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Pooling sputum samples for Xpert® MTB/RIF and Xpert® Ultra testing for TB diagnosis

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BACKGROUND: The use of molecular amplification assays for TB diagnosis is limited by their costs and cartridge stocks. Pooling multiple samples to test them together is reported to have similar accuracy to individual testing and to save costs.

METHODS: Two surveys of individuals with presumptive TB were conducted to assess the performance of pooled testing using Xpert® MTB/RIF (MTB/RIF) and Xpert® Ultra (Ultra).

RESULTS: A total of 500 individuals were tested using MTB/RIF, with 72 (14.4%) being MTB-positive. The samples were tested in 125 pools, with 50 pools having ⩾1 MTB-positive and 75 only MTB-negative samples: 46/50 (92%, 95% CI 80.8–97.8) MTB-positive pools tested MTB-positive and 71/75 (94.7%, 95% CI 86.9–98.5) MTB-negative pools tested MTB-negative in the pooled test (agreement: 93.6%, $\kappa = 0.867$). Five hundred additional samples were tested using Ultra, with 60 (12%) being MTB-positive. Samples were tested in 125 pools, with 42 having ⩾1 MTB-positive and 83 only MTB-negative samples: 35/42 (83.6%, 95% CI 68.6–93.0) MTB-positive pools tested MTB-positive and 82/83 (98.8%, 95% CI 93.5–100.0) MTB-negative pools tested MTB-negative in the pooled test (agreement: 93.6%, $\kappa = 0.851$; $P > 0.1$ between individual and pooled testing). Pooled testing saved 35% (MTB/RIF) and 46% (Ultra) of cartridges.

CONCLUSIONS: Pooled and individual testing has a high level of agreement and improves testing efficiency.

Despite intensive efforts since 1993, when the WHO declared TB a global emergency,[1] TB is still today a major cause of adult death due to infection, second only to COVID-19. In 2021, over 10 million people fell ill with TB, and despite being preventable and curable, 1.6 million died from the disease.[2]

The WHO recommends using molecular assays as the first test for examination of individuals with presumptive TB;[3] the assays most widely used are the Xpert® MTB/RIF (MTB/RIF; Cepheid, Sunnyvale, CA, USA) and Xpert® MTB/RIF Ultra (Ultra; Cepheid) assays.[4] These tests are more sensitive than smear microscopy, and major efforts are being made to expand their use worldwide. However, despite these efforts, these tests are rarely available at primary healthcare centres, which are the first point of contact for most people with presumptive TB. This is because the assays are expensive (US$9.98/test for low- and middle-income countries, FIND negotiated price) and because the GeneXpert platform requires an infrastructure that is often only available at major laboratories. Testing sputum samples of people attending primary healthcare requires transporting sputum or reference to centralised laboratories. A major impediment to improving the TB management is therefore the limitation of current diagnostics.

A recent systematic review indicated that molecular testing of samples could be more efficient if samples were tested using the pooling method.[5] In this method, clinical samples from several patients are mixed (combined in a pool) and tested together using a single cartridge. If the pool test is negative, all samples in the pool are considered negative; if positive, the individual samples are re-tested to identify the positive samples. Pooling can reduce the cost of testing, the time required to process samples and increase the diagnostic capacity of the laboratory.[6–8] However, the review suggested that pooling performance varies between MTB/RIF and Ultra, as the latter has higher sensitivity; further studies are therefore needed.

Nigeria (population: over 206 million[9]) has the second highest TB burden in Africa, with an estimated 467,000 people with TB in 2021.[2] However, under-detection is a major problem, and only 204,700 (43.8%) people with TB were notified.[2] The country is thus one of the 10 countries accounting for 77% of the global gap in TB detection and notification;[2] increasing detection is therefore a major priority.

The present study aimed to compare the accuracy of the MTB/RIF and Ultra assays when using the pooling method and individual testing in Nigeria.

METHODS

This was a cross-sectional survey of consecutive adults with signs and symptoms of presumptive pulmonary TB attending the TB diagnostic clinics of the Federal Medical Centre and Keffi District Hospital, Keffi, Nasarawa State; and Nyanya General Hospital, Federal Capital Territory (FCT) in Nigeria. Eligible participants were asked to provide demographics, medical history and clinical information, and to submit one sputum sample for examination. Samples were transferred the same day to Zankli Research Center TB Reference Laboratory, Bingham University, New Karu, Nigeria, and tested using MTB/RIF for the initial 5 months (March–August 2020) and subsequently, using Ultra, once the National TB Programme had recommended the test to be used in all diagnostic centres.

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†Deceased.

KEY WORDS
sputum samples; tuberculosis diagnosis; pooling; Xpert MTB/RIF; Xpert Ultra; Nigeria

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Samples with remnant sputum (i.e., those which would have been discarded after routine testing) were selected for pooled testing.

One pooled specimen was created for each four consecutive samples, before the results of the individual tests were known. A minimum of 0.75 ml of each sputum sample were added to an empty cup, up to a minimum of 3 ml per pool. All pooled samples were tested using either MTB/RIF or Ultra to match the assays used for individual samples. Samples with an error, invalid or no MTB/RIF test result (individual or pooled) were retested. All procedures were performed by trained personnel within a containment laboratory.

The individual and pooled Xpert results were compared to assess the agreement of the tests and the direction of disparities. The individual Xpert test was considered the reference test to estimate the sensitivity and specificity of the pooled method with 95% confidence intervals (95%CIs). Pooled test results were not used for clinical management. Xpert semiquantitative cycle threshold (CT) values were used to describe differences in bacilli DNA concentrations between the individual and pooled tests. Trace results were considered negative in this analysis.

The study was approved by the Health Research Ethics Committees of the Liverpool School of Tropical Medicine, Liverpool, UK, and the FCT, Nigeria (numbers 20-037 and FHREC/2020/01/29/10-04-20, respectively). All patients attending the centres were asked to read and confirm that they had understood the study information leaflets and the consent procedures. Individuals were included if they provided written informed consent.

RESULTS

The study included 1,000 participants, of whom 500 were tested using MTB/RIF and 500 with Ultra (Table 1). Of these, 567 (56.7%) were females and 433 (43.3%) males. The largest age group was under 35 years old (n = 559, 55.9%), followed by adults aged 35–54 years (n = 338, 33.8%). In total, 958 (95.8%) participants knew their HIV status, 141 (14.1%) were people living with HIV (PLHIV), 817 (81.7) HIV-negative and the HIV status for 42 (4.2%) was not known or not disclosed. Thirteen (9.2%) of 141 PLHIV had TB. A total of 751 (75.1%) sputum samples were mucoid, 156 (15.6%) salivary, 68 (6.8%) mucopurulent and 25 (2.5%) purulent. Males were more likely to be MTB-positive than females (83/433, 19.2% vs. 49/567, 8.2%; $P < 0.001$). Test positivity was not associated with the quality of sputum, with 16/156 (10.3%) salivary, 100/751 (13.3%) mucoid, 12/68 (17.6%) mucopurulent and 4/25 (16%) purulent samples being MTB-positive (χ^2 for trend, $P > 0.1$). Tests with errors reported on the initial test were repeated, and there were no errors reported for individual MTB/RIF tests and only one error for Ultra after re-testing (Table 2).

Xpert MTB/RIF survey

Of 500 individuals who underwent MTB/RIF testing, 72 (14.4%) were MTB-positive and 428 MTB-negative (Table 1). Seven (9.7%) of the MTB-positive tests had very low, 17 (23.6%) low, 27 (37.5%) medium and 21 (29.2%) high MTB grades. All 500 samples were tested in 125 pools, of which 50 (40%) contained ⩾1 MTB-positive sample and 75 (60%) contained MTB-negative only samples. Thirty-six (72%) pools had one, nine (18%) had two, two (4%) had three and three (6%) had four MTB-positive samples (Table 2). Forty-six (92%, 95% CI 80.8–97.8) of the 50 pools containing ⩾1 MTB-positive samples tested Xpert MTB-positive and 71 (94.7%, 95% CI 86.9–98.5) of the 75 pools containing Xpert MTB-negative only samples tested MTB-negative (Table 3). The overall agreement was 93.6% (n = 117/125, κ = 0.867; Table 4).

Thirty-six pools included only one MTB-positive sample, with 3 (8.3%) of the individual samples having very low, 9 (25%) low, 8 (22.2%) medium and 16 (50%) high MTB grades (Supplementary Table S1). The MTB grades of the individual and pooled samples were the same for 17 (47.2%) tests. The

TABLE 1 Demographic characteristics of participants

		Xpert MTB/RIF		Xpert Ultra	
		All (n = 500) n (%)	Positive (n = 72) n (%)	All (n = 500) n (%)	Positive (n = 60) n (%)
Sex	Male	214 (42.8)	48 (66.7)	219 (43.8)	35 (58.3)
	Female	286 (57.2)	24 (33.3)	281 (56.2)	25 (41.7)
Age, years	Mean ± SD (range)	33 ± 14.6 (1–80)	30 ± 10.8 (14–68)	35 ± 15.1 (2–98)	34 ± 12.7 (13–75)
	<35	288 (57.6)	51 (70.8)	271 (54.2)	36 (60.0)
	35–54	163 (32.6)	20 (27.8)	175 (35.0)	20 (33.3)
	⩾55	49 (9.8)	1 (1.4)	54 (10.8)	4 (6.7)
Sputum quality	Saliva	126 (25.2)	14 (19.4)	30 (6.0)	2 (3.3)
	Mucoid	314 (62.8)	48 (66.7)	437 (87.4)	52 (86.7)
	Mucopurulent	58 (11.6)	10 (13.9)	10 (2.0)	2 (3.3)
	Purulent	2 (0.4)	0 (0.0)	23 (4.6)	4 (6.7)
Sputum blood	Yes	0 (0.0)	0 (0.0)	1 (0.2)	0 (0.0)
	No	500 (100.0)	72 (100.0)	499 (99.8)	60 (100.0)
Tested for HIV	Yes	424 (84.8)	67 (93.1)	495 (99.0)	60 (100.0)
	No	32 (6.4)	4 (5.6)	1 (0.2)	0 (0.0)
	Not known	44 (8.8)	1 (1.4)	4 (0.8)	0 (0.0)
HIV status		(n = 500)	(n = 72)	(n = 495)	(n = 60)
	Positive	97 (19.4)	6 (8.3)	44 (8.8)	7 (11.7)
	Negative	366 (73.2)	65 (90.3)	451 (90.2)	53 (88.3)
	Won't say/not known	37 (7.4)	0 (0.0)	0 (0.0)	0 (0.0)

SD = standard deviation.

TABLE 2 Number of pools with 0, 1, 2, 3 and 4 positive results

	Individual Xpert results included in a pool					
Pooled results	Four negatives *n* (%)	One positive *n* (%)	Two positives *n* (%)	Three positives *n* (%)	Four positives *n* (%)	All *n* (%)
Xpert MTB/RIF, *n*	75	36	9	2	3	125
Detected	4 (5.3)*	32 (88.9)	9 (100.0)	2 (100.0)	3 (100.0)	50 (40.0)
Not detected	71 (94.7)	4 (11.1)*	0 (0.0)	0 (0.0)	0 (0.0)	75 (60.0)
Invalid	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Error	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
No result	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Xpert Ultra, *n*	83	27	13	1	1	125
Detected	1 (1.2)*	21 (77.8)	12 (92.3)	1 (100.0)	1 (100.0)	36 (28.8)
Not detected	82 (98.8)	6 (22.2)*	1 (7.7)	0 (0.0)	0 (0.0)	89 (71.2)
Invalid	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Error	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
No result	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

* Disagreements between individual and pooled testing.

MTB grade of 19 (52.8%) individual and pooled tests were discrepant, with the pooled MTB grade being lower than the individual test in six (31.6%) tests, two grades lower in three (15.8%), one grade higher in four (11.1%) and two grades higher in two (5.6%) pools. Four pools with individual MTB-positive samples tested pooled MTB-negative. The individual samples of two of these pools had very low MTB grades, one had low and one medium MTB grades; all contained just one positive individual sample. The median CT values for pooled and individual tests are shown in Supplementary Table S2. The A–E probes of individual test results had median CT values ranging from 18.2 to 19.7 for individual tests and from 18.0 to 19.4 for pooled tests, with ΔCT (the difference in CT) value between the pairs ranging from –1.15 to +0.4.

Xpert Ultra survey

Of 500 individuals tested using Ultra, 60 (12%) were MTB-positive and 440 (88%) MTB-negative (Table 1), including 13 samples testing MTB-trace. Five (8%) MTB-positive individual samples had very low, 17 (28%) low, 18 (30%) medium and 20 (33%) high MTB grades (Table 2). The 500 individual samples were tested in 125 pools, of which 42 (33.6%) contained ⩾1 MTB-positive samples: 27 (64.3%) contained one, 13 (31.0%) two, 1 (2.4%) three and 1 (2.4%) four MTB-positive samples (Table 2). Thirty-five (83.3%, 95% CI 68.6–93.0) of the 42 pools with MTB-positive samples tested MTB-positive. Eighty-two (98.8%, 95% CI 93.5–100.0) of the 83 pools with only MTB-negative samples tested MTB-negative. The overall agreement was 93.6% (n = 117/125, κ = 0.851; Table 4). There was no significant difference in the sensitivity (n = 46/50 and 35/42, 92.0% vs. 83.3%, Fisher's Exact P = 0.33) and specificity (n = 71/75 and 82/83, 94.7%, vs. 98.8%, P = 0.19) of pooling with MTB/RIF and Ultra (Table 4).

Twenty-seven pools had only one Ultra MTB-positive. Of these, 6 (22.2%) were not detected, 2 (7.4%) had very low, 6 (22.2%) low, 9 (33.3%) medium and 4 (14.8%) high MTB-grades (Supplementary Table S1). The MTB grades of the pooled and individual tests were the same in five (18.5%) and discrepant in 22 (81.5%) pairs. The pooled MTB grade of the discrepant samples

TABLE 3 Results of pooled and individual Xpert testing

	Xpert MTB/RIF		Xpert Ultra	
	Individual *n* (%)	Pooled *n* (%)	Individual *n* (%)	Pooled *n* (%)
MTB result, *n*	500	125	500	125
Detected	72 (14.4)	50 (40.0)	60 (12.0)	36 (28.8)
Not detected	428 (85.6)	75 (60.0)	439 (87.8)*	89 (71.2)
Invalid/error/no result	—	—	1 (0.2)	—
MTB grade				
Trace	—	—	13	10
Very low	7 (9.7)	4 (8.0)	5 (8.3)	6 (16.7)
Low	17 (23.6)	10 (20.0)	17 (28.3)	12 (33.3)
Medium	27 (37.5)	16 (32.0)	18 (30.0)	11 (30.6)
High	21 (29.2)	20 (40.0)	20 (33.3)	7 (19.4)
RIF resistance				
Detected	8 (11.1)	5 (10.0)	9 (15.0)	4 (11.1)
Not detected	63 (87.5)	42 (84.0)	48 (80.0)	31 (86.1)
Indeterminate	1 (1.4)	3 (6.0)	3 (5.0)	1 (2.8)

* Includes 13 specimens reported as 'trace'.
MTB = *M. tuberculosis*; RIF = rifampicin.

TABLE 4 Agreement of Xpert individual and pooled tests

	Xpert MTB/RIF (n = 125)		Xpert Ultra (n = 125)	
Individual test	Positive	Negative	Positive	Negative
⩾1 positive	46	4	35	7
All negative*	4	71	1	82
Agreement, n/N (%)	117/125 (93.6)		117/125 (93.6)	
κ	0.867		0.851	
Sensitivity, % (95% CI)	0.920 (0.808–0.978)		0.833 (0.686–0.930)	
Specificity, % (95% CI)	0.947 (0.869–0.985)		0.988 (0.935–0.998)	

* Trace results were considered negative in this analysis.
CI = confidence interval.

was one grade lower than the individual sample in 7 (31.8%), two grades lower in 5 (22.7%), three grades lower in 1 (4.5%), one grade higher in 2 (9.1%) and two grades higher in 1 (4.5%) sample. One pool with an individual sample with very low MTB and five pools with an individual sample with low MTB tested negative in the pooled test (Supplementary Table S1). Five pools contained a sample with trace MTB results (and three MTB-negative). All of them tested MTB-negative in the pooled test. The median CT values for pooled and individual Ultra results are shown in Supplementary Table S2. Individual insertion sequence (IS) 1081/IS6110 and rpoB1–B4 probes had median CT values ranging from 16.4 to 22.0, while the pooled probes ranged from 16.8 to 23.5, with ΔCT ranging from 0.4 to 1.75.

Cartridge costs of individual and pooled tests

The potential savings in cartridges costs were estimated when using pooled testing to screen the 500 individuals in each survey compared to individual testing (Supplementary Table S3). In the Xpert MTB/RIF survey, testing 125 pools and then re-testing the 50 MTB-positive pools would require 325 cartridges: 125 plus 200 (50 × 4) for positive pools, corresponding to saving 175 (35%) of the 500 cartridges compared to testing all samples individually.

Pooled testing with Ultra required 125 cartridges to test the pools plus 144 cartridges to re-test individually the 36 MTB-positive pools, for a total of 269 cartridges. This represents a saving of 231 (46%) cartridges compared to individual testing. Similarly, using the pooling approach, a stock of 500 cartridges could be used to test respectively 770 and 929 individuals.

DISCUSSION

Data presented here add to the emerging body of literature on the performance of molecular assays for the diagnosis of TB using the pooled method. In this study, there was no significant difference in the performance of pooled MTB/RIF and pooled Ultra, with similar sensitivity and specificity. Moreover, although the agreement between single and pooled testing was slightly lower than reported from studies elsewhere, these differences were not statistically significant. These were unexpected findings, as a systematic review had indicated that pooling samples with Ultra resulted in a higher sensitivity than pooled testing with MTB/RIF (98% vs. 91%, respectively), and a greater agreement when using Ultra.[5] Moreover, recent studies in Cambodia[6] and Lao PDR[10,11] reported that pooled testing with Ultra could achieve full agreement with individual testing, while pooled testing with MTB/RIF could lead to samples with low bacilli concentrations being missed due to the lower sensitivity of the test. This is supported by our findings, as discrepant tests were more often observed among individuals with trace or very low MTB grades; in Lao PDR, discrepancies occurred only with MTB/RIF and only in pools that included a single MTB-positive sample with a very low bacilli load.[10,11] False MTB-negative pool tests can be attributed to a dilution effect on the bacilli below the limit of detection.

Not all discrepant results, however, were associated with low MTB grades. Among samples tested using MTB/RIF, one low and one medium MTB-positive samples tested MTB-negative in the pooled assay. Similarly, among samples tested using Ultra, one sample with low MTB grade tested MTB-negative in the pool. Although previous studies have suggested that samples with low and medium MTB grades are usually above the limit of detection, these discrepancies may reflect the low resolution of the MTB semi-quantitative scale with unprecise limits between grades. Moreover, the process of pooling and testing samples require further steps than individual testing, which could result in operational errors, such as the poor mixing of samples before pipetting, with only a few or no bacilli present in the pool. Moreover, we also observed four pools with MTB-negative samples only that returned an MTB-positive pooled result. False-positive results in pooled samples lead to the use of more test cartridges, but do not negatively impact diagnosis. These apparently false-positive results have not been reported in previous studies. However, false-positive pooled tests have infrequently been observed when testing for other infections (e.g., testing for Xpert Xpress SARS-CoV-2; Iem et al., verbal communication), which are attributed to human error or cross-contamination during sample handling. An alternative explanation is that the combination of multiple samples in a pool may increase the amount of genetic material and compensate for the dilution effect of pooling, as others have reported reduced CT values (i.e., higher RNA/DNA) for pooled samples containing a single SARS-CoV-2 positive, hypothesising a 'carrier RNA' effect caused by increased total cellular RNA in the samples.[12,13] Furthermore, pooling samples can lead to improved polymerase chain reaction (PCR) efficiency and sensitivity in the case of a single positive sample containing PCR inhibitors, which are then diluted by pooling. Although these apparent errors may have an impact on the practitioner's confidence in the method, these spurious results have no impact on the clinical management of the patients, since all positive pools would have been re-tested individually. Ideally, further evidence generated by future implementation studies will document the performance of the tests under routine conditions.

Using the pooling method would have identified 94.4% (68/72) and 86.7% (52/60) of the people with MTB-positive results using MTB/RIF and Ultra, respectively, while saving 35% and 46% of the test costs. Our assumptions indicate that pooling has the potential to optimise the cost-effectiveness of testing, reduc-

ing the unit cost from USD9.98 per patient tested (FIND negotiated price) to USD6.48 and USD5.28 for MTB/RIF and Ultra, respectively.

Our results demonstrate a high level of agreement between individual and pooled testing. Pooled testing can generate significant time and resources savings; during health system crises, such as during the Covid-19 pandemic when replenishing cartridge stocks was difficult, integrating pooled approaches could increase testing capacity to identify people with TB.

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CONTEXTE : Le coût et les stocks de cartouches des tests d'amplification moléculaire limitent leur utilisation pour le diagnostic de la TB. Regrouper plusieurs échantillons afin de les tester en même temps aurait une précision similaire à celle des tests individuels et permettrait de réaliser des économies.

MÉTHODES : Deux enquêtes ont été menées auprès de personnes avec une TB présumée afin d'évaluer la performance des tests groupés en utilisant le test Xpert® MTB/RIF (MTB/RIF) et le test Xpert® Ultra (Ultra).

RÉSULTATS : Au total, 500 personnes ont été testées par test MTB/RIF, dont 72 (14,4%) étaient MTB-positives. Les échantillons ont été testés dans 125 groupes, dont 50 groupes avaient ⩾1 échantillons MTB-positifs et 75 uniquement des échantillons MTB-négatifs : 46/50 (92% ; IC 95% 80,8–97,8) groupes MTB-positifs ont été testés MTB-positifs et 71/75 (94,7% ; IC 95% 86,9–98,5) groupes MTB-négatifs ont été testés MTB-négatifs dans le test groupé (concordance : 93,6% ; $\kappa = 0{,}867$). Cinq cents échantillons supplémentaires ont été testés par test Ultra, dont 60 (12%) étaient MTB-positifs. Les échantillons ont été testés dans 125 groupes, dont 42 avaient ⩾1 échantillons MTB-positifs et 83 uniquement des échantillons MTB-négatifs : 35/42 (83,6% ; IC 95% 68,6–93,0) groupes MTB-positifs ont été testés MTB-positifs et 82/83 (98,8% ; IC 95% 93,5–100,0) groupes MTB-négatifs ont été testés MTB-négatifs dans le test groupé (concordance : 93,6% ; $\kappa = 0{,}851$; $P > 0{,}1$ entre les tests individuels et groupés). Les tests groupés ont permis d'économiser 35% (MTB/RIF) et 46% (Ultra) des cartouches.

CONCLUSIONS : Les tests groupés et individuels présentent un niveau élevé de concordance et améliorent l'efficacité des tests.

Public Health Action (PHA) welcomes the submission of articles on all aspects of operational research, including quality improvements, cost-benefit analysis, ethics, equity, access to services and capacity building, with a focus on relevant areas of public health (e.g. infection control, nutrition, TB, HIV, vaccines, smoking, COVID-19, microbial resistance, outbreaks etc).

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