

## Abstract

### Background.

Establishing diagnosis of latent and active histoplasmosis is challenging. Interferon-gamma release assays (IGRAs) may provide evidence of latent and active infection.

### Methods.

An ELISpot assay was developed using yeast cell lysate (YCL) antigen prepared from a representative North American *H. capsulatum* strain. Assay parameters were optimized by measurement of responses in healthy volunteers with and without *Histoplasma* infection. Assay performance as an aid to diagnose histoplasmosis was assessed in a prospective cohort of 88 people with suspected or confirmed infection, and 44 healthy controls enrolled in two centers in N. America (2013-18).

### Results.

Antigen specificity of IFN $\gamma$  release was demonstrated using ELISpot and ELISA. Antigen-evoked, single-cell mRNA expression by memory T cells was shown using flow cytometry. Receiver operating characteristic curve AUC estimated 0.89 (95% CI 78.5-99.9%). Likelihood ratios were maximal ( $\geq 26.2$ ) using the cut-off of 4 ASC (Adjusted Spot Counts), where the sensitivity was 77.2% (95% CI: 54.6-92.2%) and specificity was 100% (95% CI: 89.7-100%). Sixteen of 44 healthy volunteers (36.4%) from a hyper-endemic region had positive responses, altering specificity in the entire cohort of people tested (80%, supporting unrecognized latent infection).

### Conclusion.

This ELISpot assay is sensitive and specific as an aid to diagnose *H. capsulatum* infection and disease, warranting further development as a routine diagnostic test performed in clinical microbiology labs.

**Table 1. Demographics and Clinical characteristics of Subjects Enrolled in the Study (n=88)**

VARIABLE	Number (%)
Gender (male, %) <sup>1</sup>	55 (64.7)
Age (median years, range) <sup>1</sup>	54 (9-91)
Race <sup>1</sup>	
White	68 (80)
Black / African American	11 (12.9)
Asian	6 (7.1)
Ethnicity Hispanic (number, %) <sup>1</sup>	6 (7.1)
Region of Endemicity	
Southeast U.S.	48 (54.5)
Northeast U.S.	16 (18.2)
Midwest U.S.	5 (5.7)
Rocky Mountain U.S.	1 (1.1)
Southwest U.S.	1 (1.1)
Central or South America	9 (10.2)
Unknown	8 (9.1)
Underlying condition <sup>2</sup>	
Hematological malignancy	22 (25)
Solid organ transplant	20 (22.7)
Autoimmune / Immunologic	11 (12.9)
HIV/AIDS	4 (4.5)
Solid tumor	4 (4.5)
Pulmonary disease	4 (4.5)
None	20 (23.5)
Other	3 (3.5)
Absolute lymphocyte count (median, range) <sup>1</sup>	690 (0-6470)
Diagnoses <sup>3</sup>	
Histoplasmosis, proven	14
Histoplasmosis, probable	23
Histoplasmosis, possible	14
Aspergillosis, proven or probable	8
Cryptococcosis, proven	1
Possible IFI, NOS	10
No IFI, other	15
Mixed infections	3
Histoplasmosis classification	
Subacute pulmonary	12
Progressive disseminated	21
Chronic pulmonary	8
Latent	7

**Table 2. Representative cases with proven or probably histoplasmosis**

Subject	Underlying disease	Clinical presentation	Histo Ag	Histo Ab	Other Ag	Culture, Pathology	Therapy	Diagnosis	ASC
1	Kidney transplant	Swollen ankle	Positive	NA	NA	Joint fluid culture + <i>H. capsulatum</i>	Amphotericin, itraconazole	Proven, disseminated histoplasmosis	45
2	None	Pulmonary nodules	Positive	Negative	NA	NA	Itraconazole	Probable pulmonary histoplasmosis	15
3	Rheumatoid arthritis	Bronchiectasis and fibrotic changes on CT chest	Positive	Positive (1:8)	NA	NA	Itraconazole	Probable pulmonary histoplasmosis	10
4	HIV/AIDS	Respiratory distress	Positive	Negative	BDG+ (366)	BAL culture + <i>H. capsulatum</i>	Itraconazole	Proven pulmonary histoplasmosis	12
5	None	Oral lesions	Positive	NA	NA	Pathology and culture + <i>H. capsulatum</i>	Itraconazole	Proven disseminated histoplasmosis	8
6	AML	Pulmonary nodules, neutropenic fever	Positive	NA	GM EIA+ (0.5)	NA	Amphotericin, posaconazole	Probable pulmonary histoplasmosis	To = IND T+1 week = 6
7	No significant medical history	Fatigue, arthralgias, sweats, pulmonary nodules eosinophilia, arachnoiditis	Negative	Positive (1:32)	BDG- (58) GM EIA- (0.05)	NA	Itraconazole	Probable PDH	T = 64 T+1 year = 140 T+2 year = 7
8	HIV, lymphoma, s/p BMT	Pulmonary nodules, myalgias, fever	Positive	Negative	bDG- (48)	Lymph node non-necrotizing granulomas	Amphotericin, posaconazole	Probable pulmonary histoplasmosis	To = 2 T+3 month = 38 BMT donor = 15
9	Systemic sclerosis	Pneumonia, bone marrow and liver dissemination	Positive	Negative	NA	Pathology + liver, bone marrow	Itraconazole	Proven disseminated histoplasmosis	To = 6 T+1 year = 5
10	Pancreatic carcinoma, treatment-related lymphopenia	Scattered patchy and speculated consolidation, fever, diarrhea	Positive	Negative	BAL GM EIA+ (1.4) BDG NEG (44)	Pathology + granulomas lung	Posaconazole	Probable pulmonary histoplasmosis	To = IND T+3 month = 21

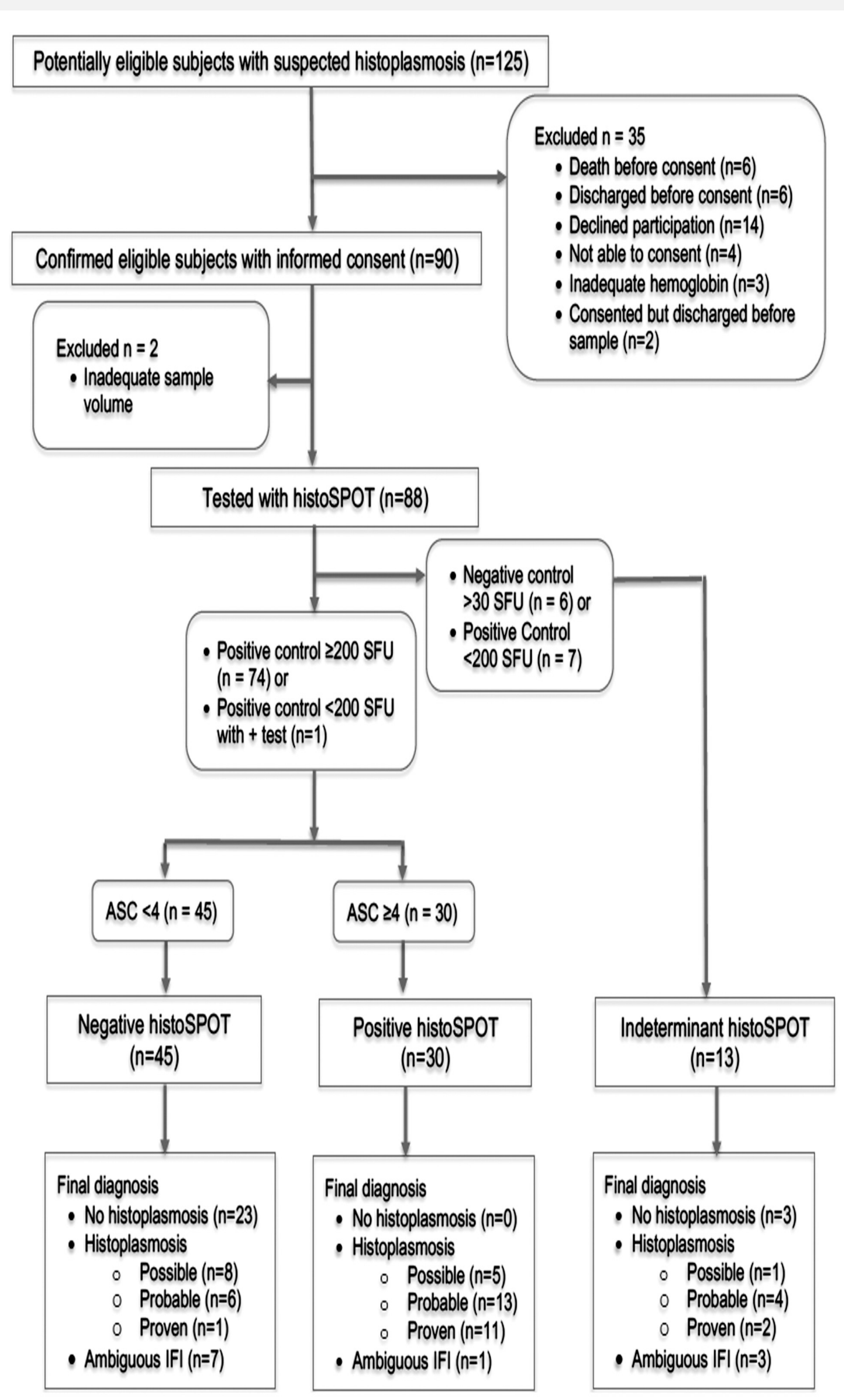
## Results Summary:

- With at least  $1 \times 10^5$  PBMC exposed to  $10 \mu\text{g}/\text{mL}$  *Histoplasma* YCL antigen for 48 h, HistoSpot spot forming units (SFU) as well as production of secreted IFN $\gamma$  were optimally detected (Fig 2). This finding was also confirmed by cytokine mRNA production using FISH-Flow (Fig 3).
- Based on frequency histograms of SFU in positive control (PHA) and negative control (no-antigen) (Fig 4), the cutoff value to accept a positive was  $\geq 200$  SFU and a negative control was  $< 30$  SFU.
- ROC curve analysis based on 79 subjects (13 proven and 9 probable Histoplasmosis cases) showed AUC at 0.89 (Fig 5), with 77% sensitivity (95% CI:54.6-92.2%) and 100% specificity (95% CI: 89.7-100%) using the cutoff value of 4 ASU. Description of 10 representative proven and probable histoplasmosis cases showed potential utility of HistoSpot assay as an aid to diagnose different forms of histoplasmosis (Table)
- Testing HistoSpot assay in 44 health subjects from the histoplasmosis endemic region showed 16 of them (36.4) had positive responses, suggesting unrecognized latent infection.

## Conclusions:

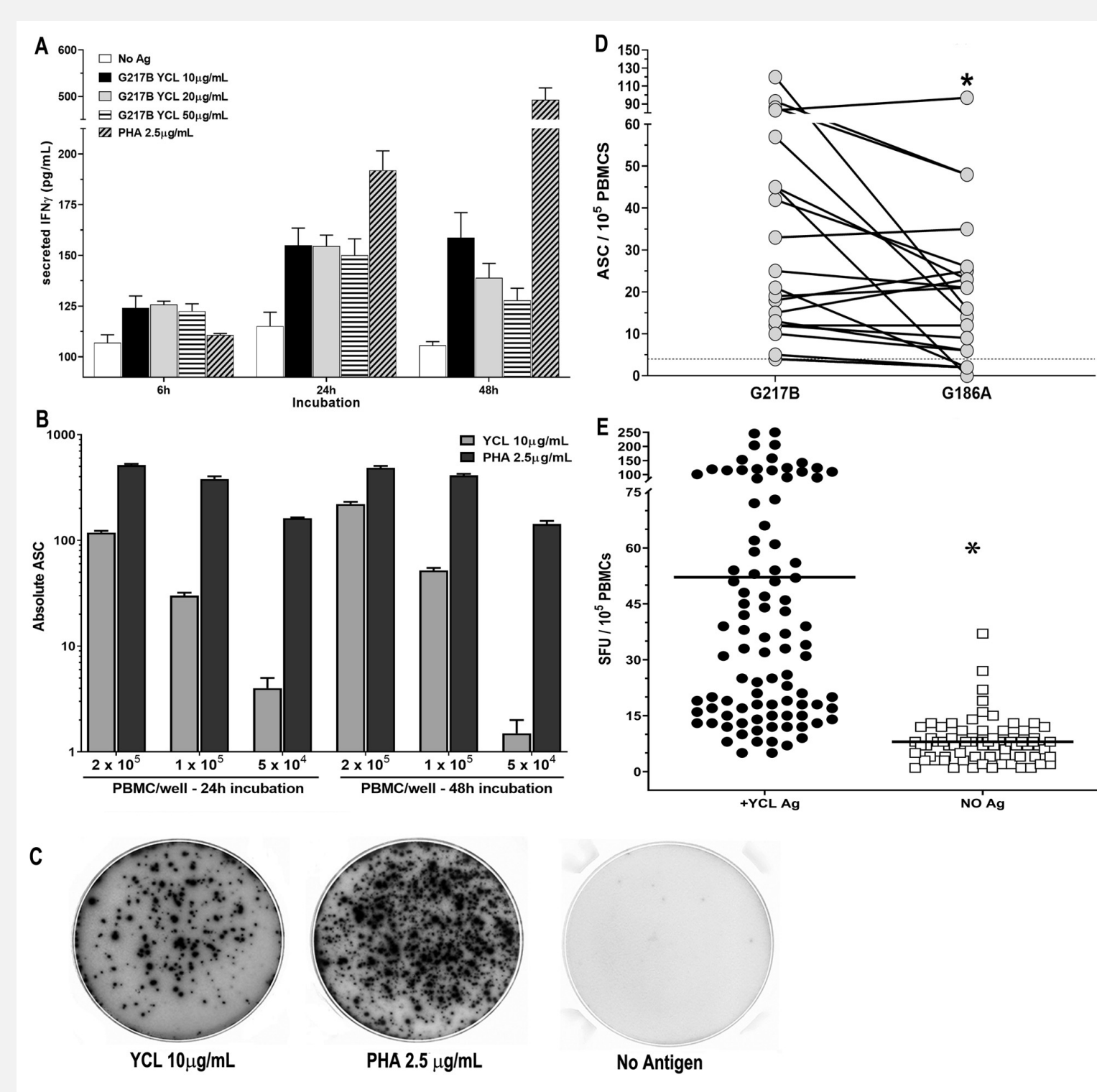
- We developed a new assay based on measuring host T-cell reactivity against *Histoplasma* antigen (HistoSpot) using sensitive and quantitative ELISpot technology.
- Our study demonstrated that HistoSpot assay could serve as an adjunct tool to aid in diagnosis of subacute and latent histoplasmosis that may be missed by current *Histoplasma* antigen and antibody based assays.

**Fig 1. STARD flow diagram of subject enrollment and adjudicated diagnoses (IFI = invasive fungal infections)**



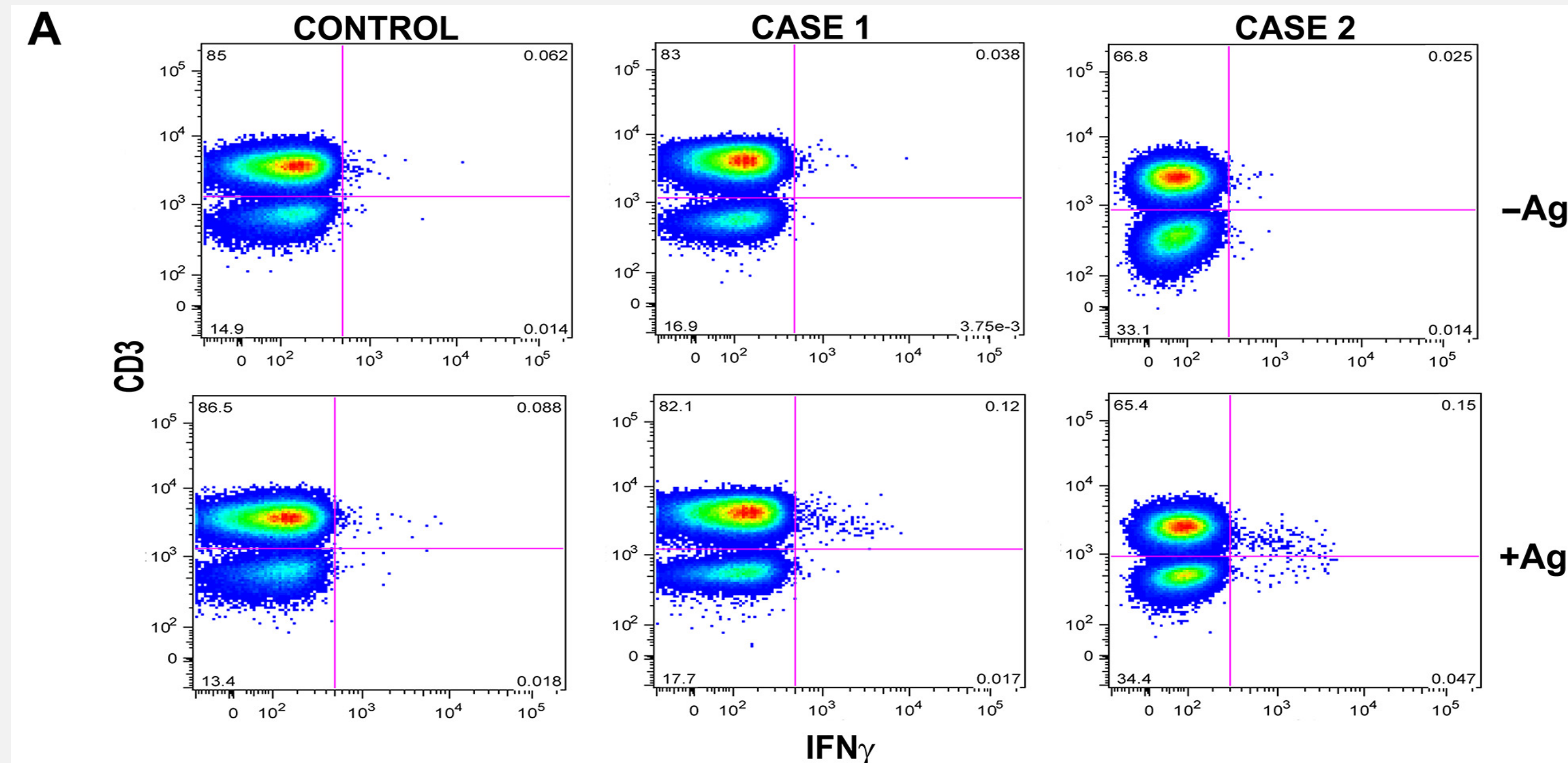
<sup>1</sup> Excludes data from 3 subjects in which demographic information unavailable. <sup>2</sup> Autoimmune / immunologic includes rheumatoid arthritis (n=1), idiopathic CD4 lymphopenia (n=1), common variable immunodeficiency (n=1), mixed connective tissue disorder (n=1), myasthenia gravis (n=1) and pauci-immune crescentic glomerulonephritis (n=1). Solid organ transplant includes kidney (n=10), liver (n=5), lung (n=2), heart (n=2) and mixed kidney/liver (n=1). Pulmonary disease includes asthma (n=1), bronchiectasis (n=1), cystic fibrosis (n=1) and sarcoidosis (n=1). Other includes Ehlers Danlos syndrome (n=1), end-stage liver disease (n=1) and congenital heart disease (n=1). <sup>3</sup> No IFI, other adjudicated diagnoses include: no definitive diagnosis (n=4), allergic bronchopulmonary aspergillosis (n=1), hemophagocytic lymphohistiocytosis (n=1), probable sarcoidosis (n=2), cytomegalovirus disease (n=1), *Helicobacter pylori* infection (n=1), Chikungunya virus infection (n=2), meningococcalitis, NOS (n=1), bacterial sepsis (n=2) and rickettsiosis (n=1). Mixed infections include possible mixed IFI + mycobacterial infection (n=2) and mixed cryptococcosis + histoplasmosis (n=1).

**Fig 2. Optimizations of assay conditions**



**A:** IFN $\gamma$  (measured by ELISA, mean of 4 replicate wells from 2 independent assays) secreted in response to different antigen or mitogen concentrations at different incubation times by PBMCs from one healthy volunteer case. **B:** Quantitative HistoSpot responses measured using different PBMC inputs and antigen exposure times, using PBMCs of same healthy volunteer in three different experiments. **C:** Representative IFN $\gamma$  spot appearance in HistoSpot wells in response to G217B YCL and PHA mitogen. **D:** HistoSpot responses in 21 subjects with proven / probable / possible histoplasmosis, comparing two N. American strains; \*  $p < 0.017$ , paired T-test. The dotted line represents ASC 4, the cutoff ASC for G217B YCL. **E:** HistoSpot responses of one subject tested over 4 years; \*  $p < 0.001$ , Mann Whitney U test. \*  $p < 0.05$

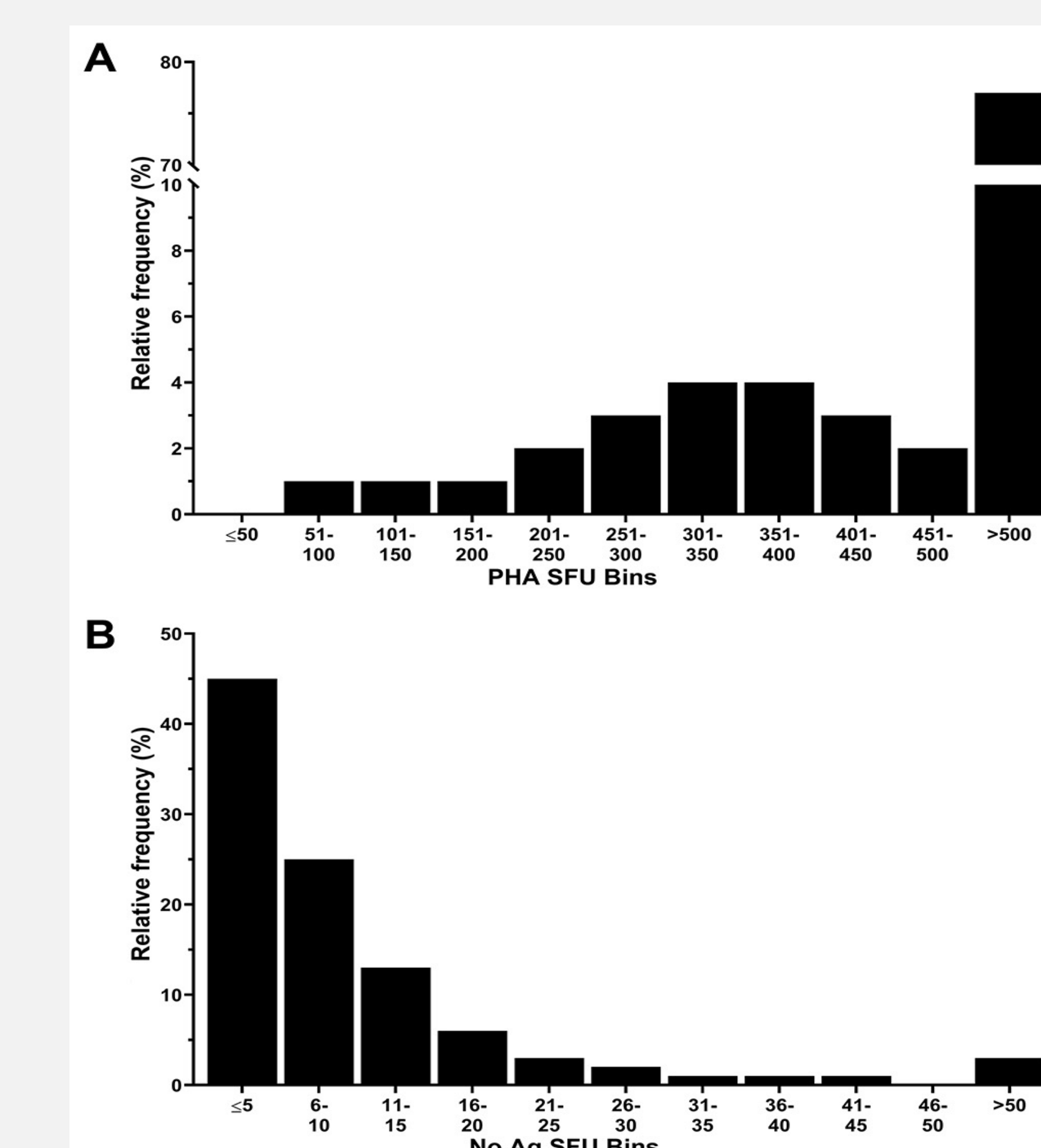
**Fig 3. Cytokine mRNA production after antigen (or no antigen control) using FISH-Flow**



	Control		Case #1		Case #2	
	-Ag	+Ag	-Ag	+Ag	-Ag	+Ag
IFN $\gamma$	0.062	0.088	0.038	0.12	0.025	0.15
IL-2	0.028	0.077	0.027	0.11	0.028	0.087
TNF- $\alpha$	0.021	0.009	0.005	0.072	0.018	0.076
IL-17	0.069	0.038	0.075	0.17	0.037	0.062

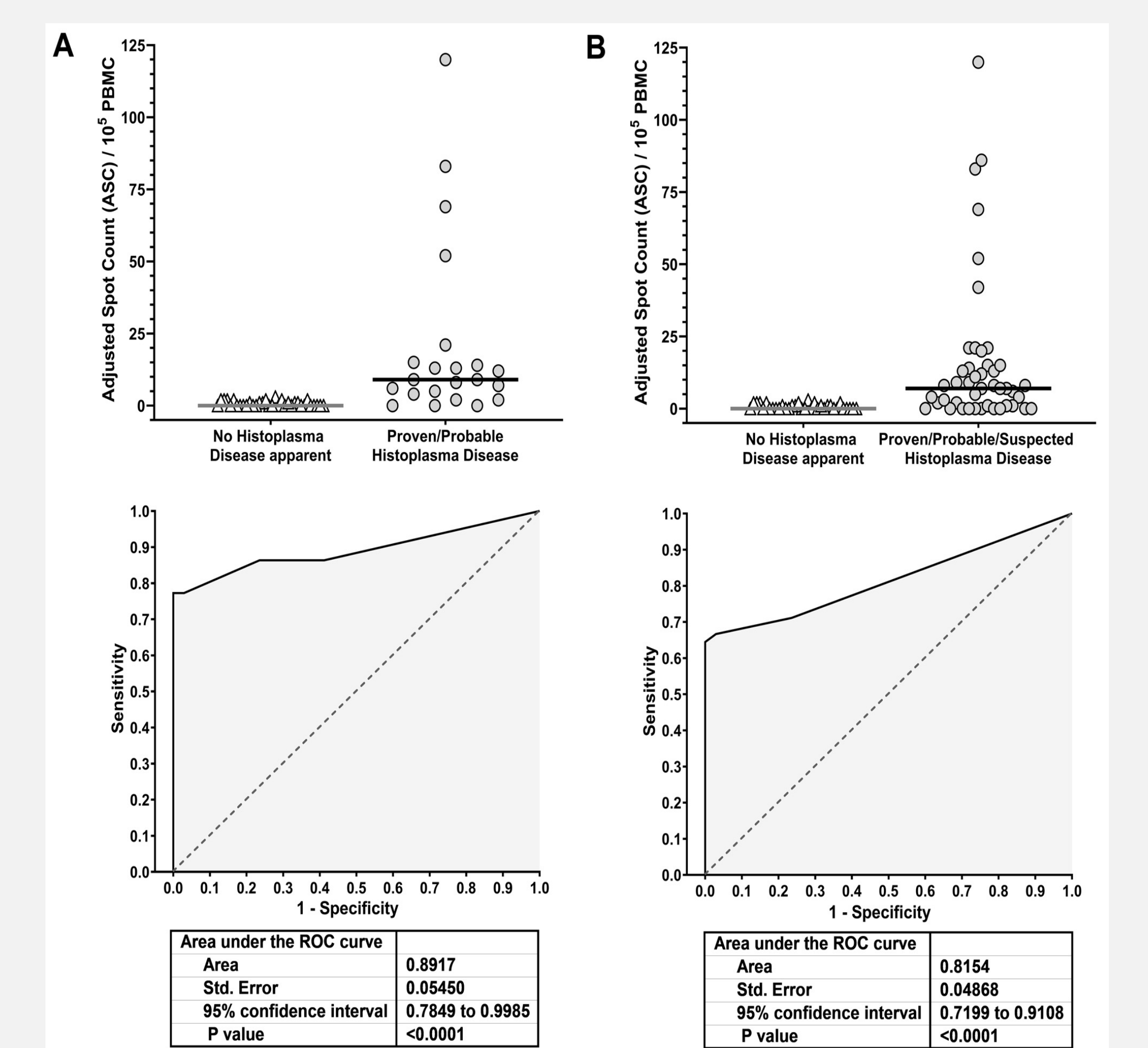
**A.** Representative flow cytometric distribution of IFN $\gamma$  mRNA producing CD3+ T-cells from one healthy control and two histoplasmosis cases. **B.** Frequency of cells expressing IFN $\gamma$ , IL-2, and IL-17 mRNAs after control and Ag-exposure from healthy control.

**Fig 4. Frequency histograms of SFUs in control ELISpot wells**



**A.** Mitogen (PHA, 2.5  $\mu\text{g}/\text{mL}$ ) assay acceptance cutoffs of PHA  $\geq 200$  SFUs and No Antigen  $\leq 30$  SFUs based on the frequencies were incorporated into assay algorithm.

**Fig 5. Receiver Operator Characteristics (ROC) curves drawn from subjects in the JHU cohort, with case definitions**



**A.** Proven/Probable infection. **B.** Proven/Probable and Suspected Histoplasmosis infection. Spot distributions and computed performance values based on different cutoffs are as shown.