFECTION	16
LEPROSY PATIENTS	96
TOTAL	910

TABLE 4 DETAIL OF VILLAGE SERA SAMPLES.

GROUP	NUMBER OF SAMPLE	% OF TOTAL
TOTAL SAMPLE	220	100.00
HISTORY OF CONTACT (NO)	112	50.90
HISTORY OF CONTACT (YES)	94	42.72
DISCHARGED TB. PATIENTS	14	6.36

TABLE 5 DETAIL OF TUBERCULOSIS SERA SAMPLES.

GROUPR		NUMBER OF SAMPLE	%OF TOTAL
TUBERCULOSIS PATIENTS		406	100.00
SMEAR	NEGATIVE	193	47.53
SMEAR	POSITIVE	213	52.46
CULTURE	NEGATIVE	184	45.32
CULTURE	POSITIVE	222	54.67
SMEAR NEG.	CULTURE NEG.	163	40.14
SMEAR NEG.	CULTURE POS.	30	7.38
SMEAR POS.	CULTURE NEG.	21	5.17
SMEAR POS.	CULTURE POS.	192	47.29
HISTORY OF	CONTACT (NO)	334	82.26
HISTORY OF	CONTACT (YES)	72	11.88
HISTORY OF TREATMENT (NO)		319	78.57
HISTORY OF TREATMENT (YES)		17	4.18
ON TREATMENT		70	17.24

END

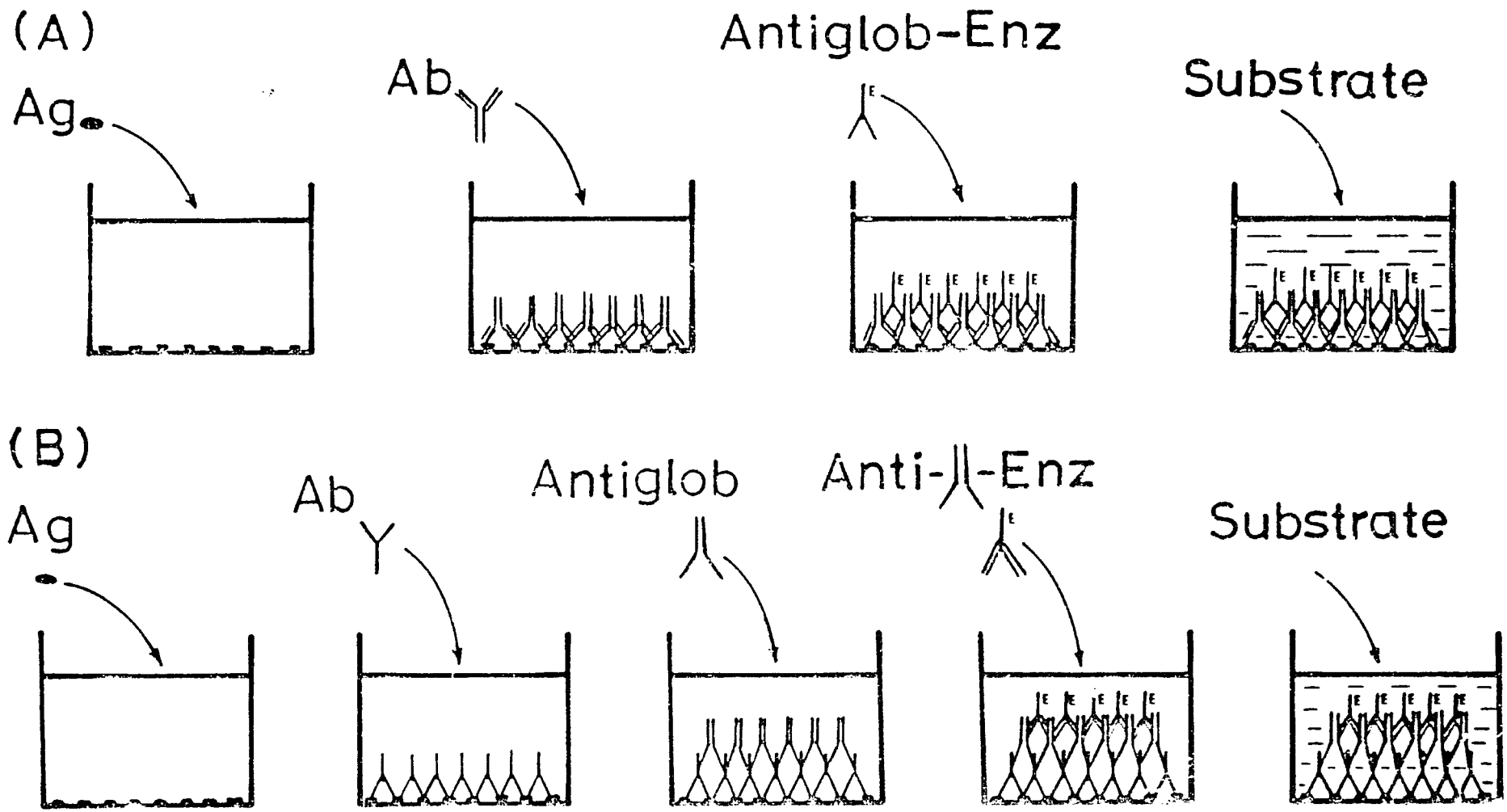

Figure 1. Principle of procedures for ELISA tests: A) ELISA procedure for detecting total lipid antigen and B) amplified ELISA for detecting antibody to purified X-antigen.

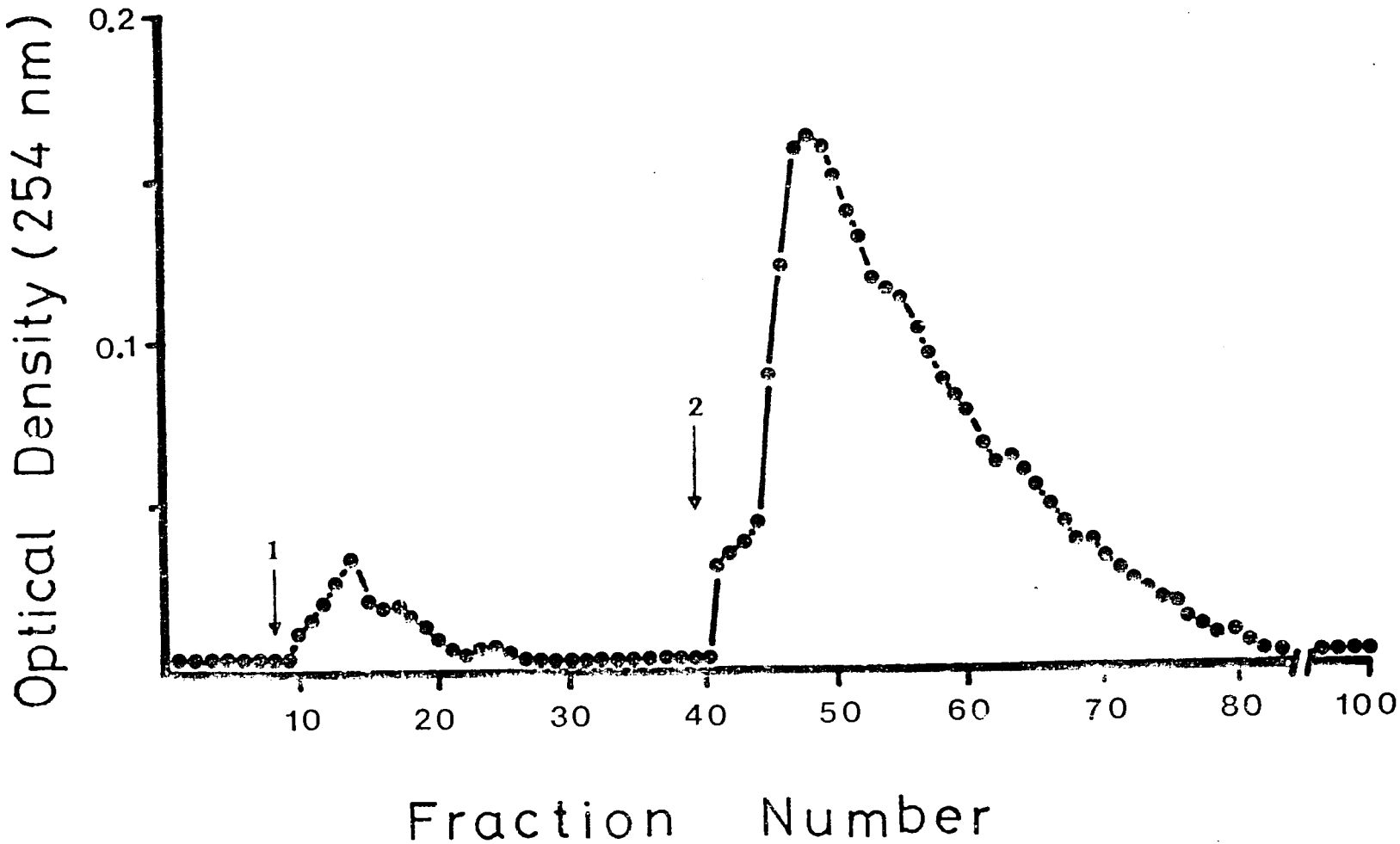

Figure 2. Chromatogram of crude lipid extract from M. tuberculosis passing through florisil column by eluting with 1% and 5% methanol in chloroform (arrow 1 and 2 respectively).

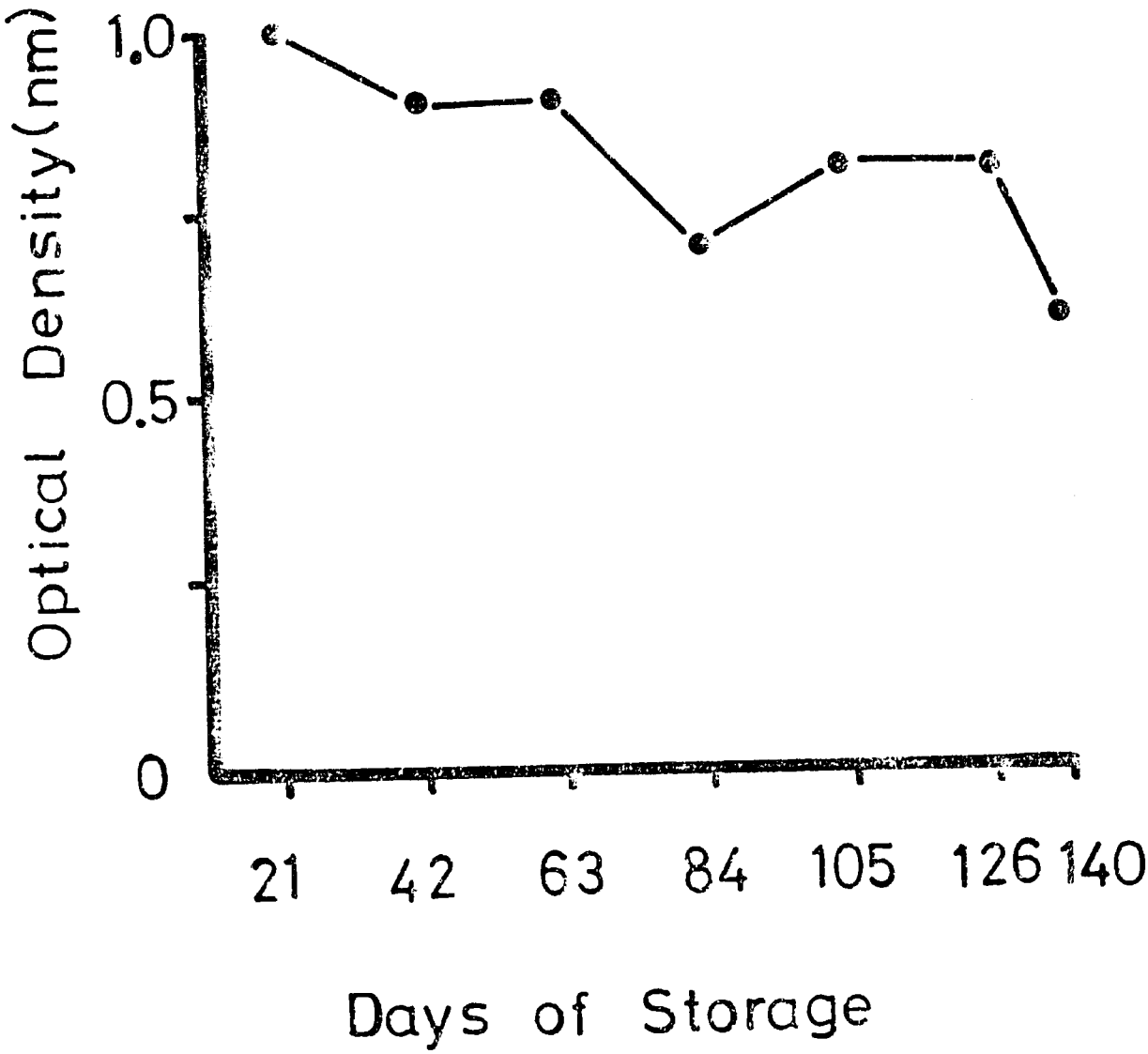

Figure 3. Preservation of antibody activity in whole blood by filter paper for various time of storage.

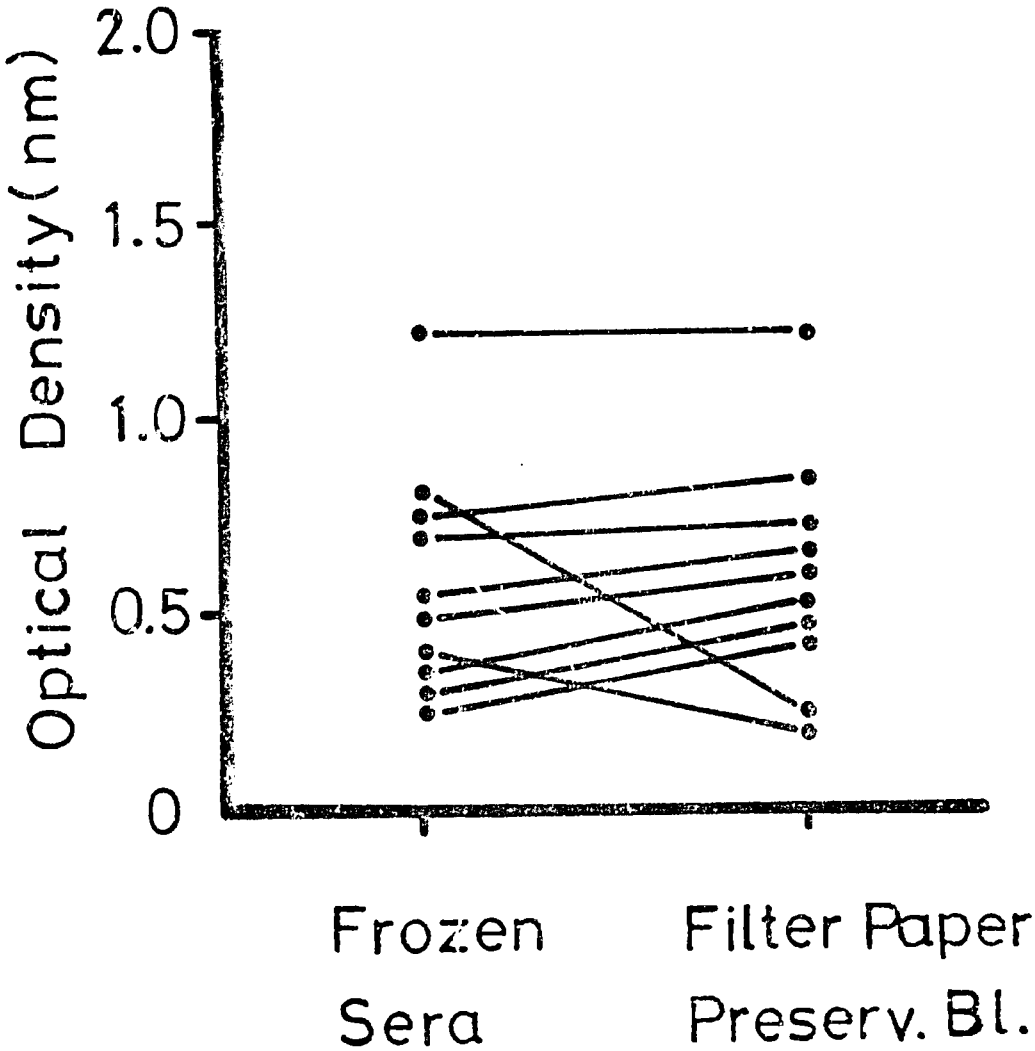

Figure 4. Comparison of antibody preservation by frozen sera and filter paper preserved whole blood (both storage for 60 days).

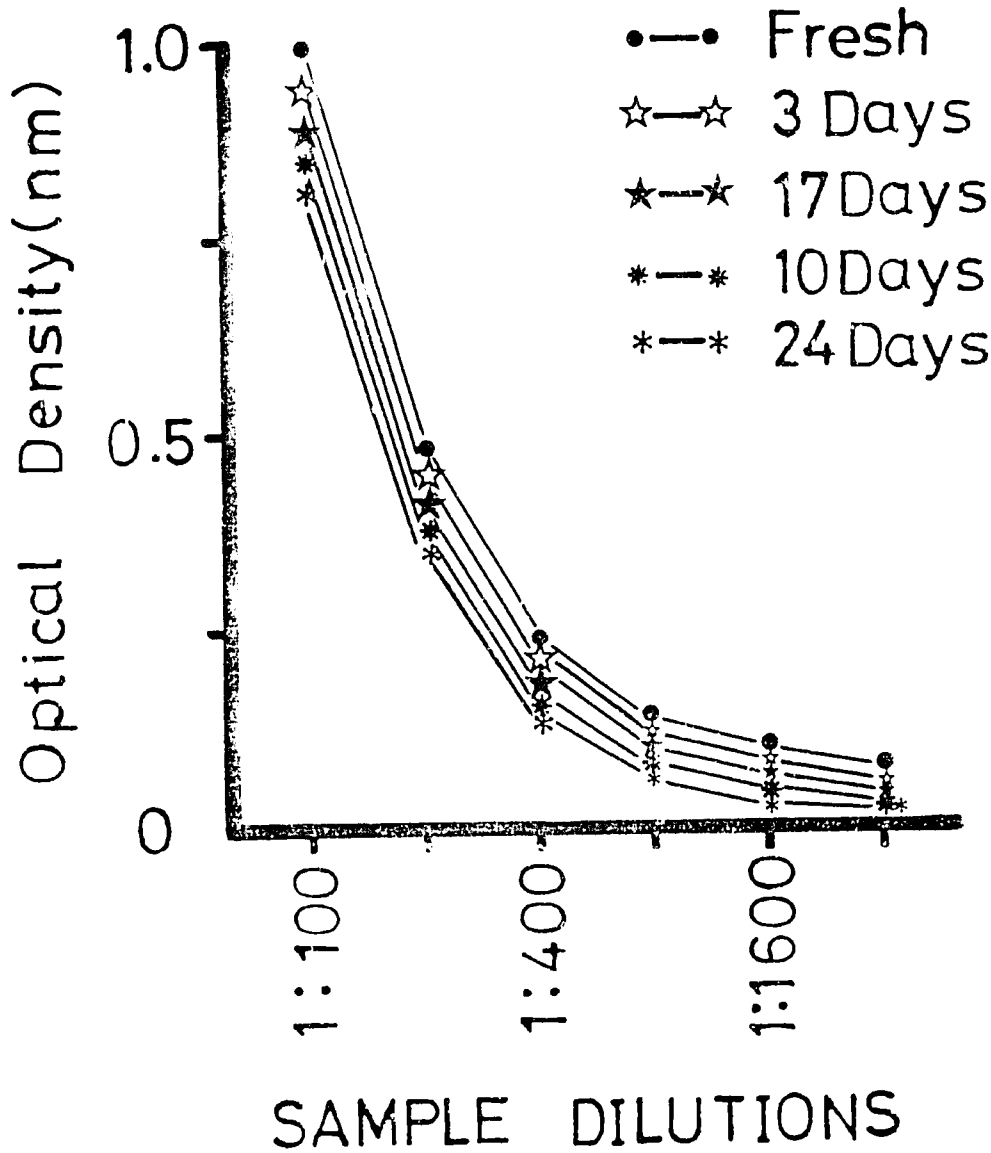

Figure 5. Comparison of ELISA values of various dilutions of fresh sera with preserved blood by filter paper storage at various period of time.

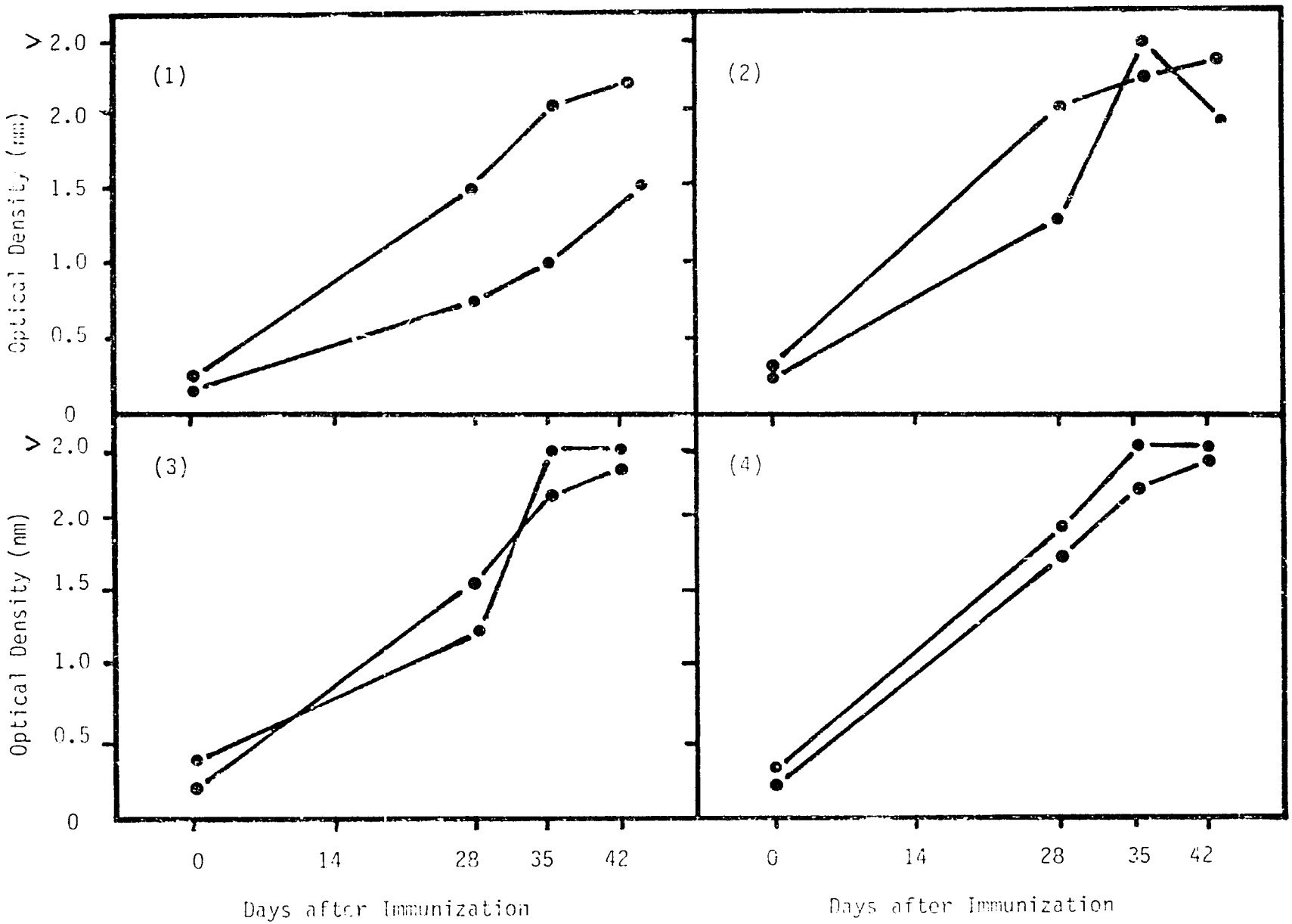

Figure 6. Antibodies response in immunized rabbit against (1) M. avium TMC706, (2) M. avium Serotype 2, NIH724, (3) M. bovis BCG,TMC1030 and (4) M. bovis NIH1608.

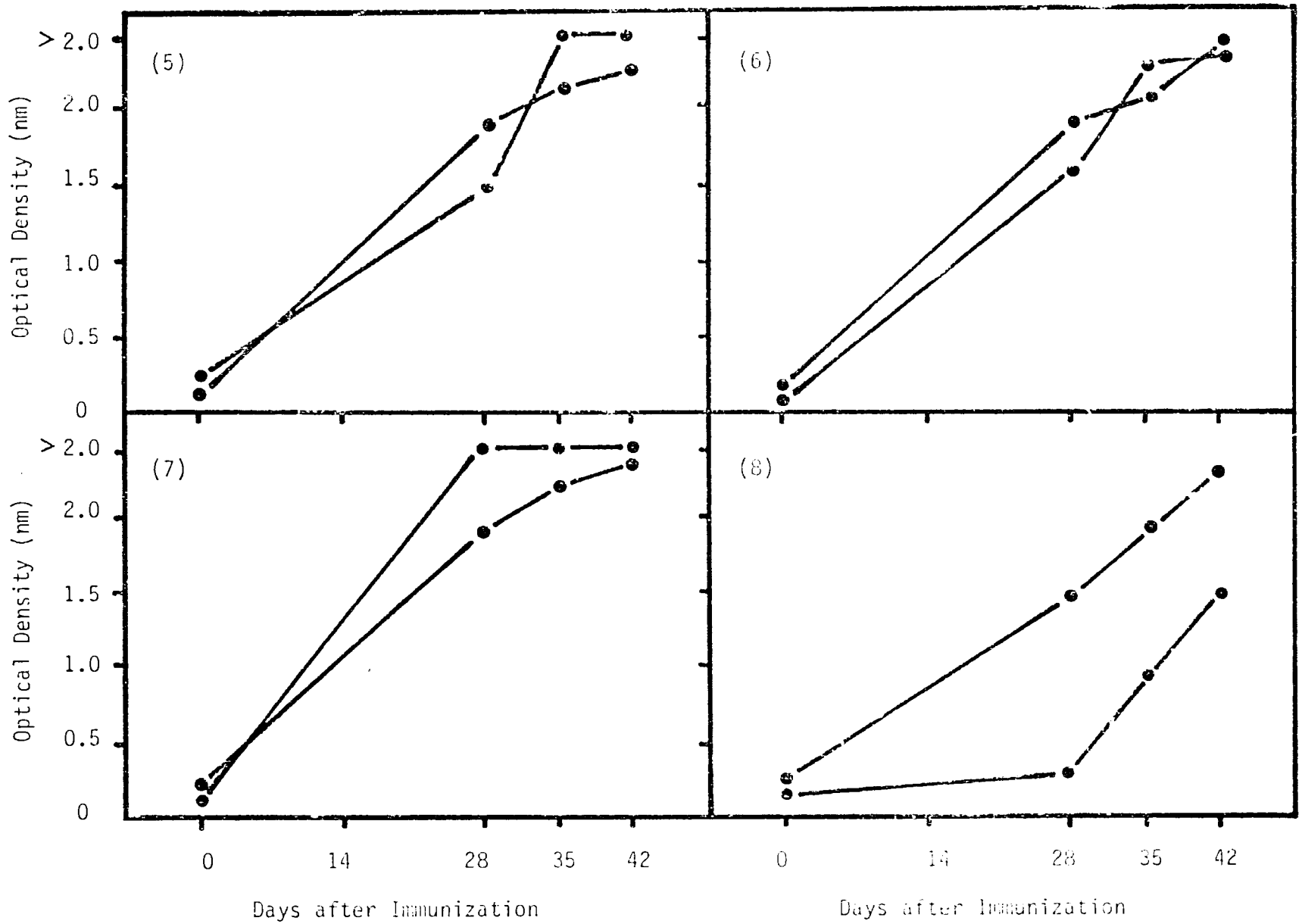

Figure 7. Antibodies response in immunized rabbit against (5) M. chelonei abscessus NIHJ1609,ATCC 19977, (6) M. chelonei chelonei NIHJ1611,ATCC19237, (7) M. flavescense and (8) M. flavescense NIHJ1614,ATCC14474.

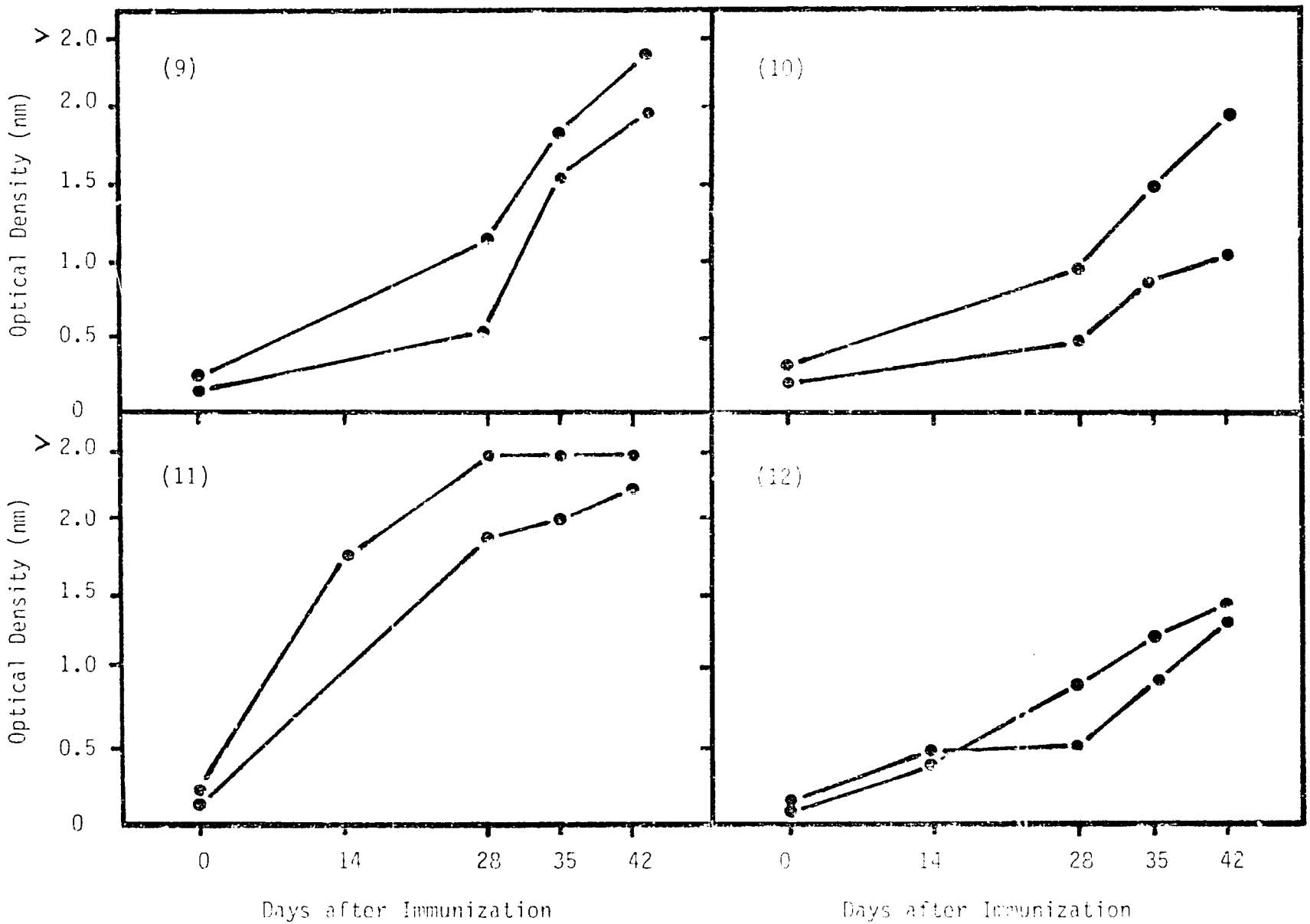

Figure 8. Antibodies response in immunized rabbit against (9) M. fortuitum NIHJ1615,ATCC19542, (10) M. fortuitum TMC1529, (11) M. fortuitum and (12) M. gastri NIHJ 1616,ATCC15754.

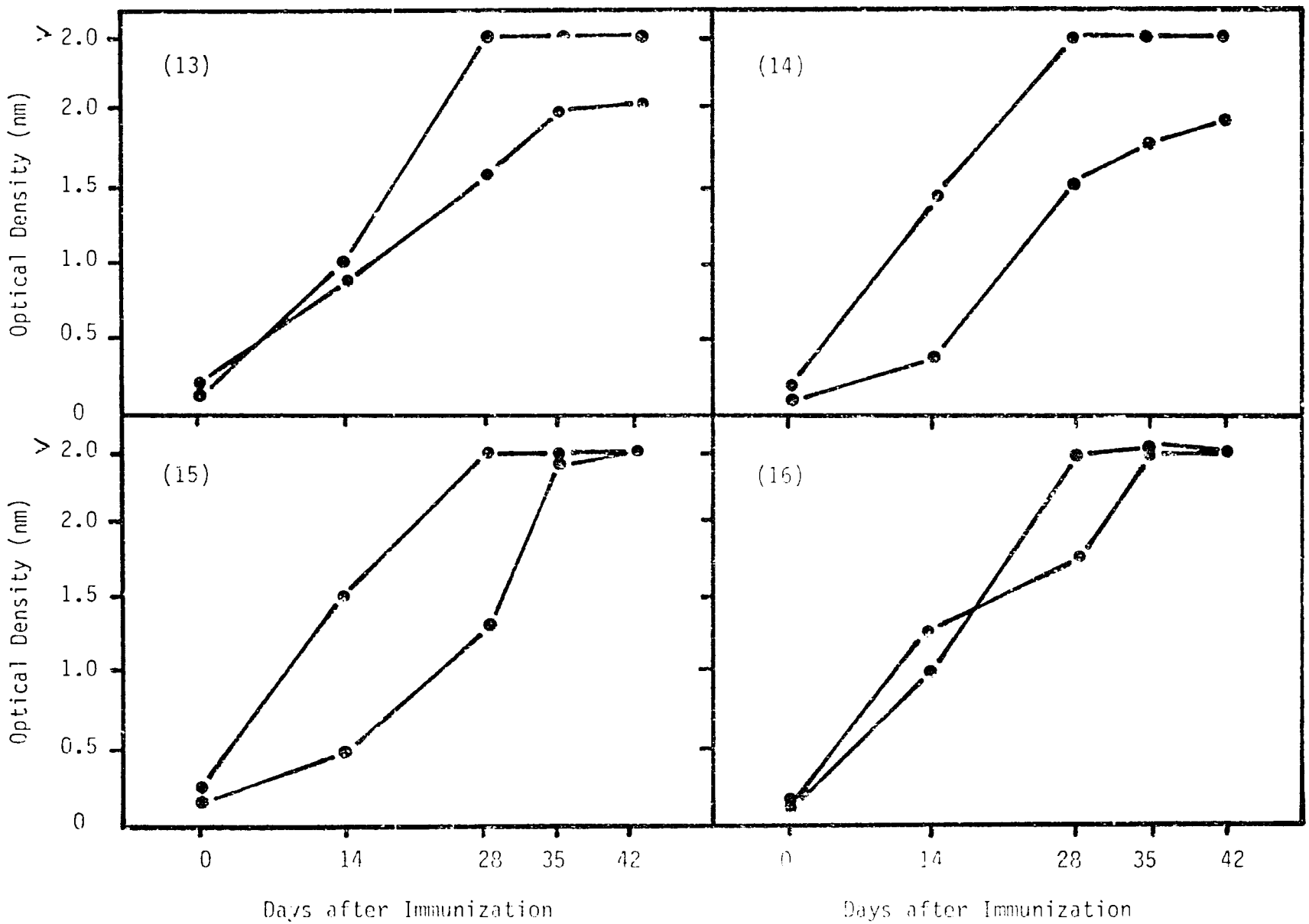

Figure 9. Antibodies response in immunized rabbit against (13) M. gordonae TMC1324, (14) M. intracellulare TMC1403, (15) M. intracellulare NIHJ1618,ATCC15980 and (16) M. kansasii TMC1204.

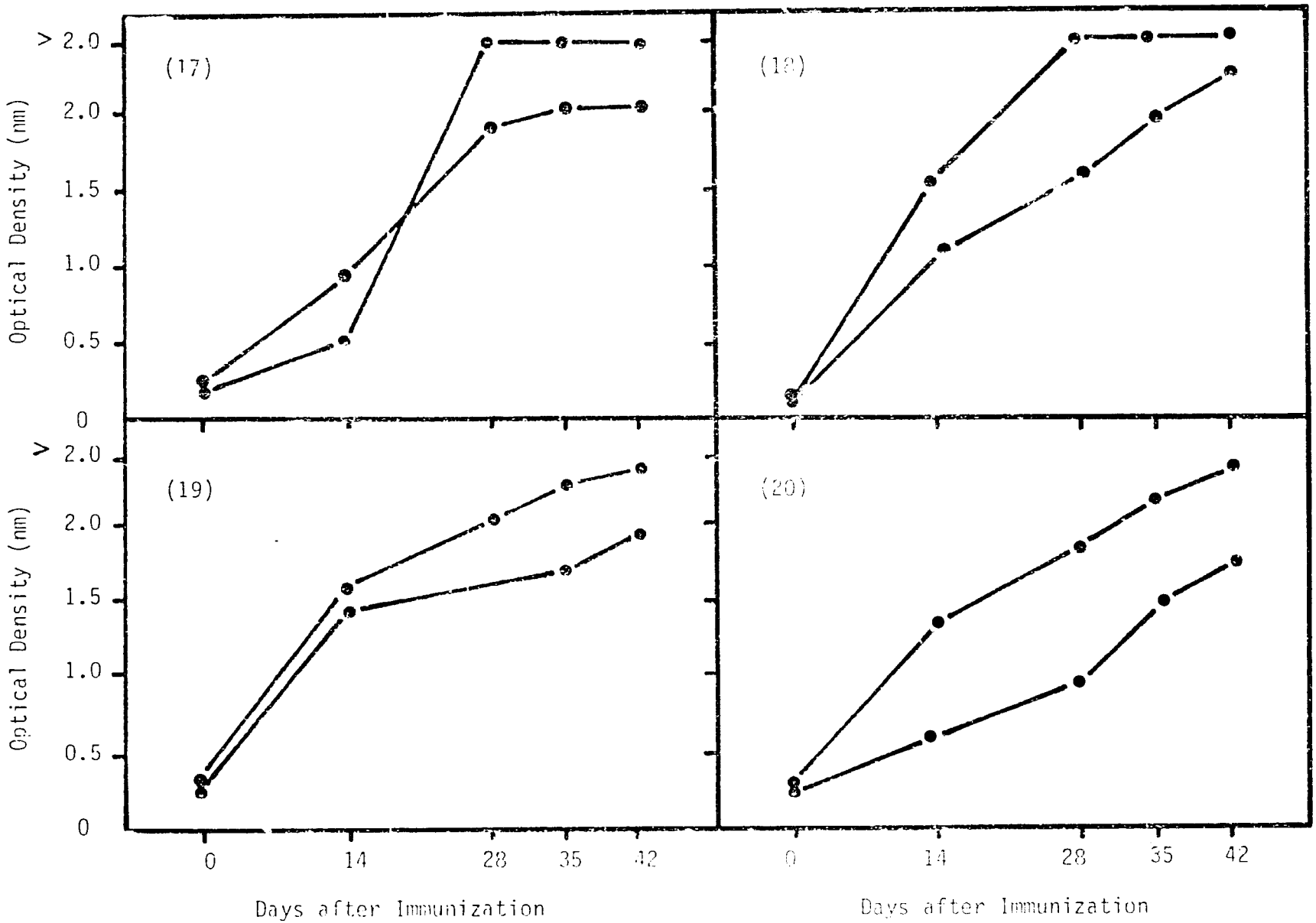

Figure 10. Antibodies response in immunized rabbit against (17) M. malmoense ATCC22620, (18) M. marinum TMC1218, (19) M. nonchromogenicum NIHJ1622,ATCC19530 and (20) M. phlei NIHJ1624,

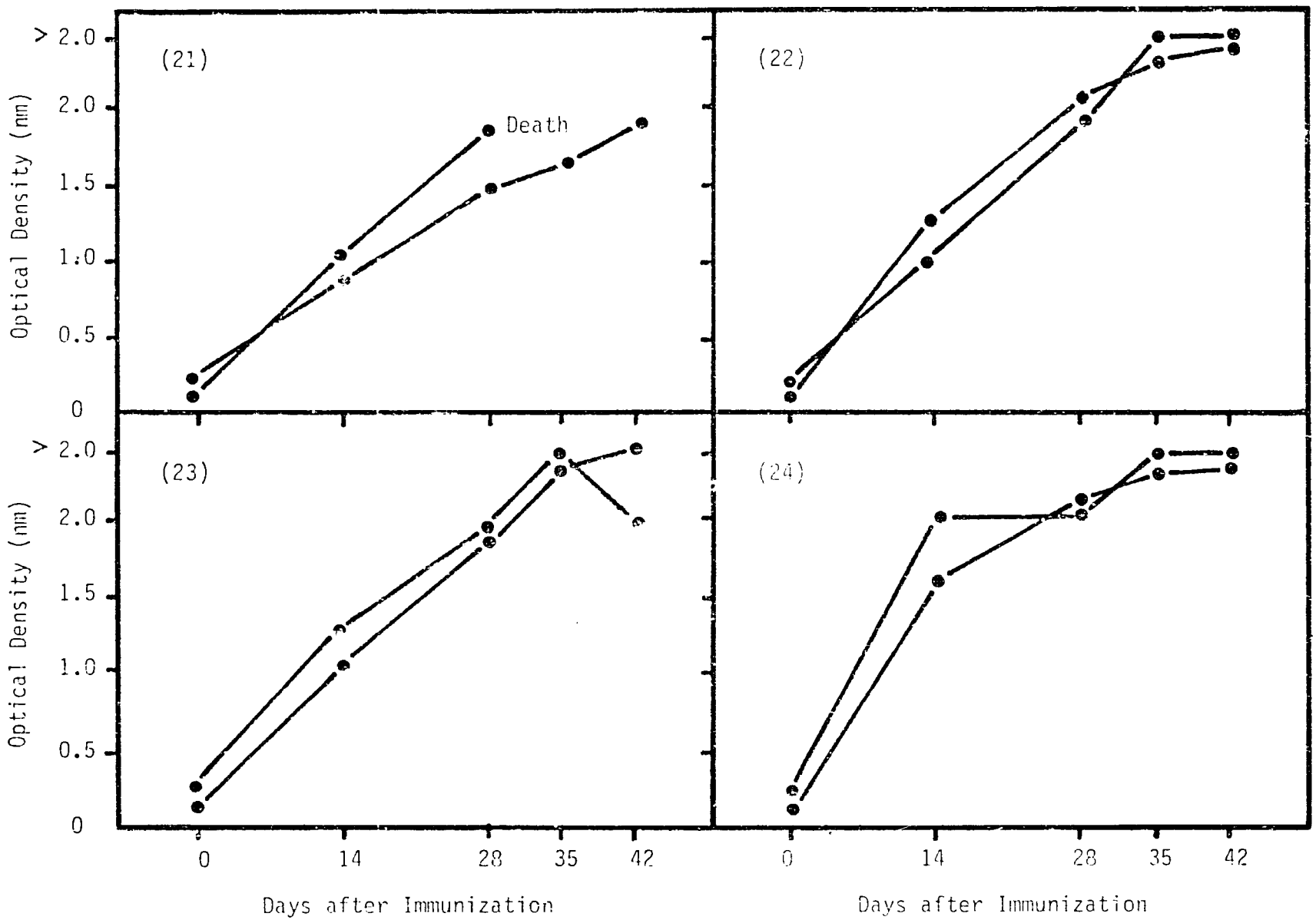

Figure 11. Antibodies response in immunized rabbit against (21) M. phlei TMC1548, (22) M. scrofulaceum, (23) M. scrofulaceum NIHJ1626,ATCC19981 and (24) M. smegmatis TMC1546.

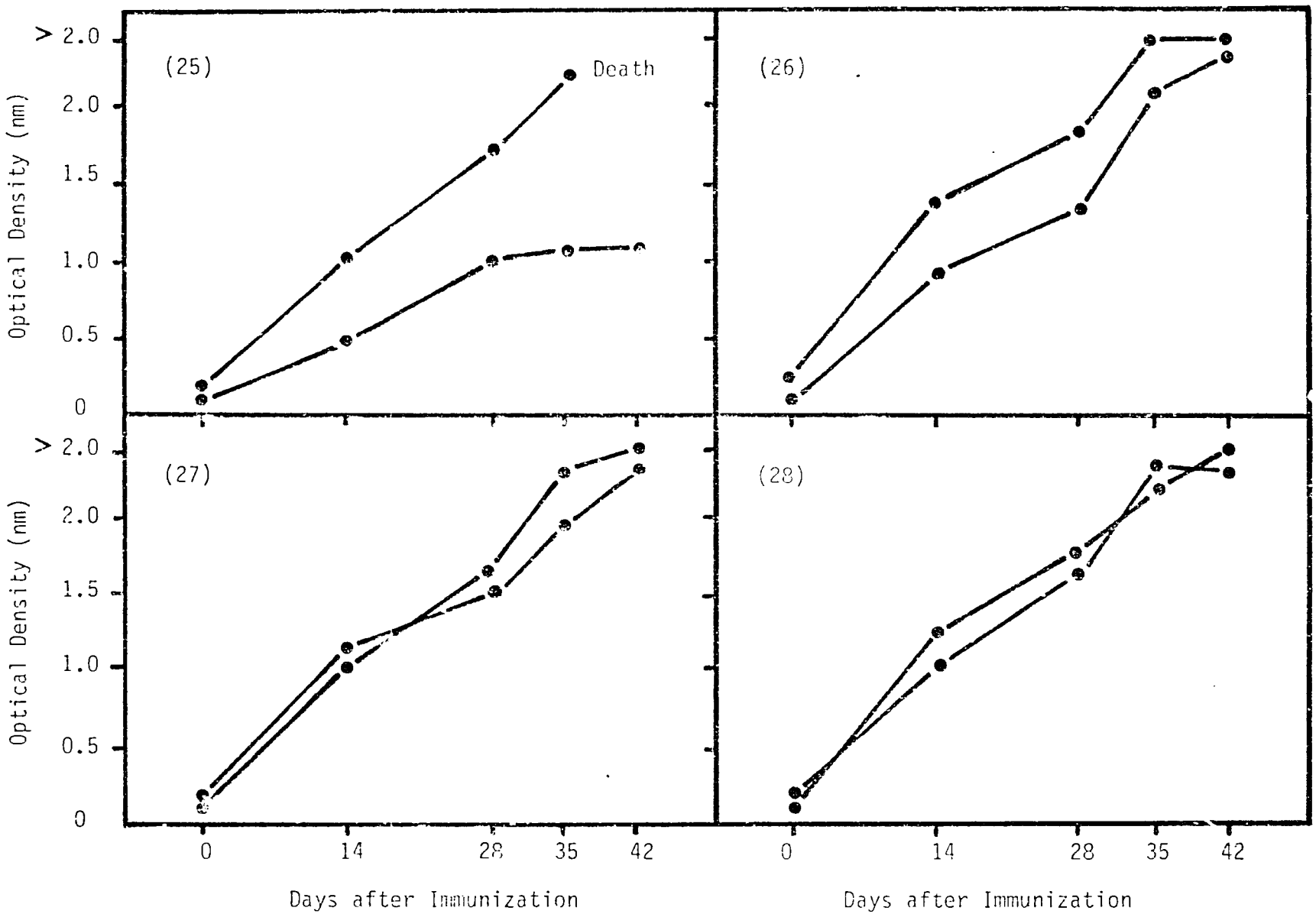

Figure 12. Antibodies response in immunized rabbit against (25) M. smegmatis NIHJ1628,ATCC14468, (26) M. tuberculosis TMC102, (27) M. tuberculosis H37Rv and (28) M. terrae.

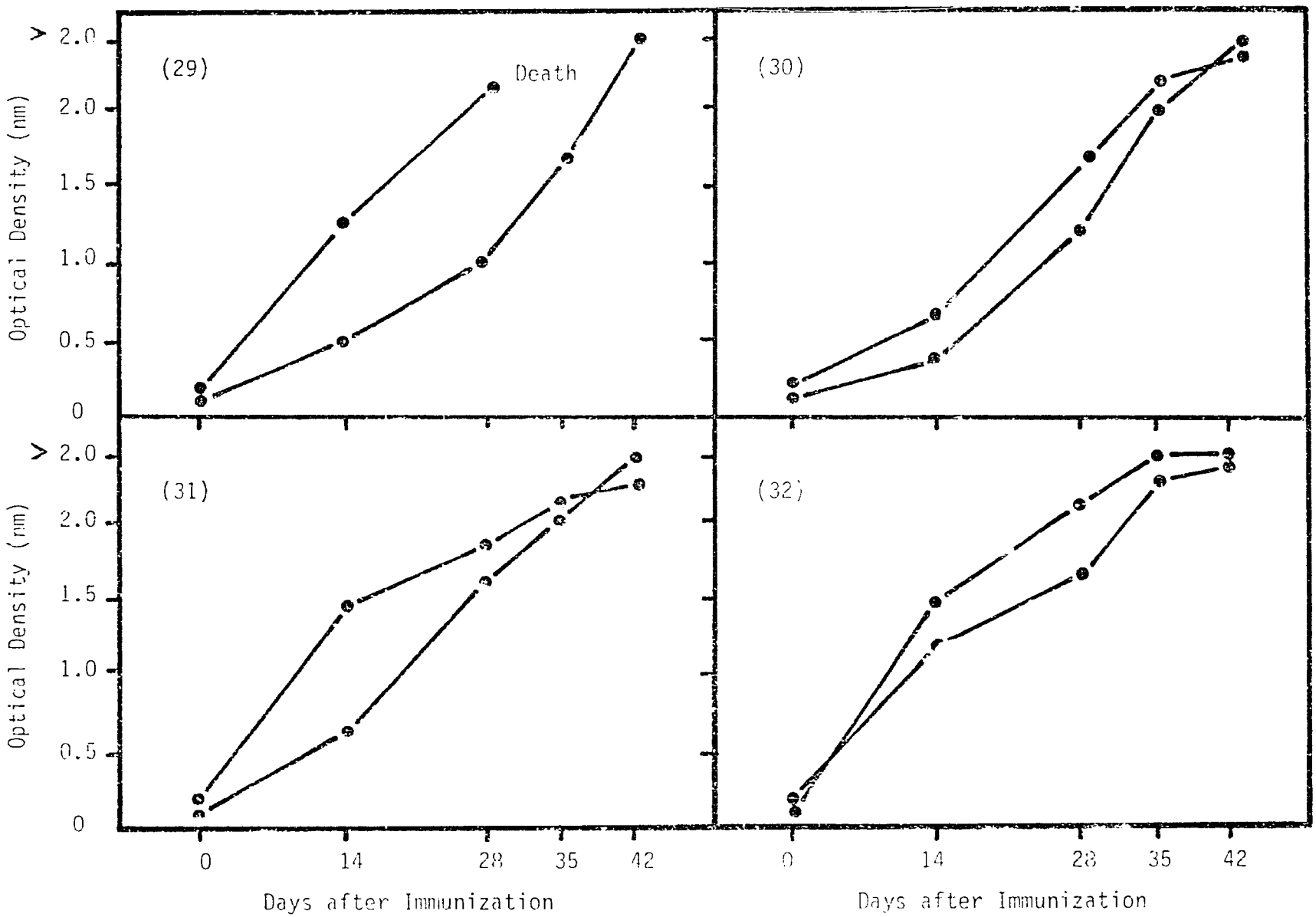

Figure 13. Antibodies response in immunized rabbit against (29) M. terrae NIHJ1603, (30) M. triviale (31) M. vaccae TMC1526 and (32) M. ulcerans NIHJ1636.

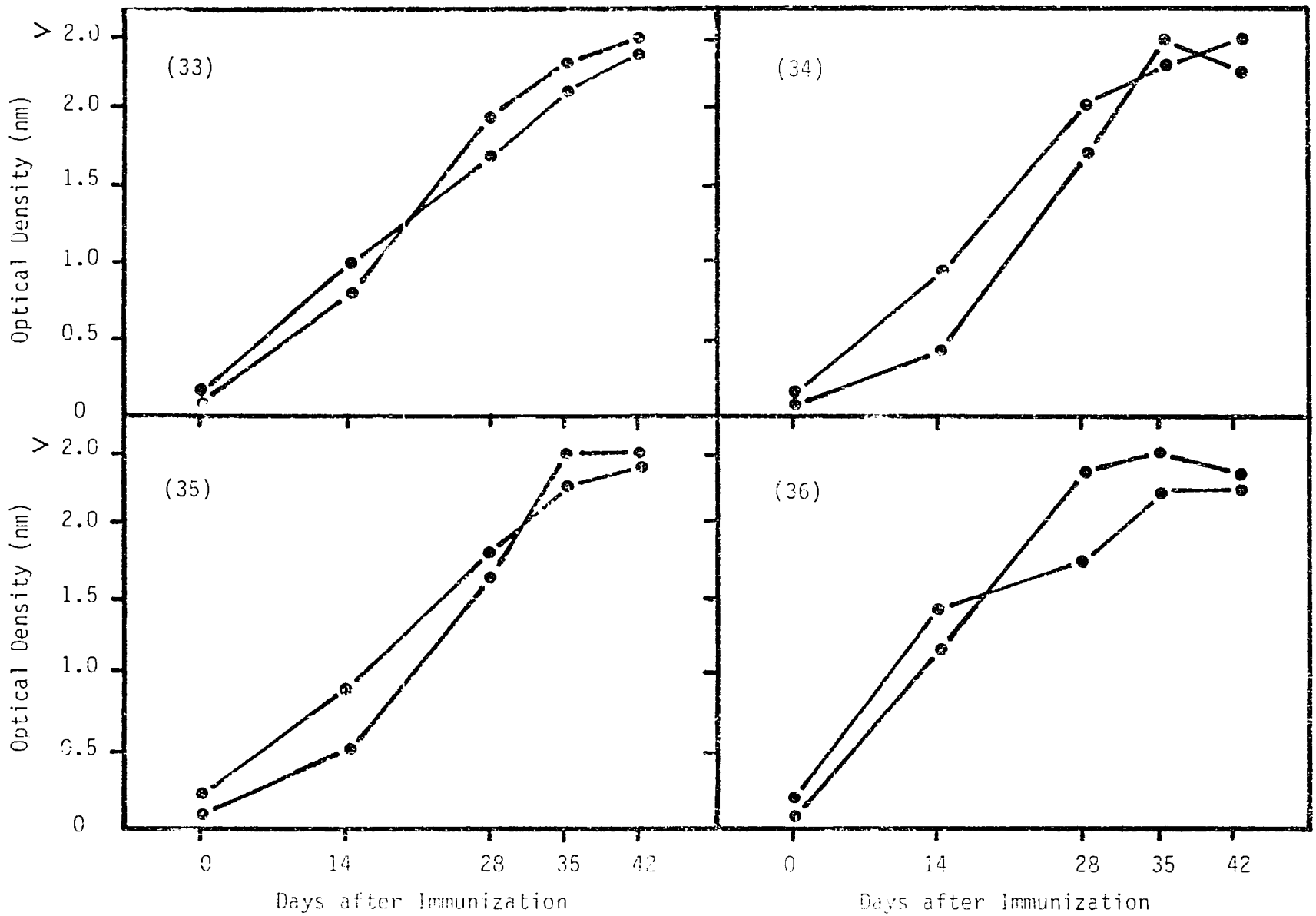

Figure 14. Antibodies response in immunized rabbit against (33) M. xenopi NIHJ1638, (34) M. simiae NIHJ1627, (35) M. szulgai ATCC10831 and (36) M. novum ATCC19621.

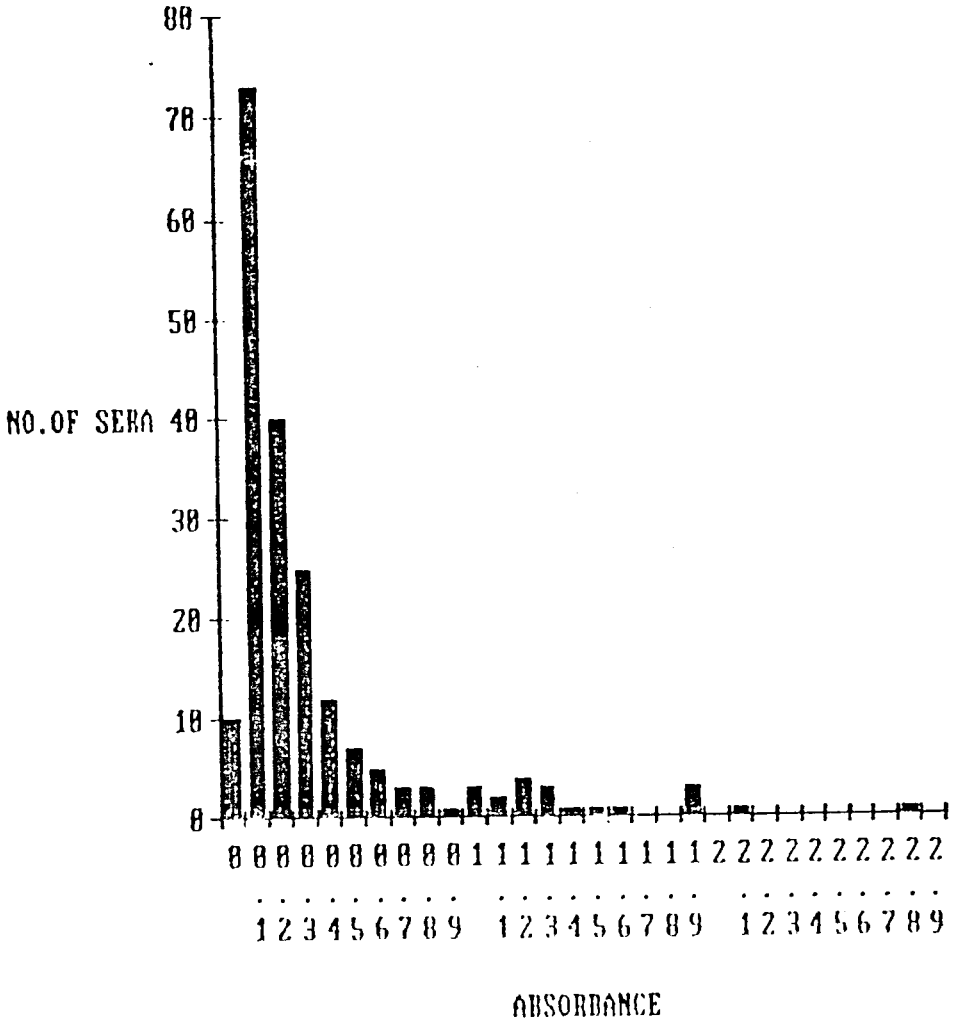

Figure 15.ELISA value of sera from smear negative of acid fast bacilli subjects against X-antigen.

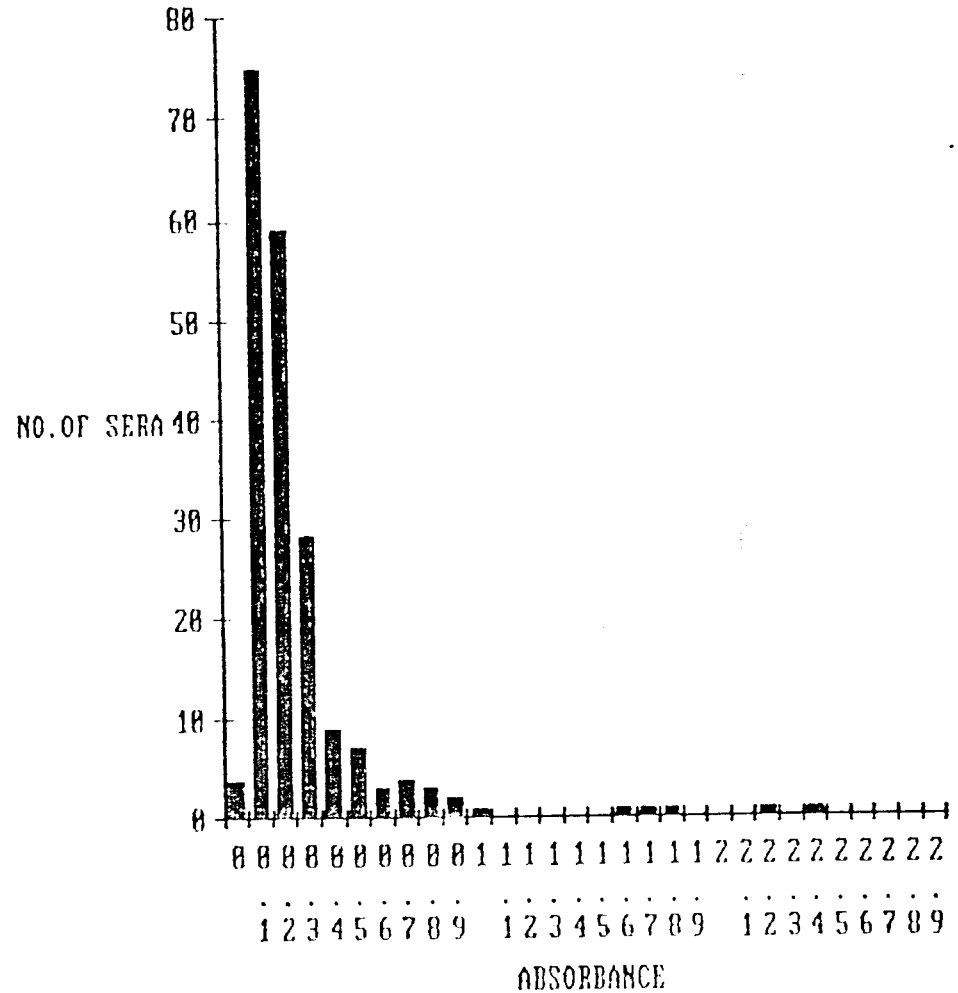

Figure 16.ELISA value of filter paper preserved blood extract from smear negative of acid fast bacilli subjects against X-antigen.

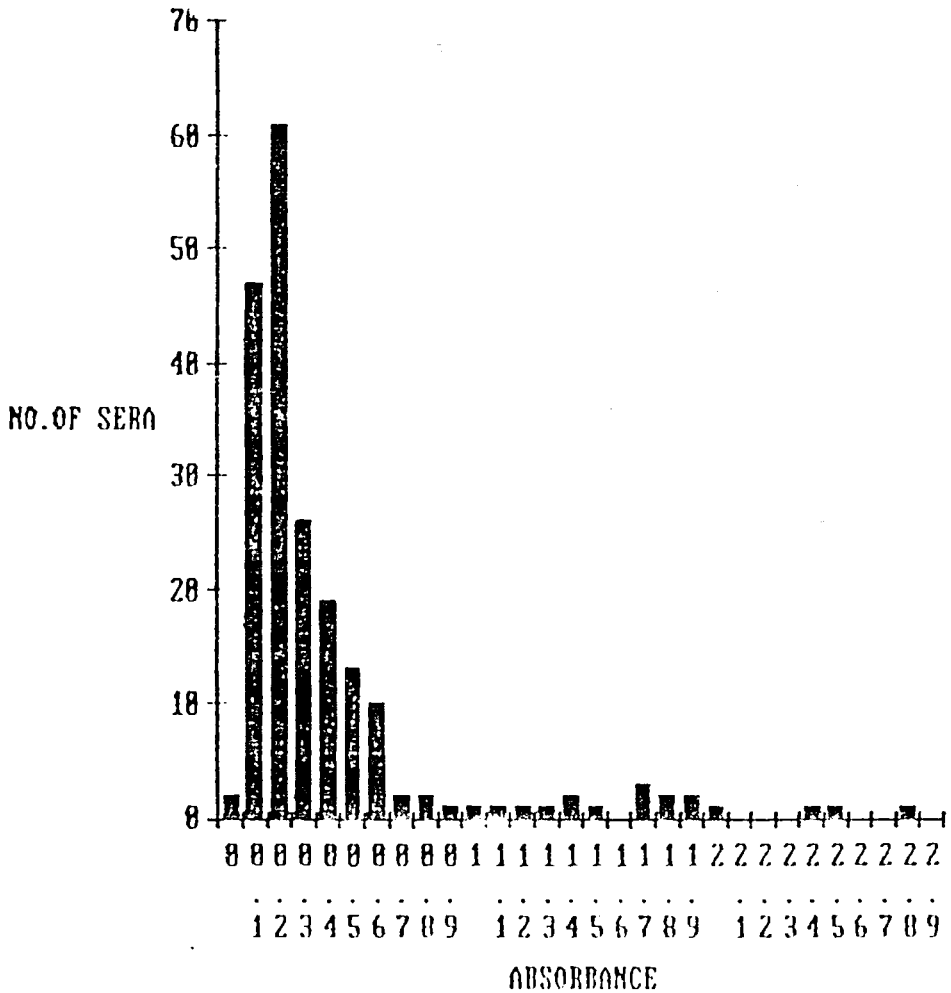

Figure 17.ELISA value of sera from smear negative of acid fast bacilli subjects against total lipid antigen.

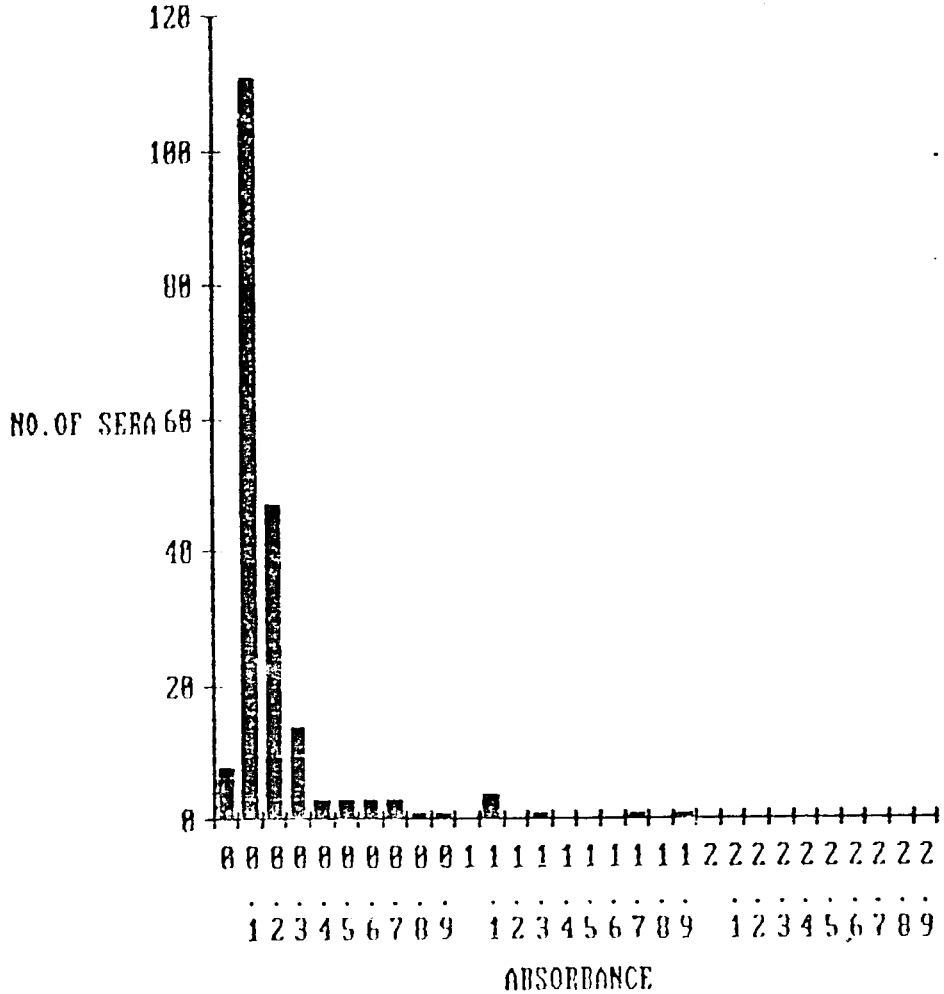

Figure18. ELISA value of filter paper preserved blood extract from smear negative of acid fast bacilli subjects against total lipid antigen.

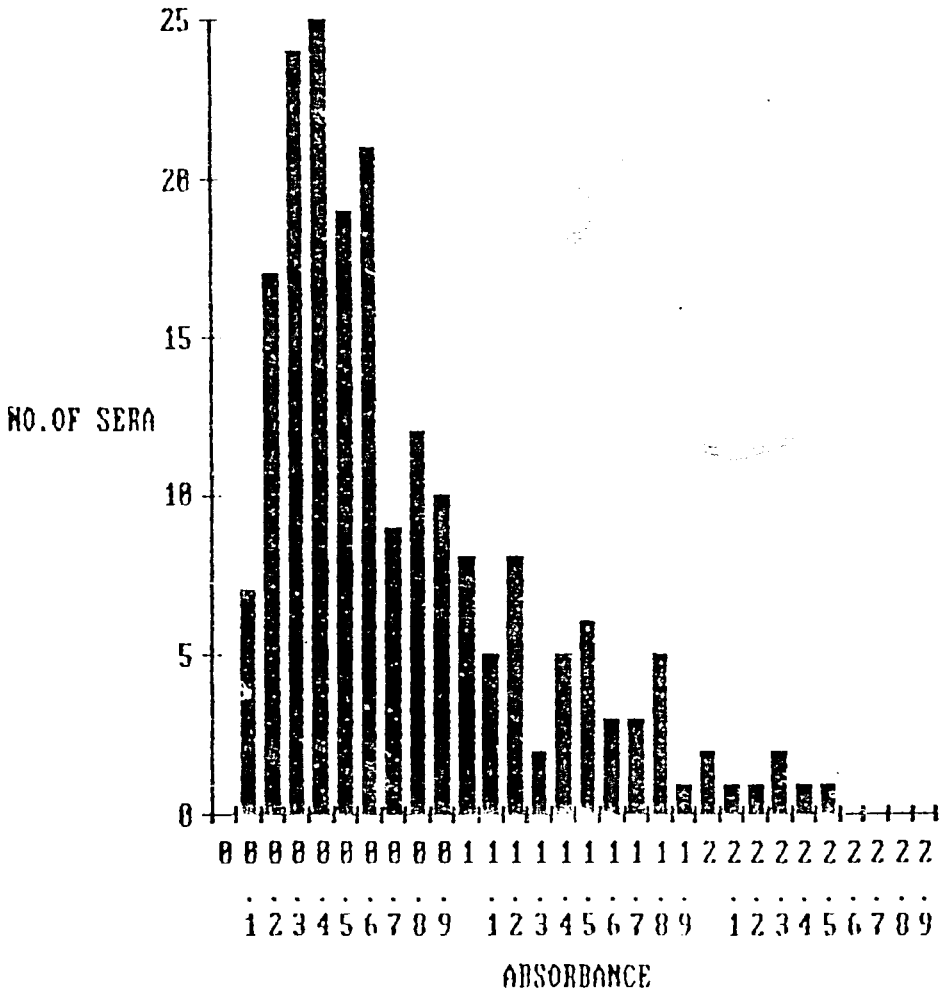

Figure19. ELISA value of sera from smear negative of acid fast bacilli subjects against crude sonicated antigen.

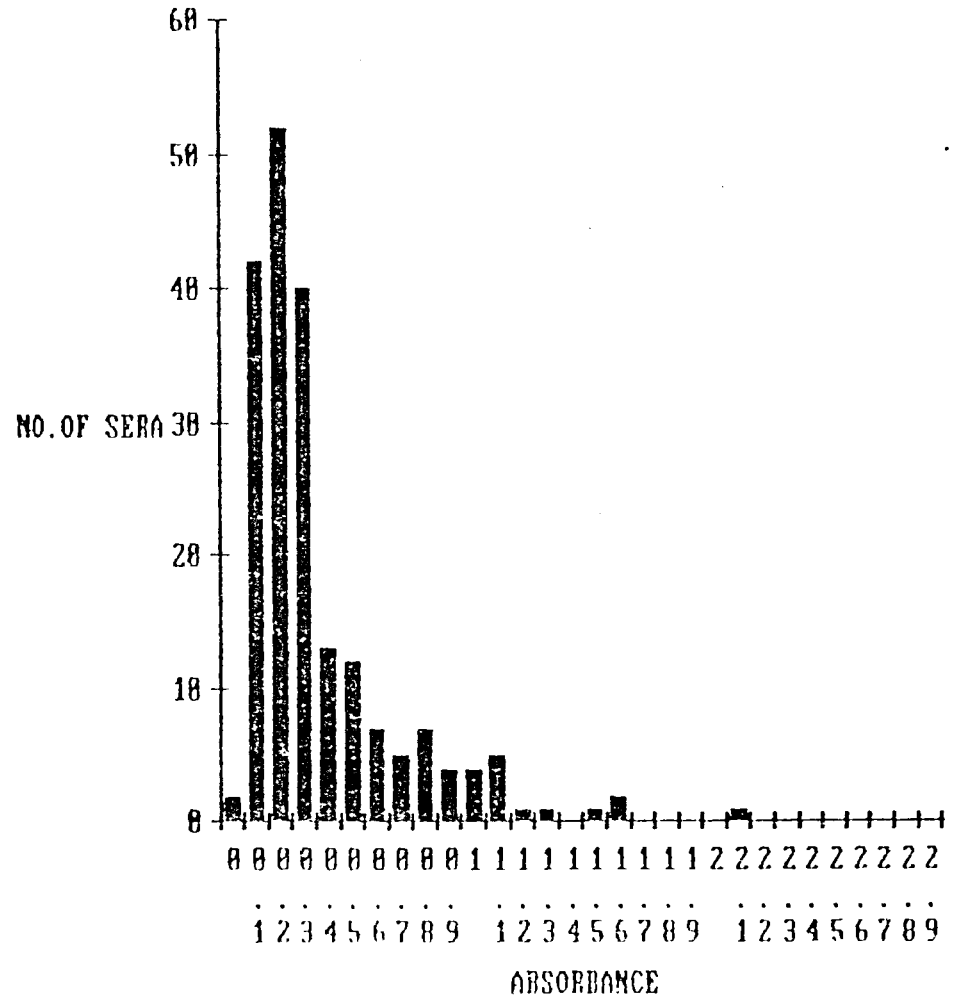

Figure 20.ELISA value of filter paper preserved blood extract from smear negative of acid fast bacilli subjects against crude sonicated antigen.

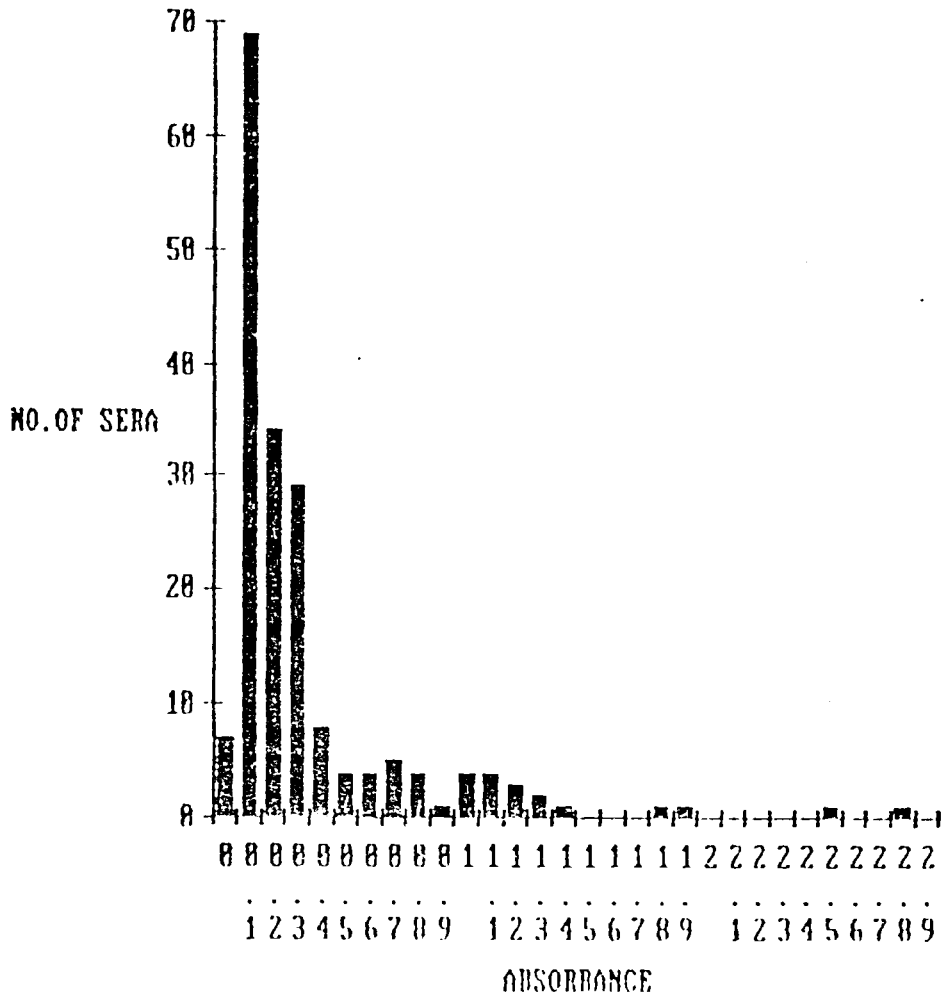

Figure 21. ELISA value of sera from culture negative of M. tuberculosis subjects against X antigen.

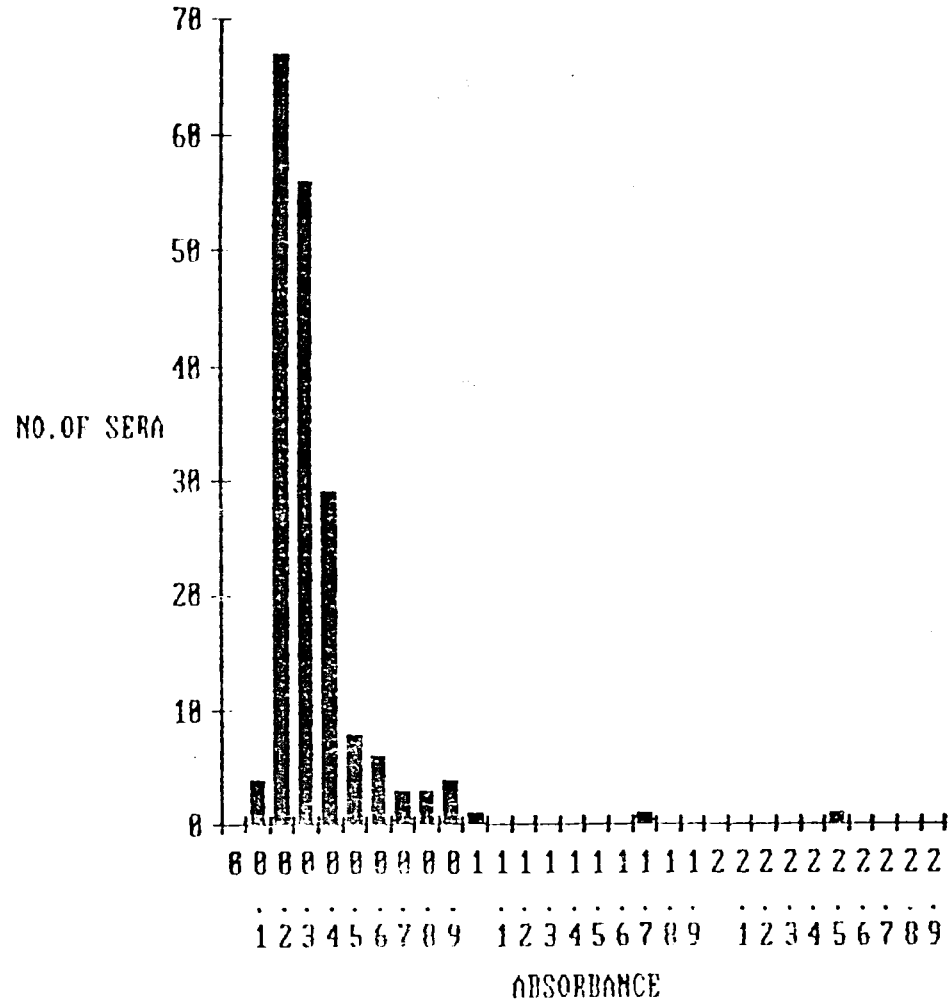

Figure 22. ELISA value of filter paper preserved blood extract from culture negative of M. tuberculosis subjects against X antigen.

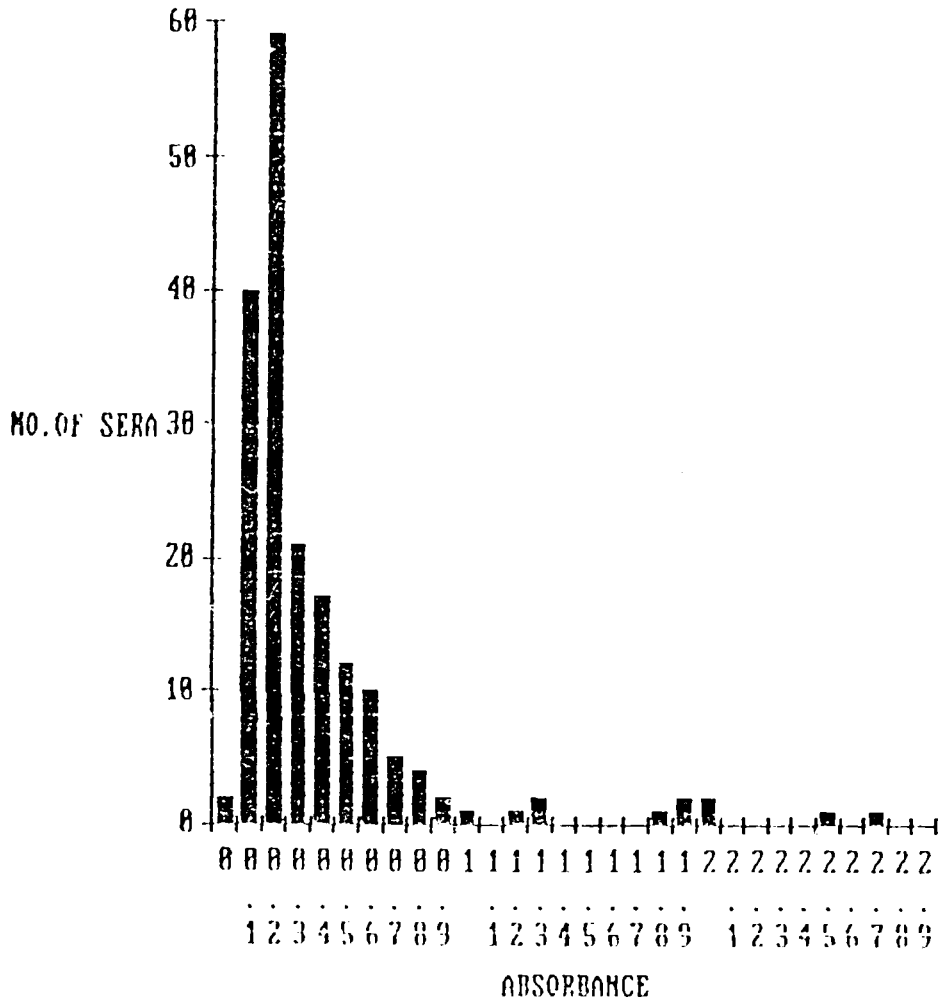

Figure 23. ELISA value of sera from culture negative of M. tuberculosis subjects against total lipid antigen.

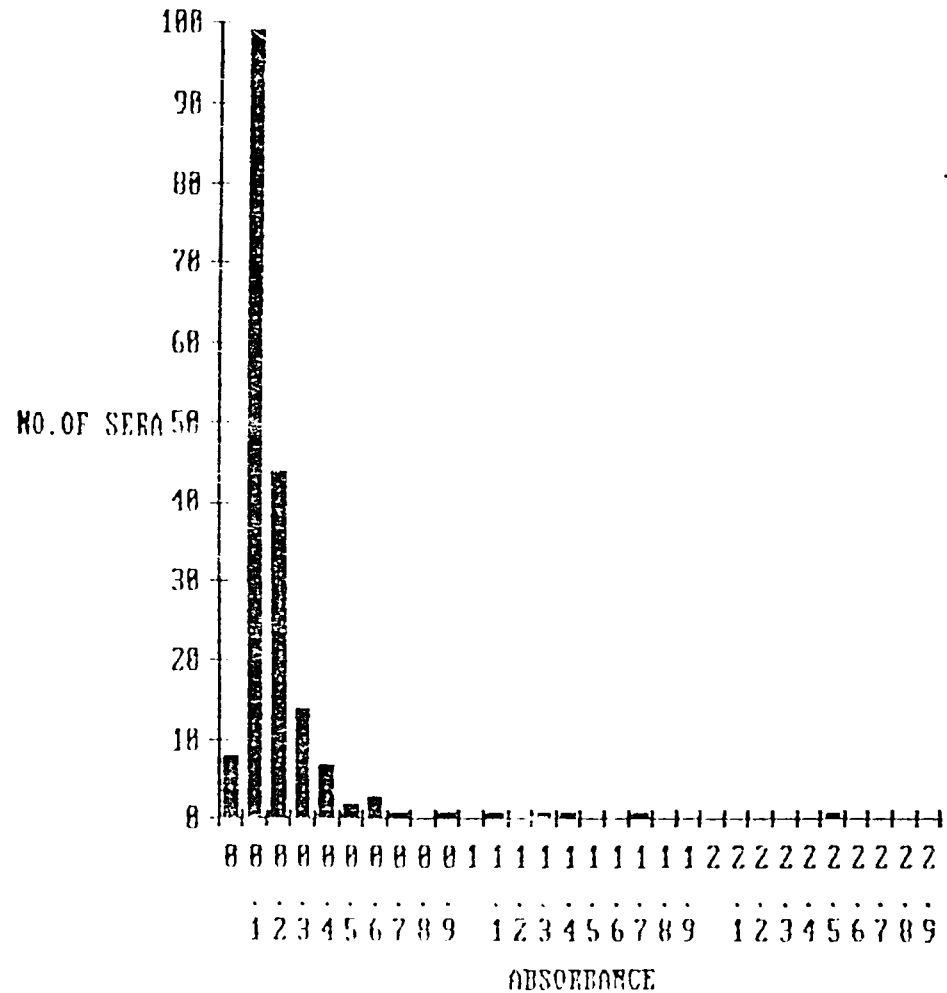

Figure 24. ELISA value of filter paper preserved blood extract from culture negative of M. tuberculosis subjects against total lipid antigen.

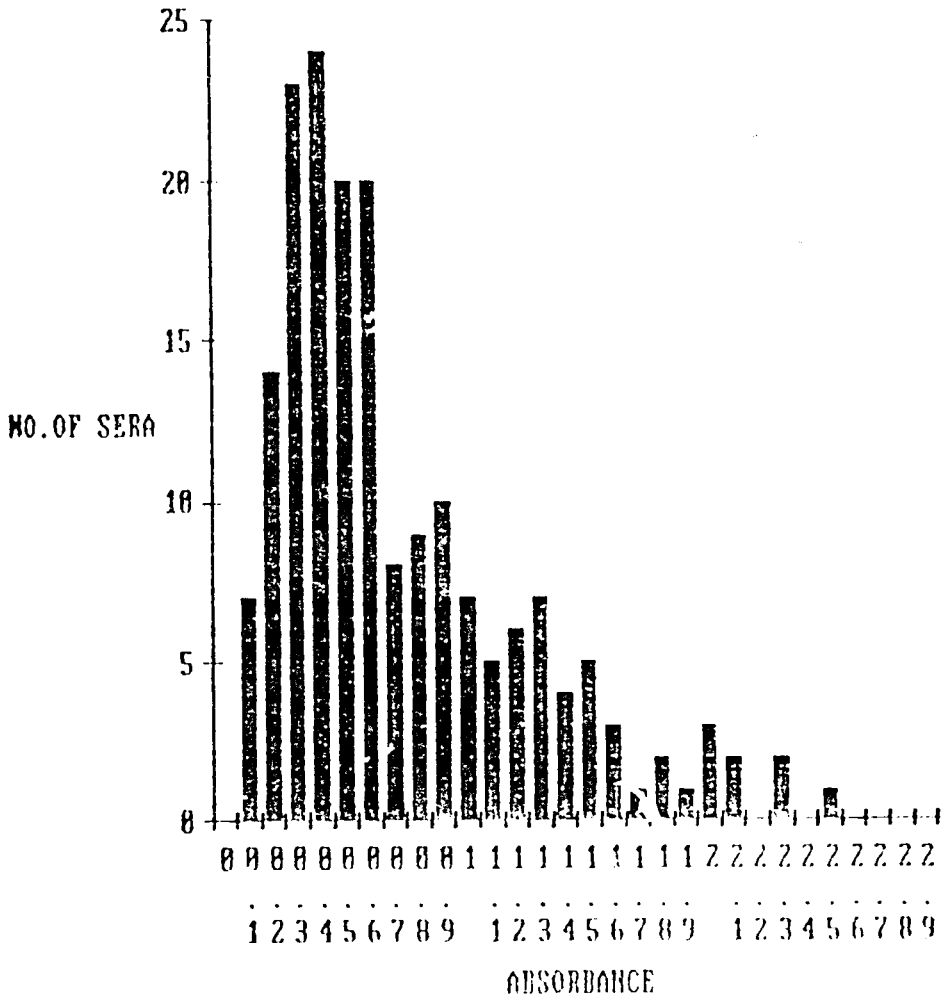

Figure 25. ELISA value of sera from culture negative of M. tuberculosis subjects against crude sonicated antigen.

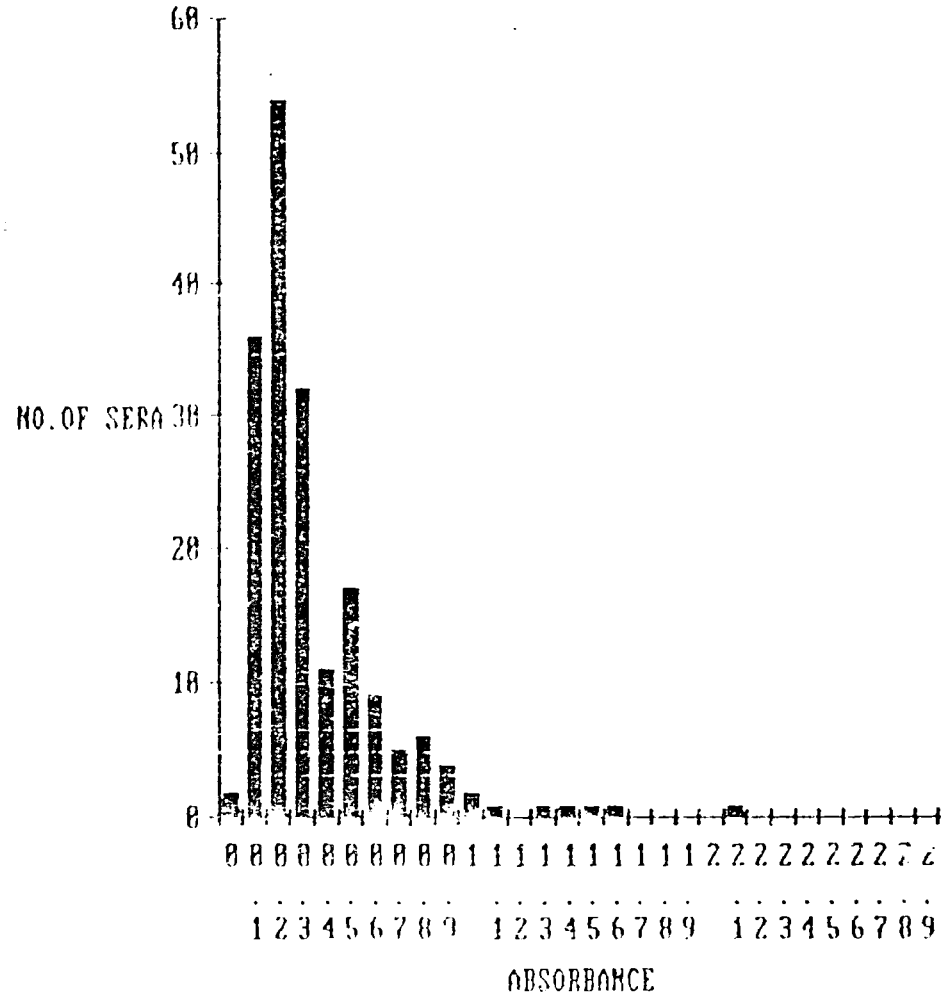

Figure 26. ELISA value of filter paper preserved blood extract from culture negative of M. tuberculosis subjects against crude sonicated antigen.

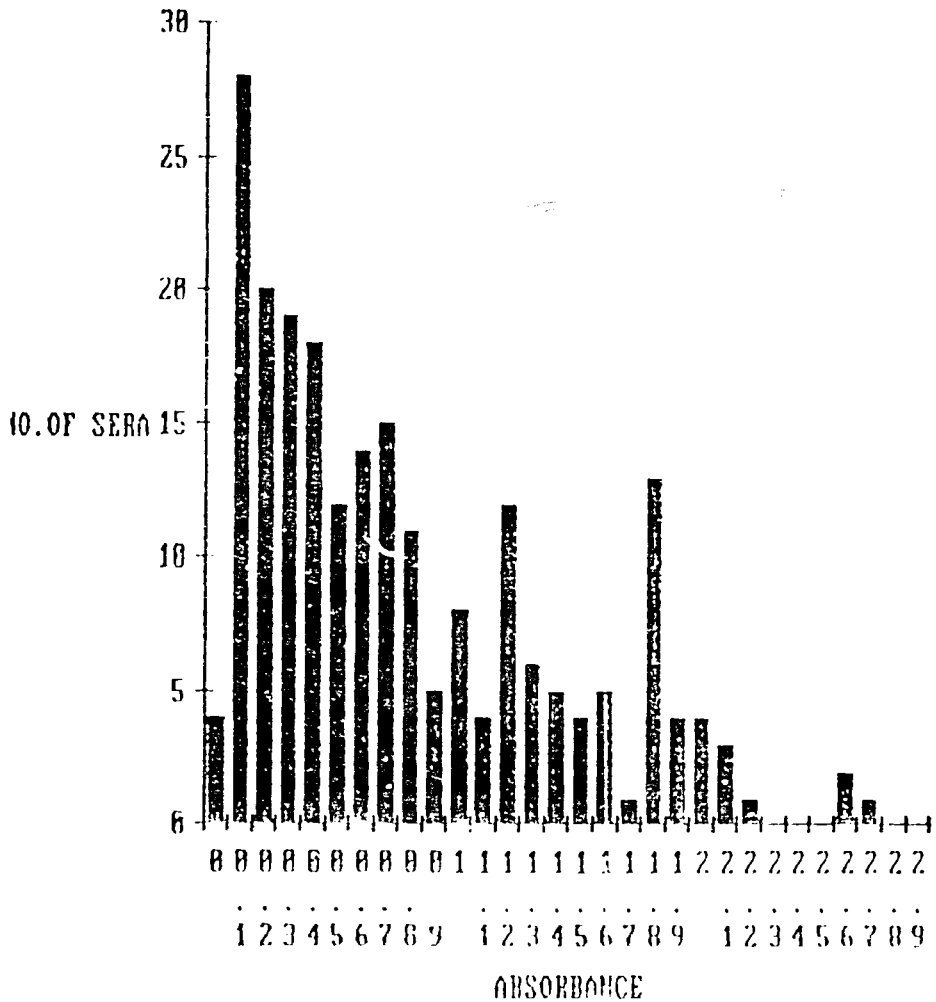

Figure 27. ELISA value of sera from culture positive of M. tuberculosis subjects against X antigen.

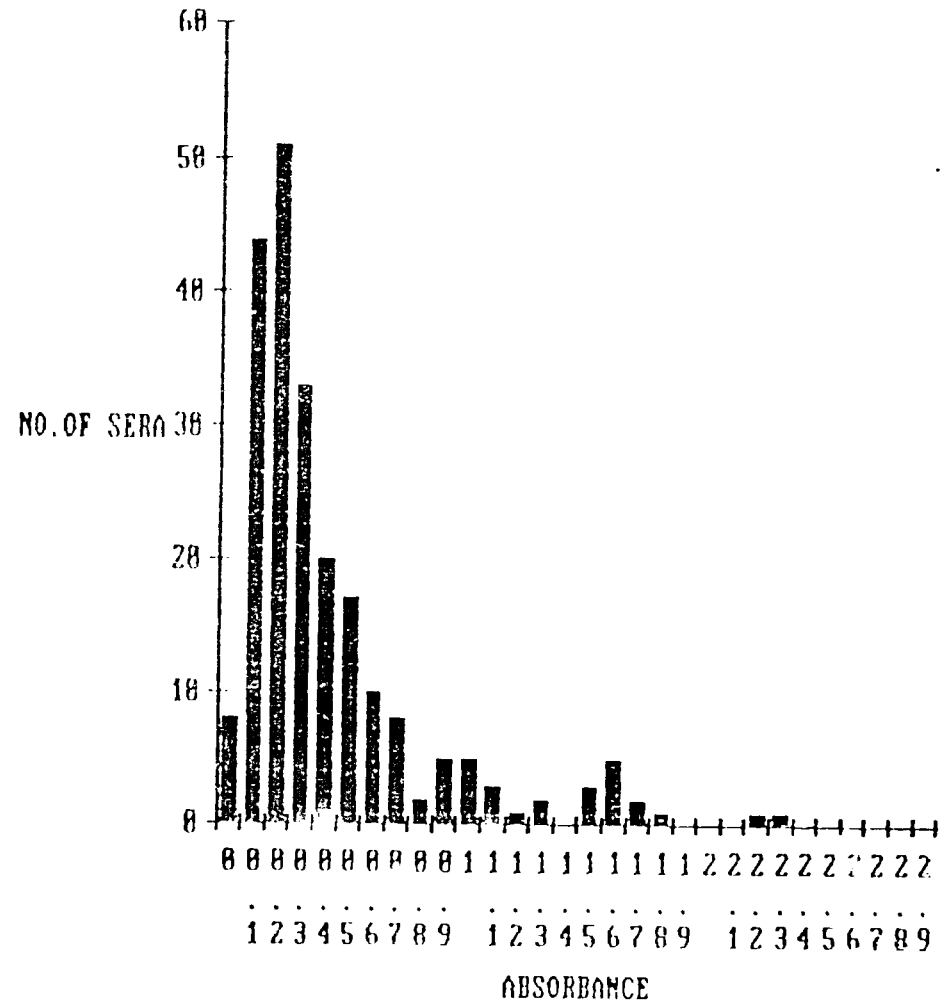

Figure 28. ELISA value of filter paper preserved blood from culture positive of M. tuberculosis subjects against X-antigen.

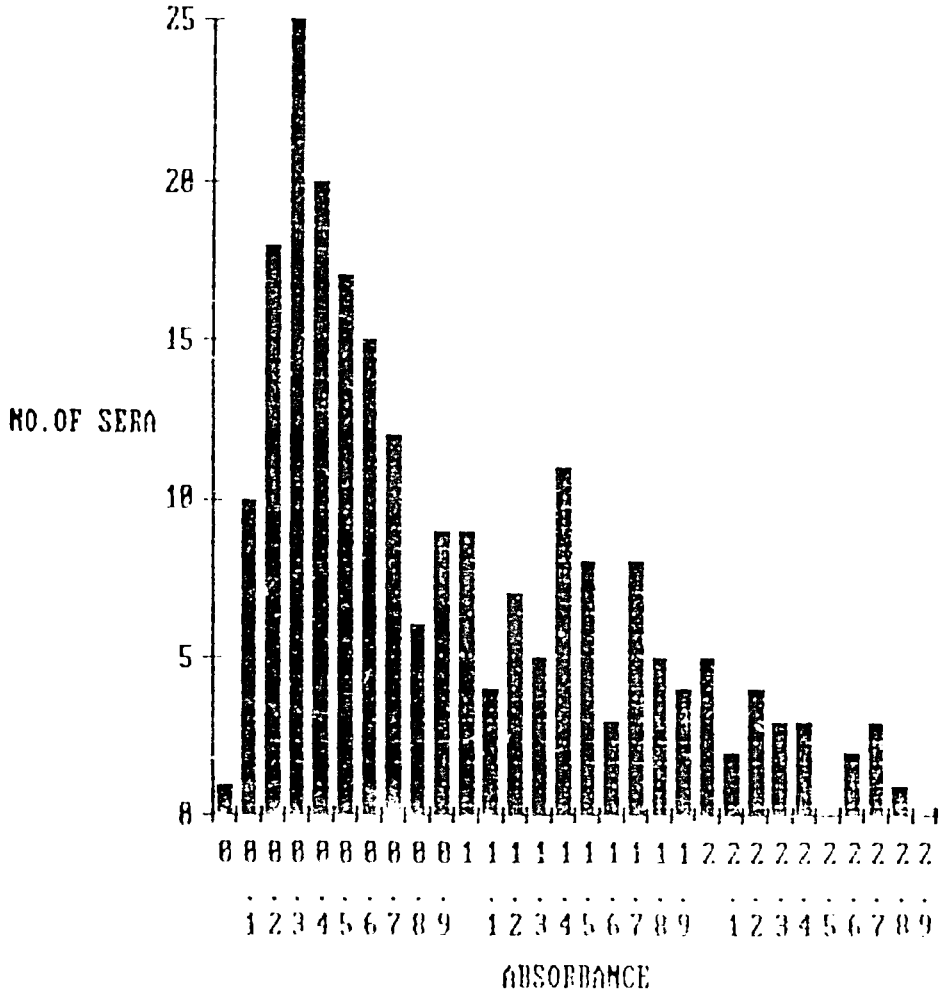

Figure 29. ELISA value of sera from culture positive of M. tuberculosis subjects against total lipid antigen.

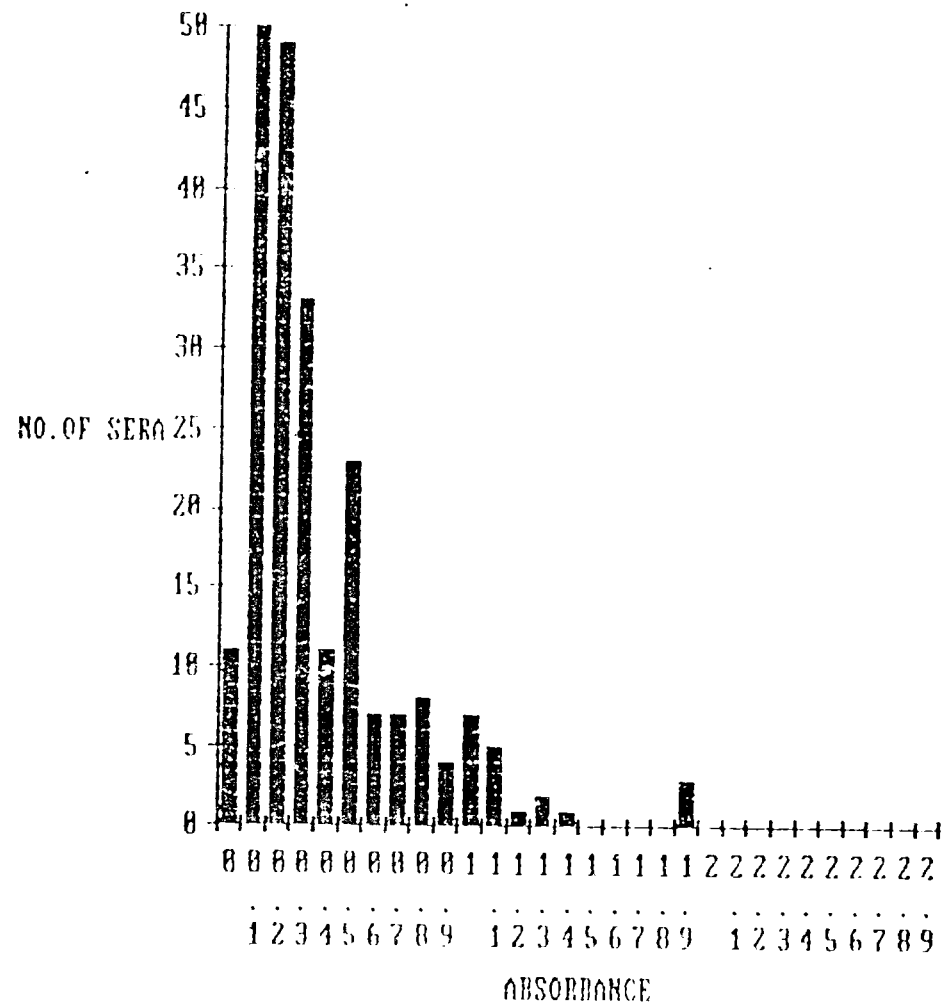

Figure 30. ELISA value of filter paper preserved blood from culture positive of M. tuberculosis subjects against total lipid antigen.

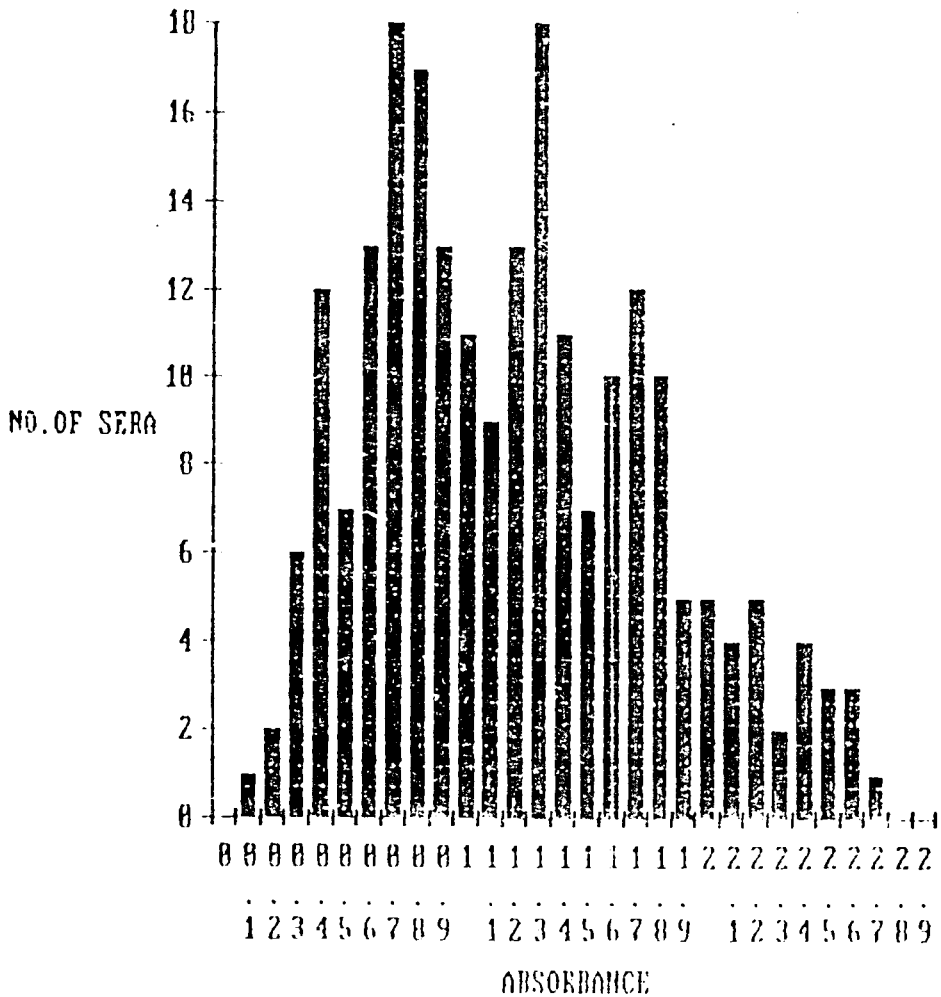

Figure31. ELISA value of sera from culture positive of M. tuberculosis subjects against crude sonicated antigen.

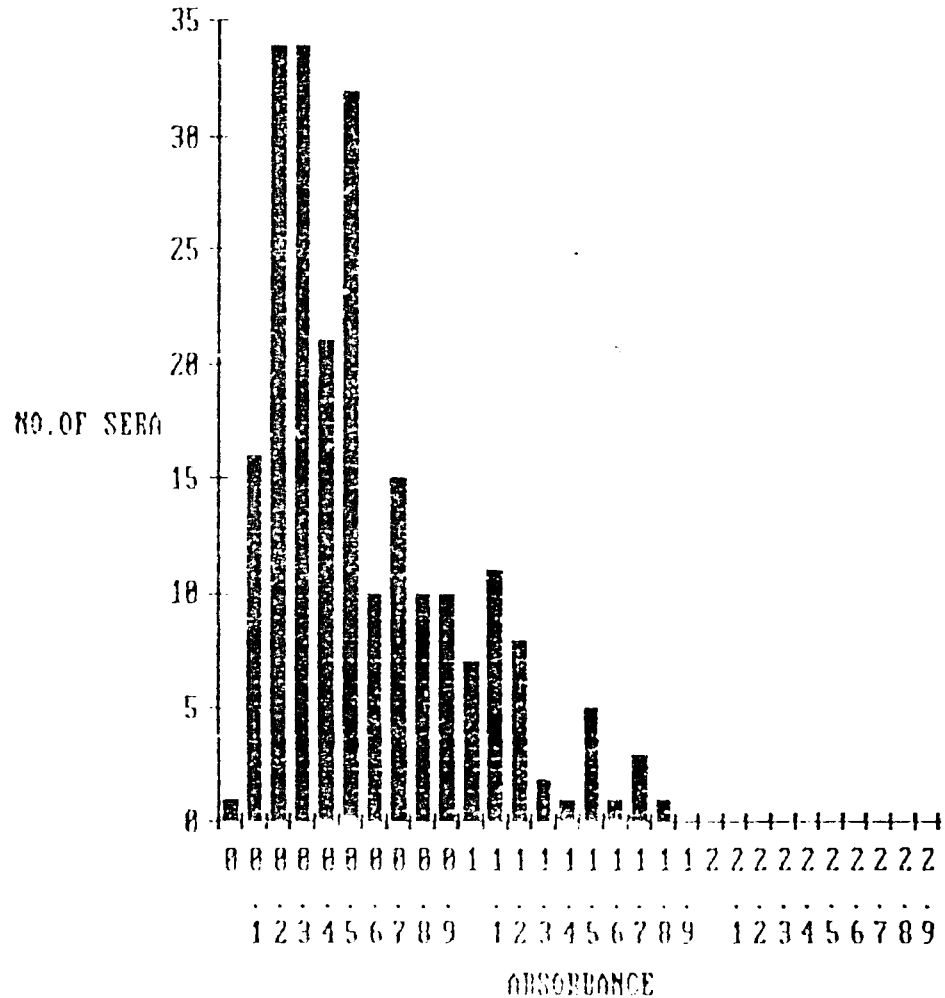

Figure32. ELISA value of filter paper preserved blood extract from culture positive of M. tuberculosis subjects against crude sonicated antigen.

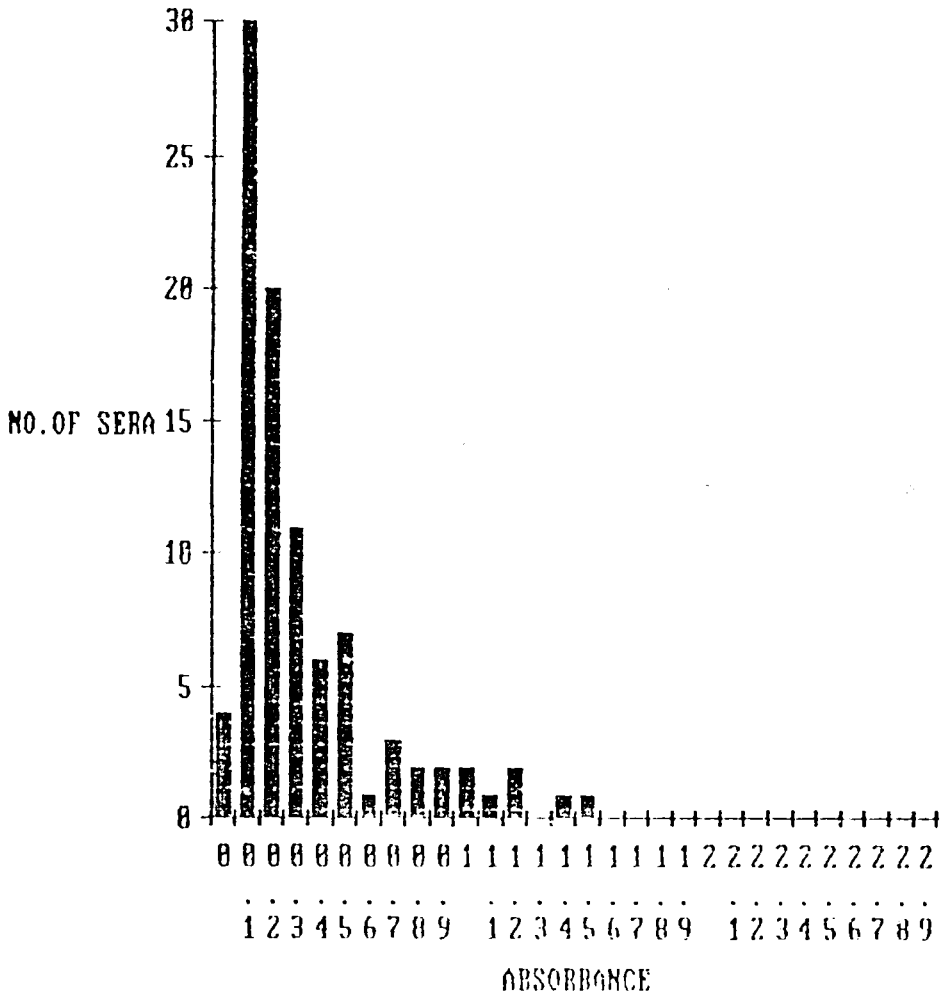

Figure 33. ELISA value of sera from T.B. contact villagers against X antigen.

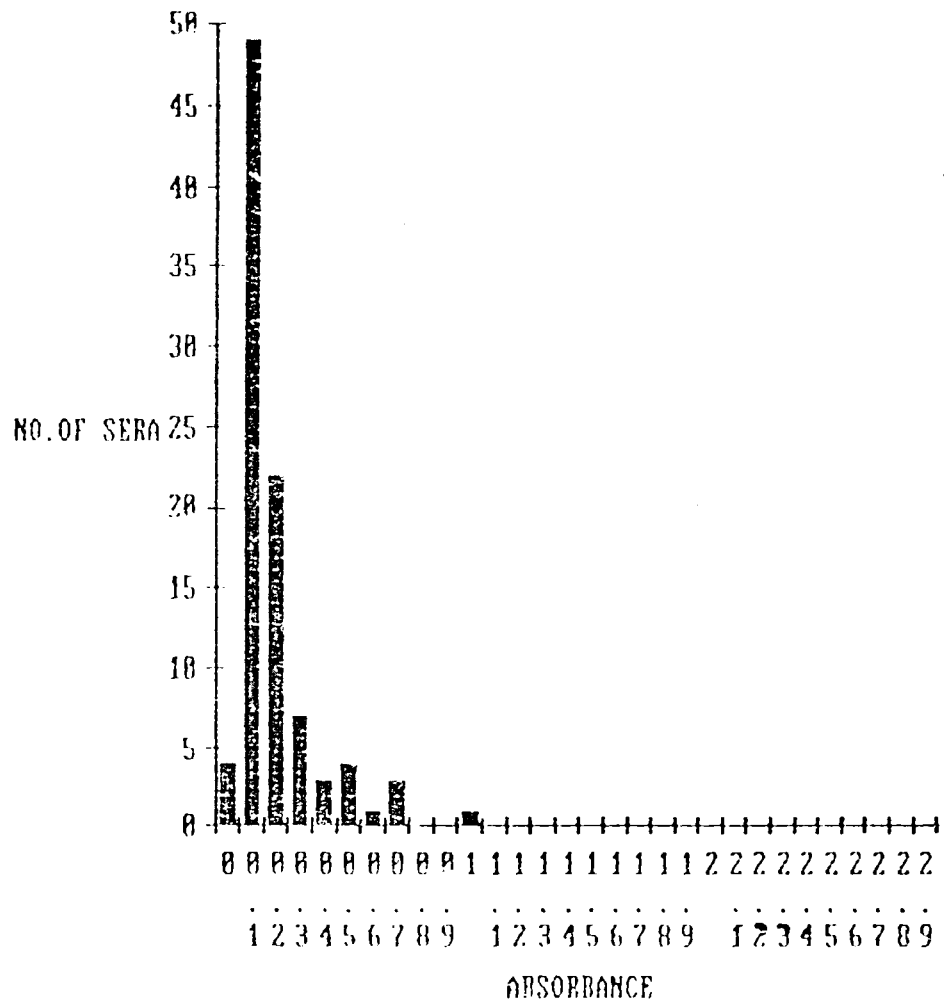

Figure 34. ELISA value of filter paper preserved blood extract from T.B. contact villagers against X antigen.

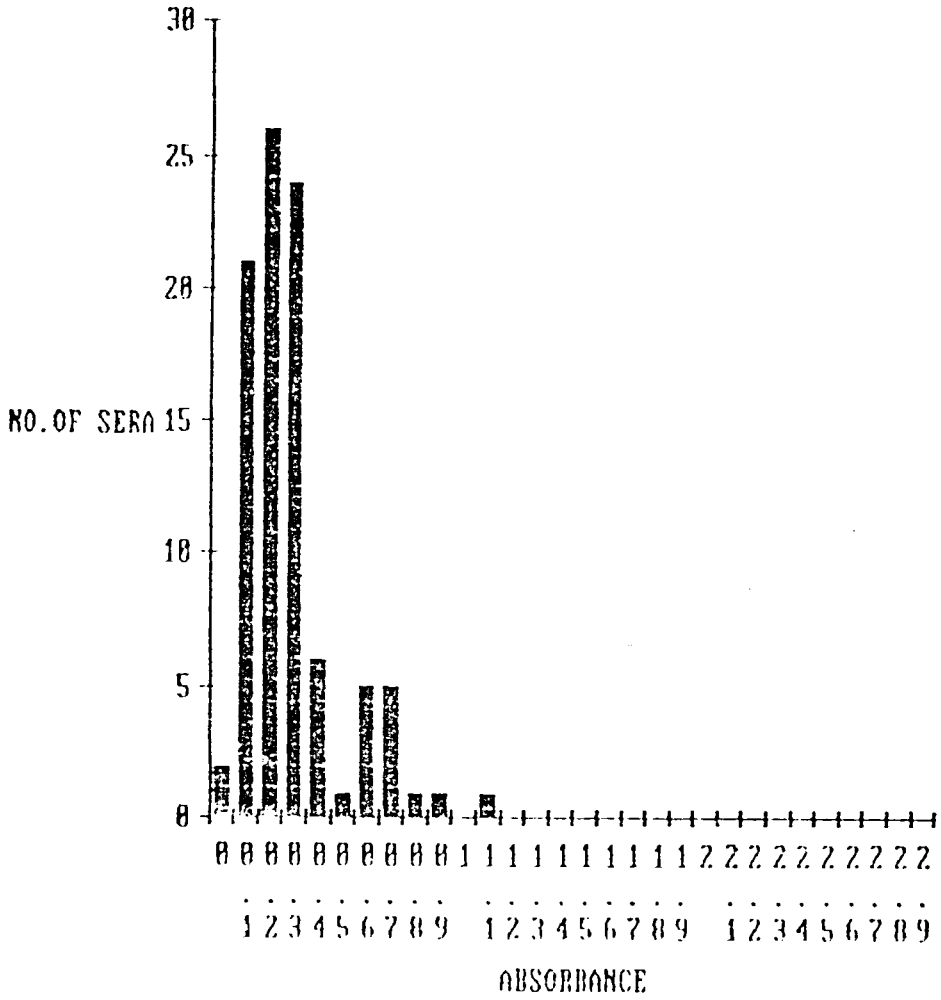

Figure 35. ELISA value of sera from T.B. contact villagers against total lipid antigen.

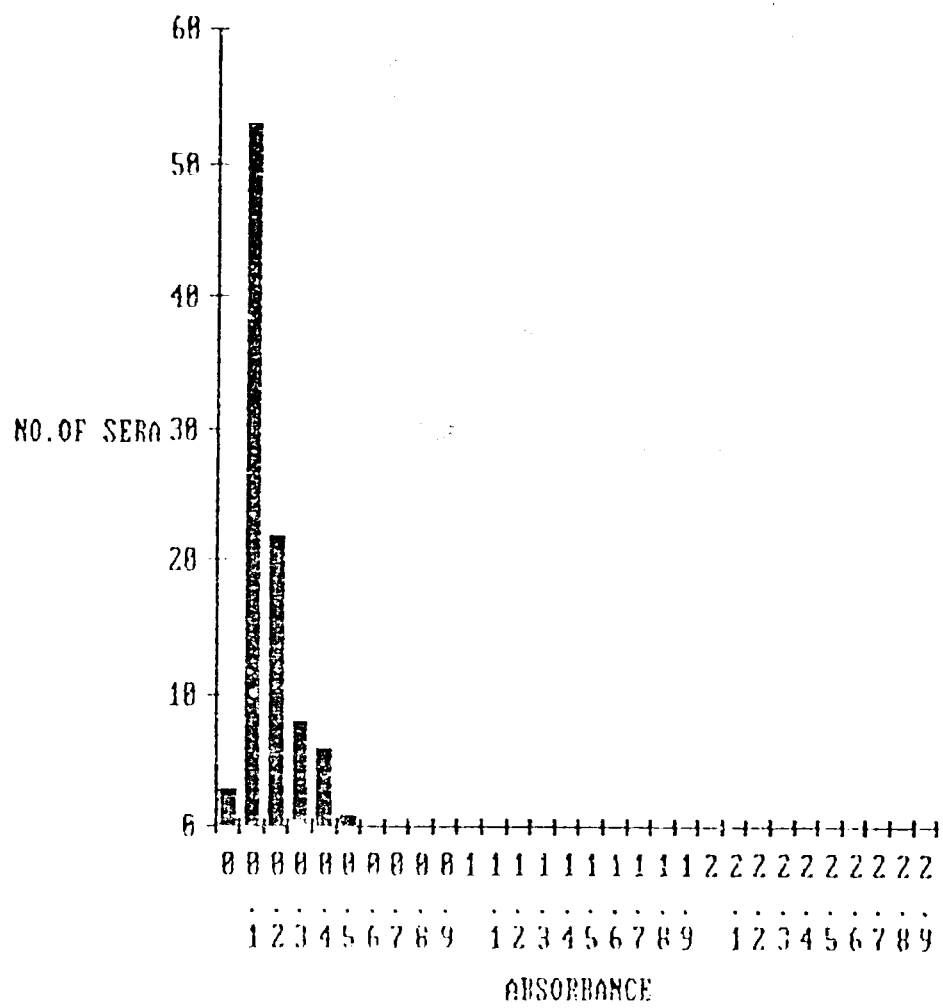

Figure 36. ELISA value of filter paper preserved blood extract from T.B. contact villagers against total lipid antigen.

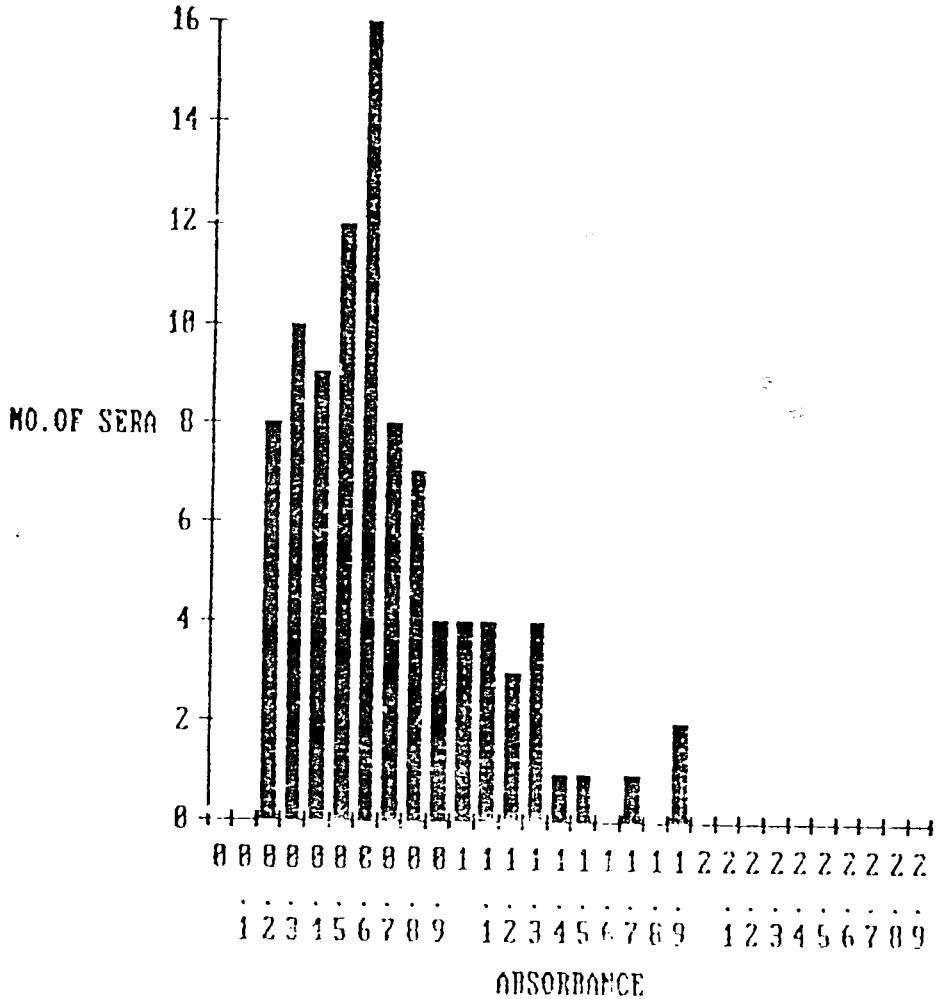

Figure 37. ELISA value of sera from T.B. contact villagers against crude sonicated antigen.

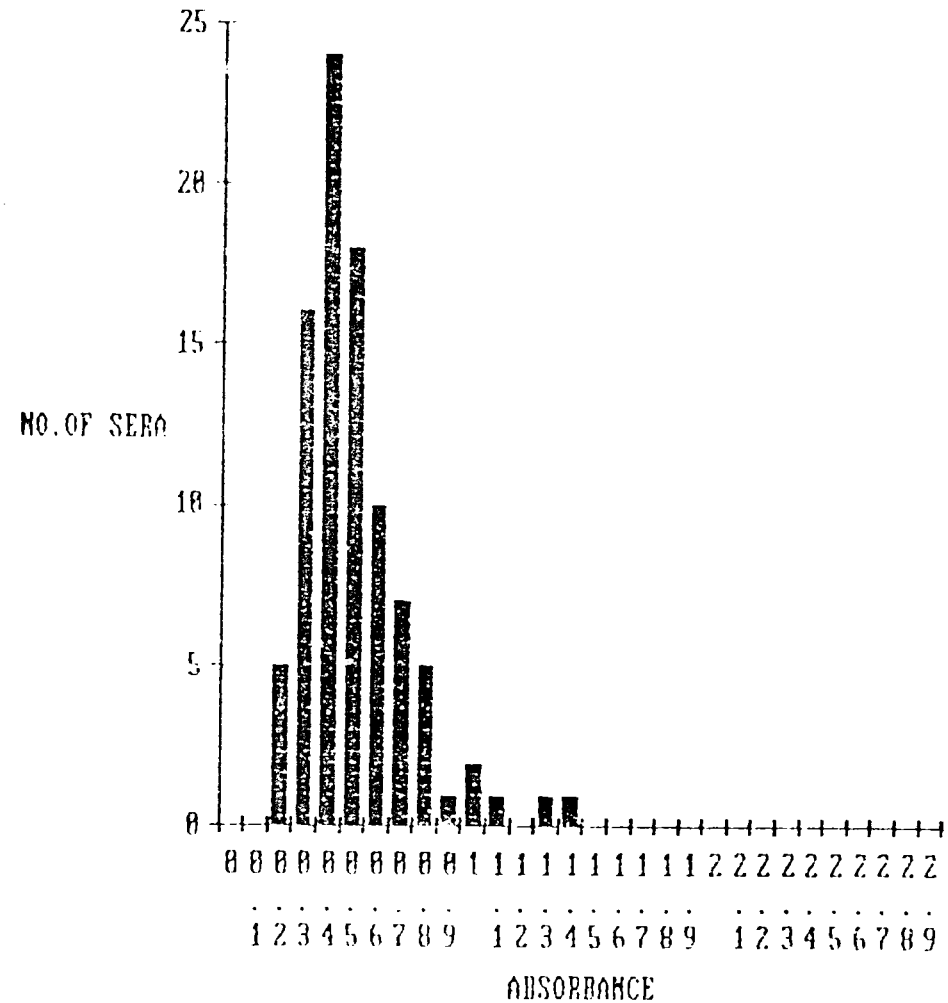

Figure 38. ELISA value of filter paper preserved blood extract from T.B. contact villagers against crude sonicated antigen.

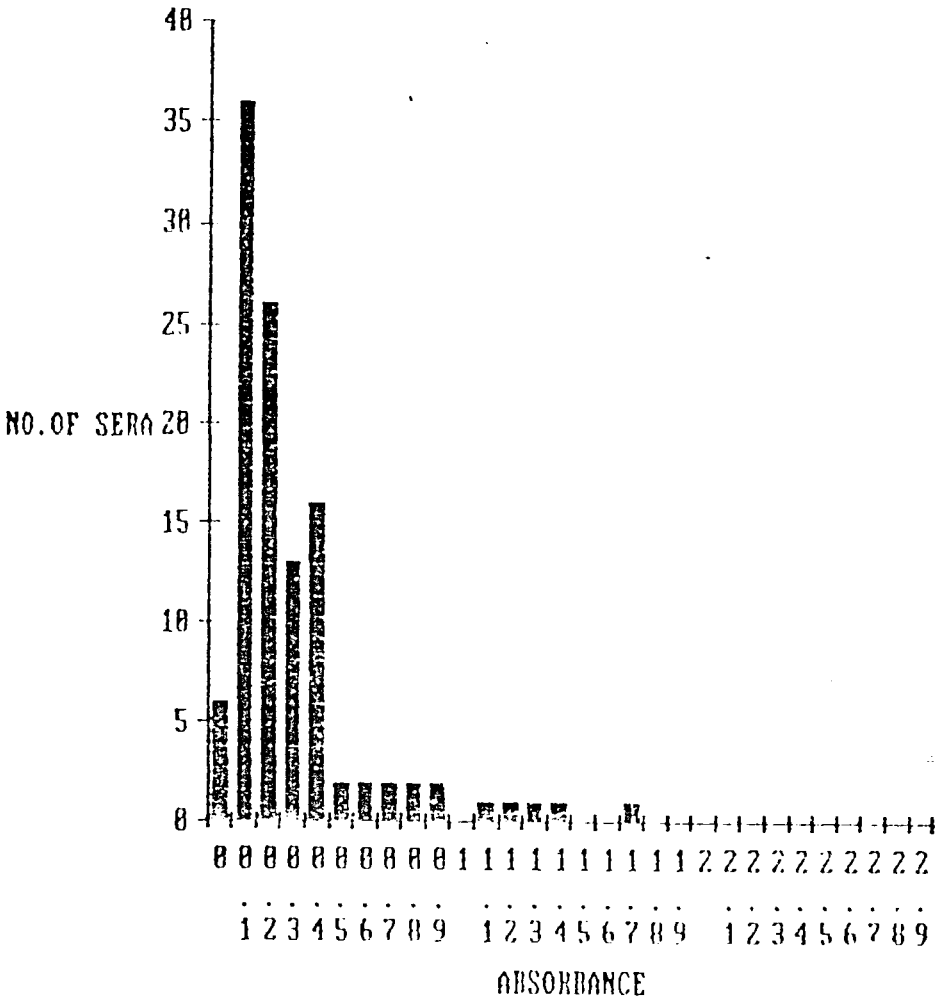

Figure 39. ELISA value of sera from normal villagers against X-antigen.

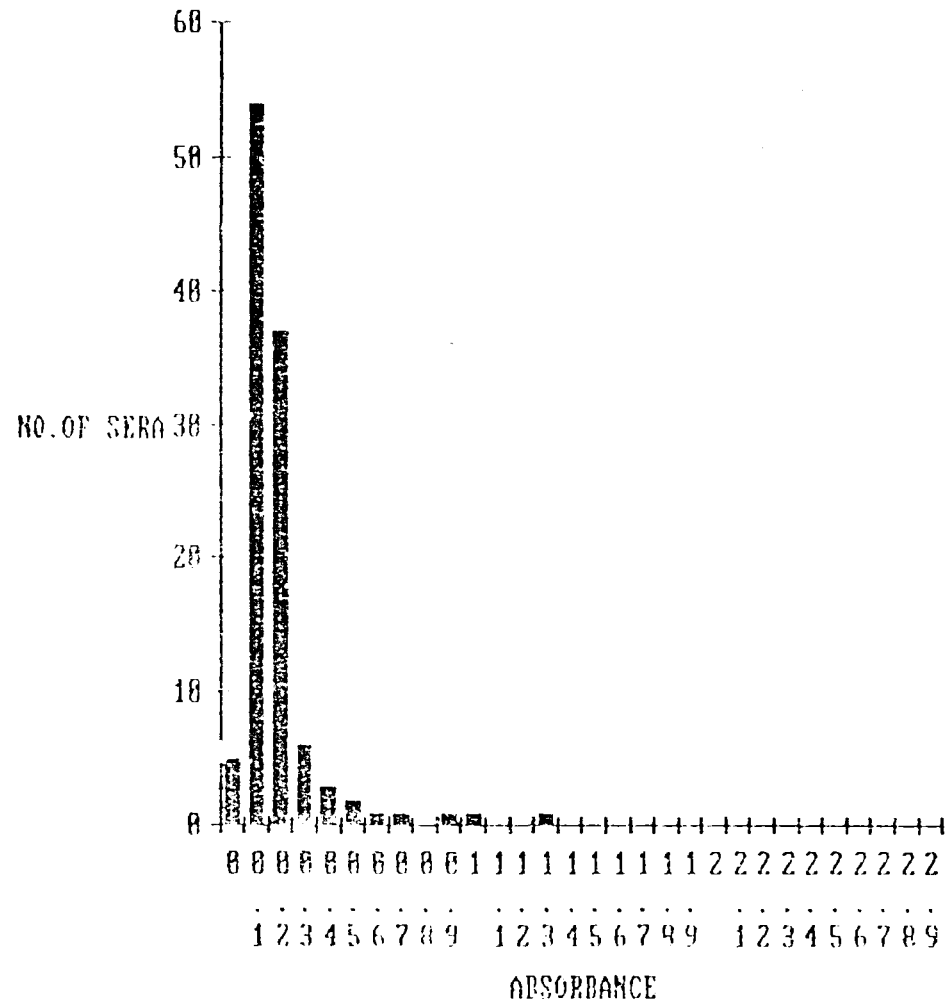

Figure 40. ELISA value of filter paper preserved blood extract from normal villagers against X-antigen.

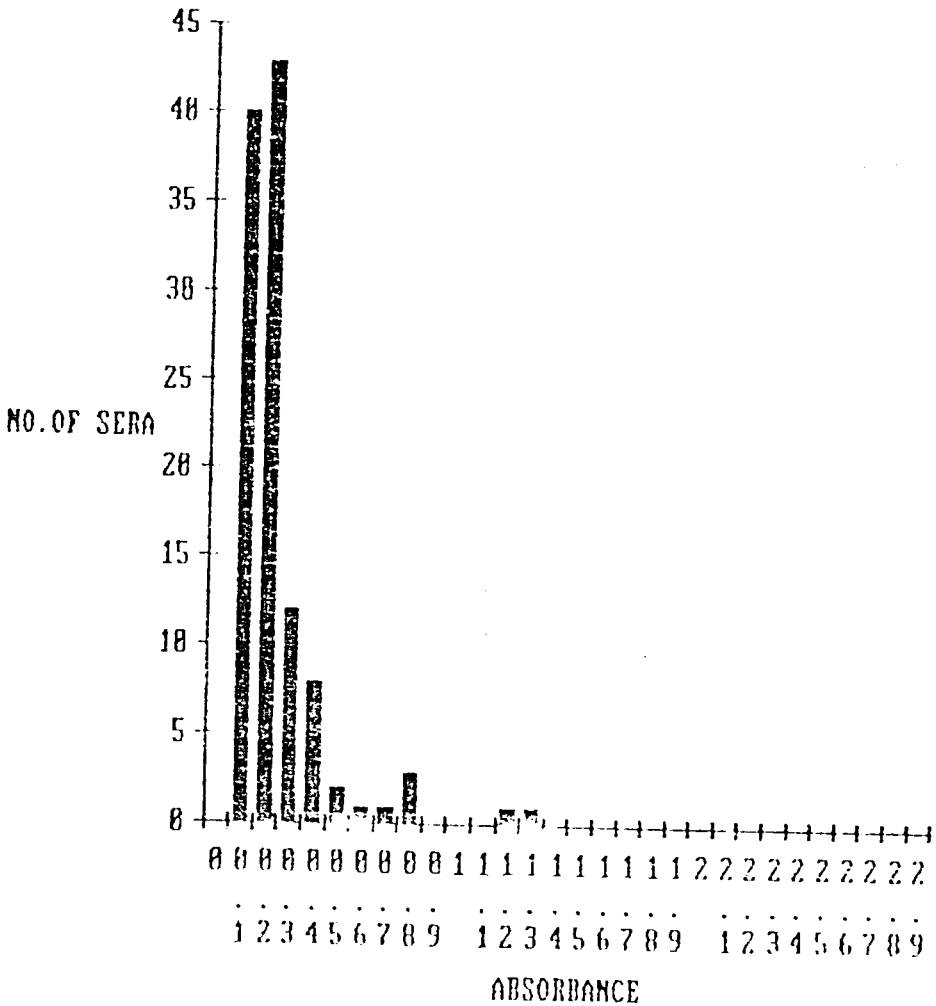

Figure 41. ELISA value of sera from normal villagers against total lipid antigen.

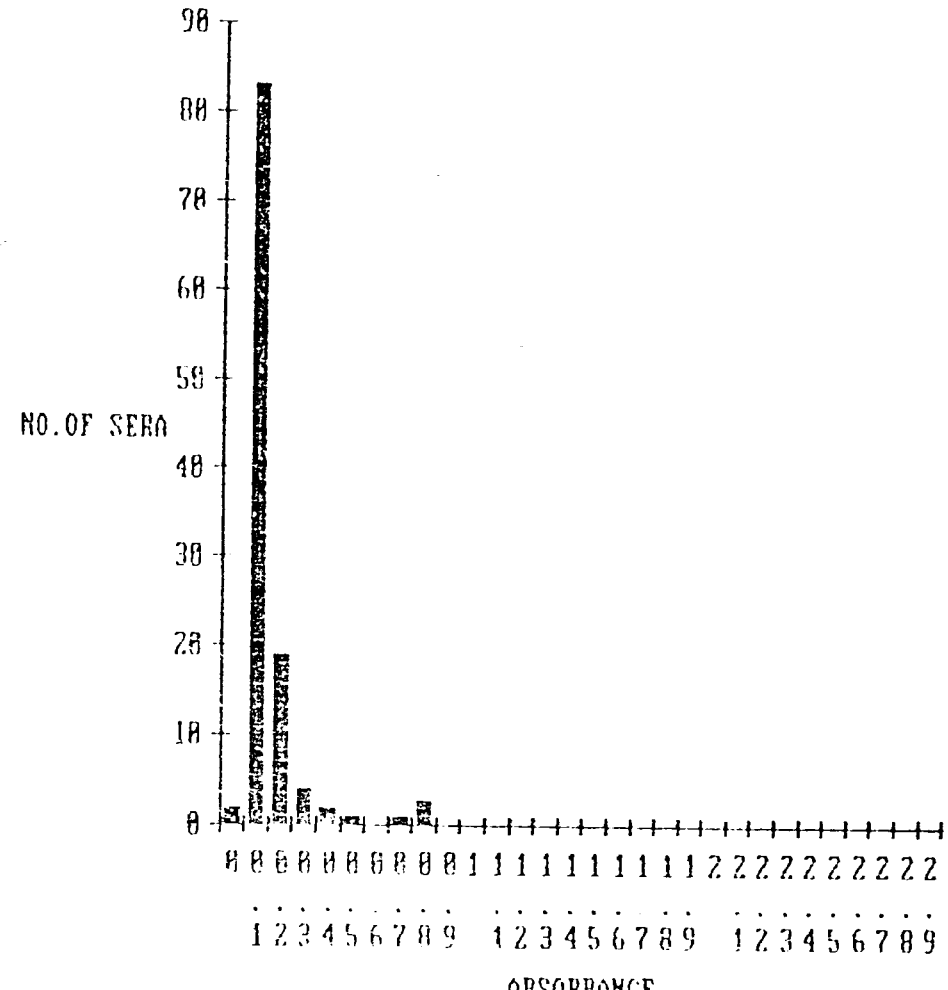

Figure 42. ELISA value of filter paper preserved blood extract from normal villagers against total lipid antigen.

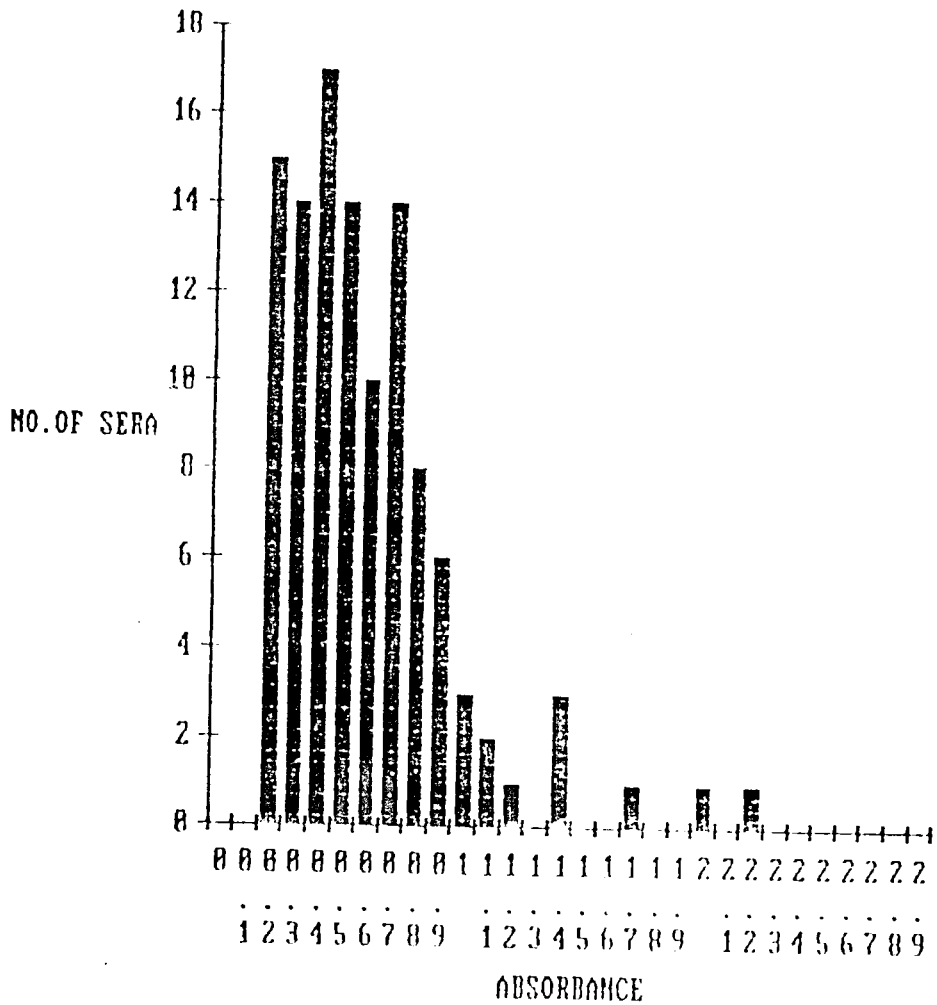

Figure 43. ELISA value of sera from normal villagers against crude sonicated antigen.

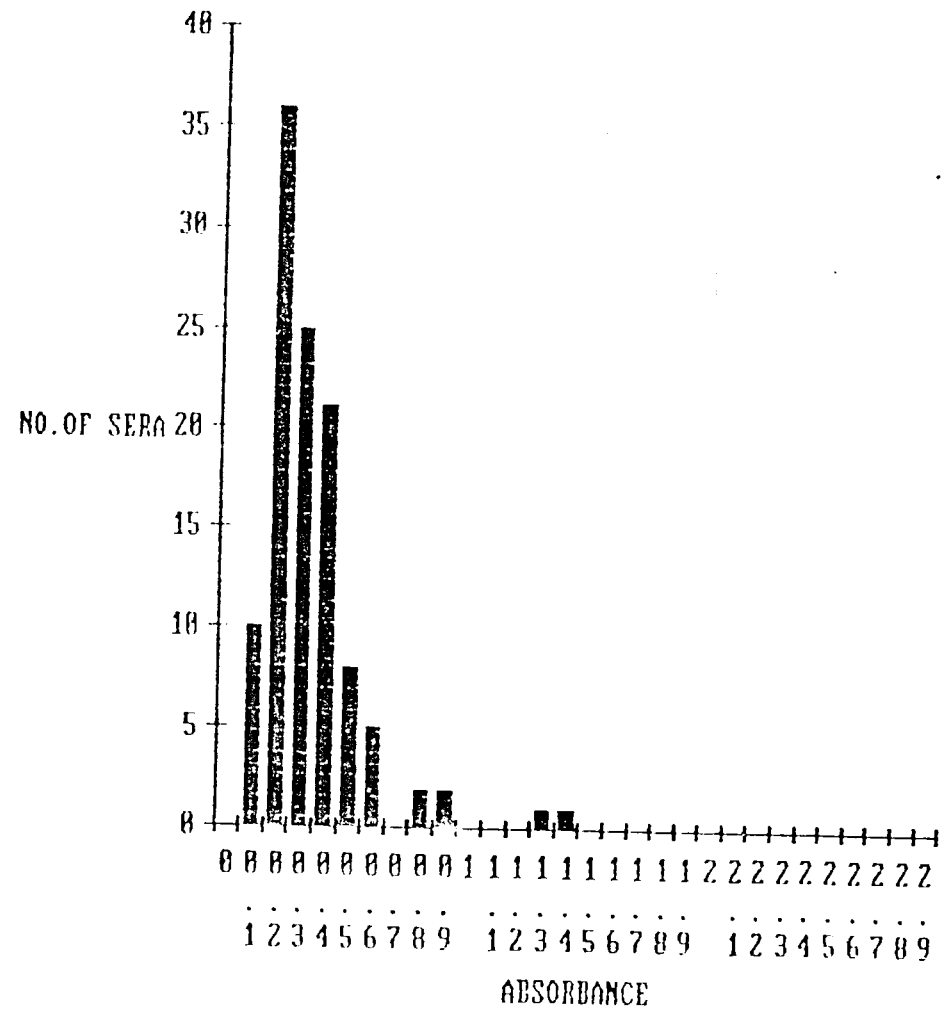

Figure 44. ELISA value of filter paper preserved blood extract from normal villagers against crude sonicated antigen.