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Dried Culture Spots for Xpert MTB/RIF External Quality Assessment: Results of a Phase 1 Pilot Study in South Africa▽

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Implementation of Xpert MTB/RIF requires quality assessment. A pilot program using dried culture spots (DCSs) of inactivated *Mycobacterium tuberculosis* is described. Of 274 DCS results received, 2.19% generated errors; the remainder yielded 100% correct *Mycobacterium tuberculosis* detection. The probe A cycle threshold (C_T) variability of three DCS batches was ≤3.47. The study of longer-term DCS stability is ongoing.

The Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA) (1, 3–5, 9, 12, 13, 15, 19, 25) for the diagnosis of *Mycobacterium tuberculosis* has recently been endorsed by the WHO (28), and recommendations for data collection to quantify the impact of this GeneXpert (GX) technology are provided (26). Guidance, however, with respect to appropriate external quality assessment (EQA) programs is lacking (17). Current international tuberculosis (TB) EQA programs focus on microscopy, culture, and susceptibility testing laboratories (24) and highlight the difficulties in expansion due to labor-intensive preparatory work and the high cost and regulations associated with shipping drug-resistant isolates (27).

Criteria for a verification ("fit for purpose") and EQA program suited to the characteristics of the Xpert MTB/RIF assay (3, 8) will require the following elements. (i) The testing material must contain whole *M. tuberculosis* (8). (ii) Transportation of EQA material needs to be safe. (iii) The testing procedure needs to be safe and compatible with the Xpert MTB/RIF current testing protocol. (iv) Health care workers who do not have laboratory skills must be able to perform the testing in nonlaboratory settings. (v) Finally, the programs will need to be cost-effective and sustainable. Such a program using whole inactivated *M. tuberculosis* spotted onto filter paper was developed and piloted in South Africa as part of the National Health Laboratory Service (NHLS) GX rollout.

M. tuberculosis was obtained from (i) pooled samples from 20 microbial growth incubation tubes (MGIT) of rifampin (RIF)-susceptible clinical isolates and tested with the MTBDR*plus* (Hain Life Sciences), (ii) 20 pooled MGIT cultures comprising American Type Culture Collection (ATCC) strain S-MYCTU-02-P2 (ATCC 25177 [H37Ra]) and well-characterized local clinical strain MYCTU 15, and (iii) the ATCC 25618 (H37Rv) laboratory strain grown for single-cell-organism suspensions (11). The MGIT cultures S-MYCTU-02-P2 and MYCTU 15 and clinical isolates were pooled in their respective batches (with strains kept separate and not mixed), centrifuged (3,000 × *g* for 15 min at 4°C) to pellet cells, and resuspended in 40 ml phosphate-buffered saline (PBS) followed by addition of 80 ml (2:1 ratio of buffer to culture) of the Xpert sample reagent (SR) buffer. For the H37Rv strain, 200 ml of culture was harvested (by centrifugation at 3,500 × *g*) at room temperature for 10 min, and cells were resuspended in PBS to 40 ml followed by addition of 80 ml SR buffer (2:1 ratio of buffer to cells). Both MGIT-grown and H37Rv strain cultures were inactivated in SR buffer for 2 h at room temperature, with intermittent mixing. The inactivated material was washed twice with sterile PBS and resuspended in final volumes of 10 ml (S-MYCTU-02-P2 and MYCTU 15) and 40 ml (H37Rv) PBS. For confirmation of inactivation, washed cultures (0.5 ml) were reinoculated into new MGIT tubes in Bactec cabinets for 42 days. These inactivated bulk stocks were enumerated by flow cytometry (FC500 using Flow count microspheres; Beckman Coulter) and tested with the Xpert MTB/RIF assay. The cycle threshold (C_T) values of the semiquantitative categories (high, C_T of <16; medium, C_T of 16 to 22; low, C_T of 22 to 28; and very low, C_T of >28) were recorded for probe A and were compared to the flow cytometry enumeration score. Dilutions that generated a medium (C_T of 16 to 22) qualitative Xpert MTB/RIF result were used to prepare the dried culture spots (DCSs).

DCSs were prepared by spotting 25-μl amounts of inactivated culture material onto Whatman 903 filter cards (Merck) together with 2 μl of DNA loading dye (Sigma-Aldrich) per spot for visualization purposes, as illustrated in Fig. 1, and dried for 1 h at room temperature before being placed in sealed plastic bags with a desiccant sachet (Sigma-Aldrich).

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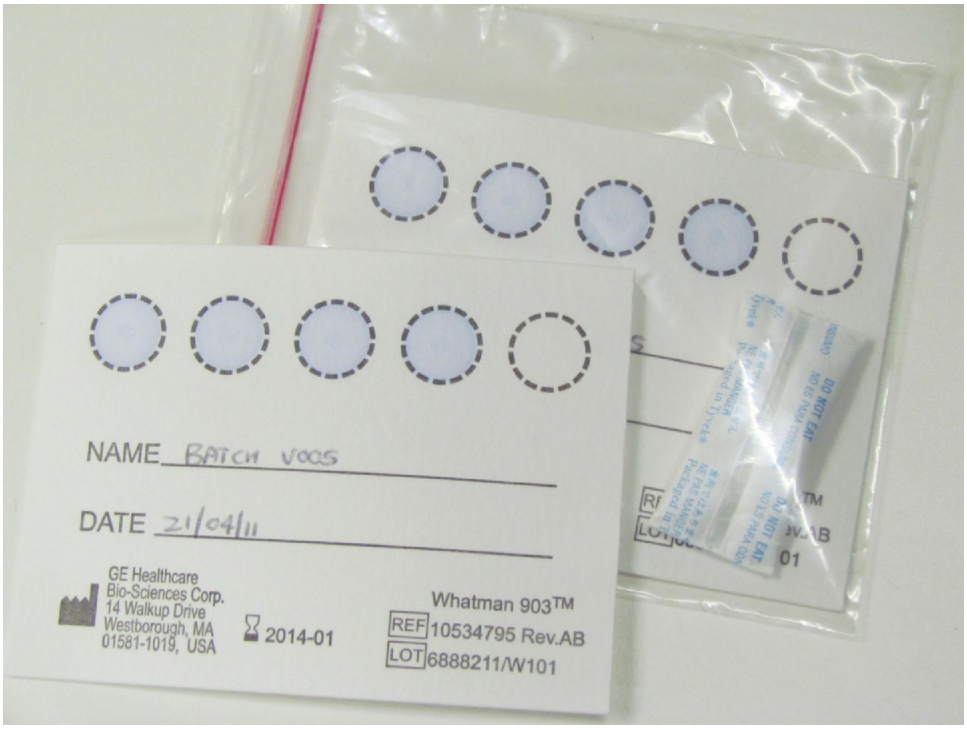

FIG. 1. A sample of the DCSs on filter cards and in plastic transport bags with dessicant sachets. Four DCSs on a card containing inactivated *M. tuberculosis* culture are visualized by the blue dye.

These were couriered ($n = 16$), hand delivered ($n = 10$), or surface mailed (repeat DCSs to 4 sites) to various participating sites, where each spot was cut (using a sterile pair of scissors) into a 50-ml standard laboratory Nunc centrifuge tube (AEC Amersham), and 2.8 ml SR buffer (to ensure there was a sufficient 2-ml concentration to pipette into the Xpert MTB/RIF cartridge after the DCS incubation) was added to the tube. The tubes were vortexed (or hand shaken by swirling

TABLE 1. Performance of the three DCS batches on 286 GX modules

Parameter	Result for DCS batch no.:		
	V002	V004	V005
M. tuberculosis bulk culture material	MGIT clinical controls (RIF-sensitive *M. tuberculosis*)	MGIT ATCC strain (RIF-sensitive *M. tuberculosis*)	H37 laboratory strain (RIF-sensitive *M. tuberculosis*)
No. of GX modules tested by DCS	49 (all RIF-sensitive *M. tuberculosis*)	173 (all RIF-sensitive *M. tuberculosis*)[a]	64 (all RIF-sensitive *M. tuberculosis*)
No. of errors[b]			
Error 5007		1	
Error 5011	1	3	1
No. of DCSs for statistical analysis	48	157	63
% of testing in qualitative category:			
Very low	0	5.1	6.25
Low	26.53	47.13	42.19
Medium	69.39	47.77	48.44
High	2.04	0	1.56
C_T for probe A			
Mean	20.75	22.58	21.89
SD	2.20	2.76	3.47
CV (%)	10.6	12.22	15.86

[a] A total of 161 modules returned results.
[b] Error 5011 refers to signal loss detected in an amplification curve, and error 5007 refers to a probe check failure.

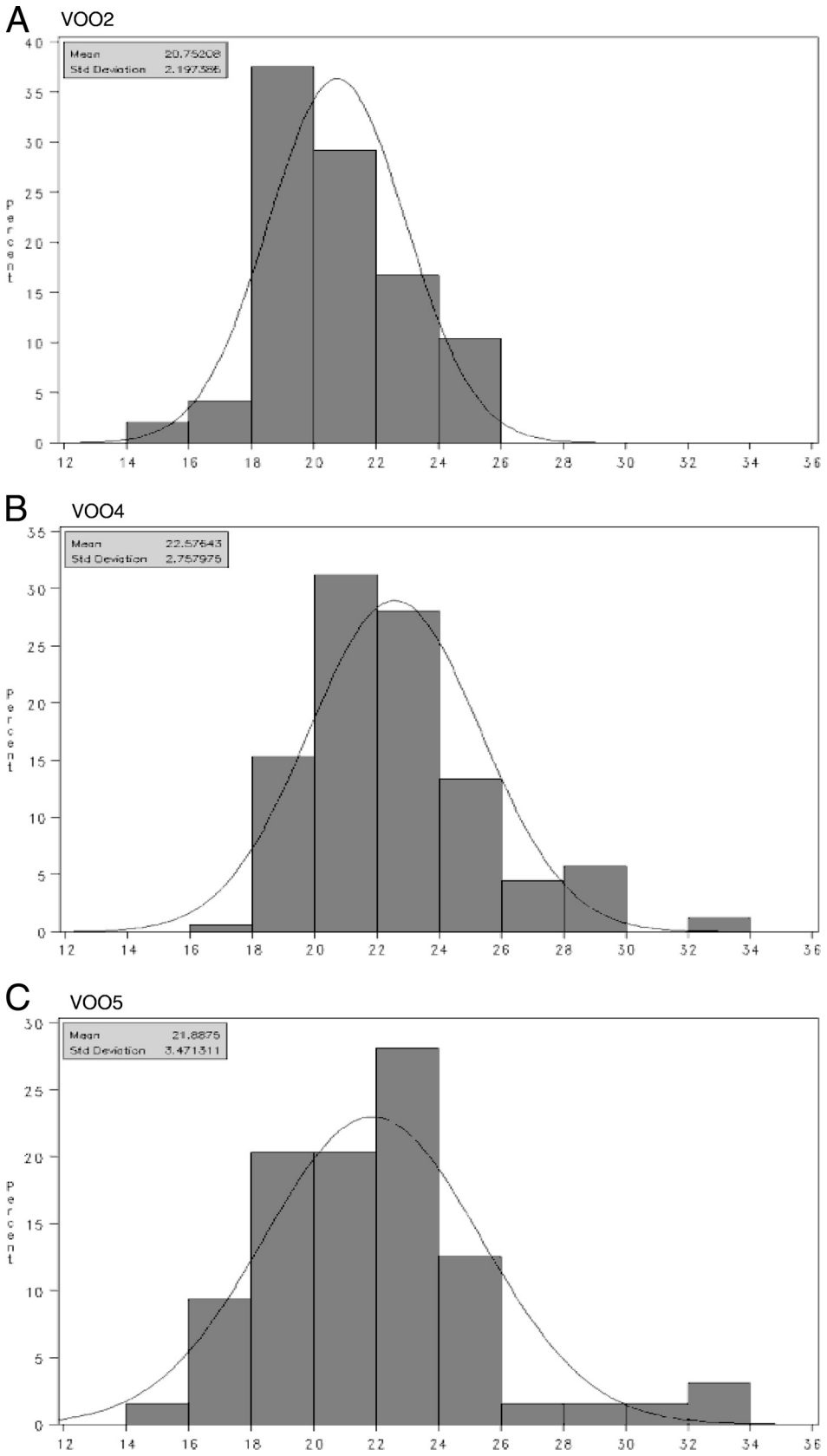

FIG. 2. Frequency distributions overlaid with normal curves of the C_T values for probe A from the three DCS batches. (A) Batch V002; (B) batch V004; (C) batch V005. The standard deviation and mean C_T values are represented in insets in each of the panels.

vigorously if no vortexer was available) and left at room temperature for 15 min with intermittent mixing. One DCS was then tested on each Xpert MTB/RIF module. The C_T mean, standard deviation, and coefficient of variation (CV) were calculated for probe A.

Three DCS batches were manufactured for 31 GXs: GX Infinity-48 ($n = 1$), GX16 ($n = 9$), and GX4 ($n = 21$). A total of 286 DCSs were distributed to the 26 participating sites, and results were received for 274 DCSs, thereby identifying sites with nonconformities. Six testing errors (error no. 5011 [$n = 5$] and 5007 [$n = 1$]) were reported, and the remaining 268 DCSs generated results with 100% *M. tuberculosis* positivity and RIF sensitivity (Table 1). Probe A was the first probe to reach the amplification C_T, with similar standard deviations across three DCS batches with a C_T of ≤3.47. Frequency distributions in Fig. 2 illustrate the greatest variability in batch V005 (CV of 15.86%) from the single-cell-generated culture.

National Xpert MTB/RIF implementation programs are challenged by determining the scope and composition of EQA panels and the infectious nature of *M. tuberculosis* material. This study provides a preliminary demonstration through the use of inactivated *M. tuberculosis* coupled with easier transportation of DCS material that an EQA program can be safely provided. The DCS material proved successful for verification of GX instruments and highlighted expected error code frequencies (2.1%) and site nonconformities.

Although this is a uniquely designed EQA program that appears so far suitable for Xpert MTB/RIF verification using different strains from different culture methods, the individual components are not unfamiliar to the field: filter paper has been used for the transportation and molecular testing of *M. tuberculosis* DNA (7, 14), and flow cytometry has been used for the analysis of *M. tuberculosis* (2, 10, 16, 18, 20–23). Flow cytometry has the advantage of rapidly and accurately identifying inactivated single whole bacterial cells, which circumvents conventional, time-consuming CFU enumeration methodologies. Enumeration of flow cytometric events can also be performed below the minimum McFarlane concentrations (1×10^7 CFU/ml) and could more accurately be used in strain mixing to test "dropout" or "delayed" C_Ts (3). Flow cytometry is also available in settings that currently perform CD4 counting of HIV patients for treatment initiation and monitoring and therefore represent a platform and infrastructure already in place (6).

The variability in C_T values may result from the spotting technique, different DCS reconstitution techniques (including vortexing/hand shaking), and variability in the amount of SR buffer added to each DCS. Other sources of variability may be explained by *M. tuberculosis* clumping from the MGIT-grown cultures being better trapped by the Xpert MTB/RIF filter membrane, whereas an *M. tuberculosis* single cell (~0.4 μm wide by 1.0 μm long) may pass through the 0.8-μm membrane pore. The advantage of single-cell-cultured material is that no sonication or declumping methods are required before flow cytometry enumeration and spotting.

Future design of an Xpert MTB/RIF EQA program could be similarly based on line probe assay programs using one pansusceptible strain, one RIF-monoresistant strain with a common *rpoB* mutation, one multidrug-resistant (MDR) strain, one nontuberculous mycobacterium (NTM) strain, and a negative control (17), each placed on a DCS card and distributed 3 times per year.

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