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Evaluation of the *Histoplasma capsulatum* 100-kilodalton antigen dot blot for the rapid diagnosis of progressive histoplasmosis in HIV/AIDS patients

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Abstract

Among people living with HIV (PLHIV), progressive disseminated histoplasmosis (PDH) represents an important cause of mortality. Since antigen detection allows a rapid diagnosis and the instauration of a specific treatment this study aimed to evaluate the analytical performance of the Hcp100 dot blot, an in-house assay that detects the *Histoplasma capsulatum* 100-kilodalton antigen in urine and compare it with two commercially available assays the *Histoplasma* Urine Antigen Lateral Flow Assay (MVD-LFA) (MiraVista®Diagnostics) and the *Clarus Histoplasma* Galactomannan EIA (*Clarus* HGM) (IMMY). Urine specimens from 23 PLHIV with PDH, 13 patients with other infectious diseases, and 20 healthy individuals were tested. The Hcp100 dot blot showed higher sensitivity (87.0%), specificity (97.0%) and accuracy (92.9%) than the MVD-LFA (73.9%, 78.8% and 76.8%, respectively) and the *Clarus* HGM (78.3%, 90.9% and 85.7%, respectively). The Hcp100 dot blot had high analytical performance and would be a valuable screening tool for diagnosing PDH among PLHIV.

Key words: *Histoplasma capsulatum*; Hcp100; progressive disseminated histoplasmosis; antigenuria; dot blot assay

Introduction

Histoplasmosis is one of the most common opportunistic fungal infections among people living with HIV (PLHIV) in the Americas and may be responsible for up to 43.6% of AIDS-related deaths every year in Argentina and other Latin American countries, where access to highly active antiretroviral therapy is limited [1–3]. In PLHIV, the extrapulmonary or disseminated form of the disease, known as progressive disseminated histoplasmosis (PDH), constitutes an AIDS-defining illness, whose diagnosis still remains challenging due the non-specificity of symptoms from other infectious diseases, especially tuberculosis, and the limited access to specific diagnostic assays [4,5]. As PDH is associated with high mortality rates due to late diagnosis, rapid detection of histoplasmosis and specific treatment are needed.

Detection of urine *Histoplasma* antigen is recommended by the World Health Organization (WHO)/Pan American Health Organization for the diagnosis of PDH among PLHIV due to their high sensitivity and

rapid turnaround time of diagnosis [6]. In addition, *Histoplasm*a antigen testing was also included in the second WHO model list of essential *in vitro* diagnostics [7]. In Argentina, the only commercially available assays for antigen detection are the *Clarus Histoplasma* Galactomannan Enzyme Immunoassay (*Clarus* HGM) (IMMY, USA) and the *Histoplasma* Urine Antigen Lateral Flow Assay (MVD-LFA) (MiraVista Diagnostics, USA). Both assays detect urinary *Histoplasma* galactomannan and demonstrated high sensitivity (72-98% for the *Clarus* HGM and 79-96% for the MVD-LFA) and specificity (91-100% for the *Clarus* HGM and 92-100% for the MVD-LFA) for the diagnosis of PHD among PLHIV [8–14]. However, as in other Latin-American countries, these assays are not routinely used, probably, due to problems with distribution, implementation and price control of the diagnostic kits [4,14–16].

In a previous study, we reported the use of recombinant Hcp100 and its derived polyclonal antibodies as novel reagents for the diagnosis of PDH [17]. The analytical performance of these reagents was compared with commercially available histoplasmin and the *Clarus* HGM in a cohort of 28 HIV/AIDS patients with proven PDH [18]. In the present study, we evaluated the analytical performance of the Hcp100 dot blot and compared it with MVD-LFA for *Histoplasma* antigen testing in urine. The MVD-LFA results were also compared with those previously obtained using the *Clarus* HGM.

Materials and Methods

Study specimens

A total of 56 urine specimens were evaluated: 23 from PLHIV with proven PDH; 13 urine samples from patients with other infectious diseases clinically-related with histoplasmosis, including paracoccidioidomycoses (n=3), coccidioidomycoses (n=1), cryptococcosis (n=2), tuberculosis (n=6) and toxoplasmosis (n=1); and 20 from healthy individuals from an endemic region for histoplasmosis. All specimens were blinded prior to testing.

A proven case of PDH was defined by the isolation of *H. capsulatum* in culture or by the microscopic demonstration of the fungus in clinical samples, according to the modified European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group/National Institute of Allergy and Infectious Diseases Mycoses Study Group criteria [19]. Other infectious diseases were diagnosed by culture or another gold standard technique.

Diagnosis of the all infectious diseases were performed at two Argentinian hospitals, “Hospital Posadas” (Buenos Aires province) and “Hospital Señor del Milagro de Salta” (Salta province). All samples were

collected from 2017 to 2022 as part of a former retrospective study and stored at –80 °C until time of analysis [18].

Dot blot assay for the detection of urinary Hcp100

Dot blot assay was performed as previously reported [18]. Briefly, 1 µl of urine samples were spotted onto a nitrocellulose membrane. Then, membranes were left to dry at room temperature for 15 minutes and blocked with 5% (w/v) dried non-fat milk in TBS containing 0.05% Tween 20 (TBST) during 1h at room temperature. Then, membranes were washed three times with TBST and incubated with 1:1,000 dilution of anti-Hcp100 mice antisera previously obtained by Toscanini et al in TBST/non-fat milk for 1 h at room temperature [17]. After washing again three times with TBST, membranes were incubated with goat anti-mouse IgG horseradish peroxidase conjugate (Jackson Immunoresearch Laboratories, USA) diluted 1:5,000 in TBST/non-fat milk for 1 h at room temperature. After washing, dots were visualized by using the ECL-Plus detection kit (GE Healthcare Life sciences, USA) and recorded with the VisionWorks® Image Acquisition and Analysis Software (Analytik Jena, USA). This assay was performed in triplicate in three different days.

2.3. Lateral flow assay (LFA) for the detection of *Histoplasma* galactomannan antigen

Urine specimens were also analyzed using the *Histoplasma* Urine Antigen LFA kit (MVD-LFA) (MiraVista Diagnostics, USA) according to the manufacturer's instructions. A positive result was interpreted as the presence of both the test line and the control line. A negative result was interpreted as the presence of the control line alone. The absence of a control line was interpreted as an invalid result. The tests were visually read by three in-dependent individuals.

Enzyme immunoassay for the detection of *Histoplasma* galactomannan antigen

Urine samples tested were also analyzed using the *Clarus* HGM (HGM201, IMMY, USA) following manufacturer's instructions for the semi-quantitative assay. Samples with EIA units above the cut off (≥1 EIA units) were considered positive.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism version 8.4.2 (679).

All samples were classified as TP= true positive (samples from patients with proven PDH that tested positive in the Histoplasma immunoassays); FP= false positive (samples from control individuals that tested positive in the Histoplasma immunoassays); TN= true negative (samples from control individuals that tested negative in the Histoplasma immunoassays); and FN= false negative (samples from patients with proven PDH that tested negative in the Histoplasma immunoassays).

The parameters sensitivity, specificity, positive and negative predictive values, and accuracy were calculated for all assays using the following formulas: sensitivity%= TP*100/(TP+FN); specificity%= TN*100/(TN + FP); positive predictive value%= TP*100/(TP+FP); negative predictive value%= TN*100/(TN+FN); accuracy%= (TP+TN)*100/(TP+TN+FP+FN). All these parameters were provided with the associated 95% confidence intervals (95%CI).

Kappa values and their respective 95% confidence intervals were calculated by a concordance analysis in order to evaluate the agreement between the *Histoplasma* immunoassays [20]. Kappa values were interpreted according to the following scale: Kappa <0: No agreement; Kappa between 0.00 and 0.20: Slight agreement; Kappa between 0.21 and 0.40: Fair agreement; Kappa between 0.41 and 0.60: Moderate agreement; Kappa between 0.61 and 0.80: Substantial agreement; and Kappa between 0.81 and 1.00: Almost perfect agreement.

Results

Of the patients with proven PDH, 20/23 were positive for Hcp100 antigenuria testing (Fig 1.), 17/23 were positive for the MVD-LFA, and 18/23 were positive for *Clarus* HGM rendering a sensitivity of 87.0%, 73.9% and 78.3%, respectively (Table 1 and Fig 2.). All 20 urines from healthy controls presented a negative result in the three assays.

Of the 13 urine samples from patients with other infectious diseases a positive result was evidenced only in one patient with tuberculosis (1/6) in the Hcp100 dot blot, whereas in the MVD-LFA a positive result was observed in patients with paracoccidioidomycosis (3/3), coccidioidomycosis (1/1) and tuberculosis (3/6) and in the *Clarus* HGM in patients with coccidioidomycosis (1/1) and tuberculosis (2/6) (Table 2). As a result, the overall specificity for the Hcp100 dot blot, the MVD-LFA and the *Clarus* HGM was 97.0%, 78.8%, and 90.9%, respectively (Table 1).

Out of the 56 urine samples tested, the urinary Hcp100 dot blot, the MVD-LFA and the Clarus HGM were able to correctly classify 52, 43 and 48 urines, respectively, yielding an accuracy of 92.9%, 76.8% and 85.7%, respectively (Table 1).

A total of 43/56 (76.8%) results of the urinary Hcp100 dot blot agreed with the MVD-LFA rendering a moderate agreement (kappa value of 0.519, 95% CI: 0.292-0.745) (Table 3), whereas 48/56 (85.7%) results agreed with the *Clarus* HGM rendering a substantial agreement (kappa value of 0.695, 95% CI: 0.500-0.890) (Table 3). Concordance analysis between the MVD-LFA and the *Clarus* HGM showed that 45/56 (80.4%) results agreed between both assays rendering a moderate agreement (kappa value of 0.593, 95% CI: 0.379-0.806) (Table 3).

Discussion

In this study we evaluated the analytical performance of the Hcp100 dot blot, an in-house assay for the diagnosis of PHD that detects the *H. capsulatum* 100-kilodalton antigen in urine, and compare it with two commercially available assays for *Histoplasma* galactomannan testing in urine, the MVD-LFA and the *Clarus* HGM, presenting moderate and substantial agreement respectively.

The dot blot assay for the urinary detection of Hcp100 showed higher sensitivity, specificity and accuracy than the MVD-LFA and the *Clarus* HGM for the urinary detection of *Histoplasma* galactomannan.

Previous studies have also reported a lower sensitivity value of the *Clarus* HGM than that declared by the manufacturer of this assay (90.5%). For instance, in a multicenter prospective study involving 123 cases of proven/probable histoplasmosis in Brazil, Falci et al reported a sensitivity value of 71.5%, attributable perhaps to previous use of antifungal drugs in some of the studied patients [11]. In a retrospective study of 226 patients with suspected histoplasmosis in Brazil, dos Santos Blan et al reported a similar sensitivity value (72%) using the cut-off described by the manufacturer (≥ 1 EIA units) [21].

Nevertheless, it is evident that combining two different antigens significantly increases the overall sensitivity in diagnosing PDH among PLHIV compared to using a single antigen, resulting in a combined detection rate of 22 out of 23 (95.6%) culture-proven PDH cases among PLHIV (Fig 2.).

It should be noted that one HIV/AIDS patient with proven PDH tested negative for urinary *Histoplasma* antigens using the three immunoassays evaluated in this study. This highlights the importance of using antigen testing as a complementary tool with other methods for diagnosing PDH, since a negative result for antigen testing does not rule out the possibility of the diagnosis of histoplasmosis.

Regarding specificity, the Hcp100 dot blot assay exhibited a putative cross-reaction observed in a HIV/AIDS patient with tuberculosis. Interestingly, this patient was culture-negative for *Histoplasma* but has tested positive for urinary *Histoplasma* antigen using the three different antigen detection assays evaluated in this study, suggesting a possible undiagnosed coinfection with histoplasmosis. In addition, absence of p100 orthologs in *Mycobacterium* tuberculosis might also suggest the possibility of an undiagnosed coinfection [17]. Tuberculosis can mimic histoplasmosis and is important to differentiate from each other in order to provide appropriate therapy. In any case, clinical suspicion of this fungal disease is still limited even though histoplasmosis is life threating and remains underdiagnosed [22,23]. It should be noted that conventional laboratory methods used for diagnosing histoplasmosis, such as culture and histopathology, present numerous challenges, including the requirement for complex laboratory infrastructure and staff with mycology training, a turnaround time of up to several weeks, and limited sensitivity [24]. Additionally, diagnosing through serologic methods is further complicated by low antibody test sensitivity when performed on immunocompromised individuals. While several molecular "in-house" tests have been developed and shown promising results, none of these tests are commercially available, and their standardization and validation are still pending [24]. However, the detection of urine *Histoplasma* antigen has emerged as the most viable option for diagnosing PDH due to its high sensitivity. As a result, the WHO/Pan American Health Organization recommends the use of urine *Histoplasma* antigen for diagnosing PDH among PLHIV, thanks to its high sensitivity and rapid turnaround time for diagnosis [6].

The lower specificity of the MVD-LFA and *Clarus* HGM might be related to the nature of the antigen detected, since the galactomannan is a polysaccharide found in the cell wall of *Histoplasma* and some other ascomycete molds, although structural variations exist [25].

In fact, according to data provided by their corresponding manufacturer, the *Clarus* HGM was found to be cross-reactive with *Paracoccidioides, Blastomyces* and some *Candida* specimens, whereas the antibodies used in the MVD-LFA cross-react with *Aspergillus* spp., *Blastomyces dermatitidis, Coccidioides* spp., *Cryptococcus neoformans, Cytomegalovirus, Enterococcus faecalis, Histoplasma duboisii, Klebsiella pneumoniae, Paracoccidioides brasiliensis, Sporothrix schenckii, Staphylococcus aureus, Streptococcus agalactiae* and *Talaromyces marneffei*.

In the present study, using the *Clarus* HGM assay, false positive results were observed in patients with coccidiodomycosis and tuberculosis, while using the MVD-LFA assay, positive results were observed in patients with coccidiodomycosis, paracoccidiodomycosis and tuberculosis.

The study performed presents some limitations including the small sample size, which took over 6 years to accumulate during the original project; the lack of the standard curve method for the *Clarus* HGM, which has been reported to have higher sensitivity [9]; and the lack of patients with blastomycosis and talaromycosis since Argentina is not endemic for these fungal diseases, as well as patients with aspergillosis and pneumocystosis, since these diseases might be clinically similar to histoplasmosis and require an ap-propriate treatment [26].

Further studies are needed to validate the usefulness of Hcp100 detection in urine for the diagnosis of PDH with a larger sample size of proven PDH individuals as well as with urine samples from individuals with other clinically-related infectious diseases. Whether Hcp100 might be detected in other body fluids of patients with PDH with high sensitivity and specificity as well as its utility in the diagnosis of other clinical forms of the disease should be further explored as well as the adaptation to an enzyme immunoassay format.

Among PLHIV, histoplasmosis represents an important cause of mortality. The screening approach using *Histoplasma* antigen detection assays allows a rapid diagnosis and the instauration of a specific treatment, and thus decreasing the mortality of the disease. This study shows the applicability of Hcp100 detection in urine for the diagnosis of PDH among PLHIV. Recombinant Hcp100 appears to be a promising antigen to be used in antigen testing showing a better performance to the commercial assays evaluated in this study. The Hcp100 dot blot would provide a rapid diagnosis of histoplasmosis with a turnaround time for results of approximately 4 hours with minimal requirements for laboratory training, equipment, and laboratory infrastructure. Particularly, the Hcp100 dot blot would be a low-cost alternative and simple assay with high sensitivity and specificity for the rapid diagnosis of PDH that can be easily applied to poor-resources laboratories.

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Statements & Declarations

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Competing interests

The authors declare that they do not anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Author contributions

Conceptualization, ADN and MLC; methodology, MAT, CRL, AVG, GBP, PC, RMV, YAC, DGM, ADN and MLC.; software, MAT and CRL; validation, MAT, CRL, AVG, GBP, PC, RMV, YAC, DGM, ADN and MLC; formal analysis, MAT, CRL, AVG, GBP, PC, RMV, YAC, DGM, ADN and MLC; investigation, MAT, CRL, AVG, GBP, PC, RMV, YAC, DGM, ADN and MLC.; resources, GBP, PC, RMV, YAC, DGM; data curation, MAT, CRL, AVG, DGM, ADN, MLC.; writing—original draft preparation, MAT, CRL, ADN, MLC.; writing—review and edit-ing, MAT, CRL, AVG, GBP, PC, RMV, YAC, DGM, ADN and MLC; visualization, MAT, CRL and MLC; supervision, ADN and MLC; project administration, MLC; funding acquisition, MLC. All authors have read and agreed to the published version of the manuscript.

Data availability

The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the institutional review committee "Dr Vicente Federico Del Giúdice" at Hospital Nacional Alejandro Posadas, Buenos Aires, Argentina (Ref. 395 EMnPES0/20) and by the institutional review committee at Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (EXP-UBA 58003/18, Resolution N° 821).

Consent to participate

Informed consent was obtained from all individual participants included in the study.

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Figure Captions

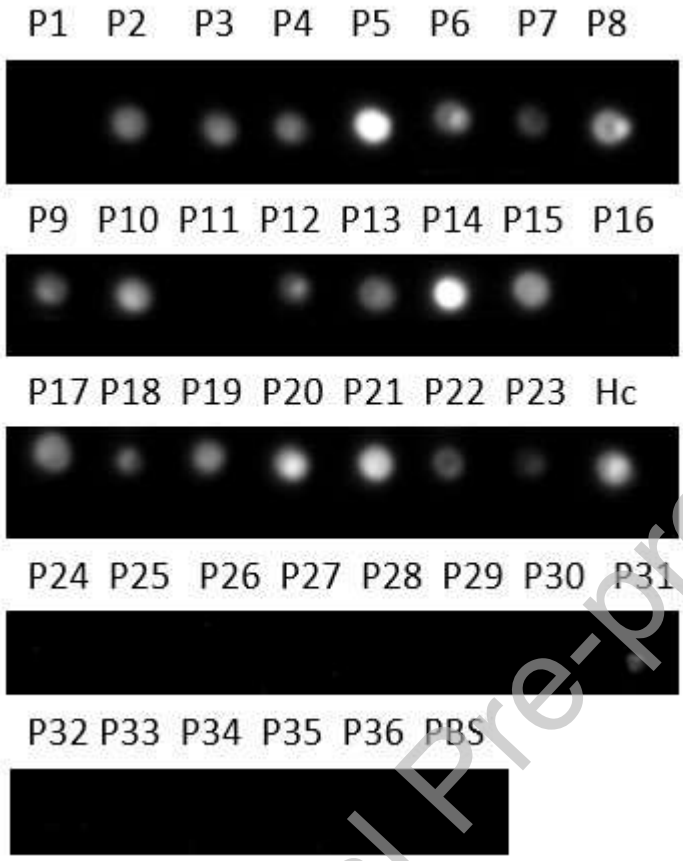

Fig. 1. Urine antigen detection by Hcp100 dot blot assay. P1-P23 are urine samples from 23 individuals living with HIV/AIDS and culture-proven histoplasmosis. Urine samples from 20 healthy individuals from Buenos Aires province (endemic region for histoplasmosis) were used as negative controls (N1-N20). Cross-reactions were evaluated using urine samples from 1 individual with coccidioidomycoses (P24), 3 with paracoccidioidomycoses (P25-P27), 2 with cryptococcoses (P28, P29), 6 with tuberculosis (P30-P35) and 1 with toxoplasmosis (P36). Hc: recombinant Hcp100 as positive control. PBS: blank.

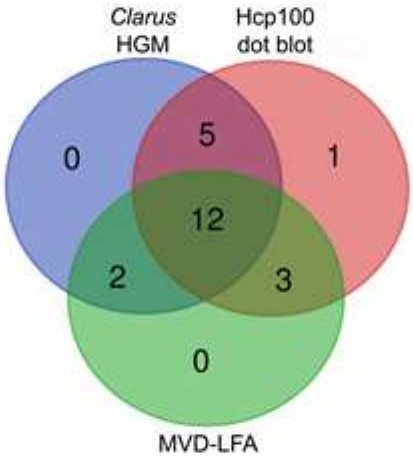

Fig. 2. Venn diagram of antigen detection assays for the diagnosis of histoplasmosis. Number of patients with confirmed PDH that tested positive by the Hcp100 dot blot, the MVD-LFA and the *Clarus* HGM.

Table 1. Performance evaluation of the three urinary antigen detection assays for the diagnosis of progressive disseminated histoplasmosis in HIV/AIDS patients.

Performance parameters	Urinary antigen detection assays		
	***Clarus* HGM**	**Hcp100 dot blot**	**MVD-LFA**
Sensitivity % (95%CI)	78.3 (56.3- 92.5)	87.0 (66.4-97.2)	73.9 (51.6-89.8)
Specificity % (95%CI)	90.9 (75.7-98.1)	97.0 (84.2-99.9)	78.8 (61.1-91.0)
PPV % (95%CI)	85.7 (66.6-94.7)	95.2 (74.3-99.3)	70.8 (54.7- 83.0)
NPV % (95%CI)	85.7 (73.3-92.9)	91.4 (78.8-96.8)	81.3 (68.1-89.8)
Accuracy % (95%CI)	85.7 (73.8-93.6)	92.9 (82.7-98.0)	76.8 (63.6-87.0)

95%CI: 95% confidence intervals; *Clarus* HGM: *Clarus Histoplasma* Galactomannan EIA; MVD-LFA: MiraVista Diagnostics *Histoplasma* Urine Antigen Lateral Flow Assay; PPV: positive predictive value; NPV: negative predictive value.

Table 2. Cross-reactivity of urinary antigen detection assays in individuals with other infectious diseases clinically similar to histoplasmosis.

Disease	Urinary antigen detection		
	***Clarus* HGM**	**Hcp100 dot blot**	**MVD-LFA**
Paraccocidioidomycosis	0/3	0/3	3/3
Coccidioidomycosis	1/1	0/1	1/1
Cryptococosis	0/2	0/2	0/2
Tuberculosis	2/6	1/6	3/6
Toxoplasmosis	0/1	0/1	0/1

Clarus HGM: *Clarus Histoplasma* Galactomannan EIA; MVD-LFA: MiraVista Diagnostics *Histoplasma* Urine Antigen Lateral Flow Assay.

Table 3. Agreement of the urinary antigen detection assays.

		Hcp100 dot blot		***Clarus* HGM**	
		+	-	+	-
MVD-LFA	+	16	8	17	7
	-	5	27	4	28
***Clarus* HGM**	+	17	4		
	-	4	31		

Clarus HGM: *Clarus Histoplasma* Galactomannan EIA; MVD-LFA: MiraVista Diagnostics *Histoplasma* Urine Antigen Lateral Flow Assay.

Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: