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REVIEW

Diagnosis of Human Immunodeficiency Virus Infection

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SUMMARY HIV diagnostics have played a central role in the remarkable progress in identifying, staging, initiating, and monitoring infected individuals on lifesaving antiretroviral therapy. They are also useful in surveillance and outbreak responses, allowing for assessment of disease burden and identification of vulnerable populations and transmission "hot spots," thus enabling planning, appropriate interventions, and allocation of appropriate funding. HIV diagnostics are critical in achieving epidemic control and require a hybrid of conventional laboratory-based diagnostic tests and new technologies, including point-of-care (POC) testing, to expand coverage, increase access, and positively impact patient management. In this review, we provide (i) a historical perspective on the evolution of HIV diagnostics (serologic and molecular) and their interplay with WHO normative guidelines, (ii) a description of the role of conventional and POC testing within the tiered laboratory diagnostic network, (iii) information on the evaluations and selection of appropriate diagnostics, (iv) a description of the quality management systems needed to ensure reliability of testing, and (v) strategies to increase access while reducing the time to return results to patients. Maintaining the central role of HIV diagnostics in programs requires periodic monitoring and optimization with quality assurance in order to inform adjustments or alignment to achieve epidemic control.

KEYWORDS CD4, enzyme immunoassay, HIV incidence, HIV rapid tests, dried blood spots, drug resistance, early infant diagnosis, point-of-care testing, quality assurance, viral load

INTRODUCTION

The Joint United Nations Program on HIV/AIDS (UNAIDS) estimated that there were 36.7 million HIV-infected persons and 1.8 million new infections globally by the end of 2016 (1). The sub-Saharan Africa (SSA) region alone accounted for approximately 69.5% (25.5 million) of the global HIV infections, with only 54.1% (13.8 million) having access to antiretroviral (ARV) therapy (ART) (2, 3) to suppress viral replication, prevent opportunistic infections, and prolong the lives of people living with HIV/AIDS (PLHIV). As part of the strategy to achieve an "AIDS-free generation" (4, 5), the 2016 World Health Organization (WHO) HIV treatment guidelines recommend that ART be initiated in all individuals living with HIV regardless of the clinical stage or CD4 cell count in order to preserve patients' immune systems, control HIV replication, and reduce further transmission (6). HIV prevention and the use of ART have reduced new HIV infections by 14%, from 2.1 million in 2013 to 1.8 million in 2016 (3, 7). However, at this rate, the decline will still fall short of the United Nations' set target of $<$500,000 new infections globally by 2020 (8). To accelerate the attainment of this goal, UNAIDS set ambitious

90-90-90 targets to be achieved by 2020, with the first 90 defined as 90% of HIV-infected persons knowing their status, the second 90 defined as 90% of patients with a diagnosis of HIV infection receiving ART, and the third 90 defined as 90% of ART-treated patients having viral suppression (9, 10). There are challenges in reaching these targets for developing countries, which include government policies, financial provision, data-driven programming, operational coordination among health care agencies and implementing partners, and laboratory infrastructure (11, 12). However, recent U.S. President's Emergency Plan for AIDS Relief (PEPFAR)-sponsored population-based HIV impact assessments have demonstrated that these targets are achievable (13). The first and third 90 targets specifically require the delivery of high-quality diagnostic testing to identify infections, monitor the effectiveness of ART, and provide timely assessment of HIV/AIDS control and the emergence of drug resistance (DR) at both the individual and population levels.

Currently, serologic testing algorithms using mostly rapid tests is used in resource-limited settings (RLS) to diagnose HIV infections. Serology-based incidence assays are widely used for estimating the rate of new infections in cross-sectional surveys, combined with viral load (VL) measurement in an algorithm. The new rapid incidence assay has generated significant excitement because it may provide an opportunity to identify hot spots in real time when used in program settings. Appropriate deployment of simple point-of-care (POC) CD4 technologies to provide same-day results to patients has had a significant impact on enrolling and retaining patients in ART programs (14, 15). Similarly, ART programs have implemented VL determination technologies in centralized and decentralized facilities to assess the effectiveness of ART. Furthermore, dried blood spot (DBS) and whole-blood-based POC methodologies have performed well in the area of early infant diagnosis (EID). Because of the rapid scale-up of ART (16), transmitted drug resistance (TDR) is on the rise in RLS. As such, testing for DR to determine the occurrence of mutations in order to provide patients with optimal treatment without an unnecessary switch to second-line and perhaps third-line ART regimens with higher costs is critical.

Accurate diagnosis of HIV infection is key and represents the entry point of infected patients into the treatment cascade. Furthermore, it has taken on renewed importance in the era of "test and treat" with ART (17). WHO guidelines for HIV testing services (HTS) underscored the importance of quality management systems. With the push to expand HIV testing to achieve the UNAIDS 90-90-90 targets, it is both programmatically and ethically imperative, and a priority for ministries of health (MOH) and national AIDS control programs, to implement robust quality management systems to support the implementation and delivery of accurate testing results to everyone tested. In this report, we review various aspects of HIV diagnostics, including advances in testing technologies to improve diagnosis and expand access, with a focus on RLS. Furthermore, we examine strategies to employ new methods and to critically assess/evaluate and support quality management systems for accurate and reliable diagnosis.

STAGES OF HIV INFECTION

There are three main stages following HIV infection in an untreated individual, characterized by clinical symptoms and biological markers that also offer the opportunity for use in diagnosis and monitoring using laboratory testing. The first stage is the acute phase, characterized by rapid multiplication and spread of the virus in the body, which may take about 2 to 4 weeks following infection (18). During this stage, there is a burst of viral replication, with shedding and peaking of p24 antigen (Ag) in blood. During the acute stage, some people experience flu-like symptoms, such as headache, fever, and rashes, for several weeks (19). The second stage is the chronic or asymptomatic stage, during which the virus continues to multiply but at low levels, and the infected individual may not experience any clinical symptoms. The host immune system also starts producing antibodies (Ab), which coincides with a decline in the VL to a steady state. Also, there is a decline in p24 antigen levels as the VL drops, due to p24 antigen bound by antibodies to form an antibody-p24 antigen complex, thereby

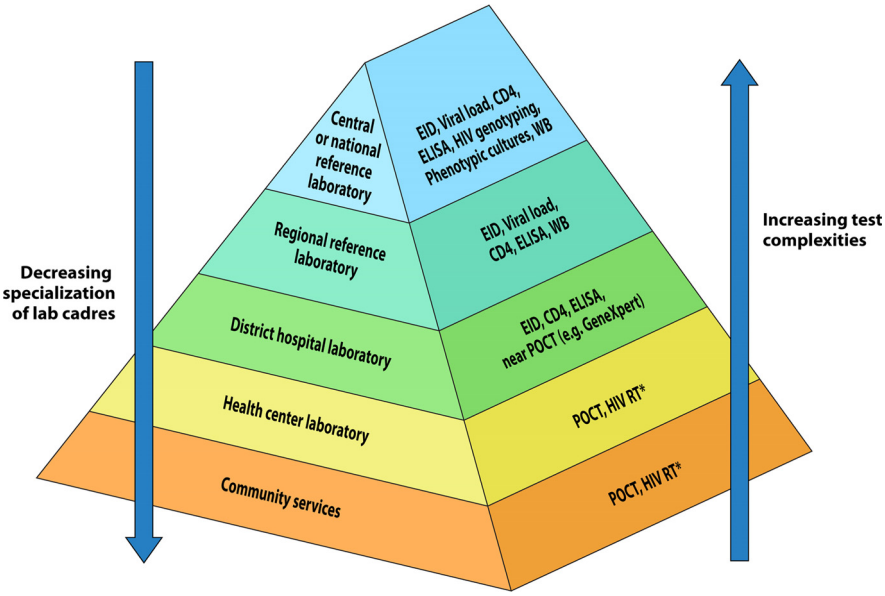

FIG 1 The tiered laboratory diagnostic network showing the different laboratory tiers, community services, and tests performed at each tier. EID, early infant diagnosis; POCT, point-of-care testing (instrument based). *, HIV RT is the HIV rapid test and refers to strip-like devices.

reducing the level of free p24 antigen in blood. The period from infection to the appearance of Ab (seroconversion) is known as the "window period." If a patient remains untreated, as viral replication continues, CD4 cells, which serve as host target cells for viral replication, are gradually destroyed, leading to a decline of CD4 cell numbers (18). The third stage, the AIDS phase, with continual viral replication and depletion of CD4 cells, leads to a weakened host immune system. The final stage is characterized by opportunistic infections and other clinical symptoms (20).

The biological markers HIV RNA, p24 antigen, HIV antibodies, and CD4 cells, appearing at various stages following infection, have been exploited for laboratory diagnostics for HIV for various applications, including (i) the determination of an individual's serostatus (antibodies), (ii) distinguishing of recent from long-term infection (antibodies, p24 antigen, and VL), (iii) EID using RNA and DNA, (iv) staging and monitoring of disease progression (CD4), (v) identification and monitoring of treatment effectiveness or failure (VL), and (vi) identification of DR mutations (DRMs) for a specific ART regimen failure (RNA and DNA).

LABORATORY DIAGNOSTIC NETWORK

The laboratory diagnostic network is key in each country's health care delivery services in order to improve the health of its population. The laboratory diagnostic network is typically organized in a tiered structure that aligns with the different layers of health care delivery (e.g., referral hospitals, regional hospitals, district hospitals, and health centers). The tiered laboratory network is a hierarchical structure, with the national reference or central laboratory at the top of the structure, followed by regional/provincial, district, and health center laboratories. This tiered structure matches the level of test complexities that can be carried out at each level, with complex and advanced tests usually being reserved for national and regional laboratories (Fig. 1). Similarly, training and the levels of specialized laboratory cadres align with this structure. At the community level, lay counselors and community health care workers primarily perform POC testing. POC testing is an extension of the tiered laboratory diagnostic network to the community where facility-based laboratories are typically absent. Coordination and oversight from the national reference laboratory are crucial to ensure standardized training of the different cadres within the tiered laboratory network. Furthermore, evaluations or verification of new diagnostics, including

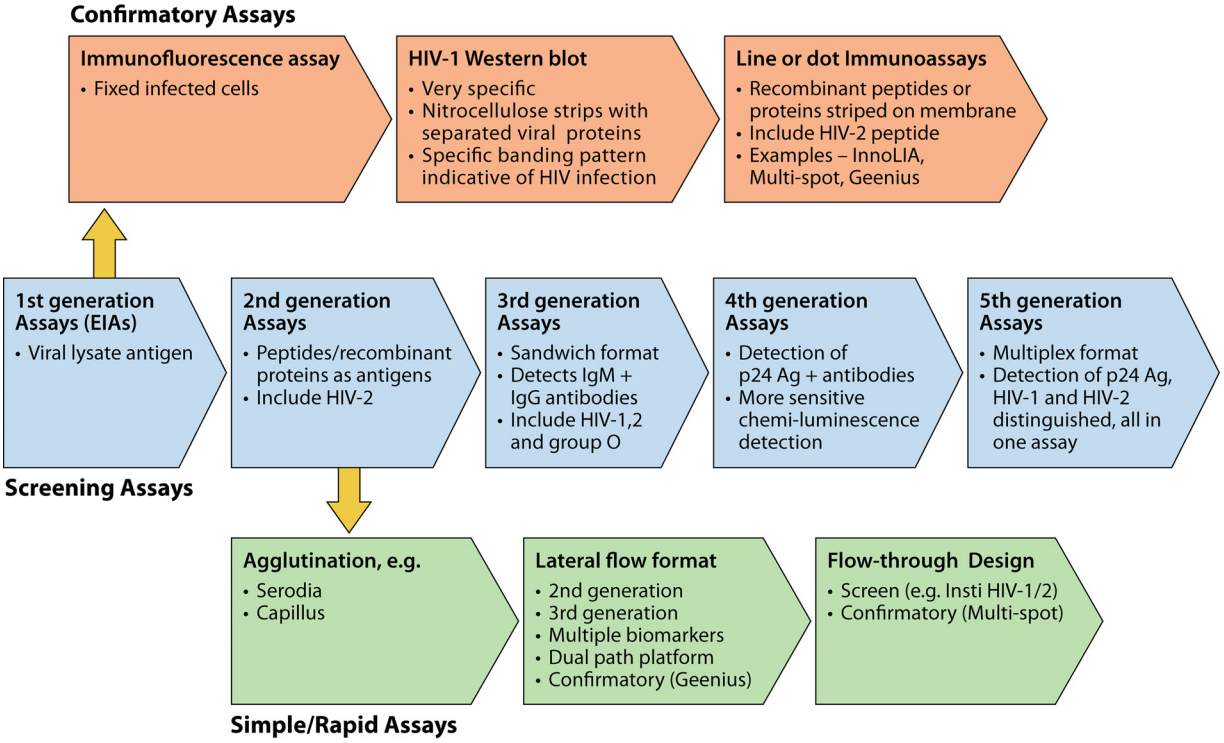

FIG 2 Historical evolution of serologic assays for HIV diagnosis. Shown are five generations of screening assays using an EIA format for high-throughput processing. Supplemental assays for confirmation of infection used immunofluorescence, WB, and, more recently, simple line or dot immunoassays. Rapid assays for POC testing were initially agglutination tests and later of a lateral flow format and flowthrough design.

POC testing, is usually performed at the national reference laboratory prior to their placement within the laboratory diagnostic network. Also, the national reference laboratory should provide oversight and monitoring of quality assurance (QA) testing within the diagnostic network.

ADVANCES IN SEROLOGIC DIAGNOSIS

AIDS was first recognized in the early 1980s. Initially, simple serologic tests for HIV antibodies using culture-derived viral antigen preparations were developed to diagnose HIV infections and to safeguard blood and blood product supplies (21). Over the next 3 decades, a wide spectrum of serologic assays was developed for simple/rapid testing (22, 23), high-throughput screening (24, 25), supplementary confirmation (26), epidemiological surveillance (27–29), and incidence determination (30–33). Assays with different testing formats, antigen designs, and signal detection amplification chemistries were formulated and evaluated in the field to assess their testing accuracy, viral strain coverage, and field applicability to ensure that infected patients could be quickly and accurately identified and linked to health care services, with minimal loss to follow-up. The progress in the development, application, and benefit of these serologic methodologies has been extraordinary, as evidenced by the identification of HIV-contaminated blood from blood supplies in the developed world (34), reduced testing times for rapid tests and supplemental confirmatory tests, and their direct impact (e.g., early ART initiation and reduced loss to follow-up) on improving health care delivery in RLSs.

Generations of Enzyme Immunoassays

Since the mid-1980s, there have been five generations of enzyme immunoassays (EIAs) using different antigen preparations and detection chemistries to provide accurate screening for blood banks and centralized laboratories with a high specimen volume (Fig. 2). The first-generation assays used antigens derived from whole viral lysates from HIV-positive cultures for the detection of IgG antibodies. Due to the

presence of impurities in the crude antigen lysate preparations, these assays had relative low specificity and high false positivity. Later confirmatory tests, such as immunofluorescence assays or Western blotting (WB), with high specificity were introduced to eliminate false positivity (35–37). In the second-generation assays, synthetic peptides or recombinant proteins derived from the immunodominant regions (IDR) of HIV-1 proteins and gp36 of HIV-2 were used to increase sensitivity and reduce false positivity. The third-generation assays, such as the Genetic Systems HIV-1/HIV-2 Plus O EIA, used a sandwich format and a variety of antigens to capture HIV-1 and -2 antibodies in serum. These antigens included recombinant p24, gp160 derived from HIV-1 group M, a recombinant peptide from HIV-2 gp36 IDR, and a synthetic peptide from HIV-1 group O (38). In addition to IgG, the third-generation assays also detected early HIV-1 IgM antibodies and further reduced the window period. The fourth-generation EIAs, such as the Abbott Architect HIV Ag/Ab Combo assay, used fully automated chemiluminescent microparticle technology to simultaneously detect HIV-1 p24 antigen and antibodies to HIV-1 (groups M, N, and O) and HIV-2. The detection of p24 antigen by the Abbott Architect HIV Ag/Ab Combo assay shortens the window period and increases the chances of early detection of HIV infection. The detection instrument provides random-access capability so that specimens can be tested on arrival without delays and generates results in 30 min with a throughput of $>$150 tests per h. These assays are best suited for facilities handling high volumes of blood bank screening tests (39–41). However, in these tests, the p24 antigen and HIV-1/2 antibodies detected are not individually distinguished. Fifth-generation EIAs, such as the Bio-Rad BioPlex 2200 HIV Ag-Ab assay, used multiple sets of magnetic beads coated with p24 monoclonal antibodies and epitopes specific for HIV-1 (groups M, N, and O) and HIV-2 (42). Acutely infected persons with p24 antigen in the window period can be identified in a single test and can be referred specifically for early intervention to further prevent HIV transmission. Individuals infected with HIV-1 or HIV-2 can also be identified for quick confirmation and linked to HIV-1- or HIV-2-specific ART.

Supplemental Confirmatory Tests

Despite progress with the operational characteristics and high sensitivity of EIA-based screening technologies, challenges with false positivity persisted (39–41). As such, for diagnosis of HIV infection, supplemental testing of an initially EIA-reactive specimen was required for confirmation of HIV infection prior to initiating patients on ART (Fig. 2). Specimens that were reactive by an EIA were retested using a more specific supplemental test. A common supplemental test used was WB. A WB assay was performed using a gradient-purified HIV lysate electrophoresed and impregnated onto nitrocellulose strips (Fig. 3A). For a specimen run on a WB to be considered positive, it had to contain antibodies reactive to p24 antigen and one or more antibodies against the envelope glycoproteins (gp160/gp120 and/or gp41). The WB banding patterns varied depending on the extents of antigen glycosylation and antibody maturation.

In general, the WB banding pattern is complex, and its interpretation requires well-trained, experienced, and competent laboratory staff. To avoid misinterpretation, new line blots that used purified HIV-1 and HIV-2 recombinant proteins and peptides were developed (Fig. 3B). The Inno-LIA HIV-1/2 strip (43) contains three positive-control lines (strong, moderate, and weak), five HIV-1 antigen lines (gp120, gp41, p31, p24, and p17), and two HIV-2 antigen lines (gp105 and gp36). Optimal clarity of banding is often produced after overnight incubation. The test cartridge of the Bio-Rad Multispot test contains only four spots, a procedural control, HIV-1 recombinant gp41 protein, HIV-1 gp41 peptides, and HIV-2 gp36 peptides, to simplify interpretation of results. In 2013, the U.S. Centers for Disease Control and Prevention (CDC) proposed the use of the Multispot test on samples that were reactive with third- or fourth-generation EIAs in order to confirm and differentiate HIV-1 and HIV-2 infections (44). Bio-Rad recently developed the Geenius HIV-1/2 supplemental assay to replace the Multispot assay (43, 45, 46). The new Geenius assay uses a closed lateral flow cartridge with a dual-path platform (DPP) to detect antibodies to recombinant or synthetic peptides for HIV-1

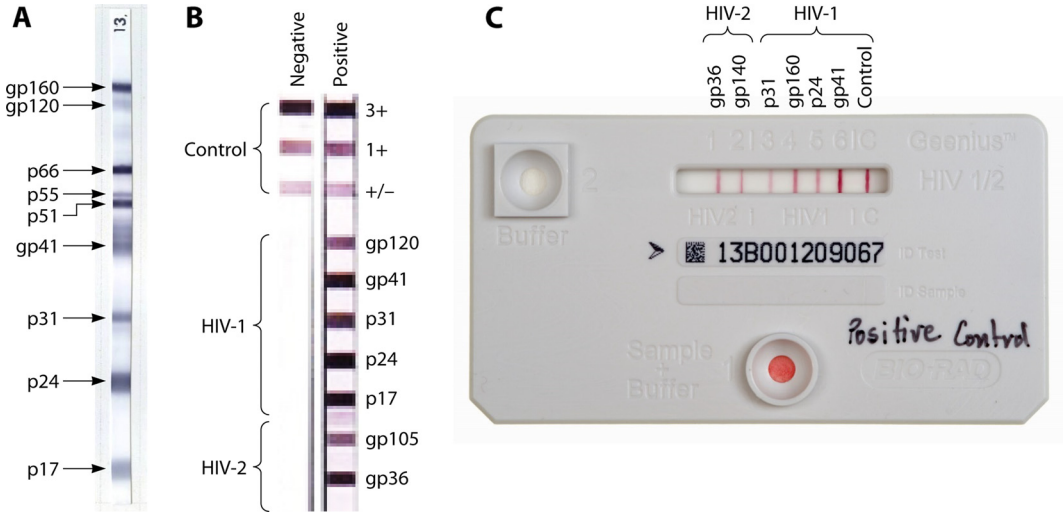

FIG 3 Illustration of three supplemental confirmatory assays, Western blotting (WB) (A), the Inno-LIA line blot (B), and the Geenius cartridge (C). WB uses gradient-purified viral lysates as separated antigens impregnated onto nitrocellulose strips. The lateral flow Inno-LIA uses combinations of five and two synthetic or recombinant antigens from HIV-1 and -2, respectively, and the Geenius assay with the dual-path platform (DPP) uses four and two synthetic or recombinant antigens for HIV-1 and -2, respectively. Three and one internal control bands are included in the Inno-LIA and Geenius assay formats, respectively. The banding patterns of a negative specimen and an HIV-1 and -2 dually positive specimen for Inno-LIA is shown in panel B, and the banding pattern of a dually positive specimen for Geenius is shown in panel C.

(p41, gp160, p24, and p31) and HIV-2 (gp36 and gp140) (Fig. 3C). It has a simple 3-step procedure with a total testing time of 30 min. Interpretation can also be made with the aid of an automated Geenius reader.

HIV RAPID TESTING

Development of Rapid Tests

The first HIV rapid test assays to be developed were the MicroGenesys agglutination test and the Abbott Murex single-use diagnostic system. A primary concern of these early HIV rapid tests was the considerably high rates of false-positive results (47) and false-negative results (48). The subsequent use of different formats, such as lateral flow devices (Determine HIV-1/2, Unigold, StatPak, and OraQuick HIV-1/2), flowthrough cartridges (Insti HIV-1/2), and modified agglutination assays (Serodia and Capillus HIV-1/2), greatly simplified and improved the testing procedure, with increased sensitivity and specificity. Rapid testing is usually completed in about 20 to 30 min, thus making them ideal for testing and counseling in primary health care sites and mobile clinics. The Insti HIV-1/HIV-2 test requires only 1 min, with an accuracy comparable to those of other rapid tests (49). Nonlaboratory staff can perform most rapid assays with standard training (with a minimum set of competencies as defined by the national program) using finger-pricked blood, plasma, or serum (22, 50–54). Assays such as the OraQuick Advance rapid HIV-1/2 assay use oral fluid, an alternative specimen type to finger-pricked whole blood, to provide the HIV serostatus of an individual.

In 2017, more than 85 million people were tested in PEPFAR-supported countries alone using mostly HIV rapid tests, and the majority of tests were performed by lay counselors or nurses (55). Although rapid tests, in general, are slightly less sensitive than EIAs because of the shorter duration of antigen/antibody incubation, their specificity is higher than that of EIAs (56). In RLS, HIV testing algorithms include a combination of two or three rapid tests, usually in a serial algorithm, depending on HIV prevalence; this has produced suitable sensitivities and specificities and improved the accuracy of testing results in POC settings (57). Countries decide on a testing algorithm, serial or parallel, to apply to their programs and use the right combination of HIV rapid tests to improve testing accuracy. To reduce costs, a serial algorithm is often adopted, with specimens initially reactive by the first test being subjected to a second test (a

different rapid test) with higher specificity. The quick turnaround time (TAT) of less than an hour allows the provision of counseling and testing during the same visit. Together with other HIV POC testing for VL, EID, and CD4, health care providers at the district or community level can provide multiple testing and treatment services to patients during same-day visits (49, 58, 59). Achieving this requires comprehensive implementation of national policies, resources, training, education, health care infrastructure, and strategic partnerships with stakeholders.

Decentralization of HIV Rapid Testing To Increase Access

HIV rapid tests became available in the early 1990s to fulfill the needs to promptly determine the serostatus of persons prior to surgical operations, organ transplantation, and maternal labor/delivery. Simple rapid tests offered the advantage that nonlaboratory staff in primary health care centers could also provide diagnostic services to patients residing in remote areas in RLS. The decentralization of HIV diagnostic services with POC testing has been transformative, as this has significantly increased access to services; however, it also posed huge implementation challenges with the sheer scale involved. There are constraints related to decentralization, including the lack of national guidelines, training, waste disposal, human resources, cold chains, inventory management, and QA monitoring (Table 1). With a high burden of HIV in many RLS, it is critical that the population have access to HIV diagnostic testing and other related services. Recent data demonstrate an increased risk of acquiring HIV for women during pregnancy and postpartum periods; therefore, it is critical to establish a retesting policy and ensure that resources are in place to allow the identification of recently acquired HIV infections in women during pregnancy and breastfeeding periods (60). Decentralization of services, including the use of POC testing for CD4, VL, and EID, further increases the opportunity to achieve the UNAIDS 90-90-90 goals.

HIV Self-Testing

Achieving the UNAIDS target of 90% of people living with HIV knowing their status will require innovative strategies outside traditional facility- and community-based testing approaches in order to reach all populations. HIV self-testing is an innovative approach to reach groups at high risk of HIV infection or youth populations that do not have access to facility-based services. It will allow individuals who want to know their HIV status to collect their own specimen (oral fluid or whole blood), perform an HIV test, and interpret the results privately or in the presence of someone whom they trust (61). HIV self-testing empowers patients and will allow access to populations that cannot be reached using existing services. Many at-risk persons refrain from testing due to fear of discrimination or stigmatization (62); thus, home-based testing provides them an avenue to access testing in privacy. In Malawi, acceptability was high in antenatal clinics where self-testing was offered to pregnant women and their male partners (63). The WHO has recommended HIV self-testing as an additional testing approach, and 40 countries have adopted and included it in their national policies (64). The WHO has prequalified three HIV rapid tests for HIV self-testing, the Autotest VIH and Insti HIV-1/HIV-2 antibody tests, which use whole blood as the specimen type, and the Oraquick rapid HIV-1/2 antibody test, which uses oral fluid (65). Similarly, the FDA has approved the Autotest VIH and Insti HIV-1/HIV-2 antibody tests for whole blood and the Oraquick Advance rapid HIV-1/2 antibody test for oral fluid. The level of access to HIV self-testing kits can vary with settings and countries. The kits can be provided to clients in health facilities, or some users can procure the kits over the counter in pharmacies or grocery stores to perform finger-pricking at home using a lancet provided in the kit. The test kits include inserts with instructions on how to conduct the test and interpret the result. Individuals who test reactive following HIV self-testing should seek further testing from a trained person using an approved algorithm for confirmation of a positive status and then be linked to treatment and care services. Individuals who have nonreactive results but have known recent HIV exposure or are at a high risk of

TABLE 1 Comparison of strengths and challenges for testing conducted in centralized laboratories and POC sites[a]

	Centralized laboratories		POC sites	
Testing consideration	**Strength(s)**	**Challenge(s)**	**Strength(s)**	**Challenge(s)**
Patient care	High throughput	Limited rural access Long TAT High rate of loss to follow-up	Increased patient access Short TAT Early ART initiation	Low throughput
Implementation	Access to LIS	Sophisticated instrumentation Specialized training Less hands-on time	Fewer infrastructure needs Remote QC potential with connectivity Simple test performance	Difficulty in instrument service Limited access to LIS More hands-on time per test
	Sample referral strengthens integrated disease system	Associated cost to maintain specimen referral network	No specimen referral required	
Procurement and supply chain	Procurement system easy to establish for relatively small numbers of laboratories	More system strengthening needed Instrument maintenance contract usually inadequate and not adhered to	Reagents do not require cold chain	Strategic planning for scale-up Complicated reagent delivery to vast number of POC sites
QA	Standard enrollment into EQA program or ability for interlaboratory comparison	Associated cost with maintaining QA program	Simple testing; may have built-in internal control	Tester training Acquisition of QC materials Dedicated QA staff False sense of simplicity equated to not requiring QA checks
Waste management	Easy to manage centrally for facility-based management, e.g., with availability of incinerator	Bulky and more waste	Less bulky; smaller quantities of waste	Usually lacks facilities for processing of waste prior to disposal
Human resources	Small number of staff needed	Special training and QA on sophisticated instrument operation	Simple testing could be performed by nonlaboratory staff	Multitasking of staff on testing and clinical care Need for proper training

[a]ART, antiretroviral treatment; LIS, laboratory information system; QC, quality control; QA, quality assurance; TAT, turnaround time; POC, point of care; EQA, external quality assessment.

infection should repeat self-testing 6 weeks later to exclude the possibility of being in the window period (61).

Rapid Test Prequalification and Postmarket Surveillance

Prequalification of *in vitro* diagnostics, such as HIV rapid test kits, is an important quality step to ascertain claims by the manufacturer on the performance of a particular test kit and whether or not it meets set standards. It is usually performed by an independent and credible institution. For example, WHO prequalification of a rapid test kit entails the quality assessment of the test using standardized procedures to ensure that it is in compliance with WHO prequalification requirements. The WHO prequalification procedure for a particular rapid test kit includes (i) presubmission of a form by the manufacturer of the rapid test kit to apply for WHO prequalification, (ii) review of the dossier by the WHO to understand the product, (iii) inspection of the test kit manufacturing site to assess compliance with quality standards, and (iv) laboratory evaluation of the test kit to assess operational and performance characteristics (66). The WHO uses its Collaborating Centers of Excellence or independent laboratories, including the Centers for Disease Control and Prevention, to perform laboratory evaluations. Following the completion of the above-mentioned procedures, a determination is made regarding whether or not the test kit meets WHO prequalification requirements. Test kits conforming to WHO prequalification requirements are included in the list of prequalified products (67). The MOH together with partners such as the PEPFAR and the Global Fund can access the WHO-prequalified list of rapid test kits for use in their HIV programs in RLS. HIV programs procuring these test kits perform in-country evaluations to determine the suitable combination of rapid tests for their national testing algorithms for diagnosis of HIV infection. These different steps ensure that test kits are safe and reliable and perform optimally for use in programs.

Once the test kits have been procured, postmarket surveillance (PMS) should be implemented to monitor the performance of the test kits after they have been shipped by the manufacturer. PMS should be done once test kits arrive in the country, prior to the shipment of test kits to different sites for programmatic use. This can be achieved by using quality control materials and performance indicators to monitor each new lot received in the country and whether it is performing as expected. Following the distribution of the test kits to different program sites, PMS should be routinely conducted and monitored as test kits are used in the field to ensure that they are performing as intended (68). The importance of PMS was demonstrated when the WHO removed the Standard Diagnostics Bioline HIV-1/2 3.0 rapid HIV test kit from its list of prequalified rapid test kits due to multiple batches that yielded excessive invalid results (69). PMS is an important quality assurance monitoring process that should be routinely performed. PMS also challenges manufacturers to maintain high standards in ensuring a quality product and thereby minimizes disruption of services due to recalls of poorly performing products.

INCIDENCE TESTING

HIV prevention and treatment programs underwent a significant global expansion in the past decade. Based on the UNAIDS 90-90-90 targets, two-thirds of individuals living with HIV knew their status at the end of 2016. Of the two-thirds who knew their status, 77% were on ART with 82% viral suppression (70). Despite this progress, the decline in the number of new infections has been slow. The number of new infections in 2010 (1.9 million) fell by only 11% to 1.8 million in 2016, as modeled by UNAIDS (71). In order to achieve the UNAIDS goal of eliminating HIV as a public health threat by 2030, individuals who do not know their status need to be diagnosed and put on ART, and populations with high rates of new infections need to be identified and initiated on ART to break the cycle of transmission.

HIV incidence testing requires recent infections to be distinguished from long-term infections to facilitate measurement of the incidence rate. Furthermore, this approach is a useful tool to allow country programs to monitor the impact of the scale-up of

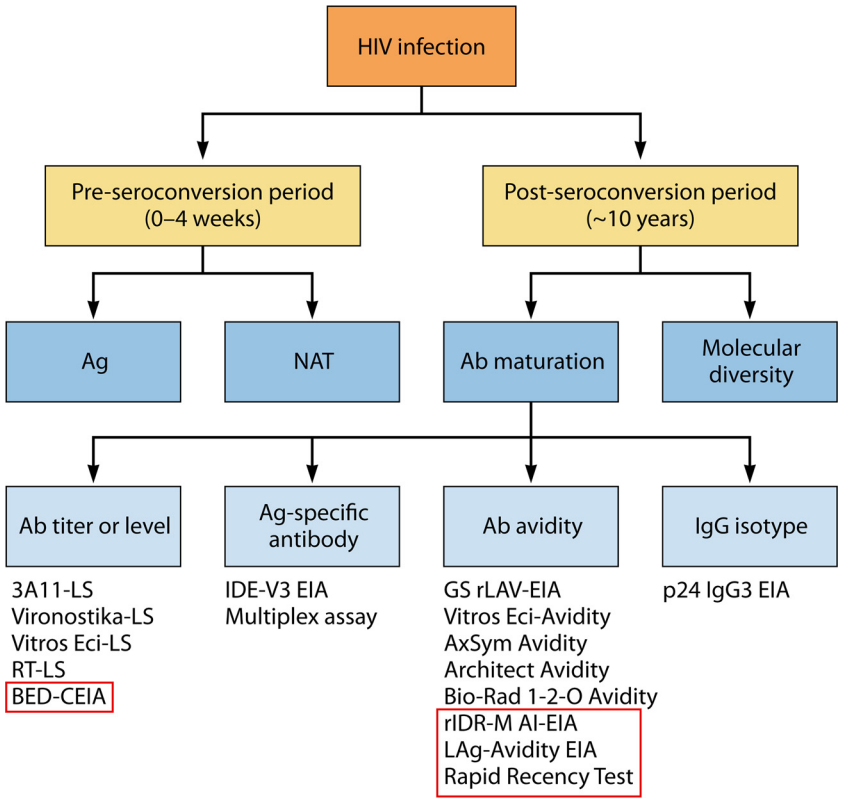

FIG 4 Methods used for incidence measurement cover pre- or postseroconversion periods. Methods used for preseroconversion specimens include detection of p24 antigen (Ag) and RNA by nucleic acid amplification tests. Methods used for postseroconversion specimens are based on antibody (Ab) maturation and viral genetic diversity. Commercial or in-house assays for the four antibody maturation groups are listed. Assays highlighted in red boxes are methods that use peptides or recombinant antigens derived from multiple subtypes to broaden subtype coverage. Abbreviations: LS, less sensitive; EIA, enzyme immunoassay; BED-CEIA: BED-Capture EIA; IDE, immunodominant epitope; rIDR-M, recombinant immunodominant region of HIV-1 gp41 group M; LAg, limiting antigen; NAT, nucleic acid testing.

combination prevention packages (HIV testing and counseling, ART, and voluntary medical male circumcision) to prevent new infections. Identifying recent infections in a given high-risk population, including those in defined geographic areas, provides reliable data for better planning, resource allocation, and targeted interventions (72, 73). Longitudinal follow-up of at-risk HIV-negative individuals, with retesting to detect seroconversions, is considered the gold standard to estimate incidence. However, this approach is very expensive and difficult to implement in most countries. Alternative methods include modeling, back-calculations from AIDS case reporting, age-based prevalence determinations, and prevalence determinations with multiple rounds of longitudinal surveys to estimate HIV incidence (74–76). In last few years, several laboratory-based approaches have been developed to distinguish recent from long-term HIV infections in cross-sectional surveys based on the evolution of maturating antibodies following infection. In 1998, a serologic laboratory-based strategy, using cross-sectionally collected specimens and sensitive/less-sensitive (LS) EIAs, was used to measure antibody titers to estimate incidence (30). Subsequent methodology optimization, based on pre- and postseroconversion specimens, resulted in a series of laboratory-based assays to detect recent HIV-1 infections (Fig. 4).

Limited Usefulness of Assays during the Preseroconversion Period

The preseroconversion phase is usually 2 to 4 weeks following exposure and is characterized by the presence of viral RNA and/or p24 antigen. Methods for detecting these two viral components (viral RNA or p24 antigen) for incidence

determination have been reported (77, 78). However, the duration of p24 antigen/viral RNA prior to detection of antibodies is short during the preseroconversion period. Therefore, the use of this method would require a large number of specimens to attain the needed confidence in incidence measurement. For instance, even in Swaziland (now known as the Kingdom of eSwatini), with an HIV-1 prevalence as high as 32% and an incidence of 2.5 per 100 persons per year, only 1 person in every 1,000 seronegative persons was found to have HIV-1 RNA (79). If the HIV-1 incidence in a given study population is low, the probability of finding individuals during this acute phase with viral RNA or p24 would be even lower. The large sample size requirement and operational cost limit the utility of this approach.

Detection of Recent Infections during the Postseroconversion Period

Detection of recent infections using postseroconversion specimens can be achieved by examining antibody maturation and viral genetic diversity (Fig. 4). Antibody maturation can be further characterized based on the development of antibody titers/levels, antibody avidity, and IgG isotypes. Early in incidence assay development, the antibody titer was the primary target based on the premise that recently infected persons would have lower antibody titers than persons with long-term infections. Initially, the Abbott HIV-1 subtype B-based 3A11 assay was modified to be "LS" by using a 20,000-fold-diluted serum specimen with a reduced antigen/antibody reaction time (30). An optical density (OD) value of the diluted specimen together with a predetermined cutoff obtained from a calibrator specimen were used to distinguish recent from long-term infections. This assay was found to work well with specimens obtained from regions with predominantly subtype B viruses but poorly with specimens from countries with predominantly non-subtype B viruses. The assay overestimated the incidence in specific populations due to antigenic differences between subtypes, resulting in significant differences in mean durations of recent infection ("recency window period") and misclassification rates (31, 33). However, several additional commercial assays were modified as less-sensitive assays to detect recent infections, including Vironostika-LS, Vitros-ECi-LS, and multiple rapid tests, such as Serodia, Unigold, Determine, OraQuick, and SeroStrip (80–84). None of them were widely adopted due to limitations of commercial assays using antigens derived primarily from HIV-1 subtype B, which led to ongoing research into and development of several new approaches. The first one included the development of the BED-Capture EIA, a *de novo* assay specifically developed to detect and distinguish recent from long-term infections using increasing levels of HIV-specific gp41 antibodies as a proportion of total IgG (31). This assay included the design of a multisubtype branched gp41 peptide (termed "BED") to address the antigenic variability of subtypes based on data from the Los Alamos sequence database. An additional unique feature was the capture format of the assay, which captured both HIV-specific IgG (HIV-IgG) and non-HIV-IgG, followed by the detection of gp41-specific antibodies using a biotin-labeled BED peptide. The capture format allowed measurement of increasing proportions of HIV-IgG during antibody maturation. This approach changed the dynamic range of the assay, compared to diagnostic assays, making it less sensitive during the early phase of seroconversion, when the ratio of HIV-IgG to total IgG is low. Since the ratio of HIV-IgG to total IgG in the blood determined the signal, the outcome was not affected by dilution of the specimen, a major advantage over LS assays, which depended heavily on the precision of a 1:20,000 dilution, which was difficult to achieve. Following optimization and calibration of the assay (32), it was commercialized as a kit and was widely used in the United States and several other countries, including South Africa, China, Thailand, Ethiopia, India, Indonesia, and Kenya, for surveillance of recent infections and estimating HIV-1 incidence (85–95). However, ongoing studies also indicated that the BED assay results were confounded by individuals with low CD4 levels (those with AIDS), elite controllers, those with high levels of total IgG (as found in Africa), and those on ART (96–99), resulting in elevated HIV-1 incidence rates. UNAIDS recommended that the BED assay not be used for incidence surveillance (100). Due to the absence of any other good

laboratory-based methods, solutions were proposed, including the use of the BED assay in case-based surveillance, which allowed the removal of misclassified cases or adjustments based on misclassification rates in a given population (96, 101–110). However, misclassification rates, which contribute to overestimations of incidence, are not constant but vary with time, place, and population (99). Parallel developments of other approaches include a two-well assay based on the differential detection of antibodies to gp41 IDR and gp120 V3 loop antigens (111, 112). Recognizing the diversity of HIV-1 antigens and the corresponding immune responses, the assay incorporated 2 sequences of IDR peptides and 5 sequences of V3 peptides. The investigators used the in-house assay to detect recently infected persons in France (112–114). However, the assay is not commercially available due to the complexity of the assay, limiting its wider application or evaluation. In 2004, Wilson et al. described the development of an EIA to detect IgG3 antibodies to p24 as a marker to detect recent HIV-1 infection (115). However, the presence of p24-IgG3 was not consistent among recently infected persons, limiting the use or commercial development of the assay.

Antibody Avidity

Antibody avidity (binding strength of antibodies) has been recognized as a reliable marker for maturation of antibodies and has been used to identify and distinguish recent infections (with low Ab avidity) from long-term infections (with high avidity) for many viral infections, including cytomegalovirus (CMV), rubella, hepatitis B/C, and HIV infections (72, 116–122). Most early efforts were focused on modifying commercial HIV assays to measure avidity indexes using different chaotropes or dissociation reagents. Although these assays provided useful information, limitations included the use of antigens that are derived mainly from HIV-1 subtype B, the need for an automated platform such as Abbott Axsym or Architect, the requirement for duplicate wells to calculate the avidity index, and increased variability coming from two wells (122).

To address antigenic diversity, a chimeric recombinant gp41 protein (rIDR-M) that included multiple gp41 sequences in a single protein was developed (123). A novel, single-well avidity assay was developed using wells coated with a limiting amount of antigen. Limiting the amount of antigen forced bivalent antibodies to bind monovalently, thus facilitating the separation of recent infections (weak antibodies) from long-term infections (strong antibodies), all in one well (Fig. 5A). The limiting-antigen (LAg) avidity EIA worked as well as or better than the two-well assays that measured antibody avidity indexes but benefited from a calibrator specimen to minimize variability and determine the cutoff for recency classification (33, 124). Again, the assay was optimized, characterized, and subsequently transferred to commercial partners for development of an LAg-Avidity EIA kit.

The LAg-Avidity EIA was found to have clean antibody kinetics, separating recent from long-term infection with less persistence of low avidity beyond 1 year (33). It also had the lowest misclassification rate compared to other incidence assays. The assay was validated in Swaziland, where the LAg-based incidence in a cross-sectional survey was compared to the observed incidence in a cohort (125). That study as well as work from independent groups, such as the Consortium for Performance and Evaluation of Incidence Assays (CEPHIA), demonstrated that almost all antibody-based assays will misclassify a certain proportion of persons as having been recently infected who otherwise are infected for a long time but are either on ART or identified as elite controllers (126). Both these groups are characterized by a low VL, which contributes to low antibody avidity, resulting in misclassification in some cases. Therefore, a recent infection testing algorithm has been proposed, which includes LAg EIA testing followed by VL testing of those classified as having been recently infected. Final classification of recency is based on recent LAg (normalized OD [$OD_{normalized}$] of <1.5) combined with a VL of $\geq$1,000 copies/ml to identify true cases of recent infection. Other investigators have used the presence of antiretrovirals (ARVs) in the blood, in addition to VL, to reclassify recent LAg cases as part of the algorithm. Application of these approaches has shown that estimated HIV-1 incidence rates are plausible and provide meaningful data

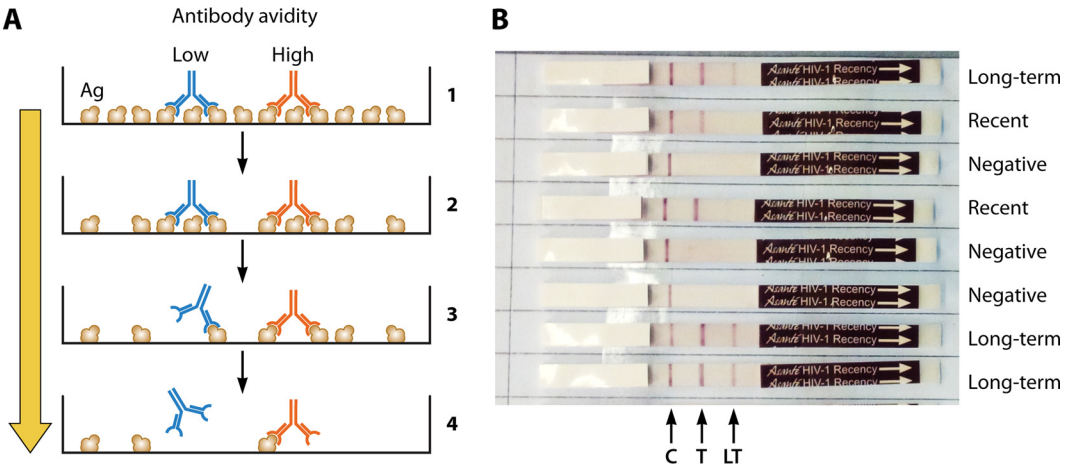

FIG 5 Schematic representations of the assay principle for a limiting-antigen (LAg) assay to differentiate low- and high-avidity antibodies (A) and a rapid incidence-prevalence assay (B). (A) At high antigen concentrations, bivalent antibodies of both low avidity (blue) and high avidity (red) bind to microwells coated with antigen, whereas at limiting antigen concentrations, only the high-avidity antibodies bind due to monovalent binding and are retained after washing. (B) Two empirically optimized antigen concentrations are incorporated onto the nitrocellulose strip for diagnosis of HIV infection and detection of recent infection. C, control line; T, test line at a high antigen concentration; LT, low antigen concentration to differentiate recent from long-term infections. The binding of antibody is determined with lateral flow technology. The control band indicated by the arrow is an internal IgG procedural control. Seronegative samples are reactive only for this control band. Persons with recent infections will show C and T lines but not the LT line, and those with long-term infections (>1 year) are reactive to all three lines, including the LT line.

for risk factor analysis of the acquisition of new infections in multiple studies. Currently, the LAg-Avidity EIA, in an algorithm with VL testing (and, in some surveys, with ARV), is the preferred method for detection of recent infections and estimation of incidence. The assay is widely used in many countries for population-based HIV impact assessment (PHIA) surveys with promising results (127, 128).

Rapid Incidence Assay

A rapid incidence test that can diagnose HIV infection and distinguish recent from long-term infections in a single test was recently developed (129), using the same concept of limiting antigen. To overcome limitations of commercial rapid tests, used primarily in a less-sensitive format, rIDR-M was used in limiting concentrations, in addition to a diagnostic line, in a rapid, lateral flow strip format (Fig. 5B) (129). The assay is now commercially available as the Asanté HIV-1 rapid recency assay (Sedia Biosciences Corporation). The assay was validated in laboratory settings, with sensitivity and specificity of >99% and high correlation with LAg-Avidity EIA results (130). As a POC test, the rapid recency assay has the potential to significantly change the outcome in the fight against HIV/AIDS and play a key role in prevention and treatment programs. For instance, the use of this test would allow diagnoses and identification of recent infections of individuals using HIV testing services, in antenatal clinics, and in surveillance. The use of such a tool in case-based incidence surveillance, with a focus on persons with newly diagnosed infections, can help identify geographic hot spots and high-incidence populations in real time for targeted prevention and interruption of ongoing transmission. Additionally, it would allow quick initiation of contact tracing of partners of the index case for testing and initiation of treatment. Several pilot projects are under way in Malawi, Vietnam, Central America, and other areas to assess the utility of the rapid recency assay. The rapid incidence assay is being recommended for wider use in many countries to detect new infections among adolescent girls and young women and in routine programs to detect hot spots of new infections.

Role of Genetic Diversity Approaches in Incidence

The premise of the genetic diversity approach is that only a small subset of HIV variant founders is established in the new host after transmission, followed by the

expansion of the genetic profile with increased accumulation of mutations in the HIV genomes with viral replication. Persons with recent infections are thought to possess less HIV genetic diversity than persons with long-term infections. Genetic diversity can be estimated by direct sequencing, which is labor-intensive and cost-prohibitive. Simplified assays, such as evaluation of ambiguous nucleotide calls (131, 132), the Hamming distance assay (133), and the high-resolution melting diversity assay (134), have been recently shown in many small studies to be promising (135). However, there are complications due to infections with multiple variants in newly infected persons (e.g., blood transfusion-related infections or infections in intravenous drug users) and among those with advanced AIDS. Moreover, none of the methods has been widely applied or commercialized.

HIV TESTING USING DBS SPECIMENS

DBS specimens are an alternative specimen type to whole blood and plasma for diagnosis and monitoring of patients infected with HIV. DBS specimens are easy to collect, process, and package and can be transported at ambient temperature (136, 137). DBS specimens have been evaluated and showed results comparable to those with plasma and are widely used in programs for HIV antibody testing (138), HIV incidence determination (139), HIV DR monitoring (140–143), surveillance (144), VL testing (145), EID (146–148), and proficiency testing (PT) (149). Different types of commercially available filter paper, including Ahlstrom 226, Munktell TFN, and Whatman 903, have been assessed and shown to be suitable for the collection of DBS specimens for EID, thus providing options for use in RLS (150). However, only Whatman 903 filter paper is optimized and recommended for testing for recent infection with BED or the LAg-Avidity EIA. The use of DBS specimens would increase access for populations where laboratory services are not available. For example, within the tiered laboratory network, where complex molecular tests such as VL tests, DR tests, and EID are found mostly in laboratories at the national, provincial, and district levels, the use of DBS specimens will ensure that the specimens can be collected by trained health care workers in remote areas and transported to a specialized laboratory for testing.

ENSURING QUALITY OF TESTING

Current Status of Quality Assurance Programs in RLS

The rapid expansion of HIV testing in the past decade has not been matched by quality assurance. The lack of adequate quality assurance practices could negatively impact the accuracy of tests results, especially those conducted in remote areas (151). In line with WHO recommendations on task shifting to support universal access to HIV/AIDS prevention, treatment, and care services, many RLS countries have revised their national policies to use community counselors to perform HIV rapid testing (152–154). Countries have the primary responsibilities to develop policies to implement and monitor QA of HIV testing. An unfortunate consequence of low-quality HIV rapid testing is wrong diagnosis, with an enormous adverse impact on individuals, families, communities, health care workers, and programs. The resulting unnecessary anxiety and treatment undermine the credibility of entire HIV programs (155–157).

To prevent enrolling misdiagnosed persons into lifelong ART, the WHO recommends the retesting of all clients determined to be HIV positive with a second specimen prior to ART initiation (57). Factors often attributed to misdiagnosis include staff inexperience due to high turnover rates, the use of a suboptimal algorithm, clerical errors, inadequate training and competency assessments, and a lack of supervisory oversight (158–160). Improper or inadequate documentation of testing records could also lead to erroneous result reporting and is often found in lower-level health care facilities. To document testing properly, the use of a standardized paper-based HIV testing logbook nationwide is a useful approach (68). The logbook captures much relevant quality information, including selection of preprinted simple test results (i.e., without the need for handwriting for reactive or nonreactive test results), retesting decisions, test kit names, lot numbers, and expiration dates. The logbook also provides a summary

tabulation of the collective results entered for a page to allow testers to better self-monitor the concordance or discordance between results of two rapid tests and to identify and capture abnormalities. Since the log is paper-based, the information captured from different testing sites cannot be aggregated in a timely manner for regional or national program managers to analyze for any trends and corrective actions, if needed. Thus, when applicable, the country should progress to the use of electronic filing and reporting methods to better record and report testing results.

Periodic site assessments and refresher training using standardized tools can narrow the quality gaps at primary sites. Due to limited resources and workforce constraints, reference laboratories at the regional and national levels should focus on newly established testing sites and those with known low-performance records. Participation in a PT program, in particular, has been found to greatly enhance problem-solving skills of testers. Some countries emphasize the importance of the use of PT materials to train testers under the supervision of experienced staff (161, 162). National reference laboratories can produce and distribute PT materials. PT programs using a cost-effective alternative approach, such as the dried tube specimen (DTS) approach, have been properly documented (151). DTS are an alternative to serum or plasma samples for use in PT panels to monitor the quality of testing. DTS are prepared from well-characterized specimens by mixing small volumes of the characterized plasma or serum sample with a color dye and allowing the mixtures to air dry. The resultant pellet is visible and is then reconstituted with phosphate-buffered saline (PBS)–Tween 20 and used for testing according to the instructions (151). DTS are temperature stable, and results obtained are comparable to those with plasma or serum samples. DTS are also used as PT materials to monitor the quality of other HIV biomarkers, such as VL (163).

Enabling Environment for Quality-Assured HIV Rapid Testing

Although HIV testing services (HTS) QA guidelines for rapid testing existed for many RLS countries (5, 6), they were deficient in policies for implementation (159, 164–166). In 2015, the WHO released a handbook, *Improving the Quality of HIV-Related Point-of-Care Testing*: *Ensuring Reliability and Accuracy of Test Results* (167), to address QA program weaknesses (8, 168). This handbook emphasizes the importance of continuous quality improvement for POC testing, the need to strengthen quality practices by quality officers and testers, the provision of new quality strategies, and the use of a comprehensive package with established new tools. The handbook describes and emphasizes the processes of the quality assurance cycle (QAC): planning, implementing, and sustaining (167). These new recommendations are being translated into national policies and guidelines in many RLS countries (161, 169). For instance, South Africa has developed an HTS policy and guidelines with emphasis on the importance of completing the QAC to prevent testing errors along the "diagnostic continuum" (169). Kenya highlighted the importance of competency demonstration and certification of testers by the National AIDS/STD Control Program and defined the specific tasks of stakeholders at the facility and country levels to address problems of personnel shortages (161).

The U.S. CDC and the WHO developed a systematic and multimodular training curriculum (170, 171). Most RLS countries have adopted this curriculum with revisions to suit their respective implementation needs. These country-specific revisions include training processes, durations of training (1 to 10 days), teaching approaches (lectures versus practical sessions), and delivery formats (158, 159, 166, 172). Due to the large number of persons to be trained, most RLS countries opted for a top-down approach through a training-of-trainers (TOT) model that allows the rapid expansion of standardized training to staff in the region, district, community, and outreach program. This training includes pre- and posttraining quizzes to assess the performance skills and knowledge gained. For example, in a health facility in eastern Kenya, a training assessment showed that of the 91% of nurses who received HTS training, 79% reported self-perceived competency and 70% demonstrated competency in performing rapid testing (173). While the study sample size was small, this report identified the existence

of a gap between training and testing performance and the need to enhance training methods, including hands-on sessions, and to ensure that core competencies are adequately assessed.

Many countries established guidelines recommending the provision of periodic refresher courses to maintain tester competency. Refresher training can involve a high work burden and requires extra resources, and thus, it is important that the country's policies are supported with adequate financial and human resources. Because Internet- and mobile-based technologies have become widely accessible in RLS, distance-learning programs can greatly improve training by reducing the time that trainees need to be away from their health facility while receiving off-site training. Examples of these cost-effective programs include the Project Extension for Community Healthcare Outcomes (ECHO) (174) and Supercourse (175) public health training programs currently in use in several African countries. Project ECHO, an example of a virtual community of practice, brings together subject matter experts, health care practitioners, and peer groups virtually to share knowledge and practical experience, including problem-solving skills, in real time to impact programs (176).

Promoting Community Partnerships for the Uptake of Quality Measures

The severe shortage of government health workers in RLS countries has highlighted the need for the participation of community volunteers. Many of the health care tasks that do not require specialized training could be delegated to community volunteers to increase access to HIV services in underserved or hard-to-reach communities (177). In 2012, a systematic review described a wide range of nonprofessional cadres of health workers to assist HIV treatment programs (178). These volunteers included care coordinators, peer health workers, field officers, health extension workers, HIV/AIDS lay counselors, adherence supporters, and home-based care workers. The WHO has recommended the involvement of community volunteers or quality corps (Q-Corps) volunteers to support the rollout of quality activities and to ensure that the QAC is completed (167, 179). Several PEPFAR-supported countries have implemented this initiative, with improved HIV testing in remote areas. Q-Corps are further stratified into two tiers, level I and level II, based on different roles, with Q-Corps level II being higher than level I and having more responsibilities (Table 2). The Q-Corps volunteers may be new health science graduates or part-time laboratory volunteers who are trained on specific QA tasks under the guidance of the MOH, implementation partners, and other managerial agencies at various health care levels (Table 2). The Q-Corps can be involved in the distribution and collection of PT results, supportive site supervision, training and competency assessment of site staff, and distribution and review of a standardized logbook or electronic system.

In Cameroon, Q-Corps were successfully employed to help distribute DTS-based rapid test PT panels to remote testing sites and contributed to reducing the PT result turnaround time from 30 days to 5 days (59, 180). This was a remarkable accomplishment, especially in areas with poor transportation and road infrastructures. With the proper training and guidance, Q-Corps can be used to help advance laboratory testing quality.

In countries where there are established partnerships with nongovernmental organizations (NGOs) for health care service delivery, HIV testing quality-related needs may not be delegated to nonprofessional community volunteers. It is critical for the managers of the MOH and the national reference laboratory to develop a supportive policy environment with appropriate regulations for NGOs to support the scale-up of QA programs. Q-Corps volunteer programs should be guided and built through sound work regulations and quality improvement frameworks. It is also beneficial for governments to establish collaborative partnership with NGOs, community or professional associations whose members can be trained to oversee the program and provide supportive supervision and input (181). Moreover, while partnering with communities and other NGOs, appropriate coordination should be built at either the regional, district, or community level with delegated responsibilities.

TABLE 2 Responsibilities and working relationship between Q-Corps volunteers and the supporting MOH or other managerial authorities[a]

	Working relationships and/or responsibility(ies)		
Organization or program	**Program activities/management**	**Q-Corps level II**	**Q-Corps level I**
Responsible organizations	MOH, NRL, and regional and district agencies	Local implementation partners, district laboratory staff, QA officers, and coordinators	Local implementation partners
PT program	PT network establishment PT panel preparation PT outcome assessment		Panel distribution to testing sites Assistance with data report and corrective action
HTS program QA	National testing logbook customization, outcome review, and corrective action plans	Logbook utilization and review for data integrity	Logbook distribution and entry assistance Monthly data assembly collection
Training programs	Planning, development, and conduct of national training curriculum and programs	Assistance with training material distribution	Participation and assistance with simple training courses
	Supervisory site visits and corrective action provision	Assistance with internal audits Assistance with competency assessment	

[a]Q-Corps level II is higher, with additional responsibilities for data review, than level I. Abbreviations: MOH, ministry of health; NRL, national reference laboratory; Q-Corps, quality corps; HTS, HIV testing services; PT, proficiency testing; QA, quality assurance.

National Program for Competency Assessment and Tester Certification

A review of POC testing outside the traditional laboratory environment clearly demonstrated gaps that should be addressed by implementing stepwise quality programs to improve tester and site competency. Implementation of a national certification program ensures adherence to quality standards across POC sites, lends credence to the sites, and inspires confidence in patients and clinical staff. Currently, only a few RLS countries have competency-based certification programs. For instance, a 2007 act to provide for the registration and regulation of the health laboratory practitioners of Tanzania specifies requirements for certification, recertification, and decertification of testers (182). However, this act was not fully implemented due to the lack of resources. National guidelines for HIV counseling and testing in Namibia also outlined certification and recertification requirements (162). It was intended for nonlaboratorians and staff conducting HIV testing in all facilities. Zimbabwe's national policy mandating HIV testing by laboratory technicians has recently been revised to extend to nonlaboratorians performing rapid HIV testing (183). When establishing a national certification program, it is critical for the government to allocate resources, infrastructure, and personnel for implementation and to identify independent organizations, such as laboratory professional associations, academic partners, or a public health institute, with demonstrated high standards to act as the certifying body. The certifying bodies can assess the competency of testers, maintain the certification data, and provide reports to ensure impartiality and transparency.

Optimizing the Process for National Certification of Rapid Testing Sites

Until recently, there have been few or no programs to systematically monitor and assess compliance to the quality of HIV rapid testing sites. Although in most countries, periodic supportive supervisory visits have been conducted using program-specific assessment tools, they are often not standardized, and they do not comprehensively cover quality processes. Furthermore, the site supervision teams often do not include trained technical staff. In order to identify gaps for targeted intervention and resource allocations needed by national programmers, the CDC, the WHO, and several implementing partners developed a stepwise process for improving rapid testing (SPI-RT) checklist (167). The checklist contains 70 audit inquiries with preassigned scores and covers seven QA areas, including personnel training and certification, the physical facility, safety, the pretesting phase, the testing phase, the posttesting phase, and external QA (EQA) assessment. The checklist is categorized into five levels to represent the extent of readiness of the sites for national site certification, level 0 ($<$40%), level 1 (40% to 59%), level 2 (60% to 79%), level 3 (80% to 89%), and level 4 (90% to 100%). Level 0 indicates that the site is in poor readiness and requires immediate remediation in all seven quality system essentials, while level 4 indicates that the site is eligible for national site certification. Sites are assessed using the checklist and scored as one of the different levels.

In 2014, nine countries in Africa and the Caribbean (Cameroon, Ethiopia, Uganda, Kenya, Malawi, Tanzania, Zambia, South Africa, and Jamaica) initiated training of MOH staff and Q-Corps volunteers and used an earlier version of the SPI-RT checklist that covered the seven areas described above, including a section on documents and records, to audit 1,740 testing sites. The quarterly audited sites included several testing modalities, such as voluntary counseling and testing (VCT), provider-initiated testing and counseling (PITC), prevention of mother-to-child transmission (PMTCT), and treatment centers and laboratories. The median SPI-RT score at the baseline audits of these sites was found to be 55.6% (level 1). Of these sites, 968 (55.6%) sites in six countries (Cameroon, Uganda, Malawi, Tanzania, Zambia, and Jamaica) had corresponding scores at baseline and 12 to 15 months later to allow for pairwise comparison over time. It was found that at the baseline audits, 20% of the sites were at level 0, 46% were at level 1, 31% were at level 2, 3% were at level 3, and none of the sites were at level 4 (Fig. 6). After 12 to 15 months of implementation of corrective actions with provision of supervisions, 38%, 39%, and 16% of the sites had moved up to levels 2, 3, and 4,

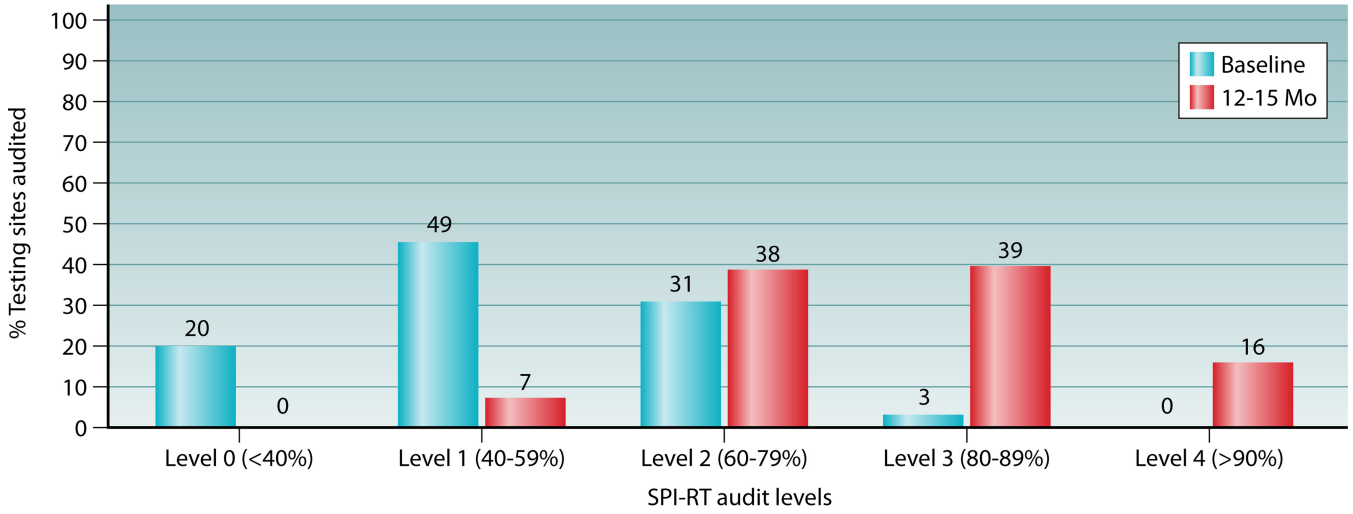

FIG 6 Performance levels from the baseline audit to the 12- to 15-month audit among 968 testing sites in five African countries (Cameroon, Uganda, Malawi, Tanzania, and Zambia) and one Caribbean country (Jamaica). Shown are SPI-RT scores at level 0 (<40%), level 1 (40 to 59%), level 2 (60 to 79%), level 3 (80 to 89%), and level 4 (>90%). The numbers on the top of each bar represent the percentages of the total sites.

respectively, and none of the sites remained at level 0. At the baseline audits, high scores were obtained for the physical facility (77%), safety (60%), the pretesting phase (66%), and the posttesting phase (70%) (Fig. 7). After 12 to 15 months of remediation, there was a >20% increase in the audit score for the testing phase (77%), the posttesting phase (93%), documents and records (84%), and the EQA program (74%). The areas with moderate improvement were the physical facility (88%), safety (77%), and the pretesting phase (83%). Although noticeable improvement was observed for EQA, there were inconsistent PT panel distributions by the national PT provider and deficiencies in the documentation of PT participation, which were previously found to be prevalent in many RLS countries (157–159, 166). The inconsistent PT panel distribution was corrected in Cameroon by using the innovative Q-Corps (180).

Our experience in RLS, including expansion of testing at thousands of testing sites, demonstrates that QA measures should be appropriate for the environment where testing is being conducted. Laboratory accreditation, offered by the College of American Pathologists (CAP) and the ISO, may not be feasible in RLS where HIV diagnosis is offered. However, development of novel approaches, including a strong training cur-

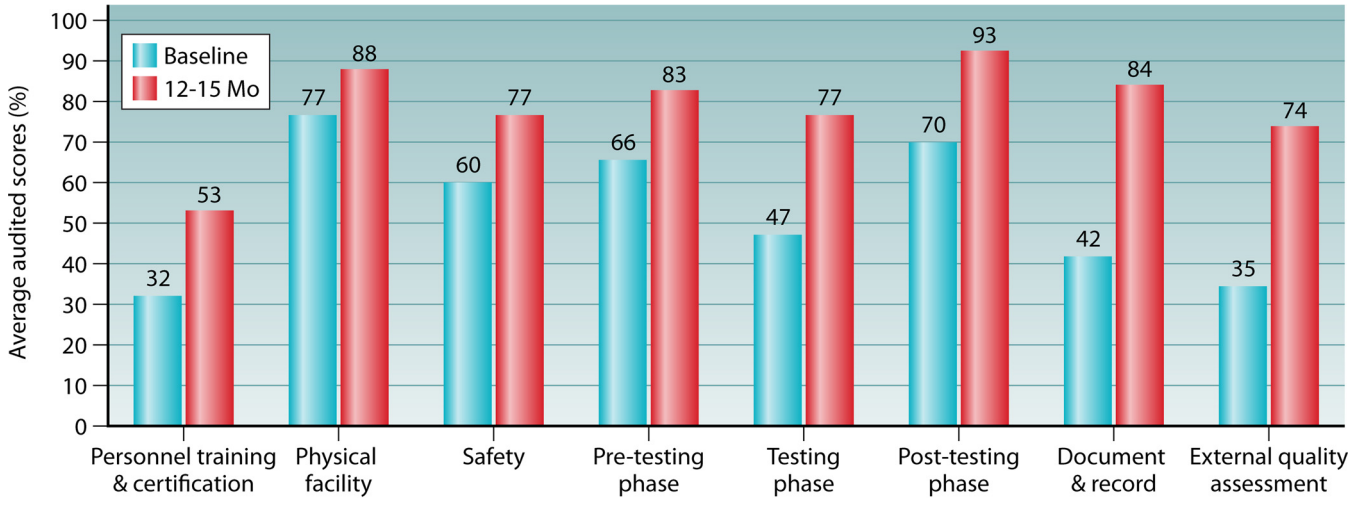

FIG 7 Average audit scores (percent) of 968 HIV testing sites in five African countries (Cameroon, Uganda, Malawi, Tanzania, and Zambia) and one Caribbean country (Jamaica) using the SPI-RT checklist on eight quality components.

riculum and ongoing monitoring, can ensure that HIV testing is accurate. Data from multiple countries in PEPFAR-supported programs indicate that the positive predictive value of HIV diagnosis can be 99% or higher.

MOLECULAR METHODOLOGIES FOR DETECTION AND QUANTIFICATION OF VIRAL LOAD

Viral Load and ART

The efficacy of ART for individuals infected with HIV can be monitored by using parameters such as increases in CD4 cell counts, VL suppression, and improvements in clinical symptoms. The 2013 WHO consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection strongly recommended VL testing for use in monitoring of patients on ART and for confirmation of treatment failure (184). VL monitoring is the preferred approach because it is an early and accurate marker of ART success compared to immunological and clinical monitoring markers and can facilitate detection of DR mutations early (185–187). VL monitoring of a patient's response to treatment is also a good indicator for assessing adherence to ART and allows the effective use of differentiated service delivery models to manage virally suppressed and nonsuppressed patients (188). In a multicountry observational study, patients with adherence counseling showed viral resuppression when the VL was used to identify patients requiring adherence support, while delayed implementation of routine VL monitoring was associated with the accumulation of DR (189). In the United States, VL is assessed at multiple time points, prior to ART initiation, within 2 to 4 weeks post-ART, and every 3 to 4 months thereafter for the first 2 years, which can be extended to every 6 months after 2 years if viral suppression is consistently observed (190). In contrast, in 2013, in SSA, only 20% of ART-treated patients had access to routine VL testing, and clinicians often received results too late to assist in better patient management (9). The WHO has recommended that VL testing be carried out 6 and 12 months after ART initiation and once every 12 months thereafter, with $>$1,000 copies/ml being the threshold for treatment failure in RLS (6). However, some countries have implemented routine VL monitoring with different thresholds for virologic failure. For example, Botswana treatment guidelines recommend a threshold of $>$400 copies/ml as virologic failure (191). A systematic review of adult virologic outcomes in response to an ART program in SSA showed 78%, 76%, and 67% viral suppression after 6, 12, and 24 months, respectively, suggesting that the first-line regimens were effective (187). Also, in a recent PEPFAR PHIA, adult viral suppression rates of 85%, 78%, and 71% were observed in Zimbabwe, Malawi, and Zambia, respectively (192).

Providing timely VL results has been challenging in RLS, partly because VL assays are complex and performed at higher-tiered laboratories in urban areas, in addition to some patients residing in hard-to-access areas. Currently, whole blood or plasma specimens, when collected from distant sites, are shipped within a few hours through an established specimen referral transportation network to centralized laboratories, where they are tested in a cost-efficient manner (193, 194). The scale-up of HIV VL testing is key for routine monitoring of patients on ART, especially as programs transition from CD4-based monitoring to VL monitoring requiring advanced molecular laboratories. One key factor that can limit access to VL testing is the high cost for testing. VL testing costs range from US$30 to US$125 per test (195, 196), and more than half of the cost can be attributed to the cost of regents and consumables (197). To increase access to VL testing and improve patient management, the Roche Global Access Program significantly reduced the price of VL testing reagents for low- and middle-income countries in 2014 (198). The Global Fund also negotiated significant cost reductions with major manufacturers for instruments and VL testing and EID reagents (199). One of the challenges with some of the agreements is that manufacturers sometimes require the ordering of guaranteed volumes of test reagents. Although welcomed as progress, the guaranteed-volume approach tends to exclude countries with lower prevalences or those countries making significant strides to curb their epidemic. It is almost counterintuitive and could be perceived as punitive. There should

TABLE 3 Characteristics of viral load platforms commonly found in centralized laboratories[a]

Purpose	Manufacturer and trade name	VL range (copies/ml plasma)	Viral genome(s) detected	Testing time	Random access	Plasma vol (μl)	DBS utility
Real-time RT-PCR	Abbott RealTime	150–10^7	*int*	7 h	No	200	Yes
		75–10^7				500	
		40–10^7				600	
		40–10^7				1,000	
	Beckman Coulter DxN Veris	235–1.06 $\times$ 10^7	*pol*	1.5 h	Yes	175	NA
		35–1.06 $\times$ 10^7				1,000	
	Biocentric Generic HIV Charge Virale	390–5 $\times$ 10^6	LTR	7 h	No	250	Yes
	Cepheid GeneXpert	40–10^7	LTR	1.5 h	Yes	1,000	Yes
	Qiagen Artus RG	45-4.5 $\times$ 10^7	LTR	5 h	No	200	NA
	Roche Cobas CAP/CTM, v2.0	20–10^7	LTR, *gag*	7 h	No	1,000	Yes
	Siemens Versant kPCR 1.5	35–11 $\times$ 10^7	Pol	6 h	No	500	NA
T7 RNA polymerase	bioMérieux NucliSENS Easy Q, v2.0	25–10^8	*gag*	2 h	No	100	Yes
		20–2 $\times$ 10^7				500	
		10–10^7				1,000	
	Hologic Aptima Quant Dx	13–10^7	LTR, *pol*	3 h	Yes	500	NA
Signal amplification	Siemens Versant bDNA, v3.0	75–500,000	*pol*	7 h	No	1,000	NA
Enzyme based	Cavidi ExaVir, v3, or Ziva	200–6 $\times$ 10^5		2 days	No	1,000	No

[a]Abbreviations: VL, viral load; RT-PCR, reverse transcriptase PCR; bDNA, branched DNA; DBS, dried blood spot; *pol*, polymerase region of the HIV genome; *int*, integrase region; *gag*, group-specific antigen region; LTR, long terminal repeat region.

be harmonization of these prices for universal access that would lead to sustained VL testing scale-up, and the cost reduction would render the monitoring of ART programs more cost-effective (200).

Commercial Assays Suitable for Centralized Laboratories

There has been great progress in the development of assays to quantify HIV loads in blood since the beginning of the pandemic. During the early phase of the pandemic, quantitative cultures of peripheral blood mononuclear cells or plasma were used to measure VL (201, 202). This methodology was labor-intensive, lacked reproducibility, and often yielded negative cultures for patients with CD4 counts of $<$200 cells/μl (201). The limitations of culture techniques led to the development of simple and more-accurate PCR-based and other molecular methodologies to accurately measure VL.

Since the first PCR-based commercial VL assay in 1996, several commercial assays have been manufactured. These assays are FDA approved, "Conformite Europeene" (CE) marked, and/or WHO prequalified. In earlier stages, the users often experienced amplification contamination-associated false positivity and large VL variations. However, the methods had been greatly improved by the utilization of contamination control strategies, internal calibration controls, process automation, and real-time detection technology approaches (203–205). With a common set of standards of known VL employed by all manufacturers, VL determination standardization became feasible. Manufacturers also examined assay sensitivity and specificity using collections of clinical specimens. The quantification of HIV-1 VL in plasma was thus standardized and expressed as the number of HIV-1 RNA copies present per milliliter of plasma or as $\log_{10}$ units per milliliter of plasma. Independent collaborative evaluations by end users in different geographic locations with different subtypes and recombinant circulating forms further validated the assay performance and the comparability of results using different assays.

A list of 12 major commercial VL assays with their respective performance characteristics, including detection ranges, gene targets, testing times, random-access modality, plasma volumes, and DBS application, is shown in Table 3. With the exception of the Cavidi assay, the other 11 assays are nucleic acid tests (NATs), since detection is based on the amplification of nucleic acid or signal amplification with the initiation target derived from viral RNA. The detected VL range is smaller with the Cavidi ExaVir load assay (200 to 6 $\times$ 10^5 copies/ml) and the Biocentric Generic assay (300 to 5 $\times$ 10^6

copies/ml), while the other 10 NAT-based assays have a wider range of approximately $<$50 to 10^7 copies/ml. The low detection limits according to all manufacturers enable the threshold of $>$1,000 copies/ml recommended by the WHO for ART monitoring in RLS.

The NATs can be divided into three groups according to their amplification principles and signal detections. The first group contains seven assays using the reverse transcriptase PCR (RT-PCR) principle to copy viral RNA and amplify the resultant cDNA. The assays using RT-PCR include Abbott RealTime HIV-1 PCR, the Beckman Coulter DXN Veris HIV-1 assay, the Biocentric Generic HIV-1 viral load assay, the Cepheid GeneXpert HIV-1 viral load assay, the Roche Cobas CAP/CTM HIV-1 viral load assay, and Siemens Versant HIV-1 RNA 1.5 (kinetic PCR [kPCR]). The second group, with the bioMérieux NucliSens EasyQ HIV-1 v2.0 assay and the Hologic Aptima HIV-1 Quant assay, uses the T7 RNA polymerase for amplification. The third group, with the Siemens Versant HIV-1 RNA 3.0 (bDNA [branched DNA]) assay, uses signal amplification methodology.

In the RT-PCR-based assays, plasma RNA is first reverse transcribed. The resultant cDNA is amplified by PCR using HIV-specific primer pairs derived from several conserved long terminal repeat (LTR), *gag*, *int*, and *pol* regions of the viral genomes to broaden subtype coverage. The generation of nascent DNA in each cycle of amplification is quantified in real time using sequence-specific fluorescent probes. The two most widely used assays in RLS are the Abbott RealTime HIV-1 PCR and Roche Cobas TaqMan assays. Abbott, Roche, bioMérieux, and Siemens provide proprietary automated plasma RNA processors and amplification/detection devices. Barcoded specimens are tracked and recorded by the instrument to minimize hands-on time and transcriptional errors. Typically, the footprints of these automated RNA processors are large. Specimens are processed in a batchwise manner to yield VL results in 6 to 8 h. Improvements have been made to the Beckman Coulter DxN Veris (206) and Cepheid GeneXpert (207) systems using cartridge-based plasma RNA processing and PCR to achieve the one-step "sample-in and result-out" process in 1.5 h. Individual plasma specimens can be loaded into the cartridge and run in a random-access manner without a time delay. This kind of instrument may be useful in district laboratories near primary health care locations with technical staff trained to prepare plasma. Further modification of the cartridge to include the plasma preparation step will make these instruments directly applicable to POC testing. The Biocentric Generic assay is run in an "open" manner. The manufacturer provides PCR reagents and quantitation controls but not the automated RNA extractor and PCR/detection device. The end users must procure their own real-time amplification/detection device. Although this open system allows end users the flexibility to conduct in-house assays to study other biological agents of interest, it requires the end users to manually calculate VL results and may be open for result variability.

The second group includes the bioMérieux NucliSens and Hologic Aptima assays. The NucliSens and Aptima assays are commonly known as a nucleic acid sequence-based amplification (NASBA) assay and a transcription-mediated amplification (TMA) assay, respectively. The RNA is first reverse transcribed using the reverse transcriptases derived from avian myeloblastosis virus and murine leukemia virus for the NucliSens and Aptima assays, respectively. The primers used for reverse transcription contain a sequence complementary to HIV-1 and a sequence complementary to the T7 RNA polymerase transcription promoter. Thus, the ensuing cDNA molecules serve as the templates to generate hundreds of nascent RNA molecules in each run of the amplification process catalyzed by T7 RNA polymerase at a constant temperature of 41°C. Quantifiable signals are produced within 1 h. The importance of the isothermic temperature used in this assay cannot be overemphasized. At 41°C, double-stranded DNA molecules do not serve as a template for T7 RNA polymerase. Thus, when DBS specimens are used for VL determination with NASBA and TMA assays, only the viral RNA, not the proviral DNA, is quantified.

The Siemens Versant HIV-1 RNA 3.0 bDNA assay is the sole NAT listed in Table 3 that does not use nucleic acid amplification. Instead, it uses a signal amplification process to

yield a VL range of between 75 and 500,000 copies/ml. The viral RNA is first immobilized by capture oligonucleotide probes on the microwell surface. The size and structure of the captured RNA grow after three steps of cross-hybridization with oligonucleotide probes to form a large immobilized branched complex containing multiple alkaline phosphatase (AP) moieties. The three sets of probes are (i) target probes complementary to multiple regions of the *pol* gene, (ii) amplifier probes, and (iii) AP label probes. AP catalyzes the added enzyme substrate to generate chemiluminescence to derive the VL using a Bayer system 340 analyzer.

Finally, the recent Cavidi ExaVir load assay distinctively measures the enzymatic activity of RT from plasma HIV particles. The viral RT in the plasma is isolated using a gel separation step, and its enzymatic activity is measured using an inexpensive enzyme-linked immunosorbent assay (ELISA) reader. The measured enzymatic activity is then translated into viral RNA copies using a special conversion factor provided by the manufacturer. The VL detection dynamic range is between 200 and 6×10^5 copies/ml, a range smaller than that of NAT-based assays but adequate for ART monitoring. In a longitudinal follow-up study of ART patients with RNA levels of $>$400 copies/ml in Kenya, ExaVir v.2 was found to exhibit 100% sensitivity compared to the reference Roche v 1.5 assay (208). Recently, the sensitivity of a new ExaVir v.3 system was 96 to 100% at 2,000 copies/ml (209–211). The reagent cost is low compared to those of most VL test kits, and the ELISA equipment used in this assay is commonly used for various types of serologic testing. However, this assay requires cold-chain transportation to maintain the integrity of the viral enzyme prior to testing, and it takes 2 days to complete the test. Nevertheless, this assay is not affected by HIV sequence diversity and is capable of detecting both HIV-1 and HIV-2. Furthermore, the new fully automated version may be suitable for district or regional hospital laboratories with medium volumes of patients on ART.

VL Tests and Genetic Diversity

Genetic diversity may pose a challenge for the quantification of VL. The majority of the VL assays are based on PCR amplification and hybridization using primers/probes derived from the conserved regions of the HIV genome. However, the presence of one or two nucleotide substitutions at the 3′ end of a primer may cause VL underestimation by more than 100-fold (212). Earlier versions of VL tests were developed based mostly on subtype B viruses. These tests often failed with non-B subtypes (213–216). Most of the current assays have been improved by using primers/probes targeting multiple conserved regions of HIV-1 group M subtypes (subtypes A to H) and circulating recombinant forms (217, 218).

Since genetic diversity can significantly affect VL determinations, it is important that the communities of assay developers, clinicians, and laboratorians stay vigilant to quickly recognize abnormal VL determinations. Laboratories should compare different evaluation results of VL test kits and identify the ones that correctly quantify and match with strains circulating in the country. It is also recommended that one assay be used throughout the course of VL monitoring of the patient (219).

Decentralization of VL Testing

Decentralization of VL testing would be key in enabling the monitoring of all patients on ART, including those in rural areas and small treatment centers, in order to evaluate the success of the UNAIDS third 90 goal. The VL assay is complex, and current conventional platforms can be used at central reference, regional, district, or hospital laboratories within the tiered laboratory network (Fig. 1). The current conventional platform has a large footprint and a high throughput, allowing testing of several specimens a day. Decentralized laboratories for VL testing would need to be supported by strong systems, including a sample referral network, an EQA program, human resources, a quality management system, a laboratory information system (LIS), and a supply chain. For example, Ethiopia has a decentralized laboratory network for VL

TABLE 4 Characteristics of major viral load assays intended for use at POC or near POC sites[a]

Manufacturer and trade name	Targeted virus(es)[b]	HIV-1 VL range (copies/ml)[c]	TAT	Plasma vol (μl)	Cold chain needed	Reference(s)
Alere q HIV-1/2 VL plasma	HIV-1, -2	NA	1 h	50	No	233
SAMBA Semi-Q	HIV-1	1,000 (cutoff)	1.5 h	200	No	227, 228
Cavidi Ziva	HIV-1, -2	200–6 $\times$ 10^5	2 days	200–1,000	Yes	210, 211
Cepheid GeneXpert	HIV-1	40–10^7	1.5 h	1,000	No	234
Roche Liat	HIV-1, -2	57–1.5 $\times$ 10^6	0.5 h	150	Yes	231, 232

[a]Abbreviations: TAT, turnaround time from blood draw to VL report; NA, information not available.
[b]Although HIV-1 and -2 are detected in three assays, they do not yield discrete VL for HIV-1 and -2.
[c]The HIV-2 detection range of the Roche Liat and Alere q platforms have not been specified by the manufacturers.

determination and EID together with an effective specimen referral network to ensure that patient specimens are efficiently transported to viral testing laboratories (220–222).

POC testing can be used to increase access to VL testing in rural areas and testing sites near the residence of patients. POC testing should be part of an existing laboratory diagnostic network. The current POC VL instrument has a small footprint and low throughput (223). Careful consideration should be taken with the introduction of POC VL testing within an existing laboratory network. These considerations include (i) site-level patient testing volumes, (ii) accessibility to remote sites, (iii) human resources and training, (iv) EQA and connectivity coverage, (v) the supply chain and equipment maintenance, and (vi) integration with other diseases, such as tuberculosis, and operational diagnostic function, such as EID or VL testing. Near-POC VL devices would still require reasonably renovated facilities with at least some minimum requirements (running water, reagent storage capacity, electricity, and waste disposal availability) in lower-tiered laboratories or in the community for their placement. In some settings, they may still require an efficient specimen referral system to transport specimens. As POC tests are introduced into existing laboratory networks, they should be accompanied by effective quality measures to monitor the quality of testing (179).

One of the challenges to guard against with the decentralization of VL testing is the temptation of "flooding" countries with conventional or POC VL instruments that may arise from multiple partners, with each partner procuring for the program. The lack of coordination among partners can result in a costly and less efficient system and can be burdensome to the entire laboratory network (224). Country and site targets should be used in conjunction with instrument capacity to inform what type and quantity of conventional or POC instruments should be procured or leased as well as their placement. The WHO reported the underutilization of conventional VL platforms, with only a 36.5% capacity of the existing platforms being used in the reported countries (224). Similarly, other studies have reported underutilization of POC or near-POC instruments (222, 225). Decentralized VL testing, if properly planned, offers several advantages, including (i) cost-efficient, flexible, and close monitoring of patients; (ii) faster turnaround times for patient results; and (iii) early detection of treatment failures for better patient management.

POC VL Testing Platforms

If properly implemented, POC VL testing locations can improve access to VL testing and improve patient care management. The POC VL system retains some unique features, which include (i) easy operation by nonlaboratory staff, (ii) short result turnaround time, (iii) built-in plasma isolation, (iv) reagent inclusivity and a self-contained cartridge, (v) accurate detection of VL of $\leq$1,000 copies/ml, (vi) low cost, (vii) a small footprint, (viii) connectivity or the ability to be electronically linkable, and (ix) low maintenance. The pipeline of POC VL platforms is promising (223), and five assays with advanced development and their performance characteristics are summarized in Table 4. Furthermore, the WHO has prequalified the GeneXpert platform for VL testing, and it is already being implemented in some field programs (226). The features of some POC VL platforms are summarized below.

Simple amplification-based assay. The SAMBA (simple amplification-based assay) Semi Q platform manufactured by Diagnostic for the Real World (DRW) is a semiquantitative assay using the proprietary isothermic method to amply HIV-1 nucleic acid from an input volume of plasma and to capture amplified HIV targets onto the solid phase of a dipstick. Detector probes labeled with multiple hapten moieties and a colored antihapten detection conjugate are used to amplify the signal and yield a visible blue band within 90 min. It provides a qualitative one- or two-line result when the VL of the tested specimen is below or over 1,000 copies/ml, respectively, to determine ART success or failure (227, 228). It has been shown to detect all major HIV-1 group M subtypes (subtypes A to K), circulating recombinant forms, and groups N and O (228). Compared to the Roche Cobas AmpliPrep/Cobas TaqMan HIV-1 test, v2.0, with 488 clinical samples from London, Malawi, and Uganda, the accuracies of the SAMBA were 99%, 96.9%, and 95%, respectively (227). Further analysis of the Malawi and Uganda results showed that a SAMBA cutoff of 1,000 copies/ml was able to distinguish between suppressed and nonsuppressed patients. Also, a comparison of SAMBA results from 150 specimens from Ukraine with the those of the Abbott RealTime assay yielded 98% concordance (229). Importantly, the specimens were tested on-site in laboratories in district hospitals and health care facilities in Malawi and health care units in Uganda, with results being available the same day (111). Other features of the instrument include the possession of Bluetooth connectivity, and thus, the results can be electronically transmitted. To limit the interference of cellular nucleic acids, the manufacturer has developed a miniaturize leukodepletion column that can deplete more than 99.9% of white blood cells (WBCs) from 100 μl venous whole blood (230). The new device with the built-in WBC-depleting component significantly improved the assay for VL testing.

Roche Cobas Liat platform. The Liat HIV quantitative plasma assay uses a pencil-sized and self-contained Liat tube with chaotropic lysis buffer and magnetic beads to extract RNA from 150 μl plasma. The tube contains reagents for RNA extraction and real-time RT-PCR quantitation in multiple segments and is processed by an actuator motor to control each step of the VL determination process. This assay also uses an internal armored HIV RNA to help provide an accurate VL calculation. The dynamic detection range is 57 to 1.5×10^6 copies (231), with a turnaround time of 30 to 35 min, to detect HIV-1 group M (subtypes A to H) and group O and HIV-2. The evaluation results of the Liat HIV quantitative plasma assay compared to the reference Roche CAP CTM v2.0 and Abbott RealTime HIV-1 assays showed good performance. Compared with the Roche platform, the clinical sensitivity of the Liat assay at a cutoff of 1,000 copies/ml was 100%, and the specificity was 88.2% (232). One advantage of the Liat assay over SAMBA Semi Q is its quantitative output. This device has full connectivity with Health Level-7 (HL7) communication protocols and has wireless and Ethernet capabilities. Currently, the storage and shipping of Liat tubes require cold-chain transportation, and the shelf life is only 6 months.

Alere q platform. The Alere q assay uses microarray-based technology to yield results in 1 h. It utilizes a cartridge containing chaotropic salts and heat and mechanical disruption to extract RNA from 0.5 to 1 ml of plasma. HIV RNA is captured by HIV-1- and HIV-2-specific biotin-containing capture oligonucleotides and streptavidin-coated Sepharose particles. This technology uses real-time fluorescence and amplification to determine VL. In addition to target spots in the microarray to calculate VL, there are also control spots for positive hybridization and negative hybridization and internal controls to identify potential errors. No cold-chain storage is needed. The throughput of the analyzer is low because it runs only one specimen at a time. In addition to the plasma cartridge, there is also a whole-blood cartridge with a direct 25-μl capillary blood intake (233).

GeneXpert platform. The Cepheid GeneXpert platform is available in a 1-, 2-, 16-, 48-, or 80-module configuration, thus providing facilities options to purchase the module best suited for their patient volume. The hands-on time for the HIV-1 VL assay is about 1 min, and the result can be obtained in 92 min. The device can process

specimens in a random-access manner, an advantage at field sites, to yield rapid results without the need for batching of specimens prior to testing.

In Malawi, comparison of GeneXpert HIV-1 VL assay results with Abbott RealTime HIV-1 quantitative assay results showed high concordance (234). Similarly, this test also showed concordance with Roche Cobas TaqMan and Abbott RealTime assay results in India, the United States, and Europe (226, 235). Furthermore, the TAT from specimen collection to receipt of results was less than a day, compared to the 7 to 10 days that were required for the Roche Cobas TaqMan assay. During testing, the instrument encountered some problems, such as cartridge leakage; thus, there should be careful monitoring of cartridges to promptly identify defective or broken ones that can leak plasma samples (226). The Xpert HIV-1 VL assay with GeneXpert has been WHO prequalified (236).

DBS for VL Expansion

Until recently, most VL testing was often conducted in urban-based centralized laboratories because of the dedicated space, the relative ease of management, and the presence of better-trained technical staff. Whole-blood specimens from rural ART health care clinics are collected and transported at ambient temperature to laboratories within 6 h after blood draw (237). Plasma can be separated from whole blood and transported under a cold chain to centralized laboratories for testing. There is a need to scale up VL testing in order to provide access to all eligible patients on ART. One alternative sample type is DBS specimens to increase access to VL testing, especially in remote settings, because of its ease of collection under field conditions, storage, and transportation to centralized laboratories (238, 239).

Several studies have shown that DBS specimens are a convenient specimen type for VL testing. Cell-free virion-encapsulated HIV RNA is stable in a dried state for several weeks (137, 168, 240). There have been improvements in assay optimization to measure VL using DBS specimens. In a review of 13 publications on DBS VL testing, Smit and colleagues found that 52 to 100% of DBS samples yielded VL values within 0.5 log units of those of paired plasma samples (241). The sensitivity of DBS specimens for the detection of VL of $>$5,000 copies/ml was close to 100% compared to plasma VL testing. However, the sensitivity decreased to 78 to 100% when the VL threshold was 1,000 copies/ml. WHO recommendations then used of a threshold of 5,000 copies/ml to indicate treatment failure (242). The major reason for VL variation between DBS and plasma VL specimens is primarily the presence of proviral DNA and cell-associated RNA in DBS specimens (243). When obtaining VL from DBS specimens, most researchers used guanidinium-based agents from Roche to isolate nucleic acids from DBS filter paper discs. This process extracted not only RNA but also DNA from DBS specimens and resulted in an overestimation of VL. In a study with specimens from Equatorial Guinea,10.5% false-positive VL ($>$1,000 copies) were noted (244). The implications may be that when the VL is low, the amount of proviral DNA would become significant, and persons with VL lower than the threshold (1,000 copies/ml) may have elevated VL and be considered HIV nonsuppressed.

It is noteworthy that although the bioMérieux EasyMag extraction method also yields both RNA and DNA, its isothermal amplification is RNA dependent, and the copurified proviral DNA does not affect the VL results. This is well demonstrated by the higher specificities of identifying treatment failure by the NASBA than by the Roche method at a cutoff of 800 copies/ml (245). The Abbott DBS sample-processing approach (246) preferentially isolates RNA and obviates the proviral DNA problem (246). In a Kenyan study, plasma specimens were first processed with the Qiagen RNA-specific chromatographic isolation method, and although the Roche RT-PCR assay was used, the VL results obtained in this manner yielded 100% sensitivity and specificity for VL of $<$1,000 copies/ml (145). These reports underscored the importance of choosing an appropriate RNA-specific isolation or an RNA-specific amplification method to eliminate misclassification problems contributed by cellular proviral DNA.

A "free-virus elution" method was recently developed by Roche for DBS specimens (247). It uses a simple PBS solution to differentially elute plasma from DBS specimens. Using DBS specimens from 196 patients with and without ART, it was shown that the new method correctly identified 64 patients previously misclassified by using the guanidinium-based method (247). Other studies have shown a high correlation between matching DBS and plasma specimen VL results (248–251). Furthermore, DBS specimens are suitable for infants for whom a venous blood draw may be challenging, including the required volume, as opposed to a finger or heel prick. DBS VL results can be reliably used to monitor patients on ART and would increase access to VL testing, as it overcomes logistic challenges (collection, storage, and cold-chain transportation) posed by plasma samples.

HIV-2 VL Assays

HIV-1 and HIV-2 are the etiological agents of AIDS, and both viruses have similar modes of transmission, including sexual and, rarely, mother-to-child transmission. However, individuals infected with HIV-2 have slower progression to disease and have lower plasma VL (252, 253). Epidemiologically, HIV-2 is prevalent and mostly confined to West Africa (7). HIV-2 is phylogenetically classified into eight different subtypes (subtypes A to H), with subtypes A and B predominating (254). Unlike HIV-1 with several WHO-prequalified commercial VL assays, there are no WHO-prequalified HIV-2 commercial VL assays. As a result, in-house HIV-2 VL assays have been developed and used to monitor patients on treatment (253, 255).

The Cavidi assay to measure viral reverse transcriptase activity can measure HIV-1 and HIV-2. All the other commercial assays listed in Table 3 were manufactured specifically for HIV-1. HIV-2 commercial kits have been lagging, presumably because of the small number of HIV-2-infected persons. The new Liat (231) and Alere q (256) assays, ideal for POC locations, could also detect HIV-2. A couple of assays were recently developed using in-house HIV-2-specific primers with a low detection limit of 10 copies/ml (257).

VIROLOGIC METHODOLOGIES FOR EARLY INFANT DIAGNOSIS

EID Guidelines, Current Practices, and Bottlenecks

Globally, more than 2.1 million children under the age of 15 years were living with HIV and accounted for about 6% of all people living with HIV in 2016 (71). In the same year, there were 160,000 new HIV infections in children, decreases of 33% and 72% from the numbers in 2013 and 2002, respectively. Despite these achievements, eliminating pediatric HIV infections remains a major public health challenge. Progression to AIDS is aggressive in infants who acquire HIV infection *in utero* or around the time of delivery and are not on ART. Studies have shown that if these infants are left untreated, about 35% and 52% of the infants die before the ages of 1 and 2 years, respectively (258). With PMTCT interventions, including early ART intervention in infected pregnant women and prophylactic ARV treatment in exposed infants, vertical transmission of HIV was drastically reduced from 35% to less than 2% in developed countries (259, 260). Early diagnosis of infection in HIV-exposed infants (HEI) provides a unique opportunity to strengthen these infants' follow-up and linkage to ART as well as the provision of prophylaxis, such as co-trimoxazole, for opportunistic infections and ART. Early identification of infants using serologic methods cannot establish the presence of infection because of passively acquired maternal antibodies in HEI. Virologic assays targeting viral nucleic acids are the methods of choice to minimize misdiagnosis (261, 262). Over several years, the colorimetric Roche Amplicor HIV-1 DNA qualitative assay was utilized as the gold standard in RLS countries (263). However, many quantitative and qualitative assays with high sensitivity and specificity using either plasma or DBS specimens have become available (Tables 2 and 3). However, pediatric testing and treatment continue to remain a challenge. In 2016, the rate of access to EID was only 43% for HEI tested by the second month of age, and of the 2.1 million infants living with HIV globally, only 43% received ART (264). Even when EID tests are conducted, a significant proportion of

the results are not returned to the clinic or returned late, resulting in delayed diagnosis and ART initiation. Point-of-care testing and birth testing provide opportunities to improve EID services through rapid identification of infected infants and immediate initiation of treatment (57).

The WHO recommends the testing of all exposed infants at the 4- to 6-week postnatal immunization visit and requires the detection assay to have a minimum sensitivity of at least 95% and a specificity of 98% or higher (265). Importantly, ART should be initiated for children who test positive as soon as possible to minimize HIV-associated mortality. When the first EID test is positive, the infant should be initiated on ART while a second specimen is collected for confirmation of infection. Several factors can influence the identification of infected infants, including the VL in mothers, the timing of vertical transmission, ongoing exposure due to breastfeeding, the timing of the test (e.g., birth testing), maternal ART, and prophylactic intervention regimens (57). The 2016 WHO guidelines also recommended the use of birth testing (0 to 2 days) to identify infants who had *in utero* transmission in addition to testing at 4 to 6 weeks following birth. However, national programs should optimize testing at 4 to 6 weeks as a priority to identify perinatally infected infants with the greatest risk of early mortality before expanding birth testing. While a study from South Africa demonstrated the potential of birth testing in increasing the yield of HIV-infected infants being initiated on treatment, infants who tested negative were less likely to return for a follow-up EID (266). A negative result at birth testing for infected infants could be due to late transmission during labor or breastfeeding postnatally. The level of linkage of HIV-infected infants identified at birth was lower than that with routine EID testing and underscored the need to immediately initiate HIV-infected infants on ART. Since infants who test negative at birth are less likely to return for a follow-up EID, together with operational challenges following the introduction of birth testing, it is cost-effective to focus on EID at 4 to 6 weeks, which also coincides with established immunization visits and where staff have been trained to perform EID (267). WHO guidelines recommend the provision of PMTCT option B^+ of lifelong ART to pregnant and breastfeeding HIV-infected women and that all exposed infants receive a course of prophylactic ART as soon as possible after birth to suppress viral replication. The WHO reaffirmed the use of EID at 4 to 6 weeks after birth, a second EID test when breastfeeding ends, and an HIV serologic test at 18 months to provide the final diagnosis (57).

POC for EID

DBS-based EID in RLS countries are conducted mostly in centralized facilities because of the relative ease in overall support for laboratory operations and quality management (66). Most of the conventional platforms for VL testing serve a dual role and are used for both VL and EID testing. Despite concerted efforts from various donors, agencies, and implementing partners, most of the EID programs in RLS countries continue to display challenges, including (i) the low testing coverage of eligible infants, (ii) the old age of tested infants, and (iii) the prolonged TAT (239, 268). These challenges can prevent early initiation of ART and lead to high infant morbidity and mortality rates (269–276). With the availability of POC technologies that can readily use capillary whole blood, the infection status could be determined in 1 to 2 h, thus enabling the provision of prompt care and ART initiation to infected infants during a same-day visit. All POC assays have undergone or are in the process of field evaluations to determine the ease of operation and assay accuracy compared to established reference assays, such as Roche CAP/CTM or Abbott RealTime assays (Table 5). Two POC EID tests, Alere q HIV-1/2 Detect and Cepheid GeneXpert, have been WHO prequalified and are being used in field settings (277).

Alere q HIV-1/2 Detect. The Alere q HIV-1/2 Detect assay is conducted in a small portable analyzer and uses a cartridge to process 25 μl capillary blood to produce results within 1 h. In a study conducted at 4 primary health clinics and a central hospital in Mozambique, consisting of 65 infected infants with known infection and 762

TABLE 5 Performance characteristics of major EID POC methodologies[a]

Manufacturer and trade name[b]	Target	Amplification method and/or detection	Means of specimen processing	Specimen vol and type	LOD (copies/ml)	Operational time (h)	Field study site	No. of infants (median age)	Sensitivity, specificity (%)	Operator	Reference(s)
Alere q HIV-1/2 Detect	RNA	RT-PCR, fluorescence	Cartridge	25 μl blood		1	Mozambique	827 (5.6 wk)	98.5, 99.9	Nurse	278
	RNA	RT-PCR, fluorescence	Cartridge	25 μl plasma	2,491	1	South Africa	1,098 (6.7 wk)	96.8, 100[e]	Laboratory staff	279
Cepheid GeneXpert qualitative	TNA	RT-PCR, fluorescence	Cartridge	100 μl whole blood	278	1.5	South Africa	300 (0–18 mo)	98.69, 100	Laboratory staff	234, 281
				1 DBS	688		Malawi	200 (1–6 mo[d])	100[c], 100[c]		
NWGHF Lynx p24 Ag	p24	p24 antigen	NA	80 μl plasma	NA	1	South Africa	389	95.8, >99.4	NA	372
Roche Liat	RNA	RT-PCR, fluorescence	Liat tube	150 μl plasma	80	1.5	South Africa	NA	NA	NA	232
SAMBA Qual whole blood (SAMBA II[f])	TNA	Dipstick, random access	Cartridges	100 μl blood	433	1.5	NA	NA	NA	NA	227, 230

[a]Abbreviations: RT-PCR, reverse transcriptase PCR; TNA, total nucleic acid; NA, not applicable because no field study was reported at the time of preparation of the manuscript; NWGHF, Northwestern Global Health Foundation; LOD, limit of detection.

[b]In alphabetical order.

[c]Samples from 4 of the 200 infants yield invalid results and were not included in the sensitivity/specificity calculations. An Abbott RealTime assay using 2 DBS specimens was used as the reference.

[d]No median age was provided.

[e]After some testing errors were corrected upon retesting.

[f]Can be run with the less-automated SAMBA I platform with a similar LOD (Table 3).

uninfected exposed infants with a median age of 1.4 months, the sensitivity and specificity were found to be 98.5% and 99.9%, respectively (278). That study also demonstrated the feasibility of the test being performed by clinic nurses after short pretesting training of 2 h. In another study in South Africa, involving 1,065 HIV-exposed infants with a median age of 47 days, the sensitivity and specificity of the Alere q HIV-1/2 Detect assay were 96.9% and 100%, respectively (279, 280). That study also found that birth testing in a subset of infants younger than 1 week of age yielded slightly a lower sensitivity of 93.3%, compared to the sensitivity of 96.9% for the routine standard of care, with high error rates (due to sample collection or operator error) that were subsequently resolved with additional testing. Testing was carried out by laboratory staff, and this highlights the critical need to routinely monitor these assays, even simplified assays. The current Alere q analyzer processes one specimen at a time; thus, an upgrade to multiple-specimen processing and random-access capacity would greatly improve its POC utility.

GeneXpert for HIV-1 qualitative detection. The Cepheid GeneXpert HIV-1 Qual assay, similar to its quantitative VL version, has been evaluated in field settings in Malawi using DBS specimens, and results were concordant with those of the Abbott RealTime qualitative assay (234). This instrument can also use capillary whole blood, and other performance characteristics of the assay are shown in Table 5. The GeneXpert systems are manufactured in different configurations, including GeneXpert I, II, IV, XV1, Omni, Infinity-48s, and Infinity-80 (223). Although not yet commercially available, GeneXpert Omni is considered the true POC platform, with its flexibility to be moved around and used beside the patient, while the other GeneXpert systems are near-POC systems, requiring laboratory or facility settings for operability. GeneXpert has been WHO prequalified for use in diagnosing infants using whole blood or DBS specimens (281).

Lynx p24 antigen assay. The Lynx p24 antigen assay is manufactured by Northwestern Global Health Foundation and is a strip-based assay with a battery-driven processor that yields results in less than an hour. It consists of a plasma separator to isolate plasma from 80 μl capillary blood. The detection of p24 requires a heat shock step to dissociate it from immune complexes. Detection is done by a colorimetric process using monoclonal anti-p24 antibodies. Similar to the SAMBA or HIV rapid test kits, it incorporates an "internal control" line and a "test" line. For a valid test result, the control line must appear, followed by interpretation of the test line: if the test line appears, then the test is positive, while the absence of a test line indicates that the test is negative. If the control line does not appear, the test is invalid. The performance of the Lynx p24 antigen assay was evaluated in Mozambique using heel-pricked whole blood and showed a sensitivity of 71.9% and a specificity of 99.6% compared to the gold-standard Roche Cobas AmpliPrep/Cobas TaqMan (CAP/CTM 96) HIV-1 qualitative test or the Roche Amplicor HIV-1 DNA test, v1.5, using corresponding DBS samples collected from the infants (282). Similar results were also observed when the Lynx p24 antigen assay was used in Zambia, with a sensitivity of 70% and a specificity of 100% compared to the Roche Cobas TaqMan assay (283). The Lynx p24 antigen assay is promising because of its high specificity; it does not require transportation or processing of specimens prior to testing, and the test can be performed in front of parents/guardians by nonlaboratory staff in facilities.

SAMBA HIV-1 qualitative assay. The SAMBA HIV-1 Qual whole-blood test manufactured by DRW processes 100 μl of capillary blood with full automation in a SAMBA II device to produce results in less than 2 h. The working principle of using a dipstick display is similar to that of its semiquantitative VL counterparts (Table 5). It can detect HIV-1 groups M, N, and O with a limit of detection of 433 copies/ml and had 100% concordance of results with laboratory gold-standard results (284). It can process samples in a random-access manner and thus is highly advantageous for throughput and POC use.

SURVEILLANCE OF HIV DRUG RESISTANCE

There has been a rapid expansion of ART programs, with 20.9 million HIV-infected individuals having access to ART as of June 2017 (3), and with the expansion are fears

or the inevitability of the emergence of HIV DR. If treatment programs are not optimally implemented or managed, there will be emergence and transmission of drug-resistant HIV strains with significant adverse public health impacts. Widespread resistance can compromise treatment programs, as it limits treatment options, with implications for first- and second-line therapy (285). In the realm of maternal and child health, it is critical for clinicians to be aware of HIV drug resistance in the HIV-positive women whom they serve to allow them to select an ART regimen that will prevent transmission of drug-resistant strains of the virus to the child. HIV DR can be categorized into two groups: (i) acquired resistance, whereby an infected individual on ART develops DRMs and, (ii) transmitted DR, when a drug-naive individual is infected with a virus having HIV DRMs. In surveys conducted between 2014 and 2016, rates of acquired DRMs of adults failing nonnucleoside reverse transcriptase inhibitor therapy (VL of >1,000 copies/ml) after 12 to 24 months were 59.7%, 47.3%, and 76.0% in Cameroon, Zambia, and Guatemala, respectively (286). Similarly, a review of studies with children failing first-line treatment showed high levels of DRMs of 90.4%, 67.6%, 91%, and 96.5% in Tanzania, Zimbabwe, South Africa, and Rwanda, respectively (286).

Recent PHIA surveys have shown high viral suppression rates of 91.0%, 89.0%, and 86.0% in Malawi, Zambia, and Zimbabwe, respectively, and suggest that first-line regimens are effective (13). It is important to monitor and prevent the emergence of DR mutations so that patients can continue to benefit from these lifesaving medications. The WHO recommended that countries in RLS implement HIV DR early-warning indicator surveys and classify resistance to each drug class as <5%, 5 to 15%, or >15% to gather information and inform national treatment programs with ART expansion (287, 288).

HIV DR testing can be conducted phenotypically or genotypically. Phenotypic assays are performed *in vitro* and measure susceptibility to ARVs in cell culture as a function of the concentration of the ARV required to inhibit the replication of HIV-1 by 50% (50% inhibitory concentration [IC_{50}]). Due to the complexity of phenotypic assay protocols and the duration required to complete them, they are typically recommended for persons with known or suspected complex DR mutation patterns, particularly resistance to protease inhibitors (190), or for DR research (289). This assay requires 3 ml of plasma with a minimal VL of 500 copies/ml and 2 to 4 weeks for reporting, and it is not expensive (289).

Genotypic testing involves sequencing of the protease, reverse transcriptase, or integrase regions of the HIV genome and identification of DR mutations of clinical significance to ARVs. Genotype testing is recommended as the preferred method of resistance testing to guide therapy in patients with suboptimal virologic responses while on first- or second-line regimens in the United States (190). While it remains an integral part of standard HIV care and management in resource-rich countries, it is unaffordable in most resource-constrained countries due to its high cost and the need for advanced laboratory infrastructure and analytical skills. To monitor and support surveillance of DR in low- and middle-income countries, the WHO recommends population-based approaches to determine the transmitted DR threshold in recently infected populations, DR in ART-initiating populations, and acquired DR in ART-receiving populations (237).

Genotyping Methodologies

There are three basic types of genotyping methods: (i) Sanger-based sequencing, (ii) deep sequencing, and (iii) allele-specific discrimination assays (Table 6).

Sanger sequencing assay. Sanger sequencing targets the protease, reverse transcriptase, or integrase region of the HIV-1 genome for sequencing using the traditional Sanger sequencing principle, which is the selective incorporation of chain-terminating dideoxynucleotides during *in vitro* replication by DNA polymerase. The most commonly used commercial assays are the FDA-approved Trugene and ViroSeq assays. The reagent kits are costly and have been mostly used in the United States with plasma specimens or peripheral mononuclear cells. To reduce cost, in-house and DBS-

TABLE 6 Major HIV-1 drug resistance detection methodologies[a]

Principle	Methodology	DRM variant detection rate (%)	Salient features	Challenges	Reference(s)
Phenotyping	PhenoSense	20	Patient-derived PR and RT (and INT in an additional assay) gene segments are cloned into a vector, and the IC_{50} of ART drugs for new recombinant virus is measured *in vitro*; 3 ml of plasma with a viral load of >500 copies/ml is needed	Long TAT (2–4 wk) and high cost	
Genotyping					
Sanger-based sequencing	Trugene/long-read Towers sequencer	20	FDA approved, well standardized for HIV-1 subtype B, closed sequencing system to cover PR codons 4–99 and RT codons 38–248, proven DBS utility	Not optimized for non-B subtypes, labor-intensive, high cost ($170/sample), integrase gene not included	373, 374
	ViroSeq/ABI 3100	20	FDA approved, well standardized for HIV-1 subtype B, semiopen sequencing system to cover Prot codons 1–99 and RT codons 1–335, proven DBS utility	Same as above, high cost ($210/sample)	375
	In-house, ABI 3100[b]	20	Optimized for subtype B, non-B subtypes, and CRFs; lower reagent cost ($40/sample) than commercial Sanger-based methods; proven DBS utility; ABI 3100 open-system sequencer	High overall operational complexity, expensive instrument maintenance	290, 293, 294
Deep sequencing	Illumina MiSeq	2	FDA-approved sequencing platform, good for most subtypes and detects INT DRMs, uses barcoded primers to pool multiple samples for high-throughput (96 samples/run) sequencing, quick turnaround and low cost ($10/sample), can be adopted in national or multinational centralized laboratories	Relatively new, needs more validation with samples from developing countries and DBS	296, 297
	Roche/454 GS Junior pyrosequencing	5	3 amplicons to cover PR and RT genes, pools 48 samples with unique barcodes per sequencing run, rapid analysis (1 h) with a reduced cost of $20 per sample, sequencing errors near known homopolymer sites are reduced	Sequencing errors after homopolymer sites; Roche/454 may be phased out soon, and thus, the method may become obsolete	295, 298, 299
	Ion PGM 200 sequencing	1	3 PCR segments, 1,657 bp (from *gag* p2 to 5′ RT), 2,002 bp (from 3′ RT to INT), and a 480-bp *env* gene (C2V3); covers DRMs from both subtype B and non-B subtypes and includes INT DRMs; also yields HIV-1 CCR5 coreceptor tropism genotypes	Newly established and requires independent validation and applications	300

(Continued on following page)

TABLE 6 (Continued)

Principle	Methodology	DRM variant detection rate (%)	Salient features	Challenges	Reference(s)
Multiplex allele-specific assay	In-house, Luminex[b]	1.56–12.5	Uses allele-specific primer extension to estimate % DRMs using suspension array, high throughput, low cost ($40/sample), quick TAT, DBS applicable, high applicability for personnel in RLS without the need for establishing a sequencing facility and skills	Subtype sequence information prerequisite prior to optimizing the assay; current assays are optimized for 20 DRM loci in subtypes B and C	302, 303

[a]Abbreviations: DRM, drug resistance mutation; Prot, protease; RT, reverse transcriptase; INT, integrase; *env*, envelope gene; CRFs, circulating recombinant forms; DBS, dried blood spot; C2V3, constant 2 and variable 3 domains of the *env* gene.

[b]This is an in-house assay from the CDC.

applicable assays were developed using degenerate amplification and sequencing primers (290, 291). For instance, the in-house assay developed by the CDC has been used for population-based surveys using plasma specimens from Nigeria (292) and DBS specimens from Vietnam (290), Malawi (290), Nigeria (290), Kenya (142), and Uganda (293). The reagent cost of the assay is 20 to 25% lower than that of the commercial test kit method. Complete genotyping kits are available to WHO-designated and CDC-supported PEPFAR genotyping laboratories (294). It is worth noting that while the success rate of DR genotyping can be >90% using plasma with VL of >1,000 copies/ml (141, 295), the success rate with the use of finger-prick-derived DBS specimens is much lower (293). General recommendations for the preparation of DBS specimens and transportation for population DR surveillance are as follows: (i) venous blood should be used to prepare the DBS specimen; (ii) a finger-prick-based DBS specimen can be used if the VL is ≥50,000 copies/ml; (iii) the DBS specimen should be kept frozen prior to its shipment to a centralized DR laboratory in-country or overseas; (iv) postal or courier transportation can be done at ambient temperature, but the specimen should be delivered within 6 days (293); and (v) a DBS specimen received by the centralized laboratory should be kept frozen prior to genotyping.

Deep sequencing. The three different types of deep sequencing instruments currently in use include Illumina MiSeq (296, 297), the Roche 454/GS Junior pyrosequencer (295, 298, 299), and the Ion PGM 200 sequencer (300). The operational principles of these methods are similar and can be completed to yield DRM reports in 1 to 2 days. DR-associated genes are amplified using patient-specific barcoded primers, and thus, amplified products from 48 to 96 patients can be pooled in a single deep sequencing run to drastically reduce the operational cost. Because of the clonal nature of amplified viral sequences, the sensitivity for detecting minor variants can be extended down from 20% by Sanger methods to 1% to 5%. One study using the Roche 454 GS Junior sequencer documented a 35% increased finding of DRMs compared to Sanger-based assays (298). A study involving 11 laboratories in Austria, Belgium, France, Germany, Italy, South Africa, Spain, and Switzerland confirmed the robustness and reproducibility of 454-asociated pyrosequencing (299). The same 454-asociated pyrosequencing platform was also successfully used in a study conducted by the Public Health Agency of Canada using 48 DBS samples selected from a transmitted DR surveillance study in Mexico (301).

All the above-mentioned genotyping assays require the performance of conventional sequencing on individual specimens or new high-throughput next-generation sequencing on pooled specimens. A common requirement for the application of these assays is the establishment of a complex high-maintenance molecular laboratory with DNA sequencers together with highly skilled laboratory personnel, thus making these assays most suitable for centralized national or regional molecular laboratories where samples from the lower health care facilities can be pooled and DRM can be determined with high-quality management.

Allele-specific discrimination assay. Allele-specific discrimination assays do not require the use of sequencers and have been evaluated in RLS. For example, the multiplex allele-specific (MAS) array assay has been optimized for 20 DRM loci for subtype B (302). A study with a subtype C-specific MAS assay optimized for DBS specimens obtained from Honduras showed a high level of agreement (99.3%) of detected major DRMs compared with data generated by Sanger-based sequencing, and the low-abundance variants were verified by Roche 454 GS-based deep sequencing (303). The results from MAS assays are easy to interpret, and if optimized for known circulating viruses in a specific country or region, the assay can yield rapid DRM surveillance data to inform ART policy in the country or region.

Ensuring and Monitoring Quality of Molecular Testing in RLS

The implementation of a comprehensive quality management system that depends on continuous quality improvement is critical for ensuring the quality of HIV molecular testing. The scale-up of VL testing and EID has led to the decentralization of molecular

laboratories in order to meet country needs (220). These laboratories are encouraged to participate in strengthening laboratory management toward accreditation to improve laboratories and then be enrolled into the WHO/Stepwise Laboratory Quality Improvement Process towards Accreditation for formal recognition using the 5-star grading system (304–306). Furthermore, some laboratories are aiming for international ISO15189 accreditation and ensuring compliance to standards in the laboratory (307, 308). Specimen integrity tracking can be accomplished through the use of data loggers during the specimen transport and storage phases. The data logger tracks and records the temperature of specimens or reagents during transportation and flags out-of-range temperatures to prevent erroneous results.

Internal and External Quality Control Materials

Strict QA standards, including quality control samples, are required to ascertain *in vitro* diagnostic claims (309). Quality control materials, including internal and external controls, are useful for laboratories to monitor their routine operations (310). For commercial VL quality control, noninfectious armored HIV-1 RNA is usually processed with patient specimens to monitor and calibrate equipment and identify problems with reagent and amplification contamination (311–313). The internal quality control and other kit controls are usually lot specific and are not suitable for the determination of lot-to-lot consistency or relative performance among different testing platforms. The instrument software captures and flags erroneous testing processes or specimens. False-negative results due to amplification inhibition and false-positive results due to low-level contamination in isolated specimens can be readily identified (314), followed by retesting of the affected specimens.

External quality control can provide the laboratory with a better understanding of testing variations and help identify systematic and random errors. Because of the high procurement costs, the use of external quality control to monitor variation across different kit lots, instruments, and laboratories is limited in RLS. External plasma RNA controls can be obtained from the Virology Quality Assessment (VQA) program, Accrometrix, and Seracare. However, they require cold-chain transportation and are expensive (315, 316).

Monitoring laboratory performance using Levey-Jennings charts could identify some systematic and random abnormal testing events. Interlaboratory comparison of the same method can be a valuable tool to compare and monitor the collective performance of laboratories within a laboratory network in multiple countries or regions. Assay manufacturers typically have a limited number of lots released concurrently; laboratories using the same external control can compare performances. Countries with multiple and different platforms can monitor performance and identify laboratories performing out of range in real time rather than waiting for an EQA panel (317, 318). Lot monitoring is critical to ensure that reagents are performing as intended. The importance of lot monitoring was recently underscored when certain VL reagent lots were found to be defective (319, 320). National laboratory programs should put into place a mechanism for systematic lot verification and work with manufacturers to manage situations where there are lot failures. Manufacturers should also perform lot verifications prior to shipment to minimize the impact of a recall if faulty lots could have been identified before shipment.

VL and EID PT Programs in RLS Countries

PT is a useful EQA method used to monitor the quality of VL testing, EID, or DR testing in a laboratory. Laboratories participating in PT programs have been shown to consistently perform better than those not enrolled in a PT program (163). HIV-1 DNA and RNA VL PT started in the 1990s to ensure the quality of results from clinical trials and are typically provided two to six times per year. For instance, the VQA program, through the support of the U.S. National Institutes of Health, provides qualitative DNA, VL, and DR panels, which are available four, six, and two times annually, respectively. The VL panel from the College of American Pathologists is available two times annually (321).

Most of these materials are based on subtype B viruses and not easily accessible to many RLS laboratories due to the high cost and the need to maintain a cold chain during shipment. To alleviate some of these challenges, the CDC initiated a free program for DBS-based EID PT and DTS-based VL PT provisions to RLS countries in 2006 and 2010, respectively. EID PT panels are prepared using filter papers spotted with seronegative whole blood spiked with known concentrations of viral DNA copies. Similar to DTS PT for HIV serology (151), the VL PT panel tubes contain dried materials with known VL and food dye for visualization (322). The DBS and DTS panels can be shipped through regular mail at ambient temperature. In 2012, 141 laboratories in 41 countries were enrolled in the EID PT scheme, and 114 laboratories in 44 countries were enrolled in the DTS VL PT scheme (322). The success of the DBS EID PT program was replicated by the National Health Laboratory Service (NHLS) in South Africa in 2013 and the Cheikh Anta Diop University (CADU) Afriqualab PT program in Dakar, Senegal, in 2014. Currently, both the NHLS and CADU serve 50 laboratories in 20 African countries. Afriqualab provides DTS VL PT panels to 24 regional laboratories. In addition, the WHO encourages laboratories to seek WHO accreditation for HIV DR testing to ensure compliance with quality standards, including receiving PT panels (323).

VL/EID Dashboard for Program Improvement and Accountability

Most often, implemented QA programs are limited to EQA programs such as PT programs, retesting of a subset of samples previously tested and supportive supervision, and the use of QC materials by testing sites (164–166, 324–326). Monitoring and evaluation of key indicators allow the identification of deficiencies or gaps to be remedied in the laboratory or program as well as assessment of laboratory or program improvement over time, thus providing accountability in the laboratory program. There is an increasing use of VL/EID dashboards, LIS, instrument connectivity, and cloud-based remote monitoring and tracking of key laboratory or program indicators. In Mozambique, mobile phone connectivity has been used to monitor the quality of POC platforms (327).

The utilization of a VL database in Uganda allows monitoring of the number of patients with ART failure (VL of >1,000 copies/ml) as well as the specimen rejection rate by the receiving laboratories in real time (328). Web-based tools, such as the HIV Infant Tracking System (HITSystem), have been used by multiple EID hospitals in Kenya (329). HITSystem requires a full-time coordinator and one or two staff in each of the participating laboratories for daily data entry. This system tracks a set of key indicators of the EID cascade, including infant age at DBS collection, time of laboratory receipt of samples, return of PCR results, mother notification, ART initiation, and infant retesting at 9 and 18 months. The system thus could provide data analysis for each and all sites for comparative analysis on a real-time basis to identify site-specific and overall program deficiencies.

Countries have developed dashboards at the national level for overall monitoring and improvement of programs. The dashboard enables viewing data at the regional and site-specific levels. For example, Kenya, Uganda, and Malawi have developed VL and EID dashboards that allow the programs to monitor several parameters for deficiencies and improvements (328, 330, 331), and similar systems are also being used in China and Cambodia.

CD4 COUNTING METHODOLOGIES

Role of CD4 and Testing Guidelines in Staging and Monitoring Patients on ART

In 1981, the CDC described the presence of *Pneumocystis* pneumonia in three homosexual men in Los Angeles, CA (332), and Gottlieb et al. described *Pneumocystis* pneumonia and mucosal candidiasis in four homosexual men with a "virtual elimination" of the Leu-3$^+$ (CD4) helper/inducer subset (333). As reports of severe immune deficiency increased, CD4 cell counts became a critical part of the care of HIV-infected patients and were used as a surrogate marker for staging of patients and as an indicator for disease progression.

Prior to 2015, CD4 T cell count was the primary laboratory-based test used to guide the initiation of patients on ART and to monitor treatment response. WHO global ART guidelines for adults and adolescents in 2002 recommended that ART be initiated in adults with clinical stage IV disease or with a CD4 cell count of $<$200 cells/mm^3 (334). While these guidelines acknowledged that CD4 testing was not widely available in RLS and advocated for the development of new technologies that could be used in such settings, they included immunological failure, defined as a fall in CD4 counts of over 30% from the peak value or a return to or a fall below the pretherapy baseline level, as one of the criteria for switching from first- to second-line ART. In 2003, the WHO revised the guidelines and recommended ART initiation in adults and adolescents with clinical stage III disease and consideration of using CD4 cell counts of $<$350 cells/mm^3 to guide decision-making (335). These guidelines also recommended that district- and regional-level hospitals should have the capacity to perform CD4 testing and that patients on ART should be monitored by CD4 testing every 6 to 12 months, if available.

By 2006, the guidelines recommended ART initiation in all patients with WHO stage IV disease and all patients with CD4 counts of $\leq$200 cells/mm^3, regardless of clinical staging, and consideration of ART for patients with CD4 counts of between 200 and 350 cells/mm^3 before the count dropped to below 200 cells/mm^3. In these guidelines, the WHO advocated for wider availability of affordable POC CD4 testing to increase access to CD4 results. CD4 testing was recommended at baseline, ART initiation, and suspected treatment failure and every 6 months prior to and after ART initiation (336). Between 2006 and 2010, several studies demonstrated that early initiation of ART led to reductions in AIDS-related morbidity and mortality (337–342). Following this, the WHO updated the ART guidelines to recommend that, in addition to patients with clinical stage III and IV disease, irrespective of the CD4 count, asymptomatic patients with CD4 counts of $\leq$350 cells/mm^3 should be initiated on ART (343). In 2010, the WHO stated that all patients should have access to CD4 testing and recommended the use of VL testing to confirm suspected treatment failures. CD4 testing continued to be recommended at baseline, at ART initiation, during treatment, at treatment failure, and every 6 months after ART initiation. In 2013, the WHO raised the CD4 cutoff and recommended that ART be initiated in patients with CD4 counts of $\leq$500 cells/mm^3, prioritizing those with CD4 counts of $\leq$350 cells/mm^3 (184). VL testing was recommended as the preferred monitoring approach to diagnose and confirm treatment failure, with CD4 testing being recommended in areas with an insufficient VL testing capacity. The frequency of CD4 testing for HIV-infected patients remained at baseline, at ART initiation, at treatment failure, every 6 to 12 months pretherapy, and every 6 months posttherapy (184).

The evolution of the WHO guidelines on CD4 cutoffs for treatment initiation clearly illustrated the fundamental role that CD4 testing has played in HIV patient care over the years. With strong evidence from the Strategic Timing of Antiretroviral Therapy, TEMPRANO, and HPTN 052 trials demonstrating substantial clinical benefits of early ART initiation, particularly when CD4 cell counts are above 500 cells/mm^3 (5, 344, 345), the WHO released new guidelines in 2016 recommending the initiation of ART for all PLHIV irrespective of CD4 counts (57). While the guidelines no longer require a CD4 test prior to ART initiation, they recommend that individuals with CD4 counts of $\leq$350 cells/mm^3 be given priority for ART initiation, especially where resources may not be available for initiation of treatment of all infected patients. In the 2016 guidelines, HIV VL testing remained the preferred method for ART monitoring, and CD4 testing is recommended at baseline, every 6 months until patients are stable on ART, and in areas where VL testing is not available. Together, these recommendations represent a shift in the utilization of CD4 cell counts in HIV care and treatment, ultimately leading to a reduction in the volume of CD4 testing in RLS. Despite this shift in routine clinical monitoring, CD4 testing remains an important clinical test for assessing the risk of disease progression, informing decisions on prophylaxis, and prioritizing assessment for opportunistic infections.

Flow Cytometry and Pipeline of Commercial CD4 Instruments and Assays

Researchers and clinicians have developed CD4 T cell flow cytometry as a clinical laboratory test for identifying and monitoring HIV-infected patients. Flow cytometry allows the quantification and analysis of single cells using light scatter to determine cellular size and internal complexity and using laser excitation of fluorochromes bound to cellular components to identify cells of interest. The first CD4 assays utilized a dual-platform approach in which a flow cytometer was used to determine the percentage of CD4 T cells and a hematology analyzer was used to provide the total lymphocyte count. Both values were then used to calculate the absolute CD4 T cell count (346). Volumetric flow cytometry delivers a precise volume of the sample through the flow cell, and bead-based counting (i.e., adding a known concentration of fluorescent beads to each sample) enabled determinations of CD4 percentages and CD4 absolute counts to be performed on one instrument, thus reducing the processing complexity and variability of results (347, 348).

The need for robust CD4 testing platforms and assays that could be utilized in settings with limited infrastructure and human and financial resources was emphasized early in the HIV epidemic. The Becton, Dickinson (BD) FACSCount system, released in 1994, was the first dedicated instrument for clinical CD4 cell enumeration (349). It is a closed system using solely BD reagents. The BD FACSCount instrument is a color flow cytometer that is used with FACSCount reagent kits to measure CD4, CD3, and CD8 cell counts. Additional reagents, such as the BD FACSCount CD4 reagent, subsequently became available to measure both CD4 percentages and absolute CD4 cell counts. The FACSCount system was designed for ease of operation, as it featured automatic gating and premeasured reagent tubes. Due to the simplicity of the instrument and the moderate cost, the FACSCount system remains the most widely utilized benchtop CD4 analyzer in RLS (350).

One of the limitations of FACSCount was its relatively low throughput, with only 30 to 80 specimens that could be analyzed per day (350). This led to the production and standardization of high-throughput flow cytometers with dedicated CD4 reagents and software packages, such as the Beckman Coulter Epics XL instrument with the TetraONE system and reagents and the BD FACSCalibur instrument with the BD Tritest and BD Trucount tubes. These instruments are open systems, and reagents are not restricted to those provided by the manufacturer. They are also multicolor cytometers that have the capacity to accommodate CD4 testing needs in high-prevalence settings. Both cytometers have automatic loading options and off-board staining platforms available to automate processing and increase the throughput for high-volume laboratories. While these instruments remain widely used and often serve as the reference instruments for evaluations of new CD4 technologies, their suitability for use in only centralized referral laboratories limited patient access in smaller communities and rural areas.

A wave of new CD4 technology entered the market, including the Millipore-Guava Auto CD4%/CD4 system, the Apogee Auto40 flow cytometer, and the Partec CyFlow counter (223). These instruments are volumetric benchtop cytometers that are capable of measuring both CD4 percentages and absolute counts without the use of reference beads. The Apogee Auto40 cytometer was designed to be used in military environments and is therefore a practical cytometer for RLS. Similarly, the Partec CyFlow counter was designed with "align-free" technology and lyophilized reagents, reducing the level of service required and the need for cold-chain transportation and storage.

The Beckman Coulter Aquios CL instrument with Tetra-1 was released in 2015 and is marketed as the first "load-and-go" cytometer. This is a large benchtop cytometer that is fully automated and reduces hands-on time for laboratorians. All reagents are barcoded and tracked on the instrument, along with specialized quality control materials, which are analyzed onboard and flagged if out of range or expired. The Beckman Coulter Aquios CL instrument represents a new age of clinical flow cytometry that automates specimen processing and tracks the validity of quality control and the shelf-life of reagents.

Expansion of CD4 Services Using POC Assays

While complex automated instruments save time in large reference laboratories with highly trained staff and good infrastructure, they are not well suited for remote geographical regions and laboratories with nontechnical staff and minimal infrastructure (Table 1). Weak specimen transport and supply chain systems, limited infrastructure at lower-tiered laboratories, and shortages of trained staff have been challenges for flow cytometry-based CD4 assays in high-burden settings. Alternative strategies to overcome these challenges include the manufacture of more community-accessible, affordable, and simple-to-perform POC CD4 instruments that would afford quality HIV care in RLS (351–353).

The first POC CD4 instrument to reach the market was the PointCare Now system in 2007. This device was designed for RLS, as it used a light-emitting diode instead of a laser. It has heat-stable reagents, daily quality controls, and a short testing time of 8 min per sample. However, independent field evaluation of the PointCare Now system showed an unacceptable high mean relative bias of +153 cells/μl for absolute CD4 counts (354). Subsequently, many POC CD4 instruments and assays with significant improvement were developed to support HIV treatment programs in Africa (14, 355, 356). The Alere Pima CD4 system has been the most widely used and distributed POC CD4 device (14, 356–363). It is small and easily transportable and uses an all-in-one cassette and static image analysis to provide absolute CD4 counts. It is compatible with the Alere connectivity solution, which sends results across a mobile network from the analyzer to a remote server to be viewed and analyzed via the Alere data point website. The centralized data point allows comprehensive and fast program management of supply stock, error analysis, and QC monitoring for multiple Pima analyzers located at different sites. Several evaluations have shown acceptable concordance between the results of the Pima POC analyzer and reference cytometers (357, 359, 360, 362, 363). The Pima analyzer has exerted a profound impact and changed the CD4 testing landscape in Africa and other continents.

The Partec CyFlow CD4 MiniPOC and BD FACSPresto systems are POC devices that provide both absolute CD4 counts and percentages, but they are not as widely distributed as the Pima analyzer. The Partec CyFlow CD4 MiniPOC system utilizes the same volumetric cytometry principles as the Partec CyFlow counter as well as lyophilized reagents and controls, allowing room-temperature storage. A laboratory and field evaluation in Senegal demonstrated good agreement between the MiniPOC system and the reference FACSCount cytometer (364).

The BD FACSPresto system is an image-based analyzer with an all-in-one cassette that, unlike the Alere Pima system, offers an off-board incubation time of 18 min and a shorter read time of 4 min on the instrument and is able to accommodate up to 10 specimens at one time. Laboratory and field evaluations conducted in Kenya (216) and South Africa (217) showed acceptable agreement between the results of the BD FACSPresto and reference CD4 instruments. However, a positive bias of 40 to 67 cells/μl for the FACSPresto system was reported in both studies (365, 366).

Another POC CD4 technology is the EMD Millipore Muse Auto CD4/CD4% system (367, 368). This technology replaces the discontinued Guava Auto CD4%/CD4 system. It utilizes volumetric cytometry and microcapillary technology similar to those of the Guava system. The instrument is preloaded with autogating software, and its assay reagents permit the determination of both CD4 counts and percentages.

POC CD4 testing has many benefits over centralized testing, including increased access to testing, reduced TAT, and minimal infrastructure requirements (Table 1). The introduction of POC devices such as Alere Pima, Partec CyFlow CD4 MiniPOC, and BD FACSPresto provides national programs the opportunity to decentralize and expand access to CD4 testing. The use of POC devices has been shown to significantly reduce the TAT of CD4 results and subsequently the time to initiation of ART, doubled linkage to care, and increased patient retention in health care (14, 15, 355, 356, 369). There are several promising POC systems for CD4 in the pipeline (223). Following standard

evaluations of platforms that enter the market with WHO prequalification, they will offer options for use in CD4 testing.

Monitoring Quality of CD4 Testing

Despite these advantages of POC technology, significant challenges surrounding QA, implementation, service maintenance, and supply chain management persist. Ensuring uninterrupted provision of accurate and reliable CD4 testing will require a substantial and sustained effort to identify needs and gaps within the care cascade, proper implementation of POC and laboratory-based testing to fill these gaps, and continuous monitoring and oversight to identify and resolve quality issues. CD4 testing facilities should be enrolled into EQA schemes, for example, UK NEQAS (United Kingdom National External Quality Assessment Services), to monitor the quality of CD4 testing. Also, the implementation of connectivity would enable monitoring and improving QA, service, and maintenance at multiple sites. Also, the use of the WHO Stepwise Process for Improving Point-of-Care Testing (SPI-POCT) to monitor POC testing sites for CD4 would identify gaps and allow monitoring of improvement of sites to maintain quality testing (167).

Role of CD4 Counts in the Era of "Test and Start" and VL Testing

Over several years, CD4 counts have played a pivotal role in making clinical decisions in the management of HIV/AIDS. First, CD4 counts were used to determine the eligibility of patients for initiation of ART. As our understanding of better management of AIDS patients with ART improved, ART initiation moved from the use of CD4 counts of less than 200 cells/μl to counts of 350 cells/μl and, later, 500 cells/μl. The increasing threshold of CD4 counts was meant to avoid patients presenting with already advanced progression to disease before ART initiation. Second, CD4 counts were used to monitor and classify disease progression and survival outcomes. Third, CD4 counts were used to monitor the effectiveness of ART as well as inform a switch to second-line treatment after ART failure. Fourth, CD4 counts have been used to assess immune reconstitution and advise on the initiation or discontinuation of prophylaxis for opportunistic infections.

In the advent of "test and start" and VL testing, there is uncertainty as to the role of CD4 counts. The 2016 WHO guidelines recommended diagnosis and initiation of a positive individual into ART regardless of CD4 counts. Furthermore, the guidelines recommended the use of VL testing for monitoring the effectiveness of ART. As the scale-up of VL testing has increased, there has been a decline in CD4 testing in some countries (370). Nonetheless, CD4 counts still have a role to play in the management of patients. As recommended by WHO 2016 guidelines, CD4 counts should still be used for monitoring ART patients in situations where there is no capacity for VL testing. For some RLS, full transition to a test-and-start regimen remains challenging, and in these scenarios, CD4 counts can be used to prioritize patients. Also, CD4 cell counts are useful for monitoring patients with advanced disease ($<$200 cells/μl) and informing clinical decisions to prevent mortality. The number of patients presenting with advanced disease is still high and is between 34 and 41% in some SSA countries (371).

Despite the huge investment in laboratory diagnostic strengthening, including POC testing, over the past 2 decades to support CD4 testing, coupled with the era of test-and-start and VL testing, determination of CD4 counts still has its place in the care and management of patients, especially those with advanced disease. Thus, national programs will need to consider this as they make strategic decisions to provide better patient management. The rapidly evolving role of CD4 counts, especially in the last decade, can be challenging for any robust program and more so for programs in RLS, and stakeholder support would be critical.

CONCLUSION

HIV diagnostics have evolved significantly over the past 3 decades from first-generation to fifth-generation assays, with each generation aimed at overcoming a

deficiency of the previous one in order to provide improved diagnosis offered by these tests. Similarly, the advent of HIV molecular testing shortened the window period and also advanced from initial designs based primarily on HIV subtype B to encompass non-B subtypes in order to overcome barriers with HIV genetic diversity. In addition to tackling HIV genetic diversity, we have adjusted to program needs to improve access to testing, misdiagnosis, and their impact on patients. National programs routinely evaluate test kits to establish suitable testing algorithms using combinations of tests for accurate diagnosis, for example, selecting the optimal combination of HIV rapid test kits in a serial or parallel testing algorithm for improved diagnosis. New robust and simple assays are available to distinguish recent HIV-1 infection from long-term infection, permitting estimation of incidence, impact assessment, and real-time surveillance. Program challenges with decentralization have led to adaptations of approaches, including task shifting, in order to optimize the use of various diagnostic tools, such as the use of both professional laboratory and nonlaboratory personnel (nurses, lay counselors, and community workers) to provide and expand diagnosis. Ensuring the quality of diagnostic assays (at the facility and community levels) has required a combination of both traditional and innovative strategies, including positive and negative quality control materials, proficiency testing or an EQA program, site and tester certification, connectivity, and the use of Q-Corps and community volunteer involvement in monitoring quality results in nonlaboratory settings. The selection of appropriate diagnostics in an evolving field with frequently changing guidelines has been challenging but necessary in order to improve the quality of patient care with a better understanding of the epidemic. Such has been the impact of the rollout of HIV POC testing on expanding and increasing access to testing. Diagnostics have played a critical role in the fight against HIV/AIDS, and achieving epidemic control will require responsiveness and adaptation of HIV diagnostics to overcome program challenges in identifying HIV status and monitoring HIV-infected patients on ART.

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