DIAGNOSIS OF TUBERCULOSIS BY USING ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) TO DETECT ANTI-MYCOBACTERIAL SUPEROXIDE DISMUTASE ANTIBODIES IN THE PATIENTS

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SUMMARY: Tuberculosis (TB) is an ancient human scourge that continues to be an important public health problem worldwide. The risk of increasing spread of tuberculosis and development of drug resistance make early diagnosis a matter of utmost concern. Improved rapid methods for laboratory confirmation are therefore urgently required.

Superoxide dismutase (SOD), an important secretory protein of Mycobacterium tuberculosis, has been ignored in the past for the development of a serodiagnostic test. We have evaluated the performance of an ELISA based on the detection of antibody to SOD in TB patients.

The presence of antibodies to SOD has been detected in most TB patients' sera. TB sera exhibited 93-94% positivity and showed a significantly higher response ($p<0.0001$) when compared with the serum samples from The Regional Blood Transfusion Centre. A slightly lower positive predictive value (77%) was obtained when Indian TB cases were compared with Indian normals. Similarly, an 88% positive predictive value was obtained with Egyptian TB cases. This test showed a significant level of specific response ($p<0.0001$). 25% of The Indian normals also found to contain a high level of antibodies to mycobacterial SOD. This may be due to the prevalence of M. tuberculosis or other mycobacteria in the environment or they may be early TB cases.

The test has a 98% specificity with 93-94% positive predictive value. However this is preliminary data and its performance needs to be evaluated on larger numbers of confirmed positive TB patients and controls.

Key Words: Mycobacterial SOD, Mycobacterium tuberculosis.

INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a major health problem. It has been found in Neolithic remains and is still the largest cause of death from a single infectious disease. Between one-fifth and one-third of the world's population is infected with *M. tuberculosis*. However, of those who become infected, less than 20% develop clinically apparent disease (1). In infected individuals the organisms persist

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and retain the potential to become reactivated and to cause progressive disease. There are about 20 million new active cases of TB each year and there are between 3-8 million death per year from this infection. This represents greater than 25% of all avoidable deaths worldwide (2).

In most developed countries, the incidence of tuberculosis during the early part of this century was about 5% annually. In developing countries, by contrast, the rate of infection has remained constant or is only declining very slowly (3). Such countries are still confronted with a major tuberculosis problem due to socio-economic underdevelopment. In such countries, control programmes are very difficult to sustain. Thus even today, despite effective drugs, tuberculosis remains a global health problem of major importance. Skin test surveys in poor countries suggest that in some regions more than 50% of the adult population have at some time been exposed to *M. tuberculosis*.

Outbreaks of multi-drug-resistant (MDR) tuberculosis have been reported during the last few years in the USA (4,5). Emergence of multi-drug-resistant strains of *M. tuberculosis* has reduced the efficacy of treatment almost to the level of the pre-antibiotic era (6). The risk of increasing spread of tuberculosis and development of drug resistance make early diagnosis a matter of utmost concern, and improved rapid methods for laboratory confirmation are urgently required.

Primary pulmonary tuberculosis is usually symptomless in adults and is discovered only on routine screening of contacts. It is a mild illness which may resolve spontaneously without sequelae (7-9). In infants and children under 5, however, the infection may have serious immediate consequences. Children under four years of age have a high risk of mortality and morbidity, since tubercular meningitis and miliary tuberculosis are the most common in this age group. Positive bacterial isolation in children is difficult, because the disease is frequently asymptomatic and paucibacillary (10).

One of the main objectives of the research in the field of mycobacteriology is the development of new methods that will improve and expedite the diagnosis and treatment of tuberculosis and other mycobacterial infections. Some forms of tuberculosis are difficult to diagnose by the available routine diagnostic methods. In spite of new technologies, no reliable new serological test has been developed for the diagnosis of tuberculosis.

We have evaluated the performance of an ELISA, for diagnosis of tuberculosis, based on detection of anti-SOD antibodies in the TB patients sera.

MATERIALS AND METHODS

Detection of anti-SOD antibodies in serum of tuberculosis patients by ELISA

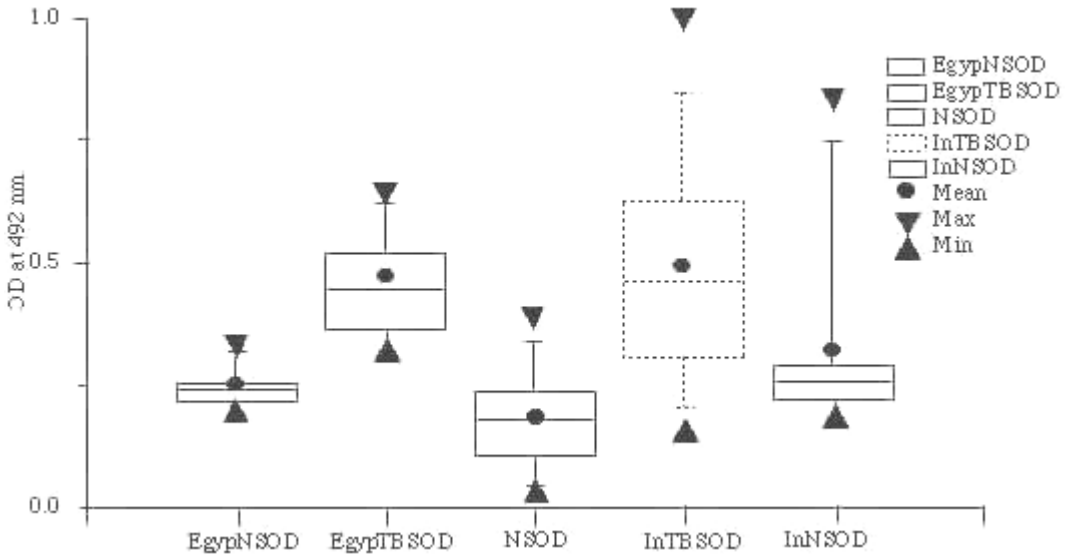
SOD, a major antigen of *M. tuberculosis* was originally identified on the basis of its ability to induce an immune response (11). SOD was found to be highly immunogenic in mice. In order to study the immune response to SOD during infection in TB patients, serum samples from TB patients and normals were tested for the presence of SOD antibodies by ELISA.

Table 1: Anti-SOD antibody level in TB patients and normal controls.

Serum	Negative	Positive	Total
TB Indian Patients	04	26	30
Normal Indian	15	05	20
Normal BTC	49	01	50
TB Egyptian Patients	03	17	20
Normal Egyptian	08	02	10

BTC : *The Regional Blood Transfusion Centre, Birmingham, UK.*

Figure 1: Antibody levels to the mycobacterial SOD in TB patients and controls.



NSOD = antibody to SOD in the Regional Blood Transfusion Centre normals.

InTB = Indian TB patients. Egyp TB = Egyptian TB patients.

In N = Indian normals Egyp N = Egyptian normals.

In figure, the single or upper box represents the distribution of OD values in which 50-75% of the subjects lie. The lower box is the 25-50% distribution.

Patients: Thirty confirmed Indian TB patients' and twenty Egyptian TB patients' serum samples were randomly selected from the samples submitted by the Lupin Laboratories, Bombay, India and Theodor Bilharz Research Institute, Giza, Egypt.

Normals: Twenty Indian and ten Egyptian normals were included in this study to compare with the patients' results. Fifty samples from The Regional Blood Transfusion Centre (external controls presumed negative) were also included in this study to compare with the patients' results.

ELISA

5 μ g/ml solutions of the purified SOD antigens were prepared in 0.05 M carbonate-bicarbonate buffer, pH 9.6, and used to coat polystyrene micro ELISA 96 well plates (Nunc:Gibco) by incubating overnight at 4°C. The plates were washed three times with washing buffer and dried.

Patients' and normals' sera were diluted 1:500, 1:750 and 1:1000 in diluting buffer (Saline + 0.05% Tween - 20) and 100 μ l of each dilution was added per well in duplicate. The plates were incubated at 37 °C for one hour. After incubation the plates were washed three times with washing buffer and dried. Anti-human Ig-HRP conjugate was diluted (1:8000) in the diluting buffer.

100 μ l of the conjugate was added per well and the plates were incubated at 37°C for one hour. The plates were washed with washing buffer and dried. 100μ l of OPD substrate in substrate buffer with H_2O_2 was added to each well. The reactions were allowed to proceed at 37°C for 30 minutes in the dark and were then stopped by adding 50 μ l of 20% (v/v) H_2SO_4 to each well. The plates were read on an ELISA reader (Labsystem Multiskan® MCC) at 492 nm.

The maximum difference between positive and negative results was obtained with the 1:500 serum sample dilution and the results with this dilution are reported in two categories:

1. Normal, 2. Positive

Data Analysis

Proven tuberculosis patients' (test) sera and control sera were used to determine:

Test Sensitivity = true positives / true positives + false negatives x 100

Test Specificity = true negatives / true negatives + false positives x 100

Test positive predictive value = true positives / true positives + false positives x 100

Test negative predictive value = true negatives / true negatives + false negatives x 100

Table 2: Data analysis of the test for anti-SOD antibody in TB patients and normal controls.

	Specificity %	Sensitivity %	Positive predictive value %	Negative predictive value %	p value
Indian TB / Indian control	75	85	77	83	<0.0001
Indian TB / BTC control	98	85	94	94	<0.0001
Egyptian TB / Egyptian control	80	88	88	80	<0.0001
Egyptian TB / BTC control	98	88	93	96	<0.0001

BTC : The Regional Blood Transfusion Centre, Birmingham, UK.

RESULTS

A direct antibody ELISA was performed to detect SOD antibodies in the TB patients' and normal human serum. A high level of anti-SOD antibodies was detected in most cases of tuberculosis (Figure 1).

Results are recorded on the basis of OD levels. A value above the mean of the normals value ± 2SD was considered as positive (Table 1).

The presence of antibodies to SOD has been detected in both the Indian and Egyptian TB patients' sera (Table 1). TB sera exhibited 93-94% positivity and showed a significantly higher response ($p<0.0001$) when compared with the serum samples from the Regional Blood Transfusion Centre (Table 2). A slightly lower positive predictive value (77%) was obtained when Indian TB cases were compared with Indian normals. Similarly, an 88% positive predictive value was obtained with Egyptian TB cases. This test showed, in both sources of TB, a significant level of specific response ($p<0.0001$). It is important to note that 25% of the Indian normals contain a high level of antibodies to mycobacterial SOD. This may be due to the prevalence of *M. tuberculosis* or other mycobacteria in the environment or they may be early TB cases.

CONCLUSION AND DISCUSSION

The field of mycobacteriology has significantly advanced during the last 10 years. Nevertheless, the diagnosis of tuberculosis is often a long and tedious process which can take up to several weeks. Despite major advances, the serodiagnosis of tuberculosis requires further development and evaluation. Recently, several new and rapid diagnostic tests have been reported.

With the development of the ELISA, serodiagnosis of TB has been studied by many investigators and each study claims some success. Most of the serological tests developed for TB diagnosis are based on the detection of anti-mycobacterial antibodies using different antigen preparation, ranging from crude mycobacterial extracts to purified antigens (12). Antigen detection test has received little attention for tuberculosis. However, this type of assay could provide useful information for monitoring the efficacy of chemotherapy.

The success of *M. tuberculosis* is dependent upon its ability to survive and replicate within the phagocytic cell of the host. Under these conditions, tubercle bacilli are exposed to a toxic form of oxygen. SOD has been implicated as an important antigen which contributes to the resistance to oxidative killing following phagocytosis (13). The possible role of SOD as a virulence factor of *M. tuberculosis* has been accentuated by Andersen *et. al.* (14). SOD has been reported to be secreted into the culture medium by several investigators (15-16). SOD, an important secretory protein of *M. tuberculosis*, has been ignored in the past for the development of a serodiagnostic test.

SOD of *M. tuberculosis* has been found to be highly immunogenic in mice. Except for Desphande *et.*

al. (17), there has been no report on serodiagnosis of tuberculosis or other mycobacterial diseases which involve the detection of the SOD antigen or anti-SOD antibody.

We have evaluated the performance of an ELISA based on the detection of antibody to SOD in TB patients. A significant level of antibodies to SOD ($p<0.0001$) has been detected in Indian TB and Egyptian TB patients' serum samples. However, antibodies could not be detected in all the TB cases. Recently, Desphande *et. al.* (17) also reported antibodies to SOD in Indian TB cases studied ($p<0.01$). However, we could detect antibodies to SOD in 85% of the Indian TB patients' sera.

In an analysis of 36 confirmed TB cases for antibody detection, the test has a 98% specificity with 93-94% positive predictive value when the data are compared with control normal samples taken from The Regional Blood Transfusion Centre. The sensitivity of the test goes down when the test specificity is determined by using local controls. We do not know the history of the Indian or Egyptian normals and what was the criteria for selection and identification of normals. The test has a highly significant value ($p<0.0001$) for anti-SOD antibody detection in TB patients. However, this is preliminary data and its performance needs to be evaluated on larger numbers of confirmed positive TB patients and controls.

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REFERENCES

1. Snider DE : Rev Infect Dis, 11:3336-3368, 1989.

2. Murray CJL, Styblo K and Rouillon A : Bull Int Union Tuberc Lung Dis, 65:6-24, 1990.

3. Bleiker MA and Styblo K : Bull un Int Tuberc, 52:235, 1978.

4. Bloom BR and Murray CJL : Science, 257:1055-1064, 1992.

5. Iseman MD and Madsen LA : Clin Chest Med, 10:341-353, 1989.

6. Snider DE and Roper WL : N Engl J Med, 326:703-705, 1992.

7. Andersen S and Geser A : Bull WHO, 22:39-60, 1960.

8. Narain RS, Nair S, Rao R and Chandraserkhar P : Bull WHO, 34:639-654, 1966.

9. Dannenberg AM : Res Microbiol, 141:192-263, 1989.

10. Wong GWK and Oppenheimer SJ : Childhood Tuberculosis. In: Clinical Tuberculosis. Ed by PD Davies. Chapmann and Hall Medical London, New York, Tokyo, pp 211-223, 1994.

11. Young RA, Bloom BR, Grossinsky CM, Ivanyi J, Thomas D and Davis RW : 1985.

12. Ivanyi J, Morris JA and Keen M : Studies with monoclonal antibodies to mycobacteria. In: Monoclonal Antibodies against bacteria. Ed by AJL Macario, EC Macario. Academic Press, pp 59-90, New York, 1985.

13. Young D, Garbe T, Lathigra R, Abou-Zeid C and Zhang Y : Bull Int Union Tubercle Lung Dis, 66:47-51, 1991.

14. Andersen P, Askgaard D, Ljungqvist L, Bennedsen J and Heron I : Infect Immun, 59:1905-1910, 1991.

15. Kusunose E, Ichittara K and Noda Y : J Biochem, 80:1343-1352, 1976.

16. Zhang Y, Lathigra R, Gabre T, Catty D and Young D : Mol Microbiol, 5:381-391, 1991.

17. Deshpande RG, Khan MB, Bhat DA and Navalkar RG : Tubercle Lung Dis, 74:388-394, 1993.

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