Looking for fungi in all the right places: screening for cryptococcal disease and other AIDS-related mycoses among patients with advanced HIV disease

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Purpose of review
As HIV treatment programmes scale up to meet the UNAIDS 90–90–90 goals, care must be taken to start antiretroviral treatment safely in patients with advanced disease (CD4 counts <200 cells/μl) who are simultaneously at risk for opportunistic infections and immune reconstitution inflammatory syndrome. Invasive fungal diseases pose a great threat at this critical time point, though the development of inexpensive and highly accurate rapid diagnostic tests has changed the approach HIV programmes are taking to reduce the high mortality associated with these opportunistic infections. This article summarizes recent advances and findings in fungal opportunistic infection diagnostics with a focus on screening to prevent cryptococcal meningitis.

Recent findings
Cryptococcal antigen (CrAg) screening using a lateral flow assay platform is cost-effective and feasible to implement as either a laboratory reflex or point-of-care test. Recent CrAg screening pilots have elucidated the varying prevalence of cryptococcal antigenemia across geographic regions, which may aid programme planning. Evidence from recently completed clinical trials provides a strong motivation for the use of CrAg titer to refine treatment options for patients with subclinical cryptococcal disease.

Summary
Although several operational barriers to programme effectiveness still need to be addressed, the utility of CrAg screening using inexpensive and accurate antigen assays has been demonstrated in real-world HIV programmes, paving the way for development and testing of other fungal opportunistic infection screening strategies and for an integrated advanced HIV disease testing package to reduce AIDS mortality and ensure safe antiretroviral treatment initiation.

Keywords
advanced HIV, cryptococcal antigen screening, cryptococcal meningitis, invasive fungal infections, rapid diagnostic tests

INTRODUCTION

The dream of bringing the decades-long AIDS epidemic to a halt has never felt as achievable as it does now. With the ambitious Joint United Nations Programme on HIV/AIDS goals of 90–90–90, epidemic control by 2030 is in reach if national HIV programmes can, in the next few years, achieve and sustain: first, 90% of people living with HIV (PLHIV) knowing their status, second, 90% of those diagnosed receiving antiretroviral treatment (ART), and third, 90% of those treated achieving viral suppression [1]. The World Health Organization (WHO) recommendation to expand ART to all patients

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KEY POINTS

- Invasive fungal diseases continue to cause significant mortality in advanced HIV patients, with cryptococcal meningitis in particular affecting hundreds of thousands of individuals each year.
- The recent development of a RDT, the cryptococcal antigen (CrAg) lateral flow assay (LFA), provides an opportunity to screen and detect cryptococcal disease early, and preemptive treatment can save lives.
- Laboratory-based reflexive screening using CrAg LFA can be integrated into routine HIV care and provides excellent coverage of target populations, though point-of-care screening may be better suited and has been shown to be feasible for areas lacking laboratory capacity.
- Even with screening and treatment, CrAg-positive patients are still at higher risk of death, and customizing treatment based on antigen titer may help to reduce this risk.
- Other IFIs such as PCP, histoplasmosis, and talaromycosis continue to cause AIDS-related mortality, and development of rapid diagnostics for these pathogens is key to the development of a more comprehensive advanced disease package of care.

diagnosed with HIV, regardless of $CD4^+$ T-lymphocyte (CD4) count will facilitate the second '90' goal [2]. Now, as countries adapt their guidelines to these recommendations and goals, the pressure is on for programmes to rapidly scale up and initiate millions of new patients on ART with a particular focus on identifying and treating individuals earlier in infection to prevent HIV transmission. However, with over one million people still dying annually of AIDS-related illnesses, we must not forget the immediate threats faced by patients initiating ART in late-stage disease [3]. In many settings, almost half of patients progress to advanced HIV disease (CD4 count <200 cells/μl) before initiating ART, placing them at increased risk for opportunistic infections. If untreated, these opportunistic infections can result in significant mortality and morbidity either directly or through manifestation of the immune reconstitution inflammatory syndrome (IRIS) as the immune system rebounds [4,5]. Because of this, early detection and treatment of opportunistic infections must be considered within HIV programmes to reduce deaths that occur within a year of ART initiation.

In addition to targeted interventions such as tuberculosis case finding, a strategy to reduce AIDS deaths must specifically address the burden of invasive fungal infections (IFIs), still responsible for an estimated 50% of all HIV-related deaths [6]. The four major IFIs driving this mortality – cryptococcosis, *Pneumocystis jirovecii* pneumonia (PCP), disseminated histoplasmosis, and disseminated talaromycosis (formerly penicilliosis) – have a combined global disease burden estimated at 1.7 million cases per year [6]. Despite this significant burden, these diseases remain relatively poorly understood, with access to diagnostics and treatment often lacking in HIV programmes. Recent development of certain rapid diagnostic tests (RDTs) has, however, created greater opportunities for large-scale prevention and effective treatment to tackle the 'neglected epidemic' of AIDS-related mycoses [6,7]. Among these, cryptococcal antigen (CrAg) testing using relatively new lateral flow assay (LFA) technology [Immuno-Mycologics (IMMY), Norman, Oklahoma, USA] has been most intensively researched [8–14,15▪]. The simple yet highly accurate CrAg LFA has not only improved diagnostic capabilities for early detection of cryptococcal meningitis, one of the most common and deadly fungal opportunistic infections, but has also opened the door to routine screening and preemptive antifungal treatment to potentially prevent cryptococcal meningitis entirely and also minimize risk of IRIS in patients initiating ART.

This article discusses recent advances in cryptococcal disease screening and the role that simple diagnostic tests for cryptococcal disease and other fungal opportunistic infections can play in reducing AIDS-related mortality and safely initiating patients on ART as we move forward into the 'treat all' era.

EARLY DETECTION OF CRYPTOCOCCAL DISEASE

Cryptococcal meningitis, the most common manifestation of cryptococcal disease, occurs in over 300 000 immunocompromised patients each year with a mortality rate of up to 70% in the first 12 months after diagnosis [16,17]. Most cryptococcal disease is acquired when *Cryptococcus neoformans* spores, which are common in the environment, are inhaled early in life and persist in the lungs, often with minimal if any clinical manifestations [18]. In immunocompromised patients, the fungus is no longer kept in check by the immune system and is able to disseminate, first into the bloodstream and later to the cerebrospinal fluid (CSF) where it causes severe neurological disease, or cryptococcal meningitis [19]. Management of cryptococcal meningitis requires hospitalization for therapeutic lumbar punctures and treatment with intravenous amphotericin B, a potent but particularly cytotoxic antifungal medication [20–22]. The high costs associated with treatment of cryptococcal

meningitis often place an undue burden on health systems, particularly in the resource-limited settings wherein prevalence is highest [23,24,25▪▪,26].

Fortunately, an alternative strategy exists to tackle the public health problem of cryptococcal meningitis. CrAg, a polysaccharide biomarker derived from the fungal capsule and shed throughout dissemination, is detectable in the blood weeks to months before the onset of cryptococcal meningitis, presenting a window of opportunity to detect disseminated infection early and to treat preemptively using inexpensive and well-tolerated fluconazole [27,28]. This CrAg screen-and-treat strategy has demonstrated improved survival of preemptively treated CrAg-positive patients in Uganda compared with CrAg-positive patients who received ART alone, and when paired with community support in a recent trial in Tanzania, a 28% reduction in cryptococcal meningitis mortality was observed [29,30▪▪]. In South Africa, the intervention was shown to be cost-effective at a prevalence as low as 0.6% in patients with CD4 count less than 100 cells/μl [24]. Routine screening and treatment of subclinical cryptococcal disease in ART-naïve patients with CD4 less than 100 cells/μl has been recommended by the WHO since 2011 and has since been incorporated into national HIV treatment guidelines in over 20 countries (Fig. 1) [2,31].

Detection of CrAg in serum and CSF is not a new concept, as latex agglutination kits and enzyme-linked immunoassays (EIAs) have been used for diagnosis of cryptococcal meningitis for several decades [32–34]. However, the notion of widespread screening for and preemptively treatment of cryptococcal disease has emerged relatively recently and has been largely propelled by the development of the CrAg LFA. Unlike its predecessors, the latex agglutination and EIA, the CrAg LFA (ImmunoMycologics, Norman, Oklahoma, USA) consists of a simple dipstick test that requires minimal, if any, laboratory capacity and costs only a few US dollars to produce [35]. Validation studies have shown the LFA to have exceptional test accuracy on serum (median sensitivity and specificity 100 and 99.5%, respectively), and have detected cases of infection not found by the latex agglutination test, making it now widely considered the new 'gold standard' [10,12,14,36–38]. The LFA has also demonstrated 100% concordance between serum and whole blood in patients with suspected cryptococcal meningitis, indicating its potential for use as a point-of-care bedside diagnostic test [15▪,39▪,40].

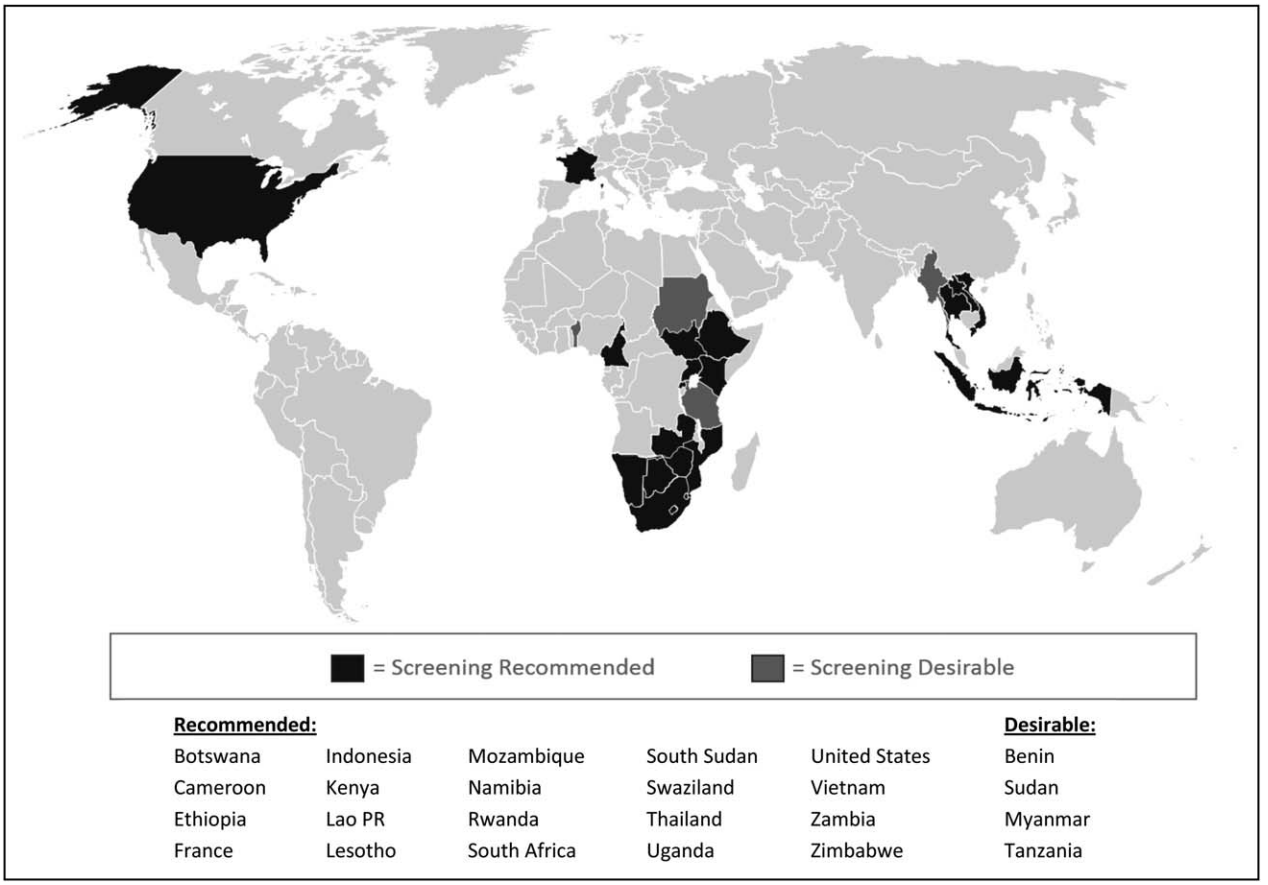

FIGURE 1. Countries that currently recommend cryptococcal antigen screening in national HIV guidelines.

The development of this simplified and accurate testing platform has likely been the single most important advance in making large-scale screening and early diagnosis feasible in resource-limited settings in line with newly adopted screening policies [41,42].

PROGRAMME PLANNING FOR ROUTINE SCREENING

As CrAg screening policies are translated into implementation strategy, pilot studies and CrAg prevalence estimates can focus public health attention and limited resources on areas with the greatest need for reduction of cryptococcal meningitis-related AIDS mortality. For example, a recent retrospective screening of over 3000 stored serum samples from several different regions in Nigeria found CrAg prevalence to be highly geographically variable, with a higher prevalence in the southern region (6.6% of CD4 <200 cells/μl) compared with the culturally and environmentally distinct north (<1%) [43▪]. Similar geographic variation is apparent in South Africa, wherein data from a pilot screening of over 50 000 samples from four districts in Gauteng and Free State provinces estimated CrAg prevalence in patients with CD4 less than 100 cells/μl to be between 4 and 5%, about half of the 9% recorded during a similar time period in an outpatient setting in KwaZulu-Natal province [44,45]. Such regional prevalence data may help with prioritizing areas of greatest need, though the lack of such data should not act as a barrier to implementation at the expense of patients with advanced HIV infection who could benefit from CrAg screening and preemptive treatment.

STRATEGIES FOR CRAG SCREENING

As an increasing number of HIV programmes integrate CrAg screening into routine care and the intervention is being adapted to different country contexts, three possible strategies for implementation have so far emerged: first, laboratory-based provider-initiated approach, second, laboratory-based reflex approach, and third, point-of-care (POC) screening approach [31]. All three of these strategies rely on health systems routinely conducting baseline CD4 tests for clinical assessment of patients initiating ART. In the provider-initiated screening approach, clinicians are responsible for ordering a CrAg test for eligible patients at their first follow-up visit, meaning a second follow-up appointment must be scheduled before appropriate treatment can be initiated. Laboratory-based reflex screening improves upon this by integrating CrAg testing into the laboratory workflow during baseline CD4 testing, instructing laboratorians to reflexively test remnant blood from all samples with a CD4 count below a determined threshold and return both CD4 and CrAg results to the provider to guide a treatment decision at the patient's first follow-up visit. In contexts wherein CD4 laboratory infrastructure is limited or unavailable and baseline CD4 is conducted using POC platforms such as the Pima (Alere Inc., Waltham, Massachusetts, USA), a point-of-care approach in which the CrAg LFA is used on whole blood during a patient's initial visit may be most suitable. This method allows for a rapid treatment decision to be made, and recent piloting of this approach in Lesotho has found screening at the POC to be feasible. Quality assurance concerns may exist in settings wherein low patient volumes mean the test is performed infrequently and regular retraining or supervision may be necessary [46].

In practice, these strategies have been assessed and compared in a handful of settings in recent years (Table 1). Following publication of WHO 2011 rapid advice guidelines for cryptococcosis, South Africa's Western Cape Province quickly rolled out provider-initiated CrAg screening. A later evaluation of this screening approach by Vallabhaneni *et al.* [47▪▪] found that, although implemented with relative ease, less than a third of eligible patients were actually screened. Meanwhile, a pilot of laboratory-based reflex screening in four districts in Gauteng and Free State provinces achieved 97% coverage [48]. Subsequent cost analysis of the two approaches revealed that, although provider-initiated screening costs significantly less from a laboratory perspective, reflex screening results in a greater number of lives saved. In addition, reflex screening costs the health system less overall by preventing many of the costly hospitalizations resulting from the development of cryptococcal meningitis in CrAg-positive patients that the provider-initiated approach would have missed because of poor coverage [25▪▪]. In light of these comparisons, reflex screening is generally recommended in settings wherein sufficient centralized CD4 laboratory infrastructure exists. To optimize the impact of the intervention regardless of the screening approach, fluconazole for preemptive treatment should be easily available at all healthcare levels.

Still, concerns exist that, even if a quick turnaround time is achieved, a reflex strategy could cause delays in fast-track ART initiation, and that South Africa's centralized approach is not likely to be universally appropriate in the rest of sub-Saharan Africa wherein the burden of cryptococcal meningitis is greatest. In these settings with poor laboratory access, CrAg screening must be conducted at

Table 1. Comparison of cryptococcal antigen screening strategies

Strategy	Time of treatment decision	Laboratory infrastructure needed	Coverage of eligible population	Benefits	Limitations
Provider-initiated: sample sent for CD4 at baseline; CrAg sample sent at first follow-up	Second follow-up visit	*Moderate* – centralized CD4 testing	25–33% in South Africa[a]	Ease of implementation	Providers forget to order tests; long turnaround times between tests and delays in treatment decisions; multiple specimen draws; high loss to follow-up
Lab-based reflex: Sample sent for CD4 at baseline; CrAg conducted automatically on remnant CD4 sample if eligible	First follow-up visit	*Moderate* – centralized CD4 testing and trained laboratorians	97% in South Africa[b]; 89% in Mozambique[c]	High coverage; quality assurance handled by laboratory; all tests performed using single specimen	Requires sufficient lab infrastructure; clinicians less involved in process; lateral flow assay not ideal for large testing volumes; turnaround time dependent on specimen transport and result relay; loss to follow-up an issue
Point-of-care: CD4 performed at point-of-care; CrAg performed on either serum or whole blood at point-of-care	Initial (baseline) visit	*Minimal* – can be conducted by lay workers	99% in Lesotho[d]	Results available quickly; treatment decisions can be made same day as presentation; minimal loss to follow-up	Quality assurance and data reporting challenges; requires frequent supervision and re-training depending on test volumes and cadre of health worker conducting test

[a]Vallabhaneni *et al.* 2016 [47▪▪].
[b]Govender *et al.* 2016 [48].
[c]Gudo *et al.* 2015 (personal communication 18 May 2015).
[d]Rick *et al.* 2016 [49].

the POC [50]. A recent opinion article published in AIDS 2016 advocating for CrAg POC screening draws on lessons learned in South Africa wherein implementation thus far has involved CrAg testing performed in central laboratories. In this setting, loss to follow-up after the initial visit is common and CrAg screening is duplicated when patients have multiple CD4 tests, leading to a small excess of resources that may not be possible in many settings [51]. Fortunately, several recent assessments of POC CrAg testing have shown promise. In Uganda, validation of the CrAg LFA on whole blood as a bedside test resulted in 100% agreement with serum and plasma testing performed in the laboratory for patients with suspected cryptococcal meningitis [15▪]. A subsequent feasibility study in Lesotho conducted by Médecins Sans Frontières found the CrAg LFA could even be performed at the POC by lay cadres of healthcare workers, though frequent re-training and supervision were needed to ensure testing proficiency was maintained [46].

The move toward a 'treat all' approach in HIV programmes dictates that new diagnostics for AIDS-related opportunistic fungal infections must either be based on POC testing or offer minimal turn-around times and rapid linkage to care in order to facilitate safe and timely ART initiation. This is especially important for patients with advanced HIV infection. Consideration must, however, be given to possible drawbacks of POC testing: additional burden on healthcare workers, lack of or limited result connectivity to allow for surveillance data collection, and quality assurance issues. Additionally, strengthening supply chains will be crucial to ensure that test kits and adequate treatments are available, especially in rural and difficult-to-reach areas wherein POC screening is likely to be employed [52]. What impact a POC CrAg screen-and-treat strategy will have on improving uptake of antifungals and ART, patient retention in HIV care, and cryptococcal meningitis prevention is yet to be evaluated in the context of large-scale public health programmes.

THE WAY FORWARD FOR CRAG DIAGNOSTICS

Recent findings have suggested several ways in which the CrAg screen-and-treat intervention can be improved, with the two most significant being: first, the introduction of differentiated care for CrAg-positive patients based on antigen titer and second, the integration of CrAg testing with other POC tests in an advanced disease package of care. When the WHO recommended CrAg screening for ART-naïve adults with a CD4 count less than 100 cells/μl in 2011, the recommendations for preemptive therapy were based largely on expert opinion, and little was known about the optimal management of asymptomatic antigenemia [53]. The WHO preemptive regimen has since been tested in a randomized controlled trial conducted in Tanzania and Zambia in which CrAg screening and treatment accompanied by 4 weeks of ART adherence support resulted in a 28% relative reduction in all-cause mortality in patients with CD4 less than 200 cells/μl [30▪▪]. However, the CrAg-positive group still experienced higher mortality than those who screened CrAg-negative [adjusted response rate (RR) = 2.87, 95% confidence interval (CI) = 1.54, 5.37], indicating room for improvement in treatment of asymptomatic antigenemia [30▪▪]. Preliminary results from an operational research study of CrAg implementation in Uganda offer a possible answer: by retrospective screening of stored samples from CrAg-positive patients, the investigators found that those with CrAg titers at least 160 were three times more likely to have died [heart rate (HR) = 2.9, 95% CI 1.25–6.63, $P = 0.012$], potentially indicating that some of these asymptomatic patients had developed subclinical meningitis and were under treated [54]. These preliminary results are backed by similar findings from a small cohort of patients in South Africa who underwent blood CrAg titer testing as well as lumbar puncture and CSF CrAg testing when initiating ART. The study found that 40% of patients with high titer (≥160) had CSF CrAg-positive results compared with none of the lower titer (<160) patients [39▪]. Further analysis of samples from patients routinely screened between 2012 and 2015 in South Africa also found a significant association between subclinical meningitis and a titer at least 160, and when using this titer as a testing cutoff, the sensitivity and specificity are moderate (75 and 71%, respectively) [55]. Subclinical meningitis indicated by high-antigen titer likely requires a more intensive treatment regimen such as amphotericin B or combination fluconazole and flucytosine (5-FC) rather than fluconazole monotherapy, though this remains to be tested.

Integration of titer testing into routine CrAg screening will likely be a critical step in improving care for CrAg-positive patients. From a practical perspective, the current IMMY CrAg LFA allows semi-quantitation only in a laboratory. A semi-quantitative POC assay that has the capability of indicating titer thresholds, will be required for the majority of resource-limited countries where laboratory infrastructure is poor. Several semi-quantitative CrAg LFA tests indicating titers above or below the 160 thresholds are now in development. Institute Pasteur and Biosynex have developed an investigational prototype CryptoPS assay (Biosynex, Strasburg, France) that is currently being evaluated in field conditions in Cameroon (Clinical Trial NCT02624453).

Pneumocystis pneumonia

Although, developments in CrAg screening are promising, reducing mortality further in a population with advanced HIV disease will require development and expansion of a standard package of care incorporating several other high-priority opportunistic infections. Of particular importance globally is PCP, a common though under-diagnosed and potentially fatal opportunistic infection [56▪]. Classically, diagnosis of PCP is difficult, as symptoms often resemble other respiratory infections and laboratory diagnosis requires fluorescent microscopy and polymerase-chain reaction (PCR) testing [56▪,57]. The recent development and evaluation of a new loop-mediated isothermal amplification (LAMP) assay has shown promise, with a sensitivity and specificity that surpasses that of traditional PCR with a test design that is simpler and faster [58]. The LAMP assay does require laboratory capacity and may not be appropriate for many resource-limited settings. A push for innovation is needed to develop bedside diagnostic tools that could facilitate early detection and treatment of PCP pre-ART initiation.

Histoplasmosis

More regionally specific, histoplasmosis in the Americas and Africa and talaromycosis in Asia together affect vast numbers of patients with advanced HIV disease. For histoplasmosis, the burden of disease has been poorly documented in most endemic areas, and modern nonculture-based diagnostic tests are rarely available, leading to the disease being commonly mistaken for and treated as multidrug resistant tuberculosis [59]. There is sufficient reason to push to make these tests more available as thousands of cases in Latin America go undiagnosed each year and mortality among those

often nears 50% [60,61]. Yet histoplasmosis is highly treatable, and sensitive and specific EIA platforms exist for detection. Innovation may be on the horizon for diagnosis of histoplasmosis as an LFA platform is being developed for the detection of *Histoplasma* galactomannan that could potentially be used with a urine sample to maximize simplicity of testing [62,63].

Talaromycosis

Not unlike histoplasmosis described above, talaromycosis in Southeast Asia, China, and India is diagnosed in an estimated 4–14% of patients admitted with advanced HIV infection and has a mortality between 10 and 30% [64,65,66▪▪]. Yet the disease often goes undiagnosed or is diagnosed too late due to the lack of a simple and RDT. Recent development of monoclonal antibody-based EIAs for detection of *Talaromyces* (formerly *Penicillium*) *marneffei* antigen offers promise for future testing strategies [67,68]. One assay was able detect *T. marneffei* antigen in 9.4% of more than 8000 archived serum specimens from patients in Guangzhou, China, with *T. marneffei* antigen prevalence as high as 24% in patients with CD4 count less than 100 cells/μl [69]. Linked clinical data were not available for this study, therefore the clinical significance of this finding remains to be assessed. If the antibodies developed for these tests could be adapted to a simple LFA platform, a screening and antifungal treatment strategy similar to the strategy for cryptococcosis could potentially be employed to reduce the mortality and complications experienced by patients initiating ART across Asia.

CONCLUSION

As monitoring of HIV disease transitions from CD4 to viral load, laboratory infrastructure for baseline CD4 testing may not be maintained in countries with limited resources and alternative technologies may need to be explored. Already several POC CD4 technologies are nearing market, from simple semi-quantitative LFA platforms indicating high or low CD4 (likely at a threshold of 200 cell/μl) to more sophisticated microchip technology contained in a handheld flow cytometer [70,71]. The development of the CrAg LFA has revolutionized the fight against cryptococcal meningitis, one of the deadliest invasive fungal opportunistic infections. This simple and inexpensive technology has made large-scale CrAg screen-and-treat programmes feasible, enabling countries to implement a WHO-recommended intervention in the resource-constrained environments wherein it is needed most. Now, with several countries implementing this intervention and landmark studies being completed, we have gained valuable insights into what strategies have worked and where improvements are needed to optimize this intervention. As the HIV community shifts focus toward substantially expanding ART programmes in the new 'treat all' era, there must still be a push for investment and innovation in simple diagnostic tests for opportunistic infections that meet the WHO ASSURED (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end users) criteria so that the risks of immediate mortality faced by patients with advanced HIV disease can be minimized, and we can achieve the goal of initiating 90% of diagnosed patients on ART with minimal deaths due to IRIS. If technology continues to progress, eventually it may be feasible to provide patients with a complete package of POC opportunistic infection diagnostic or screening tests to ensure the highest quality of care at ART initiation.

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Conflicts of interest

There are no conflicts of interest.

Disclaimer

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