In the name of God

Evaluation of Mycobacterium tuberculosis specific T cell response to ESAT-6 and PPD antigen with ELISPOT assay

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Symptoms of TB

- ✓Cough for more than two week
- ✓Bloody sputum
- ✓ fever
- ✓decreased appetite
- ✓Weight loss
- ✓sweating at night
- ✓fatigue
- ✓general weakness

Diagnosis

- ✓ Culture
- ✓ AFS (*acid-fast stain*)
- ✓ Radiography
- ✓ TST
  - *in vivo*
  - Less specific PPD
  - 2 patient visits
  - results in 2 - 3 days
  - inter-reader variability
- ✓ **IFN-γ measurement based method**
  - in vitro
  - TB specific antigens
  - 1 patient visit
  - results possible in 1 day
  - unknown variability

Materials & Methods

The first group (patients individuals)

- ✓ weight loss
- ✓ Cough
- ✓ bloody sputum
- ✓ night sweating
- ✓ AFS positive

- ✓ 30 patients (14 female and 16 mal) with mean age 33 years
- ✓ 20 percent (Afghanistan)
- ✓ Culture (all positive)
- ✓ Antibiogram test results (all isolates against first-line TB treatment drugs are sensitive) (Pyrazynamayd, Ethambutol, rifampin, streptomycin and isoniazid)
- ✓ PCR (all isolation positive)

The second group (people treated)

- ✓ Drug regimen

(Isoniazid, Streptomycin, Ethambutol, and Rifampin) to 6 months had received

- ✓ AFS negative
- ✓ 19 individual (12 males and 7 females) with mean age 31 years
- ✓ 20 percent (Afghanistan)
- ✓ AFS (negative in all these people)

The third group (people suspected)

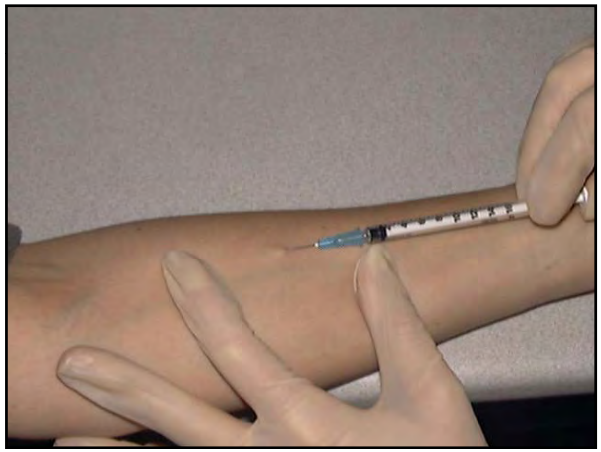
- ✓Sputum test negative after three times
- ✓22 patients (12 males and 10 females) with mean age 35 years
- ✓10 %(Afghanistan)
- ✓TST (5% positive)

The fourth group (Normal)

- ✓22 individuals (12 women and 10 men) with The mean age of this group 24/5 years
- ✓All received the BCG vaccine
- ✓no clinical symptoms were related to tuberculosis
- ✓TST (negative in all these people)

- ✓ **samples transferring (four groups)**

- ✓ **TST**



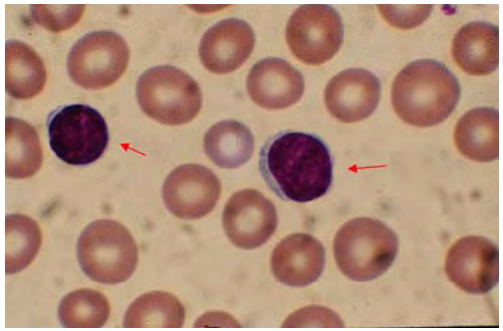
- ✓ **Blood cell separation by Ficoll gradient method**

sampling

T-SPOT.*TB*

Centrifugation

PBMC



Overnight culture in presence of ESAT-6 and PPD anti-IFN-γ antibodies; + and - Controls

Optic reading

Elispot

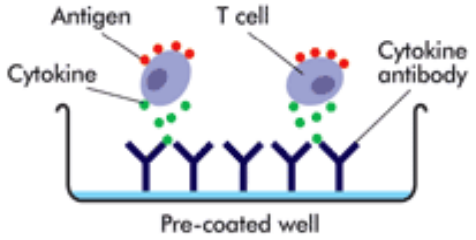
- ✓ Kit activation by ethanol 70 percent
- ✓ Adding the coating antibody (first antibody)
- ✓ Adding the Blocking buffer
- ✓ Adding the cell with antigen
- ✓ Washing
- ✓ Adding the Biotinylated detection antibody (secondary antibody)
- ✓ Washing
- ✓ GABA (ϕ-labeled anti-biotin antibodies)
- ✓ Activators

ELISPOT

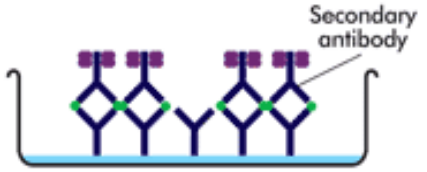
Step 1 Collect the blood sample and centrifuge to separate Peripheral Blood Mononuclear Cells (PBMCs)

Step 2 Wash and count the PBMCs

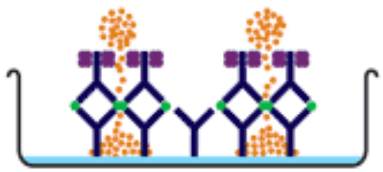
Step 3 Add PBMCs to wells with antigens and incubate overnight (37°C, CO_2)



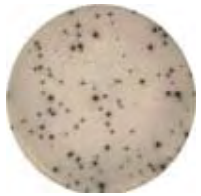
Step 4 Wash and add secondary antibody



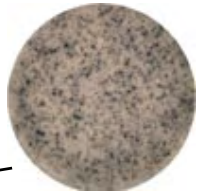
Step 5 Add substrate and count any resulting spots. One spot = one T cell



Nil Control



Infection

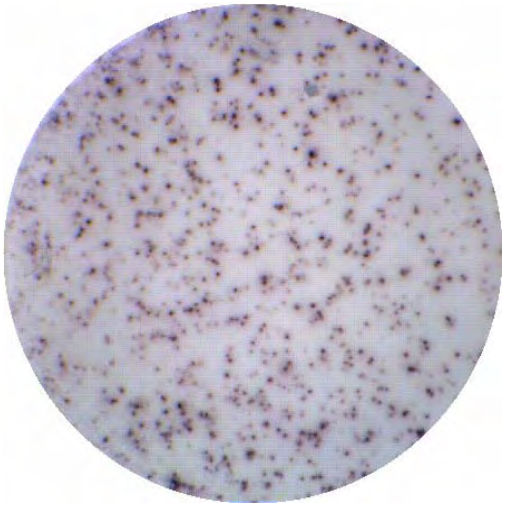


Positive Control

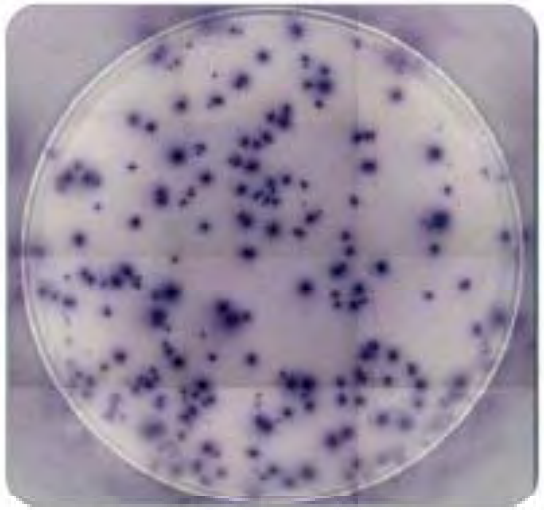
Oxford Immunotec

Results

A. Negative control

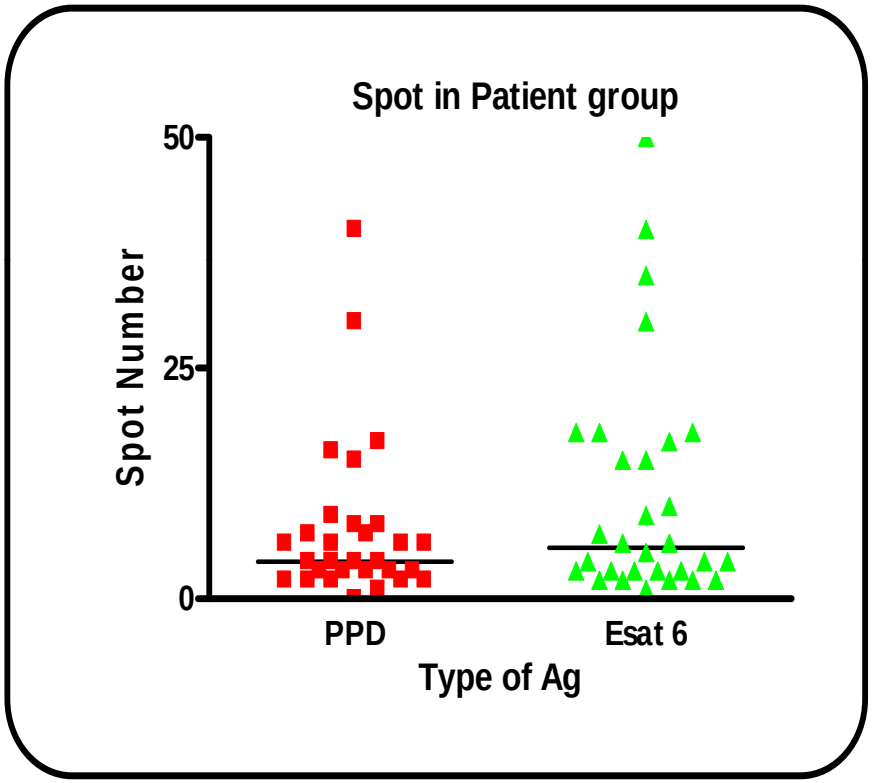


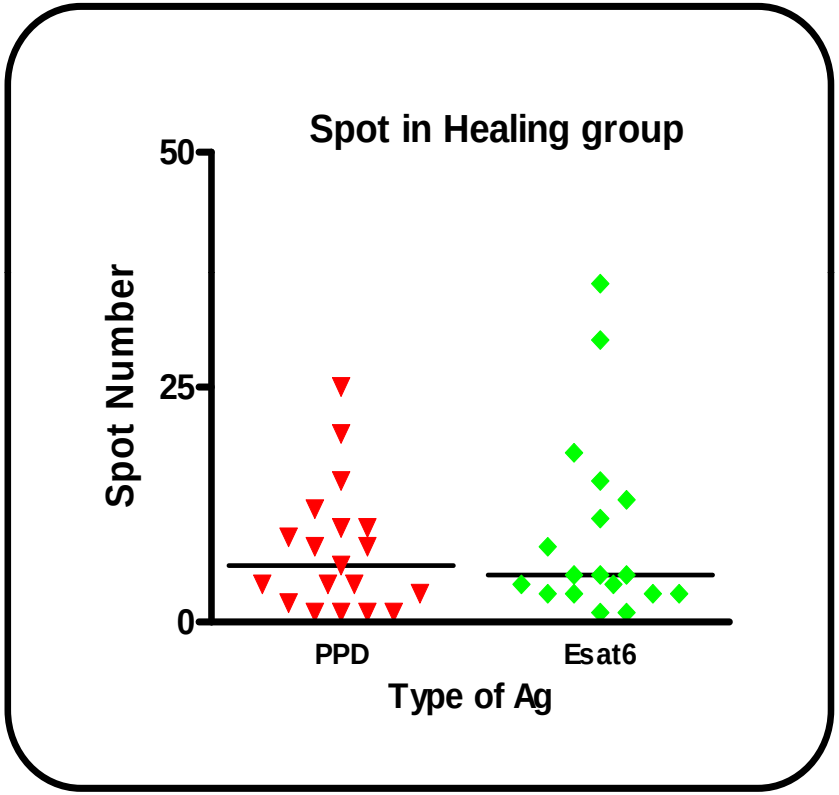
B. Positive control



C. Sample

spot number in *Patients* and *Healed* group

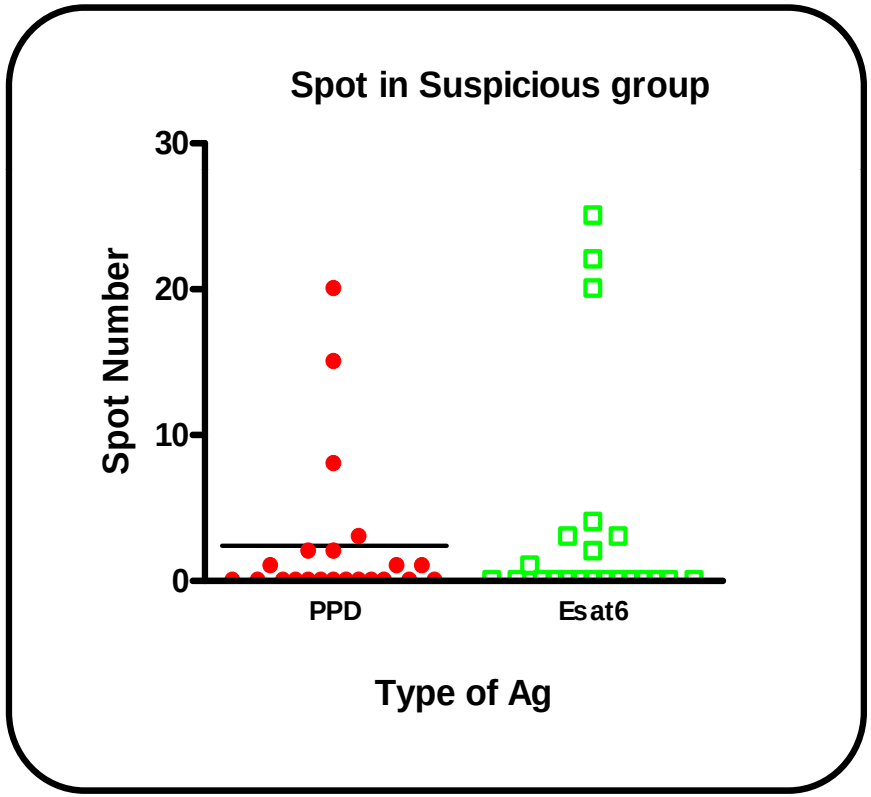




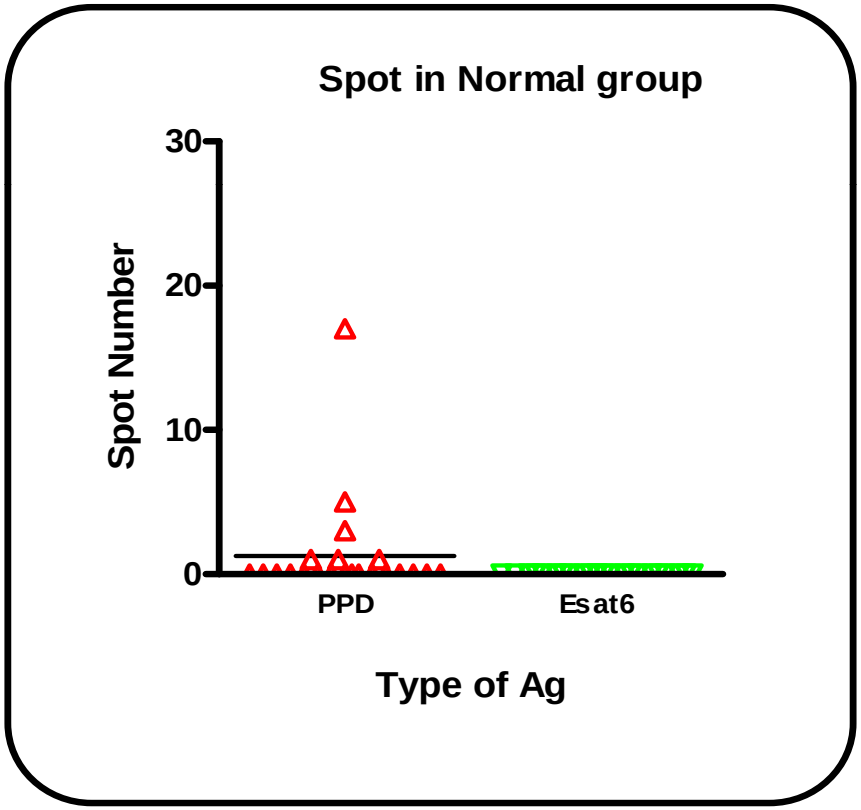
ESAT_6: Patient group: 11.23 ± 2.3
PPD: Patient group: 7.43 ± 1.5

ESAT_6: Healed group : 12 ± 3.4
PPD: Healed group : 7.75 ± 1.5

Spot number in *suspicious* and *Normal* groups

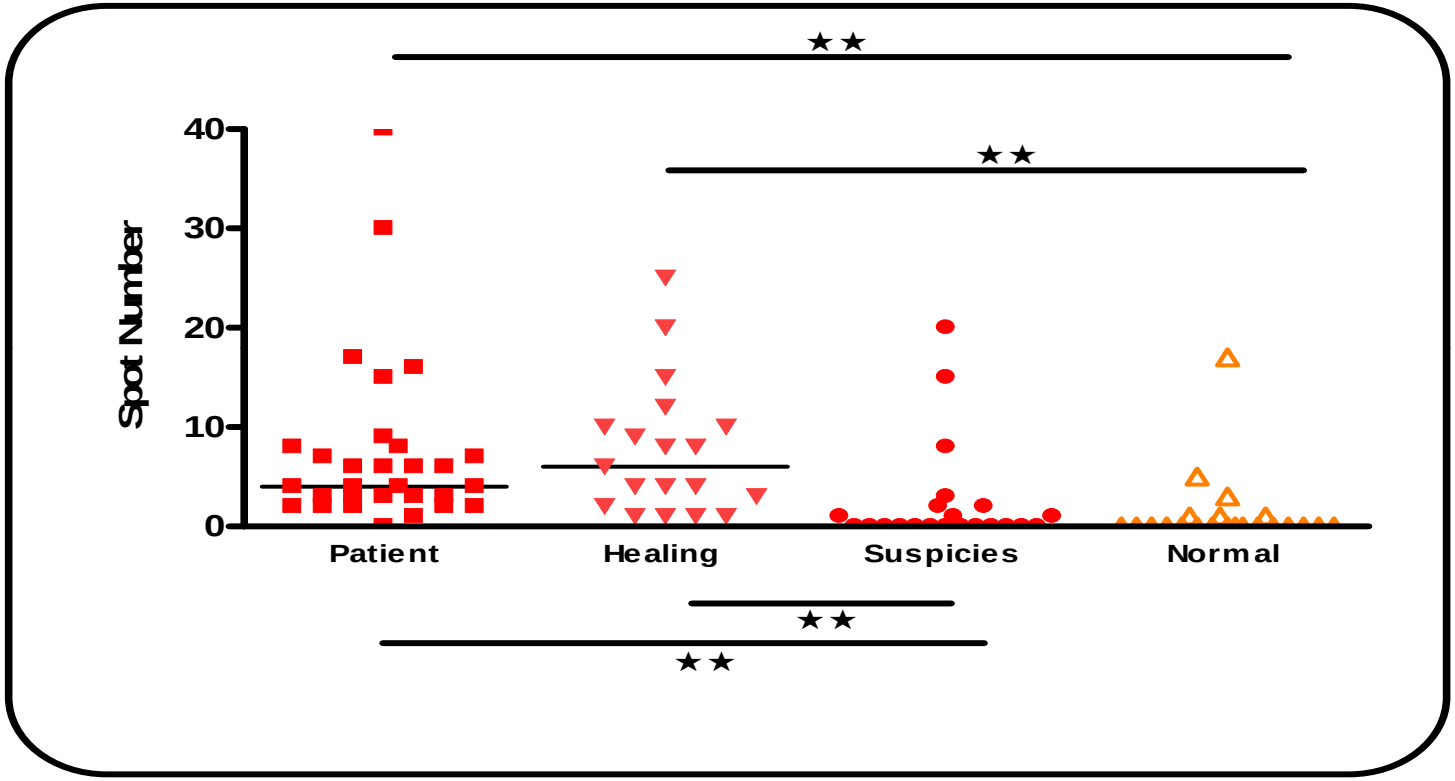


ESAT-6: 3.63 ±1.65
PPD: 2.42 ± 1.12



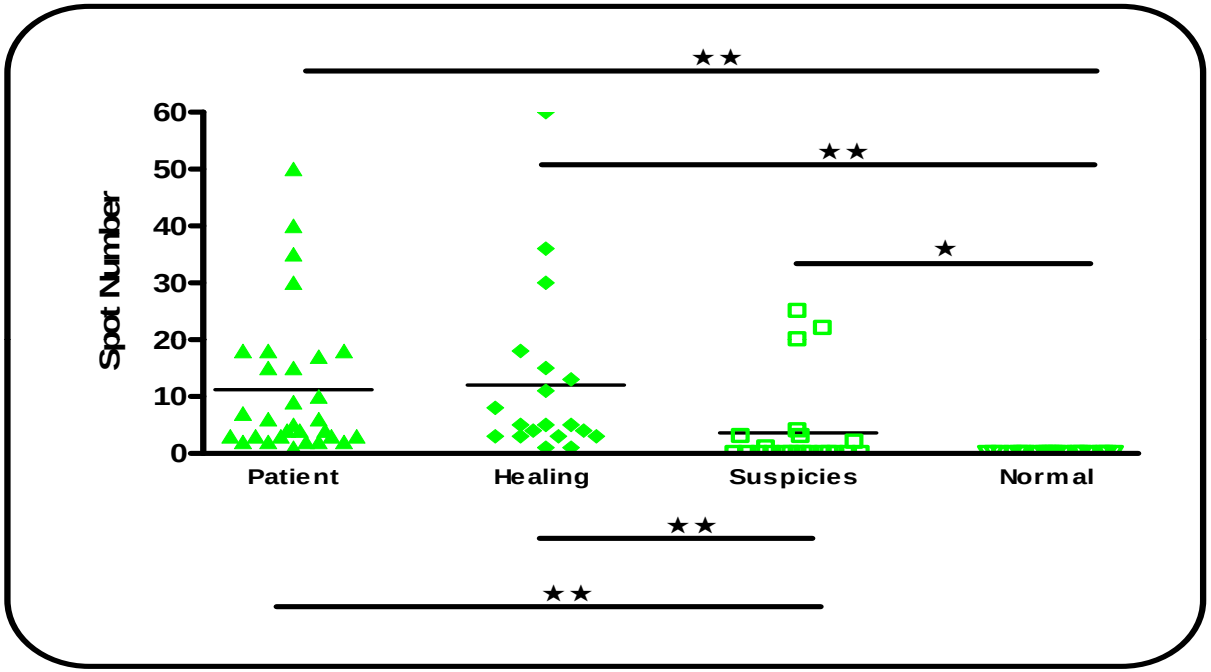
ESAT-6: 0
PPD:1.27 ± 0.79

✓ **Spot number of PPD antigen stimulated in different groups**



Suspected and the patient groups (P<0.001)
Patients and normal groups (P<0.001)
Treatment and normal (P<0.001)
patient and treated groups (P=1)
Suspected and normal (P<0.526)

✓ **Analysis of spots stimulates Esat-6 antigen in different groups**



Suspected and the patient groups (P<0.001)

Patients and normal groups (P<0.001)

patient and treated groups (P=1)

Suspected and normal (P=0.004)

IFN-γ production rate in different groups by ELISA method

Results at a minimum, maximum, standard error and mean IFN-γ secretory study groups in medium by ELISA method

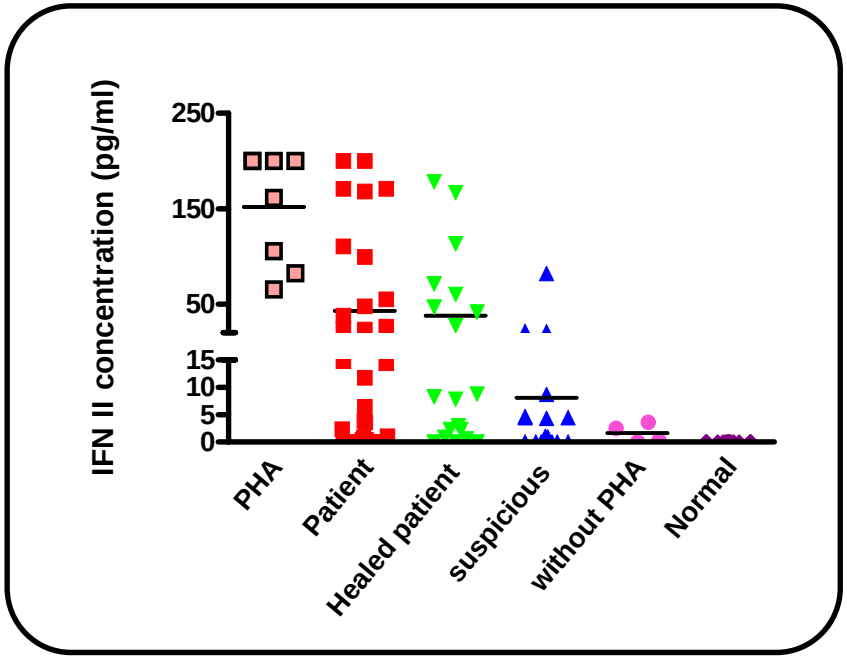
Suspected and the patients groups (P<0.018)

Patients and normal groups (P<0.003)

patients and treated groups (P<0.950)

Suspected and treated (P=0.136)

Suspected and normal (P=0.944)



groups	No.	Mean	SE	Min	Max
patient	30	47.12	12.3	0	200
Treatment	19	39.79	12.8	0	178.3
Suspected	22	7.58	3.7	0	82.17
normal	22	0	0	0	0

Discussion

IFN-γ As a marker for activated lymphocytes …

Use ESAT-6 antigens in the Elispot test:

Patients and treated groups: **All positive**

Normal group: **All negative**

Suspected group：27.3% **positive !**

False positive and Negative

Use PPD antigens in the Elispot test:

Patients and treated groups: **All positive**

Normal group: 27.3% **positive !**

Thus, results of measurement of IFN-γ secretion by lymphocytes stimulating environment with ESAT-6 to the ELISA test results to **confirm** the ELISPOT test….

- ✓ patients and treated groups: **positive**
- ✓ the Normal groups: **Negative**
- ✓ 4 patients **Negative**

No significant differences between normal and suspected whit ELISA While ELISPOT test…

overlapping between TST and Elispot test about 53% While in our study 25%....

✓Flow Cytometry

✓Real Time PCR

Role of IFN-γ in host immunity against TB, activating macrophages to induced Cytokines such as the:

- Inducible Protein10 **(IP-10)**
- Monocyte Chemo attractant Protein 2 **(MCP-2)**
- Monokine induced by IFN-gamma **(MIG)**

Dual staining ELISPOT method


Thanks for your attention