Comparative performance of the laboratory assays used by a Diagnostic Laboratory Hub for opportunistic infections in people living with HIV

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Abstract:	Objectives: We evaluated the comparative performance of different assays used in a Diagnostic Laboratory Hub that linked 13 HIV health care facilities for the diagnosis of tuberculosis, histoplasmosis, and cryptococcosis, and describing its functions in Guatemala compared with other National Reference Laboratories. Methods: The following diagnostic techniques were analyzed in 24 months (2017-2018) in a cohort of patients with HIV: smear microscopy, mycobacterial and fungal cultures, isolator blood culture, PCR assays, and antigen detection tests. Results: A total of 4,245 patients were included, 716 (16.2%) had an opportunistic infection: 249 (34.7%) tuberculosis (TB), 40 (5.6%) NTM, 227 (31.7%) histoplasmosis, 138 (19.3%) cryptococcosis, and 62 (8.6%) had multiple OIs. Two hundred sixty-three (92.6%; 95%CI, 89-95.1) of TB cases were diagnosed by PCR. Urine antigen assay detected 94% (95%CI, 89-96) of the disseminated histoplasmosis cases. A lateral flow assay to detect cryptococcal antigen (CrAg) diagnosed 97% (95%CI, 93.3%-98.7%) of the cryptococcal cases. In 85 patients (51.5%) with a CSF sample, cryptococcal

	meningitis was diagnosed in 55 (64.7%), of which 18 (32.7%) were only detected by CrAg. Conclusions: Validated commercial antigen tests, as used in this program, should be the new gold standard for histoplasmosis and cryptococcosis diagnosis. In their absence, 35% of disseminated histoplasmosis and 32.7% of cryptococcal meningitis cases would have been missed. Patients with multiple OIs were frequently diagnosed and strategies should be designed to screen patients irrespective of their clinical presentation in low resource settings, Diagnostic Laboratory Hubs can deliver quality diagnostics services in record time at affordable prices.

Table

Table 1. Baseline characteristics of the patients with OIs screening

Characteristics	**OIs cases**		**Non-OI cases**		**Total**	
	n=716 (16.8%)		n=3529 (83.2%)		n=4245 (100%)	
Gender	n	%	n	%	n	%
Male	476	66.5	2201	62.4	2677	63.1
Female	235	32.8	1291	36.6	1526	35.9
Transsexual	5	0.7	37	1.0	42	1.0
Age, mean (range)						
n	709	99.0	3517	99.6	4226	99.5
Mean (range)	38	(14-78)	36	(13-89)	36	(13-89)
Sexual orientation						
Heterosexual	592	82.7	2642	74.9	3234	76.2
Homosexual	68	9.5	602	17.1	670	15.8
Bisexual	30	4.2	207	5.9	237	5.6
Unknown	26	3.6	78	2.2	104	2.4
Category of patient						
Newly HIV infection	385	53.8	1742	49.4	2127	50.1
On ARV	191	26.7	1134	32.1	1325	31.2
Return/restart	138	19.3	641	18.2	779	18.4
Unknown	2	0.3	12	0.3	14	0.3
CD4 cell count						
n	477	66.6	2537	71.9	3014	71.0
<200	376	78.8	1104	43.5	1480	34.8
Viral Load						
n	470	65.6	2355	67.1	2825	66.5
Median (IQR)	5.1	(4.4-5.6)	4.6	(3.5-5.1)	4.7	(3.6-5.2)

Table 2. Comparative performance of the techniques employed for the OIs diagnosis

	Tuberculosis* (n=290)			**Histoplasmosis** (n=271)			**Cryptococcosis** (n=170)		
Diagnostic modality	**No. (%)**	**+ve**	**(95% IC)**	**No. (%)**	**+ve**	**(95% IC)**	**No. (%)**	**+ve**	**(95% IC)**
Single test									
Smear	284 (97.9)	40	14.1 (10.5-18.6)	---	---	---	---	---	---
Culture[1]	286 (98.6)	109	38.1 (32.7-43.9)	211 (77.8)	18	8.5 (5.4-13.0)	85 (51.5)	37	---
Isolator[2]	207 (71.4)	18	8.7 (5.6-13.3)	190 (70.1)	69	36.3 (29.8-43.3)	129 (75.8)	19	14.7 (9.6-21.8)
PCR	284 (97.9)	263	92.6 (89-95.1)	215 (79.3)	135	62.7 (56.6-69.4)	---	---	---
Urine Ag	---	---	---	260 (95.9)	188	72.3 (66.5-77.3)	---	---	---
Serum Ag	---	---	---	---	---	---	165 (97.0)	165	100 (99.7-100)
CSF Ag	---	---	---	---	---	---	93 (54.7)	64	68.8 (58.8-77.3)
Combined test									
Smear + culture	284 (97.9)	119	41.9 (36.3-47.7)	---	---	---	---	---	---
Smear + PCR	278 (95.8)	261	93.9 (90.4-96.1)	---	---	---	---	---	---
PCR + culture	280 (96.5)	280	100 (98.5-100)	207 (76.4)	138	66.7 (60-72.7)	---	---	---
PCR + isolator	---	---	---	157 (57.6)	119	75.8 (68.5-81.8)	---	---	---
Urine Ag + isolator	---	---	---	186 (68.6)	136	73.1 (66.3-79.0)	---	---	---
Urine Ag + PCR	---	---	---	206 (76.0)	198	96.1 (92.5-98.0)	---	---	---
Urine Ag + isolator PCR + Culture	---	---	---	153 (56.4)	153	100 (99.7-100)	---	---	---

*NMT were excluded because the unique diagnostic technique employed was culture [1]Culture was performed from sputum for histoplasmosis and tuberculosis, and CSF for cryptococcal meningitis; [2]Isolator blood culture

Table 3. Main activities of the National Reference Centers and the Diagnostic Laboratory Hub (DLH) in Guatemala

	National Reference Laboratories				Diagnostic Laboratory Hub (DLH) Guatemala
General area of action	Centers for Disease Control and Prevention (CDC) United States of America	National Administration of Laboratories and Institutes of Health (ANLIS) Dr. G. Malbran, Argentina	Institute Gorgas Reference Central Laboratory (Panama)	National Reference Laboratory Guatemala	
Health services	• Detect infectious organisms, food-borne outbreaks, and biosecurity threats • Identify environmental and workplace hazards • Monitor the health of communities • Invent new ways to rapidly test for infectious disease in the field • Arm state, county, and local public health laboratories with the expertise and data they need to protect their citizens	• Coordinates the national network of laboratories and provides technical assistance • Perform diagnostic confirmation • Contributes to surveillance activities • Provides scientific advice	• Design, promote, coordinate and execute research programs • Provides scientific advice to the Ministry of Health and other institutions • Evaluate new health technology and research policies • Promote cooperation agreements, for the development of health knowledge • Provide services such as the Central Laboratory of Public Health	• Provides technical advice • Produces technical guidelines, regulations and teaching materials. • Technically support research of interest for public health and epidemiological surveillance. • Provides support in the quality processes of the laboratory network.	• Provides high quality diagnosis services for fungal infections and tuberculosis for a network of HCFs • Coordinates communication activities for the network • Implements and monitor the delivery of specimens between the HCF and the DLH • Develops informatic tools to strengthening communication of the network • Collects epidemiological data
Early warning and response	• Deploy diagnostic tests and tools, such as advanced molecular detection technology that helps scientists detect health threats more quickly	• Description of possible warnings to detect outbreaks and communication to the Ministry of health	• Develop surveillance and public health programs	• Participates in research in health surveillance and public health	

Training	• Sponsors and hosts new initiatives and training to encourage laboratory scientist networking and promote cross-cutting concepts.	• Provides educational programs	• Offer health information and documentation services	• Participates in the training activities for medical and laboratory personnel in areas of virology, parasitology and bacteriology and epidemiological surveillance.	• Supports training activities within the network

Centers for Disease Control and Prevention: https://www.cdc.gov/labs/protecting-america.html
National Administration of Laboratories and Institutes of Health (ANLIS) Dr. G. Malbran, Argentina: http://www.anlis.gov.ar/mision-vision-y-objetivos/
Institute Gorgas Reference Central Laboratory: http://www.gorgas.gob.pa/objetivos/
National Reference Laboratory of Guatemala: http://portal.lns.gob.gt/

Abstract

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Short title: Diagnosis of Opportunistic Infections

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Introduction

In 2017, the World Health Organization (WHO) published their first guidelines for managing advanced HIV disease and rapid initiation of antiretroviral therapy, which were aimed to reduce global HIV morbidity and mortality [1]. These guidelines recommend a package of interventions to diagnose and treat major opportunistic infections (OIs), which continue to be an important cause of AIDS-related mortality [1,2]. Hence, identifying people who are eligible for elements of the package of care, requires quality diagnostic services which have a crucial role to improve clinical outcomes [3-5].

According to UNAIDS, Guatemala has the largest number of people living with HIV (PLWH) in Central America, the highest proportion presenting with advanced HIV disease and high viral loads which are factors that increase the risk of OIs [6,7]. Despite the development of the HIV health care program and increasing coverage of antiretroviral therapy (ART), access to accurate testing for OIs was limited. In 2017, to provide diagnostic services for mycobacterial and fungal infections, a Diagnostic Laboratory Hub (DLH) was implemented linked with a national network of HIV health care facilities (HCFs). Here, we evaluate the comparative performance of different diagnostic assays employed in the DLH and describe the functions of this Health System in comparison with those provided by National Reference Laboratories (NRL).

Materials and Methods

Setting and population

From January 2017 through December 2018, diagnostic services for tuberculosis (TB), non-tuberculous mycobacteria (NTM), histoplasmosis and cryptococcosis were provided by the DLH to a national network of 13 HIV healthcare facilities (HCFs). Screening for these OIs was performed regardless of CD4 cell count for (i) newly diagnosed patients; (ii) patients who had abandoned ART (>90 days) and returned to care, and (iii) those on ART

when an OI was suspected. Clinical specimens were obtained in the HCFs and delivered to the DLH by means of a courier service. Additionally, an electronic system was set up to capture patient data, request diagnostic services and report results.

Laboratory procedures

HCFs were requested to send sputum samples, urine, serum, and 10 ml of blood in an Isolator® tube (Wampole Laboratories, Cranbury, N.J.). Additional clinical samples were also received depending on the clinical criteria. Diagnostic methods included: (i) smear for mycobacteria; (ii) Cultures for fungi and mycobacteria; (iii) Isolator blood culture; (iv) *in-house* PCR for the detection of *M. tuberculosis* and *H. capsulatum*; (v) enzyme-linked immunosorbent assay (IMMY, Norman, OK, USA) to detect *Histoplasma* antigen in urine and; (vi) lateral flow assay (IMMY, Norman, OK, USA) to detect cryptococcal antigen (CrAg). Detailed methods are described in the supplementary material.

Role of the national reference laboratories (NRLs) and the DLH

To compare the role and functions of the NRLs with the DLH, the official websites of the Center for Disease Control and Prevention (CDC, USA), the National Administrations of Laboratories and Health Institutes “Dr. Carlos G. Malbrán” (Argentina), Institute Gorgas Reference Central Laboratory (Panama), and the National Reference Laboratory (Guatemala) were searched.

Data analysis

The percentage of the diagnostic performance of each technique, with their corresponding 95% confidence intervals, was calculated against the number of total cases diagnosed. Chi-squared test was used to compare the positive proportions and significance was set at $P = 0.05$.

Geographic Information System (GIS) and google maps tools were used to describe the location of the DLH and HCFs. Statistical analysis was performed using SPSS 19.0. software (IBM Iberica, Madrid, Spain).

Results:

Health care network

Guatemala has an area of 108,889 km^2, with an asphalted road system of 7,342 Km. The national network included 13 (81.2%) of the 16 available HCFs in the country, which are mainly located in rural areas (12 out of 13; 92.3%). Nine (69.3%) of these facilities are located within an average time of 5 hours to the DLH. Regarding the distribution, 5 (38.5%) HCFs are located in the west, 4 (30.7%) in the east, 2 (15.3%) in the north, 1 (7.6%) in south, and 1 (7.6%) in the metropolitan area.

Patients characteristic and OIs diagnosed

During 2017 and 2018, a total of 4,245 PLWH were screened for OIs. Baseline characteristics are shown in **Table 1**. A total of 2,677 (63.1%) patients were male with a mean age of 36 years (range 13-89), and 2,127 (50.1%) patients were newly diagnosed with HIV infection. Patients with OIs were slightly older than without OIs (38 vs 36 years, *P = 0.002*), and had a significantly higher HIV viral load (median Log_{10} 5.1 vs 4.6 copies/mL, *P < 0.0001*). Only 71% of patients had CD4 counts done (**Table 1**).

Seven hundred sixteen (16.9%) patients had OIs: 249 (34.7%) TB, 40 (5.6%) NTM, 227 (31.7%) histoplasmosis, 138 (19.3%) cryptococcosis and 62 (8.6%) had multiple OIs. Among patients with multiple OIs, there were 24 (38.7%) histoplasmosis plus TB, 15 (24.1%) cryptococcosis plus histoplasmosis, 11 (17.7%) cryptococcosis plus TB, 3 (4.8%) cryptococcosis plus NTM, 3 (4.8%) TB plus NTM, 3 (4.8%) histoplasmosis plus NTM, and 3 (4.8%) triple infections. Almost 79% of OIs patients had < 200 CD4/mm^3 defined as advanced HIV disease by the WHO (**Table 1**) [1].

Comparative diagnostic performance among techniques

Full screening for mycobacteria, histoplasmosis and cryptococcosis was performed in 3,448 (81.2%) patients. Partial screening was done in 797 (18.8%) patients; of those 626

(78.5%) were tested for histoplasmosis, 371 (46.5%) for cryptococcosis and 272 (34.1%) for mycobacteria. The diagnostic performance results were similar between those with full and partial screening. Therefore, all cases were included in order to have more statistical power (**Table 2**).

A total of 290 cases of TB, 50 of NTM, 271 of histoplasmosis and 170 of cryptococcosis were diagnosed. These numbers encompass single infections and cases with multiple OIs; thus, total cases were used to assess the comparative performance of the diagnostic techniques (**Table 2**).

Out of the cases tested for TB (**Table 2**), direct microscopy was positive in 14.1% (95%CI, 10.5-18.6), culture in 38.1% (95%CI, 32.7-43.9), Isolator blood culture in 8.7% (95%CI, 5.6-13.3) and PCR in 92.6% (95%CI, 89.0-95.1). One hundred and sixty-one (55.5%) were only detected by PCR. All TB cases were diagnosed by a combination of PCR plus conventional culture. NTM cases were diagnosed by culture.

Of those tested for histoplasmosis (**Table 2**), Isolator blood culture, a longstanding high volume blood culture technique employed before the appearance of urine antigen for histoplasmosis, was positive in 36.3% (95%CI, 29.8-43.3), urine antigen in 72.3% (95%CI, 66.5-77.3), culture of respiratory samples in 8.5% (95%CI, 5.4-13.0) and PCR in 62.7% (95%CI, 56.6-69.4), sputum was the sample used for PCR in 92.6% of the samples tested. Urine antigen and PCR were the only positive test in 95 (35.1%) and 64 (23.6%) cases, respectively. Combining urine antigen and PCR assay the positive rate increased by 23.8% (72.3 % to 96.1%). The median CD4 cell count of those diagnosed by urine antigen testing or isolator was 33 and 20 cells/mm^3 respectively (*P=0.667*).

For cryptococcal disease, 165 out of 170 cases were diagnosed by serum CrAg. The remaining 5 cases were diagnosed by CSF CrAg (2 cases) and Isolator blood culture (3 cases). A lumbar puncture was done to 85 (51.5%) of 165 CrAg serum positive patients. A total of 55 (64.7%) cases had cryptococcal meningitis. Out of these cases, 37 (67.3%) were

157 CrAg and culture positive, and 18 (32.7%) were only CrAg positive. Of those, 35 and 16
158 cases had CD4 cell counts, with a median of 36 and 47 cells/mm^3, respectively (*P=0.692*).
159
160 **Functions of the NRLs and the DLH**
161 A review of the main activities of NRL are summarized in **Table 3**. The functions listed are
162 consistent with the role of assessment, policy development, and assurance, which
163 contribute to the public health system. Main activities included: confirmatory/reference
164 diagnostic testing, typing of microorganisms, assessment of antibiotic resistance, and
165 standardization of methodologies. In Guatemala, the NRL perform most of these activities;
166 however, do not include a Reference Laboratory for fungal infections. For the DLH the
167 main role is to provide rapid diagnostic services and to coordinate activities to improve the
168 management of OIs in the network. Indirectly, the DLH collects epidemiological data as a
169 result of the diagnostic activities. This information is shared with the HCF network as well
170 as with the Ministry of Health.
171
172 **Discussion**
173 Access to accurate diagnosis services is crucial to guide therapy and to improve clinical
174 outcomes. In this study a DLH provided rapid diagnostic services for OIs to a national
175 network of HCFs. We used a courier service for specimen transportation and a similar
176 strategy implemented in Uganda showed an eightfold increase in referral samples and TB
177 case detection [10]. In Guatemala, this system was able to provide diagnostic access to
178 81.2% of the HCFs in the country including the most remote ones.
179 Regarding TB, a low positivity rate (14.1%) of smears was found. Lower performance of
180 sputum microscopy in HIV patients has been widely described because they frequently
181 have paucibacillary infections [11]. However, our results were lower than those found by
182 previous studies, where sensitivity ranged from 30-48% [11,12]. A low positivity rate was

183 also found for TB culture with 38.1% vs 62.6% in the literature [13]. Nevertheless, culture
184 is essential to isolate the microorganism, and determine drug susceptibility, and monitor
185 resistance rates. To increase the detection rate, it would be necessary to explore the use
186 of different transportation systems that could improve mycobacteria recovery. TB PCR
187 detected 92.6% of the cases, which is similar to other reports (90 to 100%) [14,15]. Xpert
188 MTB/RIF and lateral flow lipoarabinomannan (LAM) assay were not available at the DLH.
189 Concerning histoplasmosis, the sensitivity of the urine antigen (72.3%) and the isolator
190 blood culture (36.3%) were lower than previously reported 81-98% and 66.7-74.2%
191 respectively [16,19]. It is well-known that urine antigen is designed to diagnose
192 disseminated histoplasmosis and thus, it is expected to be negative in localized
193 histoplasmosis. Unfortunately, in this study clinical symptoms and signs that could have
194 helped to classify the histoplasmosis cases were not recorded by the DLH. Therefore, we
195 assumed that a patient with a positive urine antigen, and/or Isolator blood culture had
196 disseminated histoplasmosis. Considering this definition, the number of disseminated
197 histoplasmosis was 205 (77.3%) out of 265 patients. Of those, 94% had a urine antigen
198 positive. In patients with Isolator and urine antigen test, seven cases had a positive
199 Isolator blood culture and negative result in urine antigen, which means that the use of
200 both techniques increases the positivity rate by 3.5% but at a substantially higher cost. The
201 cost of one determination of *Histoplasma* antigen in Guatemala is $13 vs $ 22 for the
202 Isolator blood culture. Other study found that histoplasmosis may cause proteinuria [20]
203 and other kidney damage, that in some cases, could render a false negative antigen result.
204 An assay designed for TB in urine samples, LAM have shown that early morning urine and
205 sample concentration can increase the sensitivity [21]. Further studies are needed to
206 evaluate the influence of these factors. The urine antigen test has resulted in a higher
207 detection of the histoplasmosis cases. Global access to these diagnostic tests, especially

208 in Latin America, would increase awareness as well as the availability of an early
209 diagnosis and treatment which will certainly increase the patient survival.
210 For cryptococcosis, we found that the positivity rate of the CrAg test in serum was
211 consistent with previous reports (98-100%) [22,23]. Eighteen meningitis cases were
212 diagnosed only by CrAg detection, highlighting the importance of this assay in the rapid
213 diagnosis of this severe infection. CSF culture alone would have only diagnosed 67% of
214 cryptococcal meningitis cases and at a slower response rate. Similar results were reported
215 in other study with 10% of patients with a positive CSF CrAg having a negative culture
216 [24]. The DLH used CrAg CSF results as India ink microscopy is less sensitive and time
217 consuming [25].
218 Taking into account our findings, we recommend the following approaches. (i) The sensitivity
219 of direct microscopy for AFB is very low. It is a demanding technique that requires patience
220 and expertise to get good results but provides a quick answer. Laboratories should evaluate
221 the usefulness of this technique considering their available workforce. (ii) Culture is an
222 insensitive technique for both, *Mycobacteria* and fungi, but in our opinion, should be
223 maintained in order to recover the microorganisms for proper identification, susceptibility
224 testing, and typing. (iii) Isolator blood culture has a low sensitivity and we do not recommend
225 its use as a diagnostic tool. However, it could be useful for the recovery of *Histoplasma*
226 isolates when the urine antigen is positive. Therefore, if a patient had a positive urine
227 antigen, we would recommend asking for an urgent Isolator blood culture. (iv) TB PCR
228 should be used because it has a good sensitivity and delivers results in a short time.
229 However, we recommend the implementation of standardized commercial techniques,
230 already available, such as the Xpert MTB/RIF assays. For histoplasmosis there is no
231 commercial PCR system and, although our *in-house* PCR has an acceptable performance,
232 we cannot give a clear recommendation about its use until further analysis about its
233 comparative diagnostic performance in the diagnosis of localized histoplasmosis is done;

(v) Antigen detection techniques are the current gold standard for diagnosing cryptococcosis and disseminated histoplasmosis and should be available in every single laboratory dealing with OIs in PLW. Without these tests, 95 (35%) disseminated histoplasmosis and 18 (32.7%) cryptococcal meningitis cases would have been missed. Currently, these methods are included in the WHO Essential Diagnostics List [26]. (vi) The rate of patients with multiple OIs in advanced HIV in Guatemala is substantial. Therefore, optimal case-finding strategies should include screening for potential OIs irrespective of their clinical presentation, and the local prevalence of different OIs. Similar diagnostic interventions should be considered in other low resource settings.

This study also demonstrates that the DLH does not interfere with other health care systems. The European Center for Diseases Control (ECDC) classifies the main activities of microbiology reference laboratories into five core functions: i) diagnostic confirmation services, which include reference methods for specific pathogens with a low prevalence or not covered by the usual commercial portfolio; ii) scientific advice; iii) collaboration and research; iv) provision of reference materials, and v) monitoring alert and response [27]. The functions of the NRLs are similar to the core activities outlines by the ECDC, which reflect the homogeneity of the Reference Laboratory activities. Guatemala NRL aims are similar but OIs diagnosis for PLWH is not included in its activity's portfolio. On the other hand, the main goal of the DLH is to provide quick diagnostics services for improving the care and management of patients as well as to aggregate the epidemiology data obtained from the diagnostic analysis. Besides, with more automation, the DHL can manage thousands of samples without a huge increase in labor costs.

This study has several limitations. The transport time was not measured and thus we cannot determine their impact. Additionally, we did not collect data of patients' symptoms and the added value of it was not determined. Despite this, a large number of patients and events were analyzed allowing us to make clear diagnostic recommendations for other

countries and states that want to improve the diagnosis of OIs in PLWH. The DLH is a promising approach to provide diagnostic services to a large community of HCFs at an affordable cost.

Authors' contributions: N.M., A.A-I. and JL. R-T. performed the research. A.A-I. and J.L.R.T. designed the study. L.A., O.B., and JC.P. participated in data extraction and data cleaning. D.M. and B.S. contributed essential reagents or tools. N.M. and JL. R-T. analysed the data. N.M. wrote the paper. A.A-I, E.A, DW. D, JL. R-T participated in critical revisions.

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Conflict of interest:

A. A-I. has received research grants or honoraria as a speaker or advisor from Astellas, Gilead Sciences, MSD, Pfizer, F2G, Amplyx and Scynexis outside the submitted work.

E.A. has received honoraria from GILEAD for educational conferences and participation in Advisory board meeting.

D. W. D. holds Founder shares in F2G Ltd, a University of Manchester spin-out antifungal discovery company, in Novocyt, which markets the Myconostica real-time molecular assays and has current grant support from the National Institute of Allergy and Infectious Diseases, National Institute of Health Research, North West Lung Centre Charity, Medical Research Council, Global Action Fund for Fungal Infections and the Fungal Infection Trust. He acts or has recently acted as a consultant to Astellas, Sigma Tau, Basilea, Biosergen, Cidara and Pulmocide. In the past 3 years, he has been paid for talks on behalf of Astellas, Dynamiker, Gilead, Merck and Pfizer. He is also a member of the Infectious Disease Society of America Aspergilosis Guidelines and European Society for Clinical Microbiology and Infectious Diseases Aspergillosis Guidelines groups.

All other authors declare no conflicts of interest

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Lucy Franks
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July 19, 2020

Subject: Revision and resubmission of manuscript AIDS-D-20-00384
Dear Dr Franks,

Thank you for the opportunity to revise our manuscript. The suggestions offered by the reviewers have been helpful. We have included the reviewer comments immediately after this letter and responded to them individually. We also provide a point-by-point list of the changes as requested.
In attention to the reviewer's comments, we have considered remove Figure 1. We consider it is useful to show how the network is spread in the country, but the cost is prohibitive for us. You have to take into account that project resources are limited, and the priorities are the diagnosis and treatment of the patients. If you can consider to substantially decrease the costs of the publication, we would be very happy to include the figure. Sorry to be so direct but we have strong constraints and although it is mandatory to share our findings with the community, specially to prove that with the right resources for access to diagnosis and treatment everything is possible, we cannot afford to spend a lot of money in the publications. Sure, you fully understand our position. All the changes are listed at the final of the document.

Thank you very much for your attention
Sincerely,

Juan Luis Rodriguez Tudela, MD, PhD
Senior Advisor for GAFFI
Corresponding author

Reviewers' comments:

Reviewer #1: This is an interesting paper on an important initiative in central America.
i think that the discussion stays very focused on the "technical" aspects and should take a step back and reflect on the importance of such a model in Latin America, and elsewhere, where the burden of histoplasmosis is high for lack of diagnostic tests. their intervention is an interesting model that answers the ambitions stated in the manaus declaration.

R. Thank you for this observation. Concerning to the Manaus declaration, we have added (page 9, line 206-209): “The urine antigen test has resulted in a higher detection of the histoplasmosis cases. Global access to these diagnostic tests, especially in Latin America, would increase awareness as well as the availability of an early diagnosis and treatment which will certainly increase the patient survival”.

(page 10, line 241-242): “Similar diagnostic interventions should be considered in other low resource settings””.

Reviewer #2: This is an interesting paper which reports important data on the experience of using newer vs. older diagnostic methods to diagnose opportunistic infections (OI) in HIV-infected patients. It is an important paper since it addresses the real-world utility of various diagnostic tests in a resource-limited setting. The authors should consider addressing the following:

1. A minor terminology issue: Since all of the patients in this report are, by definition, "co-infected" with HIV and one or more OI's, would not call patients with multiple simultaneous OI's "co-infected." Suggest just stating that some patients had more than one simultaneous OI.

R. We agree with the better use of “multiple OIs” terminology. We have changed it through the entire manuscript. Abstract, Page 2 (line 39), Page 2 (line 49). Manuscript, Page 5 (line 122), Page 6 (line 136), Page 10 (line 238-239).

2. The authors emphasize that a significant percentage of patients with both histoplasmosis and cryptococcosis would not have been detected if only traditional diagnostic methods had been used. It would be useful for the authors to either describe in the results (possibly prepare a table) showing more details of the characteristics of the patients who were diagnosed vs. missed with traditional smears/stains/cultures vs. newer diagnostics. For example, of the histo patients- breakdown of disseminated vs. pulmonary or other site; CD4+ lymphocyte counts of patients by group. Similarly with cryptococosis- Did the patients who had negative cultures/India ink have higher CD4+ counts? Were the patients with discrepant results ill with meningitis vs. cryptococcemia without meningitis? More details

about all the patients with discrepant results would be helpful to both clinicians and microbiologists.

R. We thank the reviewer for this comment and we agree it is an important point, however we don't think that a new table could help in clarifying this point. Regarding Cryptococcal meningitis, it is important to mention that in the results section we said that all patients with a CSF negative culture but with a positive CrAg result had serum CrAg positive results. To compare these groups, we have now included (Page 6, line 158-160): "Of those, 35 and 16 cases had CD4 cell counts, with a median of 36 and 47 cells/mm^3, respectively (*P=0.692*)."

Regarding disseminated histoplasmosis, 95 patients who only had a positive antigen test were considered as disseminated cases. The remaining cases were diagnosed by different test combinations (results section). To clarify and compare cases only diagnosed with antigen or isolator we have added (Page 6, line 151-152): "The median CD4 cell count of those diagnosed by urine antigen testing or isolator was 33 and 20 cells/mm^3 respectively (*P=0.667*)."

In addition, we highlight in the discussion section the importance of these commercial antigen tests as the new gold standard for rapid histoplasmosis and cryptococcosis diagnosis. As discussed, we were not able to include clinical symptoms and signs that could have helped to classify the histoplasmosis cases as disseminated or localized

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Point-by-point list of the changes:

- Adding (page 9, line 206-209): "The urine antigen test has resulted in a higher detection of the histoplasmosis cases. Global access to these diagnostic tests, especially in Latin America, would increase awareness as well as the availability of an early diagnosis and treatment which will certainly increase the patient survival". and (page 10, line 241-242): "Similar diagnostic interventions should be considered in other low resource settings"".
- Use of "multiple OIs" terminology instead of coinfections and structure of the sentence: Abstract, Page 2 (line 39), Page 2 (line 49). Manuscript, Page 5 (line 122), Page 6 (line 136), Page 10 (line 238-239).
- Adding (Page 6, line 158-160): "Of those, 35 and 16 cases had CD4 cell counts, with a median of 36 and 47 cells/mm^3, respectively (*P=0.692*)."
- Adding (Page 6, line 151-152): "The median CD4 cell count of those diagnosed by urine antigen testing or isolator was 33 and 20 cells/mm^3 respectively (*P=0.667*)."
- Remove Figure 1 (Page 3, line 76) and (Page 5, line 110-111).
  Change the title "Acknowledgement" for "Authors' contributions" Page 11, Line 264