Pooling samples to increase testing capacity for Tuberculosis and COVID-19 diagnosis.

Thesis submitted in accordance with the requirements of the

Liverpool School of Tropical Medicine

for the degree of Doctor in Philosophy by Vibol Iem.

May 2023

Abstract

The COVID-19 pandemic created an overwhelming burden for National Tuberculosis (TB) Programs who struggled to maintain essential services while coping with diversion of equipment and personnel. This resulted in delays and stock-outs in procurement of laboratory equipment including due to lockdowns, leading to TB testing requirement exceeding the capacity of TB laboratories.

International development partners are increasingly requesting countries to commit to co-financing mechanisms from government-funded schemes for the procurement of recommended molecular TB tests (Xpert MTB/RIF or Xpert Ultra), but such tests can be expensive, especially for low- and middle-income countries (LMIC).

One strategy to improve cost-efficiency and access to these tests is through pooled testing. This approach consists of mixing (pooling) sputum specimens from several individuals into a single pool, which is then tested with a single test. If the pooled test is negative, all samples in the pool are considered negative. If the pooled test is positive, it means at least one of the individual samples is positive, and each individual sample is then re-tested to identify the positive sample(s).

The potential cost and time savings of the pooling method appear promising but there remains minimal evidence, especially from LMIC settings, on whether pooled testing can increase testing capacity while at the same time offering reliable performance with similar accuracy to individual testing.

In Lao People's Democratic Republic (Lao PDR), a LMIC with moderate TB burden, we evaluated whether pooled testing was suitable to implement within routine passive and active case finding strategies, the two main approaches for TB screening in Lao PDR. More precisely, we described the level of agreement between individual and pooled testing, investigated potential discrepancies between test results, and assessed whether the pooling approach would result in assay cost savings.

First, we reviewed the literature to assess the sensitivity/specificity of GeneXpert-based pooled testing compared to individual testing and potential cartridge/time savings. We found that testing pools with 4 sputum samples with Xpert-MTB/RIF and Xpert Ultra had 91% and 98% sensitivity, with 99%–100% specificity and 27%–31% cartridge savings. However, the review found that the number of high-quality studies available in the literature was too small to develop evidence-based guidance to inform policy and decision makers.

Second, we conducted cross-sectional surveys comparing the individual vs pooled sample methods using Xpert-Ultra and Xpert-MTB/RIF in Lao PDR and Nigeria. Surveys using Xpert-Ultra showed an agreement between individual vs pooled testing of 100% and 94%, with considerable Xpert-Ultra cartridge savings, ranging from 42% to 46%. Higher savings were observed in populations where the proportion of positive tests was lower. Pooling with Xpert-MTB/RIF in Lao PDR and Nigeria showed 98% and 94% agreement between pooled and individual testing and saved 38% and 35% in cartridge costs. Pooled testing with Xpert-Ultra had similar agreement (100%) whether active or passive case finding strategies were used.

Third, to respond in real time to an urgent public health need, we temporarily pivoted to assess the feasibility of the pooled testing strategy when applied to SARS-CoV-2 testing at the height of the COVID-19 pandemic in Lao PDR, during which demand

for SARS-CoV-2 testing outstripped capacity. Pooling samples for SARS-CoV-2 using the Xpert Xpress SARS-CoV-2 reduced the number of tests required by 67% and tripled the laboratory testing capacity.

Fourth, we conducted a cost-minimization analysis of individual vs pooled TB testing to establish the value for money of pooling. This showed that pooling can lead to significant savings related to cartridge expenditure, but the amount of savings varies significantly depending on the proportion of positive tests, with lower prevalence and positivity rates leading to higher savings.

There remains a need for high-volume rapid testing for both TB and COVID-19, especially in LMIC. Our results show that pooling samples increased testing capacity, reduced costs per positive test, improved maintenance of stocks, and enhanced essential services during the pandemic. Pooling could continue to provide cost and time savings post-pandemic for TB and SARS-CoV-2 screening and could also be of preparedness for future infectious pandemics or emergences.

Acknowledgements

This thesis is dedicated to the memory of our dear colleague, friend, and mentor Professor Luis Cuevas, to whom I would like to express my deepest gratitude. He agreed to take on the role of primary supervisor and I could not have undertaken this incredible journey without his trust in my capacity. His expertise and guidance throughout this endeavor helped me reach an unhoped-for level of excellency.

I am extremely thankful to Dr Tom Wingfield, who also agreed to join this challenging adventure and become my new primary supervisor. His knowledge and invaluable advices contributed to improving the quality of the final results and helped me get through it all.

I would like to extend my sincere thanks to Dr Jose Dominguez and Professor Jahangir Khan for all the teaching and the technical support to complete each milestone of this amazing project.

Special thanks to my colleagues from the Lao National TB Control Center and the Lao National TB Reference Laboratory for helping me on a daily basis with laboratory activities, late-night sessions during the COVID-19 pandemic and the moral support. Thanks should also go to Dr Nadia Kontogianni for helping in the project design and all the data quality assessment.

I am also grateful to my Progress Assessment Panel members, Professor Stephen Allen, and Dr Emily Adams, for their valuable feedback on the thesis, their guidance and precious recommendations that helped me remain on track.

Another special thoughts to my parents, brothers and sisters who have always believed in me, showed love, affection and motivated me to follow my dreams.

Words cannot express how grateful I am to have had my two wonderful sons Ryan and Jaeden beside me throughout this amazing journey. Their laughter and naughtiness cheered me up when I was down. And last but not least, I would like to also dedicate this achievement to my marvelous wife, Dr Silaphet Somphavong MD, PhD, for all the love and the emotional support. She inspired me to follow in her footsteps and undertake this PhD, to become a better version of myself.

Table of Contents

List of original papers 10

List of abbreviations 11

List of Tables 12

List of Figures 16

Preamble 18

Introduction 19

Chapter I - Systematic Review of Pooling Sputum as an Efficient Method for Xpert MTB/RIF Tuberculosis Testing during COVID-19 Pandemic 25

Abstract 26

Introduction 27

Materials and methods 28

Results 30

Discussion 41

Conclusions 45

Chapter II - Pooling sputum for Xpert MTB/RIF and Xpert Ultra testing during the COVID-19 pandemic in Lao People's Democratic Republic 46

Introductory section 47

Abstract 48

Introduction 49

Materials and methods 51

Results 55

Discussion 66

Conclusions 69

Chapter III - Pooling sputum in Nigeria for Xpert MTB/RIF and Xpert Ultra testing for tuberculosis during the COVID-19 pandemic 71

Abstract 72

Introduction 73

Materials and methods 75

Results 77

Discussion 87

Conclusions 89

Chapter IV - Pooled testing of sputum with Xpert MTB/RIF and Xpert Ultra during tuberculosis active case finding campaigns in Lao People's Democratic Republic 90

Introductory section 91

Abstract 92

Introduction 93

Materials and methods 95

Results 99

Discussion 107

Conclusions 109

Chapter V - Pooling samples to increase testing capacity with Xpert Xpress SARS-CoV-2 during the COVID-19 pandemic in Lao People's Democratic Republic 110

Introductory section 111

Abstract 112

Introduction 113

Materials and methods 114

Results 119

Discussion 129

Conclusions 133

Chapter VI - Pooling sputum testing to diagnose tuberculosis using Xpert MTB/RIF and Xpert Ultra: a cost-effectiveness analysis 134

Introductory section 135

Abstract 135

Introduction 138

Materials and methods 140

Results 146

Discussion 156

Conclusions 162

Final discussion and Conclusions 163

Controversies 165

Implications for the field of TB 168

Implications for other fields of study 170

Limitations of our study 172

Recommendations 175

Future directions 178

Conclusions 182

References 183

List of original papers

This thesis is based on the following six publications, which are referred to in the text by their Roman numerals.

I. Cuevas LE, Santos V, Lima SVMA, Kontogianni K, Bimba J, **Iem V**, Dominguez J, Adams E, Cubas Atienzar A, Edwards T, Squire SB, Hall PJ, Creswell J. Systematic Review of Pooling Sputum as an Efficient Method for Xpert MTB/RIF Tuberculosis Testing during the COVID-19 Pandemic. Emerging Infectious Disease journal. 2021;27(3):719. https://doi.org/10.3201/eid2703.204090.

II. **Iem V**, Chittamany P, Suthepmany S, Siphanthong S, Somphavong S, Kontogianni K, Dodd J, Khan JAM, Dominguez J, Wingfield T, Creswell J, Cuevas LE. Pooling sputum for Xpert MTB/RIF and Xpert Ultra testing during the COVID-19 pandemic in Lao People's Democratic Republic. PLOS Global Public Health. 2022;2(4):e0000116. https://doi.org/10.1371/journal.pgph.0000116.

III. Bimba J[†], Adekeye OA[†], **Iem V**[†], Eliya T, Osagie I, Kontogianni K, Edwards T, Dodd J, Squire SB, Creswell J, Cuevas LE. Pooling sputum samples for Xpert® MTB/RIF and Xpert® Ultra testing for TB diagnosis. *Public Health Action* 2023: 13(1): 12-16.
[†] **Equal contributions**
https://doi.org/10.5588/pha.22.0052

IV. **Iem V**, Chittamany P, Suthepmany S, Siphanthong S, Siphanthong P, Somphavong S, Kontogianni K, Dodd J, Khan JAM, Dominguez J, Wingfield T, Creswell J, Cuevas LE. Pooled testing of sputum with Xpert MTB/RIF and Xpert Ultra during tuberculosis active case finding campaigns in Lao People's Democratic Republic. BMJ Global Health. 2022;7(2): e007592. https://doi.org/10.1136/bmjgh-2021-007592

V. **Iem V**, Xangsayarath P, Chittamany P, Suthepmany S, Siphanthong S, Paboriboune P, Somphavong S, Kontogianni K, Khan JAM, Edwards T, Wingfield T, Creswell J, Dominguez J, Cuevas LE. Pooling samples to increase testing capacity with Xpert Xpress SARS-CoV-2 during the COVID-19 pandemic in Lao People's Democratic Republic. PLOS ONE 2022: 17(9): e0275294.
https://doi.org/10.1371/journal.pone.0275294

VI. **Iem V**, Bimba J, Santos V, Dominguez J, Creswell J, Somphavong S, Wingfield T, Khan JAM, Cuevas LE. Pooling sputum testing to diagnose tuberculosis using xpert MTB/RIF and xpert ultra: a cost-effectiveness analysis. BMC Infectious Diseases 2023: 23(1): 341.
https://doi.org/10.1186/s12879-023-08330-9

List of abbreviations

COVID-19	Coronavirus Disease-19
CT value	Cycle Threshold value
DNA	Deoxyribonucleic acid
EP	Extra-pulmonary tuberculosis
Xpert	GeneXpert MTB/RIF
Xpert Ultra	GeneXpert MTB/RIF Ultra
AFB	Acid-Fast-Bacilli
HIV	Human immunodeficiency virus
LMIC	Low- and middle-income countries
MDR-TB	Multidrug-resistant tuberculosis
MTB	*Mycobacterium tuberculosis* complex
NCLE	National Center for Laboratory and Epidemiology
NRL	National Tuberculosis Reference Laboratory
NTC	National Tuberculosis Control Center
NTG	National Technical Guidelines
PCR	Polymerase chain reaction
RIF	Rifampicin
RR	Xpert test result: MTB detected, rifampicin resistance detected
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
TB	Tuberculosis
WHO	World Health Organization
TB/HIV	TB/HIV co-infection

List of Tables

Tables numbering follows chapters.

Chapter I

Table 1.1. Characteristics of the studies, number of participants, and pool size used in a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic.

Table 1.2. Tuberculosis Xpert results of pools composed of positive and negative samples, with sensitivity and specificity, in a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic.

Table 1.3. Potential cost and time savings and positive and negative effects of pooling in a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic

Chapter II

Table 2.1. Baseline characteristics of participants with single and pooled Xpert results.

Table 2.2. Distribution of positive individual samples among pooled results, by Xpert test.

Table 2.3. Median CT values of individual and pooled Xpert MTB/RIF and Xpert Ultra probe results.

Table 2.4. Cost and savings to screen consecutive patients using the pooling method and number of patients that could be tested with Xpert MTB/RIF and Xpert Ultra cartridges.

Chapter III

Table 3.1. Baseline characteristics of participants with individual Xpert MTB/RIF and Ultra results

Table 3.2. Number of pools with 0, 1, 2, 3 and 4 positive results

Table 3.3. Results of pooled and individual Xpert testing

Table 3.4. Agreement of Xpert individual and pooled tests

Supplementary Table 3.1. Correlation of Individual and pooled Xpert MTB grades (only includes data for pools with one positive sample)

Supplementary Table 3.2. Probe results for Xpert MTB/RIF and Xpert Ultra in individual and pooled tests

Supplementary Table 3.3. Cartridge costs screening 500 consecutive patients using the pooling method and number of patients that could be tested with 500 Xpert MTB/RIF and Xpert Ultra cartridges

Chapter IV

Table 4.1. Baseline characteristics of participants and Xpert MTB results

Table 4.2. Number of pools with 0,1,2,3,4 positive results

Table 4.3. Correlation of Individual and pooled Xpert MTB grades (positive pools only include those with only one positive Xpert)

Table 4.4. Median CT values of individual and pooled Xpert MTB/RIF and Xpert Ultra probe results

Table 4.5. Cost and savings to screen consecutive patients using the pooling method and number of patients that could be tested with Xpert MTB/RIF and Xpert Ultra cartridges

Chapter V

Table 5.1. Baseline characteristics of participants with single and pooled Xpert Xpress SARS-CoV-2 results

Table 5.2. Median CT values of individual and pooled Xpert Xpress SARS-CoV-2 probe results

Table 5.3. Cost and diagnostic savings to screen 1000 consecutive patients using the pooling method and number of patients that could be tested with 1000 Xpert Xpress SARS-CoV-2 cartridges

Supplementary Table 5.1. CT values for the probes E and N2 for both individual and pooled results

Supplementary Table 5.2. Effect of dilution on CT values of positive samples

Chapter VI

Table 6.1. Resources costs assumptions for TB diagnosis by Xpert MTB/RIF and Ultra (unit costs from the Global Drug Facility products catalogs “Ordering List of TB Medicines or Diagnostics, Medical Devices and other health products”)

Table 6.2. Agreement of individual and pooled tests

Table 6.3. Cost analysis of each strategy (individual vs pooled) by country, by assay (MTB/RIF vs Ultra) using a fixed amount of 1,000 cartridges

Table 6.4. Cost analysis of each strategy (individual vs pooled) by country, by assay (MTB/RIF vs Ultra) to test 1,000 presumptive TB patients

List of Figures

Figures numbering follows chapters.

Chapter I

Figure 1.1. Flow diagram of study selection for a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic

Figure 1.2. Sensitivity and specificity for (A) pooling sputum in the ratio of 1:2 and (B) pooling sputum in the ratio of 1:4 in a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic

Chapter II

Figure 2.1. Flow diagram of sputum pooling and processing

Figure 2.2. Correlation of individual and pooled (A) Xpert MTB/RIF and (B) Ultra grades (positive pools only include those with one individual Xpert MTB-positive sample)

Chapter III

No figure

Chapter IV

Fig 4.1. Flow diagram of the sample processing

Chapter V

Fig 5.1. Flow diagram of the sample processing

Fig 5.2. CT values of samples containing 1, 2, 3 and 4 positive samples in a pool

Supplementary Figure 5.1. Correlation of individual and pooled Xpert Xpress Sars-CoV2 (positive pools only include those with single individual Xpert Xpress SARS-CoV2-positive sample)

Supplementary Figure 5.2. Effect of serial dilution on CT values of positive samples

Chapter VI

Fig 6.1. Flow diagram of the sputum processing

Fig 6.2. Parameters affecting pooled testing cost effectiveness

Fig 6.3. Effect of the prevalence of the disease on the amount of savings by pooling method

List of appendix

Appendix 1. LSTM Ethical waiver (Chapter II, III, IV)

Appendix 2. Lao PDR Ethical waiver (Chapter II & IV)

Appendix 3. Nigeria Ethical approval (Chapter III)

Appendix 4. Informed consent form for the study in Nigeria (Chapter III)

Preamble

Like many fellow students, my PhD research journey has been turned upside down by the COVID-19 pandemic. The mitigation measures worldwide such as lockdowns, curfews, travel restrictions were implemented just a few months after I enrolled on the PhD programme, when I was still in the early stage of the development of my research protocol. Due to this, I was unable to access data, samples, or conduct patient interviews as planned. Therefore, my supervisors and I had to develop a new PhD topic with consideration of the new COVID-19 context in which we were likely to be working. Our priority was to look for a topic that would be in line with international needs to support the detection of this new pathogen while, at the same time, supporting my institution, the National TB Control Center, to maintain essential TB services following the diversion of healthcare workers, laboratory commodities and financial support towards COVID-19 response.

The studies and the results presented here are therefore an adaptation of the original thesis plan prior to the COVID-19 pandemic. Despite our pooling strategy being implemented during a time of global crisis, we have concluded that the pooling method is not a strategy specific to the context of crisis and could be also used in non-crisis times. Indeed, our findings suggest that pooling can support countries to increase testing capacity, maintain essential TB services, and save resources for a more cost-effective testing strategy even beyond the pandemic. Within the thesis, we have also provided a series of practical recommendations on how countries can best implement this re-emerging approach following the pandemic in their own specific context.

Introduction

Tuberculosis (TB) is an ancient disease that remains a major public health concern. TB was the leading cause of death by a single infectious agent before the emergence of the coronavirus (COVID-19) pandemic, ranking above HIV/AIDS and Malaria combinedly [1]. In 2021, the World Health Organization (WHO) estimated 10.6 million people fell ill with TB, with an incidence rate rising by 3.6% between 2020 and 2021, reversing declines of about 2% per year for most of the previous two decades [1]. Geographically, in 2021, most TB cases occurred in the WHO regions of South-East Asia (45%), Africa (23%) and the Western Pacific (18%), with fewer cases in the Eastern Mediterranean (8.1%), the Americas (2.9%) and Europe (2.2%) [1].

In 2021, as COVID-19 became pandemic, TB claimed an estimated 1.6 million lives globally, with 82% of global TB deaths occurring in the WHO African and South-East Asia regions [1]. Globally, the estimated number of deaths from TB increased between 2019 and 2021, reversing years of decline between 2005 and 2019. Indeed, due to the impact of the COVID-19 pandemic, deaths due to TB rose for the first time in more than a decade, reaching a total equivalent to the number of deaths estimated in 2017.

In the latest World Health Organization (WHO) guidelines in 2021, nucleic acid amplification tests (NAATs) were listed as the molecular WHO-recommended rapid diagnostics (mWRD) for TB [2]. These tests include the Xpert MTB/RIF [3] and the Xpert MTB/RIF Ultra (Xpert Ultra) [4], assays that simultaneously detect both *Mycobacterium tuberculosis* complex (MTB) and resistance to rifampin (RIF), using the GeneXpert platform [5].

The global response to and funding mobilisation for the COVID-19 pandemic has adversely affected TB service provision in many countries [6]. A major direct negative impact is that, due to global demand, most in-vitro diagnosis companies have shifted their production sites towards COVID-19 diagnostic testing. For example, Cepheid production sites have prioritised manufacturing the Xpert Xpress SARS-CoV-2 assay [7] and reduced production of Xpert MTB/RIF and Xpert Ultra assays, to the detriment of TB control programmes [8]. Existing diagnostic platforms for major infectious diseases have been diverted to test for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Repurposing of systems such as the GeneXpert platform - the leading mWRD for TB diagnosis -have been responsible for an 18% decrease in TB cases notifications between 2019 and 2020, back to the level of 2012 [9].

Public health measures to limit population mobility including travel restrictions and lockdowns have also resulted in challenges for many countries to procure laboratory commodities for the diagnosis of TB. The limited means for shipping the cartridges for TB testing from production sites to low- and middle-income countries (LMICs) has resulted in significant delays in laboratory procurement, leading to prolonged turnaround time for TB diagnosis.

Consequently, National TB Programmes struggled to maintain essential TB services during this time of crisis, which is threatening to reverse up to eight years of progress, and result in an additional 6.3 million TB cases globally between 2020 and 2025 [6].

Countries have tried to stabilize this programmatic disruption and reorienting to a “new normal” by developing and implementing adaptive catch-up plans and innovative approaches to maintaining TB services. However, in some high TB burden

countries, TB case detection continues to be lower than pre-COVID-19 levels. This is especially the case in LMICs such as Lao People's Democratic Republic (PDR). Lao PDR is a landlocked country of 7.4 million people that lies between China, Vietnam, Cambodia, Thailand, and Myanmar. Lao PDR has reached the Millennium Development Goal target (MDG 6 Goal 4) of halving TB prevalence in 2015 compared to 1990. Estimated incidence of TB was 143 per 100,000 (11,000 TB cases) and mortality due to TB was 27 per 100,000 in HIV-negative TB patients and 3.2 per 100,000 in HIV-positive TB patients in 2021 [10]. While the estimated incidence is consistently declining, many cases are thought to be missed by the National TB Control Center (NTC) due to limited health access. TB burden remains high and TB treatment coverage sub-optimal, related to limited access to, and low quality of health services for a large portion of the population living in remote rural areas of the country. However, successive COVID-19 lockdowns in 2020 and 2021, further reduced the access of patients to health services and interrupted outreach case finding among high-risk populations in Lao PDR. This resulted in a large drop in the TB cases notification rate from 110/100,000 in 2020 to 89/100,000 in 2021 and a treatment coverage of only 62% equating to an estimated 2,000 TB patients being missed for TB diagnosis and care in 2021.

In 2014, the Lao PDR National TB Reference Laboratory started using the Xpert MTB/RIF assay (Cepheid, Sunnyvale, CA, USA) for rapid molecular testing of rifampicin resistance. There has been significant progress in Lao PDR in case detection over recent years, driven by a transition to upfront GeneXpert testing, improvements in clinical diagnosis, and active case finding activities. Through this combination of strategies, the TB treatment coverage increased from 30% of estimated incidence in 2011 to 74% in 2020 by increasing the use of GeneXpert rapid

TB diagnostic and scaling-up active case finding in high-risk population. However, successive COVID-19 lockdowns considerably reduced patients access to health services and outreach activities in 2021 when only 6,123 TB cases were notified (56% TB treatment coverage). With continued slow decline of incidence, the End TB 2025 target of halving TB incidence (compared to 2015 level) could not be attained. In order to accelerate progress and reach the WHO End TB target of 90% reduction by 2035 in TB incidence rate and 95% reduction in deaths from TB by 2035 compared with 2015 [11], it is essential to understand what is slowing down the detection rate. Low TB notification correlates with low attendance at health facilities and low capacity to identify respiratory symptoms and to refer people with symptoms for bacteriological sampling and confirmation.

The 2018 National Technical Guidelines for TB in Lao PDR followed WHO recommendations on using mWRDs as the initial test for bacteriological confirmation to diagnose TB for all individuals with presumptive TB. The standard method to diagnose TB is to collect samples from people suspected of having TB individually and test each sample separately. Despite being highly specific and sensitive, this individual testing method has been shown to be lengthy, complicated and resource consuming [12].

An alternative to individual testing is pooled testing. This approach consists of combining a number of specimens from several individuals into one pooled specimen and testing them as a group [13]. If the pooled result is negative, all individual samples included in the grouped specimen are considered negative. If the pooled result is positive, it means at least one of the individual samples in the pooled sample is positive. All individual samples from a positive pooled sample are then retested separately to identify positive sample(s). The accuracy of the pooling method is

dependent upon the performance of the test used (sensitivity and specificity), and the potential savings upon the prevalence of the disease in the population tested.

Historically, the pooling method originated from serological testing in syphilis [13] and has also been applied to other infectious diseases [14] and for blood bank screening [15]. The main benefit for the pooling method is that it can achieve the same performance as individual testing while saving resources and time [16, 17]. The approach has been enhanced throughout the years with an emerging body of literature on its general utility and application to various infectious diseases. However, this approach has not been considered in any national testing algorithms for routine diagnosis of infectious diseases including TB.

The objective of this PhD research is to provide the international community and decisions makers with evidence on the performance and cost savings associated with the pooling method in order to inform whether pooling can be applied with high reliability in various contexts and in real situations, especially during the COVID-19 pandemic.

The PhD is structured around a series of peer-reviewed publications in leading scientific journals reporting the thesis research findings. Each chapter addresses a specific context for the application of the pooling method under different practical conditions during the COVID-19 pandemic. The chapters go on to describe the benefits and the limitations of the pooling method under these specific conditions and in these specific contexts representing the reality of a day-to-day activity of a TB laboratory.

In Chapter I, we systematically review the related literature to assess the performance (sensitivity and specificity) of GeneXpert-based pooled testing compared to

individual testing. Chapter II to V are independent cross-sectional surveys to document the pooled testing performance under diverse specific practical conditions. We describe the agreement of pooled and individual testing for the surveys and the numbers and costs of tests that could be saved by using the pooling method for TB testing. Chapter II and III assess the reliability of the pooling method in routine practice such as passive TB case finding. Chapter IV assesses the pooling method in the frame of outreach activities in active case findings campaigns for TB diagnosis. Chapter V describes how pooled testing can be applied to other diseases such as COVID-19. In Chapter VI, we conducted a cost-effectiveness analysis of the pooled testing strategy in comparison with Xpert MTB/RIF and Xpert Ultra individual testing, during routine MTB passive case finding (PCF) activities.

Chapter I - Systematic Review of Pooling Sputum as an Efficient Method for Xpert MTB/RIF Tuberculosis Testing during COVID-19 Pandemic

As published in Emerging Infectious Diseases (Volume 27, Number 3—March 2021).

Citations as follows: Cuevas LE, Santos V, Lima SVMA, et al. Systematic Review of Pooling Sputum as an Efficient Method for Xpert MTB/RIF Tuberculosis Testing during the COVID-19 Pandemic. Emerging Infectious Disease journal 2021: 27(3): 719.

https://doi.org/10.3201/eid2703.204090

VI's contributions: given I was going to conduct a PhD study to document performance of the pooled testing, we agreed with the supervisors and the research team that I would participate in the study design, data extraction, data analysis of this systematic review. I also participated in the preparation of the initial manuscript, responses to reviewers during the publication process and the final version of the manuscript prior to publication.

The study in Chapter I represents the conceptual framework that drove the thesis. The study was conducted in early 2020 and serves as the foundation for the following chapters.

Abstract

Molecular rapid diagnostics, including GeneXpert-based testing with Xpert MTB/RIF or Ultra assays, have improved the rapidity and accuracy of tuberculosis diagnosis and are recommended by the World Health Organization. However, testing may be affected by cartridges and staff shortages. More efficient testing strategies could help, especially during the coronavirus disease pandemic. We searched the literature to systematically review whether GeneXpert-based testing of pooled sputum samples achieves sensitivity and specificity similar to testing individual samples, using the individual tests results as the "gold standard" reference; this method could potentially save time and preserve the limited supply of cartridges. From 6 publications, we found 2-sample pools using Xpert MTB/RIF had 87.5% and 96.0% sensitivity (average sensitivity 94%; 95% CI 89.0%–98.0%) (2 studies). Four-sample pools averaged 91% sensitivity with Xpert MTB/RIF (2 studies) and 98% with Ultra (2 studies); combining >4 samples resulted in lower sensitivity. Two studies reported that pooling achieved 99%–100% specificity and 27%–31% in cartridge savings. Our results show that pooling may improve efficiency of GeneXpert-based testing.

Introduction

Xpert MTB/RIF (Cepheid, https://www.cepheid.com) is a cartridge-based nucleic amplification assay for use with Cepheid's GeneXpert diagnostic instrument systems that detects both *Mycobacterium tuberculosis* complex (MTB) and resistance to rifampin (RIF). In 2010, the World Health Organization endorsed Xpert MTB/RIF for laboratory detection of tuberculosis (TB) [18], signaling a sea change for diagnosing TB. Xpert MTB/RIF increased sensitivity over microscopy and its ability to simultaneously detect rifampin resistance led to its rapid adoption in low- and middle-income countries. Within the first 5 years, 23 million cartridges were procured [19] at the negotiated price of $9.98/each (P. Jacon, Cepheid, pers. comm., email, April 2020). In 2017, the Cepheid Xpert MTB/RIF Ultra assay (Ultra) was released for use on GeneXpert instruments and results determined to be comparable to those from the Xpert MTB/RIF assay, with an even lower limit for detection [18].

Coronavirus disease (COVID-19) is severely disrupting health systems and is threatening progress made by national TB control programs. The new Xpert Xpress SARS-CoV-2 test is run on the same GeneXpert instruments as those for Xpert MTB/RIF and Ultra testing; it is being expedited for large-scale production and deployment. Consequently, TB-testing capacity, already limited by the availability of necessary staff, testing modules, and Xpert MTB/RIF and Ultra cartridges, may be further reduced by the increased demand for GeneXpert for COVID-19 testing [20]. There is an urgent need to develop laboratory testing approaches to expand TB diagnostic and case-finding services in preparation for crises, such as the COVID-19 pandemic.

GeneXpert-based testing for TB requires 1 cartridge per sputum sample. However, screening for other infectious diseases has used sample pooling methods, in which

samples from several patients are pooled together for a single test to optimize processing. If a pooled-sample test is negative, all samples in the pool are considered negative; if the pooled-sample test is positive, all samples in the pool are retested individually to identify the samples that are positive. This method is routinely used in situations where the prevalence of disease is low (e.g., blood banks screening donated blood for hepatitis and syphilis) [21-26]. The method can substantially reduce workload and cost and, for TB, could more efficiently process samples for diagnosis. We reviewed the literature to determine the accuracy of pooling for Xpert MTB/RIF and Ultra detection of pulmonary TB, with the aim of supporting TB programs as they continue to test for TB in the context of increased resource constraints during the COVID-19 pandemic.

Materials and methods

We conducted a systematic review following the Cochrane Collaboration's Diagnosis Test Accuracy Working Group protocol (https://methods.cochrane. org). Our primary aim was to describe whether testing using GeneXpert for pulmonary TB on pooled samples would result in similar numbers of patients being confirmed with TB as testing samples individually. Secondarily, we aimed to describe the advantages and disadvantages reported, such as savings in cartridges used and time required to process samples.

We searched PubMed, CINAHL, Global Health, and Web of Science for publications from January 2010–March 2020 with no regional or language restrictions. We used the terms "GeneXpert" OR "Xpert" OR "Ultra" AND "tuberculos*" AND "pool*" AND "diagnos*" with associated subject headings and search terms without filters (Appendix Table, https://wwwnc.cdc.gov/EID/article/27/3/20-4090-App1.pdf).

S.V.M.A.L. and K.K. eliminated duplicates, screened titles and abstracts, and read full texts to determine eligibility. We also searched for article references manually and for abstracts published at the 2019 Union World Conference of Lung Health. Studies were included if they presented original data, if data were not duplicated in other publications, and if the articles were not reviews or opinions. We excluded studies that pooled several samples from the same patient to increase the yield and those that included samples other than sputum. Given the paucity of studies, we included both those that directly processed patient samples and those that used leftover samples to prepare a specimen repository for bench evaluation of the pooling method. We read selected studies in full for data extraction; L.E.C. and V.S.S. resolved disagreements by consensus.

Data extracted included study identifiers (author, year, country, and setting), methods (study design, pooling methods, number of participants, pooling ratio, number of pools, and type of test), and whether the pooled positive and negative test results coincided with those obtained through individual testing. Data are presented as sensitivity and specificity values, considering the individual GeneXpert test as the reference. Sensitivity was defined as the proportion of pooled samples correctly identified as positive when the pool contained at least 1 sample with a positive individual GeneXpert test. Specificity was defined as the proportion of pooled samples correctly identified as negative when all samples in the pool were negative in individual GeneXpert tests. Data are presented with 95% confidence intervals and ranges.

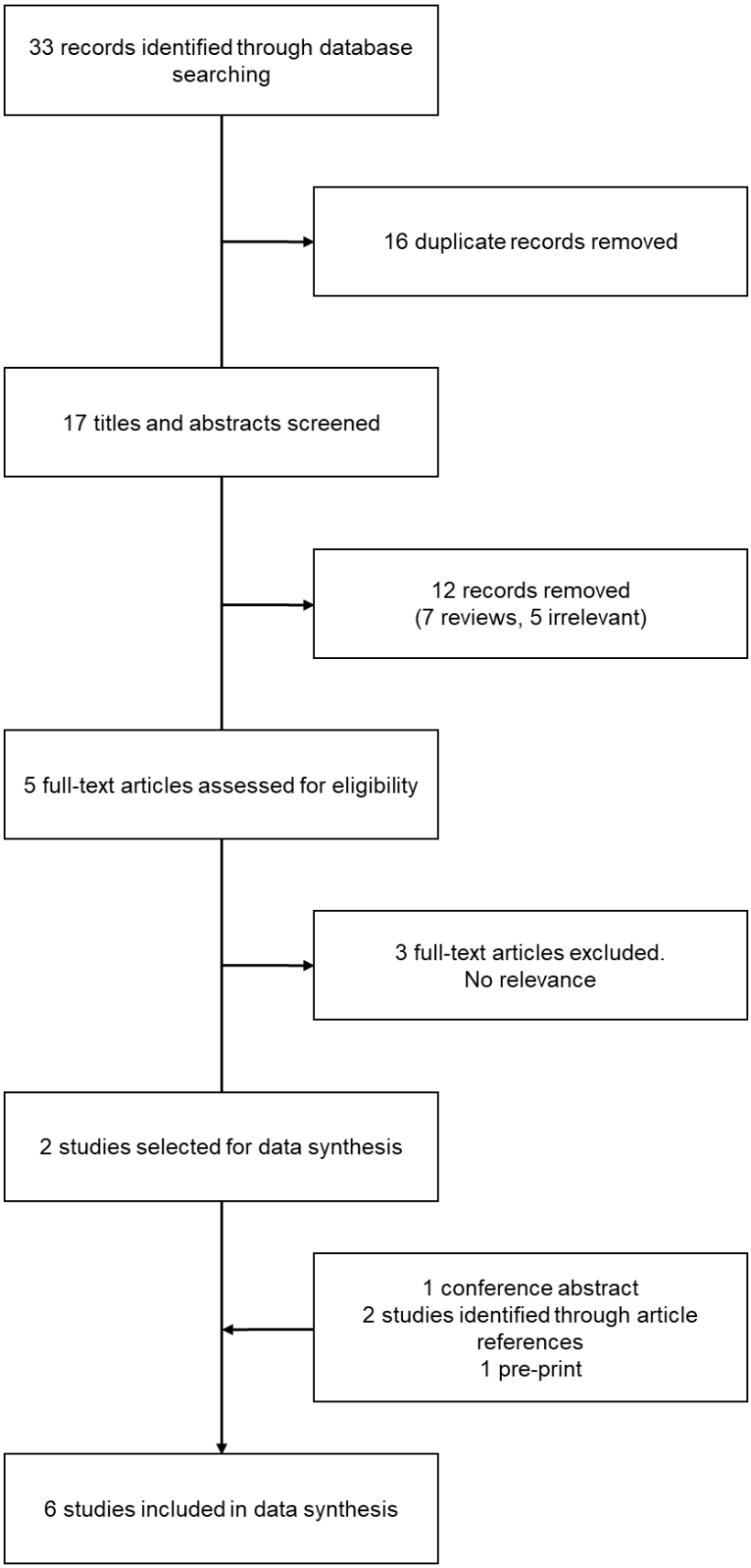
We assessed the quality of the studies based on a further reference standard, the use of TB culture by any method, whether pooled results were recorded blind to the individual results and whether participants had been recruited consecutively to

represent the range of disease severity. The quality of studies and the risk of bias were assessed by 2 independent reviewers (authors) using the QUADAS-2 (Quality Assessment of Diagnostic Accuracy Studies) guidelines (https://www.bristol.ac.uk/media-library/sites/quadas/migrated/documents/quadas2.pdf). We used Cochrane Collaboration Rev-Man 5.3 software (https://training.cochrane.org/online-learning/core-software-cochrane-reviews/revman/revman-5-download) to generate the graphs on the risk of bias (Appendix Figures 1.1, 1.2). Because the studies were highly heterogeneous and most (4/6) did not present data on specificity, we were unable to perform a meta-analysis to estimate the pooled sensitivity and specificity or to explore the reasons for heterogeneity through meta-regression. Institutional review board approval was not required because all data sources and publications were in the public domain and in aggregate format.

Results

We identified 33 publications through the initial publication search. After screening titles and abstracts, we assessed 5 full-text articles for eligibility and initially included 2 in data syntheses. In addition, 4 studies were identified from other sources: 1 conference report, 1 preprint article, and 2 articles from the reference lists of other studies. We included 6 articles in the final data synthesis (Figure 1.1). One study was conducted in South America [27], 2 in Africa [28, 29], and 3 in Asia [30-32]; all were published during 2014–2020, before the COVID-19 pandemic.

Figure 1.1. Flow diagram of study selection for a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic.



We assessed the quality of the studies and the risk of bias (Appendix Figures 1.1, 1.2). Three studies used samples collected directly from patients with presumptive TB, and 3 studies used previously collected stored samples with known GeneXpert results. Studies pooling direct clinical samples were conducted in high-burden settings in which the proportion of patients that tested GeneXpert-positive was high (15%, 16%, and 38.6%), whereas stored samples were used to prepare pools varying the proportion of positive specimens in each pool to explore the effect on sensitivity. Pools were prepared with clinical samples from consecutive patients in 5 studies and in bench-prepared spiked sputum in a laboratory setting in 1 study. The latter study had also prepared the pool using combinations of smear-positive/culture-positive and smear-negative/culture-positive samples. Generally, the studies followed a similar approach to pooling: a sample was collected from patients with presumptive TB and split into aliquots for Xpert MTB/RIF or Ultra testing following the manufacturer's guidelines. Studies that processed and homogenized sputum used the same steps for the individual and pooled GeneXpert tests. One aliquot was used to obtain an individual result, which was considered the reference result; and the second aliquot was mixed with aliquots from other patients and then tested as a pooled sample. All studies reported that laboratory technicians were blind to whether they were testing pooled versus individual samples. One study collected smear and culture results from all participants in addition to the GeneXpert result [28]. Four studies tested sputum using Xpert MTB/RIF [28-31] and 2 with Ultra [27, 32] (Table 1.1).

Table 1.1. Characteristics of the studies, number of participants, and pool size used in a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic.

Study	Country	Participants	N samples	Culture	GX cartridge used	Pooling ratio	N of pools	N patients GX-pos* N (%)	N patients GX-neg* N (%)	N RIF pos	Comments
Zishiri, 2014	South Africa	Reference laboratory	100	Yes	MTB/RIF	1:5	20	20 (20.6)	80 (79.4)	5	Culture and SM pos
			85			1:5	17	17 (20)	68 (32)	3	Culture pos/SM neg
Abdurrahman, 2015	Nigeria	OPD	729	No	MTB/RIF	1:4	185[a]	115 (15.8)	614 (84.2)	4	Compared active and passive case finding
Ho, 2017	Vietnam	Spiked samples	118	No	MTB/RIF	1:2	16	75 (63.6)	43 (36.4)	NR	
						1:4	16				
						1:6	16				
						1:8	16				
						1:10	16				
						1:12	16				
Phuong, 2019	Vietnam	Hospitals	262	No	MTB/RIF	1:2	101[b]	99 (37.7)	163 (62.3)	NR	Pools constructed one pos/one neg
Chry, 2020	Cambodia	ACF	584	No	ULTRA	1:4	125	91 (15.6)	493 (84.4)	3	Used chest x-ray to screen
						1:3	28				
Santos, 2019	Brazil	Prisons, SS	1120	Yes	ULTRA	1:4	20	100 (8.9)	1020 (91.1)	NR	
						1:8	20				
						1:12	20				
						1:16	40				

*Single tests; [a]3 had a failed result; [b]2 had a failed result.
Abbreviations: GX, Xpert; RIF, rifampicin; SM, smear; N, number; NR, not reported; OPD, outpatient department; Hosp, hospitalized patients; ACF, active case finding; SS, Spiked samples.

These 6 studies tested 1,878 individual samples. Participants were recruited from hospitals (n = 262), ambulatory clinics (n = 914), and outreach activities (n = 702). The percentage of individual patients with Xpert MTB/RIF-positive tests included in the pools ranged from 8.9% to 37%, except for 1 in vitro study, which used spiked samples and prepared pools with up to 64% of positive samples. Only 15 (0.8%) participants across all studies had rifampin resistance (Table 1.1). Overall, of the 690 pools tested, 117 pooled 2 samples, 28 pooled 3 samples, 364 pooled 4 samples, 37 pooled 5 samples, 16 pooled 6 samples, 36 pooled 8 samples, 16 pooled 10 samples, 36 pooled 12 samples, and 40 pooled 16 samples. Most of the pools with high numbers of samples (≥6) per pool were in the bench-based study. Only 2 studies reported specificity, 1 in which pools were tested with Xpert MTB/RIF (99%, 95% CI 94%–100%) and 1 in which pools were tested with Ultra (100%, 95% CI 96%–100%; Table 1.2) [29, 32].

Table 1.2. Tuberculosis Xpert results of pools composed of positive and negative samples, with sensitivity and specificity, in a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic.

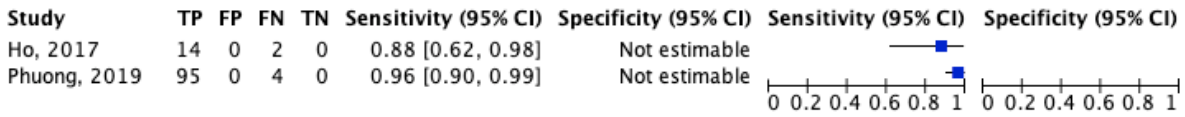
Study	**Pooling ratio**	**Test result (n)**				**Sensitivity, % (95% CI)**	**Specificity, % (95% CI)**
		True positive*	False positive**	False negative *	True negative**		
Zishiri, 2014	1:5 (Cult neg/SM pos)	20	-	0	-	100 (80–100)	-
	1:5 (Cult pos/SM neg)	13	-	4	-	76 (50–92)	-
Abdurrahman, 2015	1:4	80	1	5	96	94 (87–98)	99 (94–100)
Ho, 2017	1:2	14	-	2	-	88 (62–98)	-
	1:4	14	-	2	-	88 (62–98)	-
	1:6	11	-	5	-	69 (41–98)	-
	1:8	10	-	6	-	63 (35–85)	-
	1:10	13	-	3	-	81 (54–96)	-
	1:12	13	-	3	-	81 (54–96)	-
Phuong, 2019	1:2	95	-	4	-	96 (90–99)	-
Chry, 2020	1:4	73	0	0	80	100 (95–100)	100 (96–100)
Santos, 2019	1:4	19	-	1	-	95 (75–100)	-
	1:8	20	-	0	-	100 (83–100)	-
	1:12	16	-	4	-	80 (56–94)	-
	1:16	39	0	1	0	98 (87–100)	-

*At least one of the patients included in the pool had an Xpert-positive test.
**All patients included in the pool were Xpert-negative (in the individual tests).
Abbreviations: CI, confidence interval; Cult, culture; SM, smear.

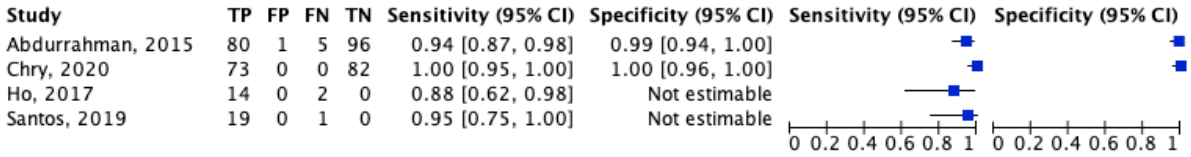
The 2 studies [30, 31] combining 2 sputum samples per pool reported 87.5% and 96.0% Xpert MTB/RIF sensitivity relative to individual testing (Figure 1.2, panel A). The 4 studies combining 4 samples per pool reported sensitivities of 88% [27] and 96% [29] for Xpert MTB/RIF and 95% [30] and 100% [32] for Ultra (Figure 1.2, panel B). In 2 studies [27, 30], pools combining >4 sputum samples reported lower sensitivity ranges for Xpert MTB/RIF (63%–81%) and for Ultra (80%–100%) (Table 1.2).

Figure 1.2. Sensitivity and specificity for (A) pooling sputum in the ratio of 1:2 and (B) pooling sputum in the ratio of 1:4 in a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic.

A)



B)



Given that all studies had <200 pools, we combined the results from all studies with similar pool sizes and test type (e.g., all studies that pooled 4 samples and test them using Xpert MTB/RIF) to evaluate the effect of the number of pooled samples on accuracy. Although this approach has limitations due to variations in study design and proportion of sample positivity, we believe the benefit of this preliminary analysis of the potential use of pooling during the COVID-19 pandemic outweighs these limitations. After combination, when using Xpert MTB/RIF, 114/117 2-sputa pools and 101/201 4-sputa pools tested contained an Xpert MTB/RIF-positive sputum; when using Ultra, 93/173 4-sputa pools tested contained an Ultra-positive sputum. If only pools containing a positive sputum sample were considered, 109/114 2-sputa

pools tested by Xpert MTB/RIF had a MTB-positive result (sensitivity 93.2%, 95% CI 87.0%–96.4%), and 94/101 4-sputa pools tested by Xpert MTB/RIF had a MTB-positive result (sensitivity 93.0%, 95% CI 86.4%–96.6%). Lastly, 92/93 of the 4-sputa pools tested by Ultra had an MTB-positive result (sensitivity 98.9%, 95% CI 94.1%–99.9%), an increase in sensitivity over those tested by Xpert MTB/RIF.

Studies reported slight changes in the cycle threshold (CT) values of the pooled samples compared with the individual tests. Most of the CT changes were relatively small, although studies were not sufficiently powered to determine statistical significance. One study reported that the pooled Xpert MTB/RIF test was negative in 5/10 samples with very low individual Xpert MTB/RIF semiquantitative results [29]. The South African study that used reconstituted processed sputa to generate pools reported that 20 pools containing 1 smear-positive and 4 smear-negative, but culture-positive, samples yielded a median Xpert MTB/RIF CT value increase of 12 (IQR 0.3–20.0), and 22 pools containing only smear-negative/culture-positive samples had a median Ct increase of 6.2 (IQR 3.2–16.0) [28]. Another study [30] also reported that Xpert MTB/RIF CT values increased slightly with increasing pool ratios and, although most pools had CT values similar to the individual sample tests, pools containing >12 sputum samples had a median increase in CT value of 2.1 (IQR 0.0–4.5).

A study from South Africa [28] reported 5 five-sample pools in which 1 was smear-positive/culture-positive and RIF-resistant and 3 five-sample pools in which 1 was smear-negative/culture-positive and RIF-resistant. All 8 pools containing RIF-resistant samples tested positive for RIF-resistance [28]. However, in Chry et al. [32], of the 3 MTB-positive/RIF-resistant samples subjected to Ultra testing, the pools containing the samples yielded MTB-positive but RIF-sensitive results. Abdurrahman

et al. [29] included MTB-positive/RIF-resistant samples in all 4 pools, of which 3 were detected by Xpert MTB/RIF as MTB-positive/RIF-resistant and 1 as MTB-positive/RIF-sensitive.

Only 2 studies [29, 32] reported on the operational effects of using a pooling method, including cartridge costs and time savings. The 2 studies [29, 32] using 4 samples per pool reported savings in cartridge costs alone of 31% ($2,295 on 230 Xpert MTB/RIF cartridges) and 27% ($2,092 on 202 Ultra cartridges). These 2 studies also reported reductions of 377 (62%) and 226 (26%) hours in the staff time required to process and run samples (Table 1.3). All 6 studies included comments indicating the pooling procedure was feasible and beneficial. The study from South Africa [28] noted the lower sensitivity found among smear-negative/culture-positive patients. Several studies mentioned the need for specific training on the pooling procedure. The only negative effect, reported anecdotally, was the need to process samples more carefully to avoid handling and reporting errors. No studies included data on patient outcomes, such as treatment initiation.

Table 1.3. Potential cost and time savings and positive and negative effects of pooling in a systematic review of pooling sputum as an efficient method for Xpert MTB/RIF and Ultra testing for tuberculosis during the COVID-19 pandemic.

Study	Cartridge savings	Time savings (hours)	Negative effects	Positive effects
Zishiri, 2014	A model of 1000 patients with a TB prevalence rate of 3% found a 67.5% cartridge savings.	NR	Lower sensitivity for smear-negative TB. Require laboratory infrastructure and training.	Process higher volume of samples with fewer materials and time savings.
Abdurrahman, 2015	11% cartridge savings for hospital-based patients	377 (62%)	The steps involved heightens the potential for errors.	High level of agreement with individual Xpert at reduced cost. Substantial time savings to process hospital samples.
	41% cartridge savings for patients identified through active case finding.		-	Higher cartridge cost and processing time savings for patients identified through active case finding.
Ho, 2017	NR	NR	-	Improved feasibility and cost-effectiveness of large-scale testing. Reduced number of cartridges.
Phuong, 2019	NR	NR	Increased in “error” results when using less buffer for pooling compared to the standard buffer technique.	Reduced costs. Reduced number of cartridges.
Chry, 2020	27% (lower savings estimate using a combination of approaches).	226/876 (26%) for all samples. 300/876 (30%) if a hybrid approach is used.	-	Method is feasible. Potential to reduce costs and higher throughput. Pooling can be used selectively if another

				screening test like x-ray is employed for additional savings (a ‘hybrid approach’).
	34.5% (if used in patients with normal X-Rays)		-	Higher savings if only samples without chest X-rays abnormalities are included.
Santos, 2019	NR	NR	-	Method sensitive and cost-effective.

Discussion

This systematic review synthesizes the available literature on the performance of the pooling method using sputum for GeneXpert testing for detecting pulmonary TB. Although the number of studies is small, the studies reported high sensitivity and specificity for 1:2 and 1:4 pooling ratios, replicating single test results, but pooling >4 samples decreased sensitivity. Studies reporting CT values consistently reported a slight increase in CT values and corresponding lower MTB/RIF semiquantitative results for pooled samples. This result is to be expected because testing samples together necessarily dilutes individual samples. Efficiency gained by pooling samples could increase the resilience of TB diagnostic services in a time when health system resources are being challenged by the COVID-19 pandemic.

The Xpert MTB/RIF Ultra cartridge was expected to help improve the sensitivity of pooled tests because the new assay has a much lower limit for detection than Xpert MTB/RIF [4]. Ultra's improved performance was confirmed by the higher sensitivities reported in 2 studies included in this review, suggesting that Ultra may be preferred over Xpert MTB/RIF for pooled sample testing [27, 32]. Moreover, the only 2 studies reporting specificity (of 99% and 100%) indicated that almost all pools containing all negative individual samples correctly reported negative results for the pooled samples [29-32]. This is an important consideration because the additional steps required to split sputum samples and the need to keep track of sputum batches with a link between individual samples could be prone to cross contamination and error. Further studies are needed to replicate these findings under operational conditions.

Regarding the reproducibility of RIF resistance results in pooled samples, in 1 study from South Africa, all 8 individual RIF-resistant results were detected as pooled RIF-

resistant [28]. However, in a study in Cambodia, 3 samples with RIF-resistant results from individual testing were reported as RIF-susceptible in the pooled testing [32] and in a study from Nigeria, pooling missed 1 of 4 RIF-resistant results [29]. Although pooling seems to be an unreliable method to detect RIF resistance, in practice all samples from MTB-positive pools would be retested individually, which should replicate RIF resistance results from individual samples.

Almost all studies reported anecdotal positive feedback from laboratory staff, and 2 studies [29, 32] quantified savings in cartridge costs and staff time required to process samples. Although both of those studies reported substantial savings, they were conducted in populations with a high proportion of patients testing positive. If a high proportion of presumptive TB patients is expected to be positive, presumably a greater proportion of pools would test positive and require follow-up testing of individual samples. Savings therefore would be more substantial when applied within outreach case-finding activities in the community, where typically around 5% of samples are Xpert MTB/RIF-positive [29] and lower in referral and congregate centers (e.g., prisons), where patients might have a higher probability of having TB. The expected proportion of positive samples may therefore guide the pooling ratio selected for evaluation. For example, in active case finding, it is likely a pool ratio of 1:4 would be highly efficient and generate substantial savings, whereas a ratio of 1:2 would be more suitable for busy TB diagnostic centers where the proportion of samples that are positive can be as high as 15%. Pooling is not likely to be useful at a much higher prevalence than 20%, because most of the pools would be positive and samples would have to be retested individually (B.G. Williams, unpublished data, https://arxiv.org/abs/1007.4903). Moreover, there are operational issues that need further study, as it is unclear whether the timing of sputum splitting could affect

results. For example, splitting samples before adding the GeneXpert buffers requires dividing thick and infectious samples, which are likely to have unevenly distributed bacilli, whereas splitting after adding the buffers could increase the risk of cross contamination but provide a safer and more liquid sample with more evenly distributed bacilli.

To inform national programs, further research is needed to determine the effects on time savings from pooled testing, from sample collection to notification and treatment initiation. Two studies quantified large reductions in testing time from pooling [29, 32], which could shorten turnaround times for patient notification, but time to notification was not reported in any of the studies. Quality management of the pooling process is critical, as reflected in discussions in the studies highlighting the importance of sample management and procedure training. As with routine testing procedures, ensuring that pooling is implemented in a biosafe and quality-assured manner would help mitigate risk to laboratorians from increased sample manipulation and prevent errors in sample handling and testing, which could reduce efficiency and benefit to both patients and programs.

Our findings are especially relevant during the ongoing global COVID-19 pandemic, which is severely disrupting health services, the availability of diagnostic and treatment resources, supply chains, and other disease control efforts. Although the diagnosis of COVID-19 takes precedence, steps can be taken to preserve key services for diagnosing and treating patients with presumptive TB. Quarantine and restriction of movement during the pandemic have limited accessibility to services and reduced the numbers of patients attending TB diagnostic and treatment centers. Confinement of the population to households and the resulting increase in contact with other household members in crowded conditions could increase TB transmission. A surge

in undetected cases, together with increases in treatment interruptions, will likely lead to increases in incident cases. Demand for testing also may cause severe resource constraints. Preparing for this scenario, such as by introducing pooling strategies, may result in more efficient use of limited resources.

Before the COVID-19 pandemic, the World Health Organization issued guidelines promoting a rapid diagnostic test, such as a GeneXpert-based test, for all persons with presumptive TB [33]. However, <20% of the GeneXpert TB tests necessary to test the estimated 100 million people who develop presumptive TB each year have been procured [19]. Individual rapid molecular diagnostic testing for all patients with presumptive TB remains the standard of care and a goal for national TB programs worldwide, but the cost of individually testing all estimated symptomatic persons using GeneXpert would have been more than US $1 billion in cartridges alone in 2018 [19], more than the total amount of funding provided by international donors globally for TB in 2019 [34]. Moreover, although passive case finding has long been the standard approach in many countries, it is becoming apparent that outreach beyond health facilities is needed to identify those with TB missed by programs [35]. Increasing outreach activities usually means more testing, requiring more cartridges, will be needed. However, a typically greater negative-to-positive testing ratio in persons identified through outreach activities means that pooling strategies might decrease costs.

Despite the potential usefulness of our findings, the quality of evidence we present remains insufficient to support wide adoption of the pooling method. Because the 6 studies were heterogeneous, we were unable to conduct a meta-analysis, and we considered all the studies together with bench evaluations of the technical sensitivity and specificity of the methods; our findings should therefore be considered

hypothesis-generating to promote and inform further studies. Moreover, all studies were underpowered for investigating the performance of the pooled testing method in subpopulations (e.g., HIV-positive vs. HIV-negative, men vs. women), and very few samples tested rifampin resistant. CT values also need to be interpreted with caution.

Although both Xpert MTB/RIF and Ultra tests report CT values, the test algorithms that determine their CT and semiquantitative results differ, which impacts the interpretation of CT-based analyses. Moreover, because CT ranges vary between multiple tests on the same homogenized sample, it would have been preferable to describe changes in positivity relative to the semiquantitative results. However, semiquantitative results were not reported in most studies. Similarly, although culture was used in some of the studies, this information was not used to stratify analyses. A second reference method would have been useful to further investigate whether discordant results were potentially due to improper sample management, cross-contamination in the laboratory, or random variation due to the bacilli not being homogeneously distributed in the sputum sample.

Conclusions

Despite these limitations, we propose that the pooling method be considered as an interim option to strengthen capacity of TB laboratories during times of crisis, such as during the COVID-19 pandemic. Our team is currently conducting accelerated evaluations of the pooling method in Lao PDR and Nigeria. We encourage the TB community to conduct studies on the pooling strategy and other resource-saving strategies for TB diagnostic testing that generates data for open access databases to inform national programs.

Chapter II - Pooling sputum for Xpert MTB/RIF and Xpert Ultra testing during the COVID-19 pandemic in Lao People's Democratic Republic

As published in PLOS Global Public Health (Published: April 4, 2022)

Citations as follows: Iem V, Chittamany P, Suthepmany S, et al. Pooling sputum for Xpert MTB/RIF and Xpert Ultra testing during the Covid-19 pandemic in Lao People's Democratic Republic. PLOS Global Public Health 2022: 2(4): e0000116.

https://doi.org/10.1371/journal.pgph.0000116

Introductory section

Findings from the systematic literature review in Chapter I show that GeneXpert-based testing of pooled sputum samples achieves sensitivity and specificity similar to testing individual samples.

Chapter II and III assess the performance of the pooled testing using both GeneXpert MTB/RIF and Ultra, with pools of four samples, in order to provide more evidence to the emerging body literature on the performance of the pooled testing. These studies were conducted under routine passive case findings condition to recreate the real workflow if the pooled testing was implemented routinely in Lao PDR and Nigeria. The study in Chapter II was conducted from March to May 2020 and from January to March 2021 just before COVID lockdown and quarantine measures were put in place in Lao PDR. The study in Chapter III was conducted from March to August 2020 in parallel with the study in Chapter II.

It is also important to demonstrate that pooled testing is appropriate in any setting where individual testing is normally implemented. Therefore, the following Chapter IV is a reproduction of the same study design but during outreach active case findings campaigns in Lao PDR to demonstrate the reproducibility of the results and the feasibility of implementing the pooled testing in a different setting.

Abstract

Background: The global COVID-19 pandemic has limited access to molecular TB diagnostics and National Programmes are struggling to maintain essential services. The pooling method (testing several samples together) could reduce the number of cartridges and staff time needed for TB diagnosis but has not been tested within the pandemic.

Methods: We conducted two independent cross-sectional surveys. Pools composed of four sputum samples were tested using either Xpert-MTB/RIF or Xpert-Ultra. Pooled and individual results were compared to determine the level of agreement.

Results: Each survey included 840 participants and 210 pools. In the Xpert MTB/RIF survey, 77/81 (sensitivity 95.1%, 95% CI 87.8%-98.6%) pools containing ≥1 positive sample tested MTB-positive and 4/81 (4.9%, 95% CI 1.4%-12.2%) tested MTB-negative. All 129/129 pools containing MTB-negative samples tested MTB-negative (specificity 100%, 95% CI 97.2%-100%), with 98.1% agreement (Kappa: 0.959). In the Xpert-Ultra survey, 70/70 (sensitivity 100%, 95% CI 94.9%-100%) pools containing ≥1 MTB-positive sample tested MTB-positive and 140/140 (specificity 100%, 95% CI 97.4%-100%) pools containing only MTB-negative samples tested MTB-negative, with 100% agreement (Kappa: 1). Pooled testing with Xpert-MTB/RIF and Xpert-Ultra saved 38.3% and 41.7% (322/840 and 350/840, respectively) in cartridge costs alone.

Conclusions: The pooling method with Xpert-MTB/RIF and Xpert-Ultra has similar performance to individual testing and can reduce the number of cartridges needed. These efficiencies can facilitate maintenance of stocks and sustain essential services as countries face difficulties for laboratory procurement during the pandemic and will provide cost and time savings post-pandemic.

Introduction

Tuberculosis (TB) is a major cause of morbidity and death worldwide, with an estimated 10 million people falling ill and 1.4 million deaths occurring in 2019 alone [34]. Despite its public health importance, three million people with TB are missed by national TB programmes (NTPs) every year [34], due to accessibility barriers, and diagnosis, treatment and notification gaps [36].

The World Health Organization (WHO) recommends testing individuals with presumptive TB with molecular assays as the first test for bacteriological confirmation [11]. These tests include Xpert MTB/RIF and Xpert MTB/RIF Ultra (Xpert Ultra), which are automated and simultaneously detect *Mycobacterium tuberculosis* complex and markers of rifampicin resistance [37] and the latter, is currently recommended in preference to Xpert MTB/RIF, based on its increased sensitivity, which improves the detection of paucibacillary forms of TB [5]. However, despite major international initiatives to increase the use of molecular assays, the majority of TB diagnoses in low and middle income countries are based on smear microscopy, which has lower sensitivity [38] and does not detect drug resistance, but is locally available and has lower costs than molecular assays [39].

Lao's People's Democratic Republic (PDR) had an estimated incidence of 155 people with TB per 100,000 population in 2019 and has improved TB case detection in recent years, with the number of people detected increasing from 44 per 100,000 population in 2000 to 95 per 100,000 in 2019. Lao PDR has a low prevalence of rifampicin resistance-TB with 1.2% (95% CI: 0.5–2.0%) and 4.1% (95% CI: 0–9.6%) among new cases and previously treated cases, respectively, and a low prevalence of multi-drug resistance TB (MDR-TB) with 0.5% (95% CI: 0–1.0%) and 2.3% (95%

CI: 0–6.7%), respectively [40]. Improved detection is partly due to concentrated efforts to identify people with presumptive TB and an increased use of Xpert as the first test for diagnosis, with 66% of people with presumptive TB tested with Xpert MTB/RIF in 2019 [41], and the country aims to provide universal Xpert testing from 2021 onwards. These ambitious targets, however, would require considerable increases in cartridges, GeneXpert instruments and human resources, resulting in higher costs.

At the end of 2019, coronavirus disease-19 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) turned into an epidemic that triggered chaos in hospitals and primary care services [42]. On the 30th of January 2020 the WHO declared this outbreak a Public Health Emergency of International Concern [43] and countries with limited laboratory resources, such as Lao PDR, were requested to share existing GeneXpert platforms for both COVID-19 and TB testing [44]. Lockdowns and reassignments of health personnel and equipment away from TB created a devastating impact on the performance of NTPs, especially in low- and middle-income countries [45].

To address these challenges, we evaluated whether combining specimens of four individuals with presumptive TB in a pool and testing the pool using either Xpert MTB/RIF or Xpert Ultra would result in the same accuracy as testing samples individually and estimated whether the approach would result in assay cost savings. In the pooling method, when a pool tests positive, all individual samples included in that pool are re-tested individually to identify the positive sample(s), while if the pool tests negative, it is assumed all samples included are negative. Pooling could then be used to test larger numbers of people with the same number of cartridges, thus increasing the efficiency of Xpert-based testing in locations with limited resources [17].

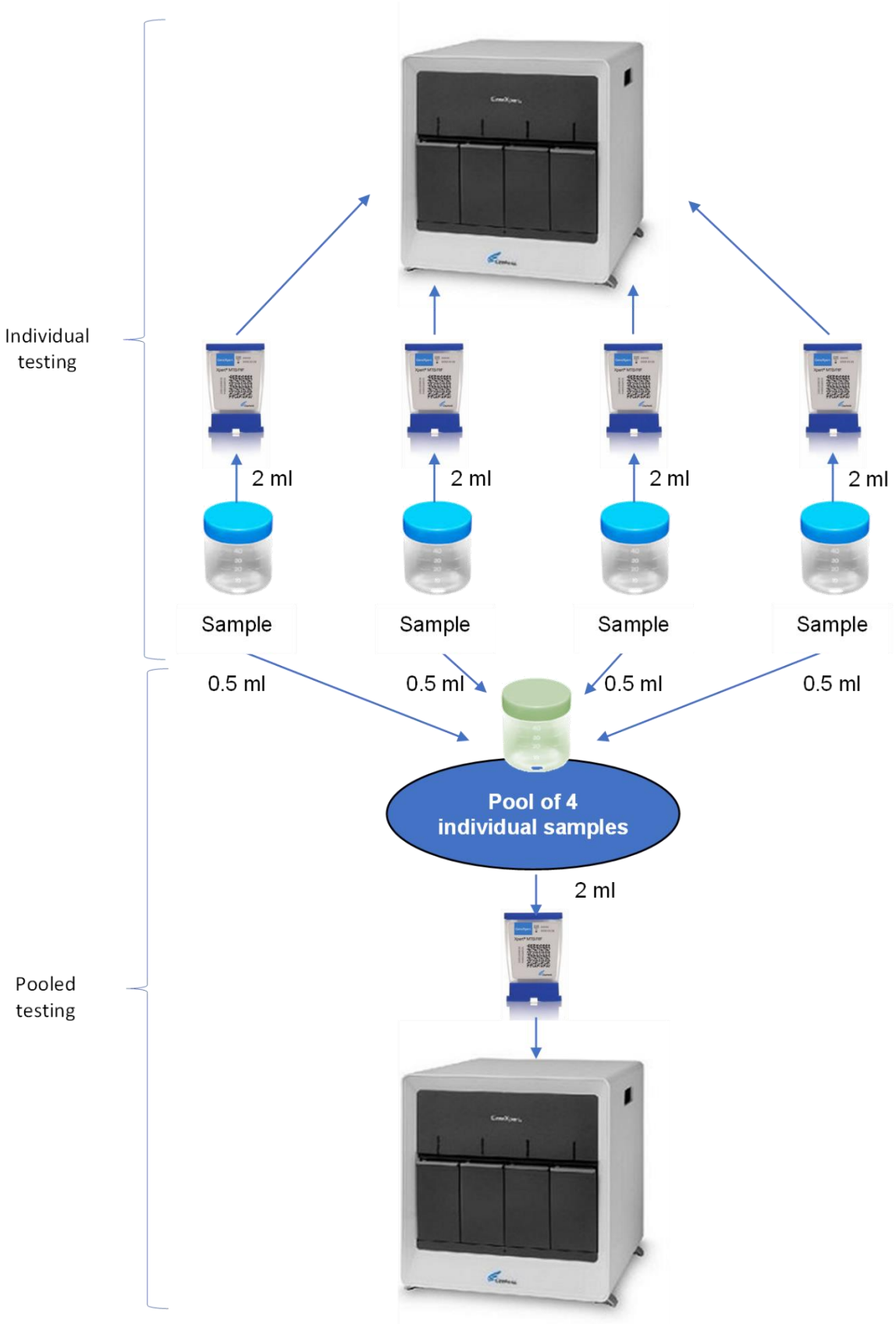
Materials and methods

The study took place at Mahosot hospital and nine district health facilities in Vientiane, the capital of Lao PDR, and included individuals presenting to the TB diagnostic services with a diagnosis of presumptive TB. The national technical guidelines (NTG) define a person with presumptive TB as an individual with cough for 2 weeks duration or with two or more symptoms including cough, hemoptysis, weight loss, fever, night sweats, tiredness, chest pain, dyspnea, or the presence of chest X-ray abnormalities suggestive of TB. The guidelines recommend requesting one sputum sample from all individuals with presumptive TB and to examine the sample using Xpert MTB/RIF or Xpert Ultra, as available. Sputum samples were collected on the spot at the time individuals presented to the clinics and were sent to the National TB Reference Laboratory at the National TB Control Center in Vientiane. The study consisted of two separate cross-sectional surveys, with the first taking place from March to May 2020 (called the 2020 survey) and the second survey from January to March 2021 (the 2021 survey). All individual and pooled samples in the 2020 survey were tested using Xpert MTB/RIF and all individual and pooled samples in the 2021 survey were tested using Xpert Ultra, at a time when Xpert Ultra had become available in the country.

All Xpert MTB/RIF and Xpert Ultra testing followed the manufacturer's instructions. Briefly, the sample reagent was added to the sample at a 2:1 ratio, mixed on a vortex and left at room temperature for 10 minutes. The sample was then vortexed again and left to stand for a further five minutes. Two ml of the sputum sample were then loaded into the Xpert cartridge for individual testing [3] and the remnant of the specimens were grouped into batches of four to prepare the pools for testing. Pools of four were selected because in settings such as Lao PDR the proportion of positive samples is

between 1% and 30% and this pool size may be close to optimal [46]. The pools of specimens were created using consecutive samples without knowing the results of the individual tests. An equal volume of 0.5 ml of each of the four individual pre-treated samples was added to a new cup and the cup was vortexed and loaded into a new Xpert cartridge (Figure 2.1). Pooled samples were tested in batches, independently of individual tests.

Figure 2.1. Flow diagram of sputum pooling and processing



Xpert trace calls were considered as MTB-positive as per the NTG, and patients were re-tested with a new sample to determine the rifampicin resistance status. Individual Xpert results were communicated back to the diagnostic centers and were the only Xpert test result used for patient management, while pooled sputum results were only used for research purposes and were not reported to the clinicians nor the patients.

Statistical analysis

Categorical data were summarized using descriptive statistics, with chi-squared tests used to test for statistically significant differences, where appropriate. Results obtained with the pooled samples were compared with the four individual results for both Xpert MTB/RIF and Xpert Ultra. The agreement of the pooled and individual tests was tested using kappa statistics. We compared the CT values and the Xpert grades (trace, very low, low, medium, and high) for individual tests for concordance with the results from pools containing a single positive test. Cost differences were calculated on the basis of the number of cartridges that would have been required to test all specimens when using either a pooled or an individual testing strategy. We then modeled the potential cost savings from our results for testing 1,000 consecutive individuals with Xpert at the FIND negotiated cartridge cost of USD 9.98 [47] and calculated the additional people tested for TB when using 1,000 cartridges with the pooling method.

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable requests for guideline development and systematic reviews.

Need for ethical approval and informed consent were waived by the National TB Control Center of Lao PDR and the Liverpool School of Tropical Medicine Research Ethics Committee, UK (Ethical waiver 20-037).

Results

A total of 1680 participants were included, with 840 participants tested with Xpert MTB/RIF in the 2020 survey and 840 with Xpert Ultra in the 2021 survey (S1 Data).

Xpert MTB/RIF survey

In the 2020 survey, 491/840 (58.5%) participants were male and 349 (41.5%) female and 102/840 (12.1%) were Xpert MTB/RIF MTB-positive (Table 2.1). Males were more likely to be Xpert MTB-positive than females (72/491 (14.7%) and 30/349 (8.6%), p-value < 0.1, Chi-Square test), respectively.

Table 2.1. Baseline characteristics of participants with single and pooled Xpert results.

		Xpert MTB/RIF	Xpert Ultra
		All n= 840 (%)	**All n= 840 (%)**
Sex	Male	491 (58.5)	500 (59.5)
	Female	349 (41.5)	340 (40.5)
Age (Mean (sd, range))		49 (19.3, 1-98)	51 (19.6, 1-96)
Age group (years)	<35	237 (28.2)	210 (25.0)
	35-54	238 (28.3)	207 (24.6)
	>=55	365 (43.5)	423 (50.4)
Individual Xpert Result	Detected	102 (12.1)	100 (11.9)
	Not detected	738 (87.9)	740 (88.1)
	Male MTB detected	72/491 (14.7)	59/500 (11.8)
	Female MTB detected	30/349 (8.6)	41/340 (12.1)
		n= 102	**n= 100**
MTB Grade	Trace	NA	14 (14.0)
	Very low	17 (16.7)	10 (10.0)
	Low	22 (21.6)	27 (27.0)
	Medium	44 (43.1)	9 (9.0)
	High	19 (18.6)	40 (40.0)
		n= 102	**n= 100**
Rif Resistance	Detected	0 (0.0)	1 (1.0)
	Not detected	102 (100.0)	85 (85.0)
	Indeterminate	0 (0.0)	14 (14.0)
		n= 210	**n= 210**
Samples tested in the pool	≥ 1 MTB-positive sample	81 (38.6)	70 (33.3)
	4 MTB-negative samples	129 (61.4)	140 (66.7)
Pooled Xpert MTB result	Detected	77 (36.7)	70 (33.3)
	Not detected	133 (63.3)	140 (66.7)
		n= 77	**n= 70**
Pooled MTB Grade	Trace	NA	11 (15.7)
	Very low	19 (24.7)	12 (17.1)
	Low	27 (35.1)	24 (34.3)
	Medium	24 (31.2)	6 (8.6)
	High	7 (9.1)	17 (24.3)
		n= 77	**n= 70**
Pooled Rif Resistance	Detected	0 (0.0)	2 (2.9)
	Not detected	77 (100.0)	57 (81.4)
	Indeterminate	0 (0.0)	11 (15.7)

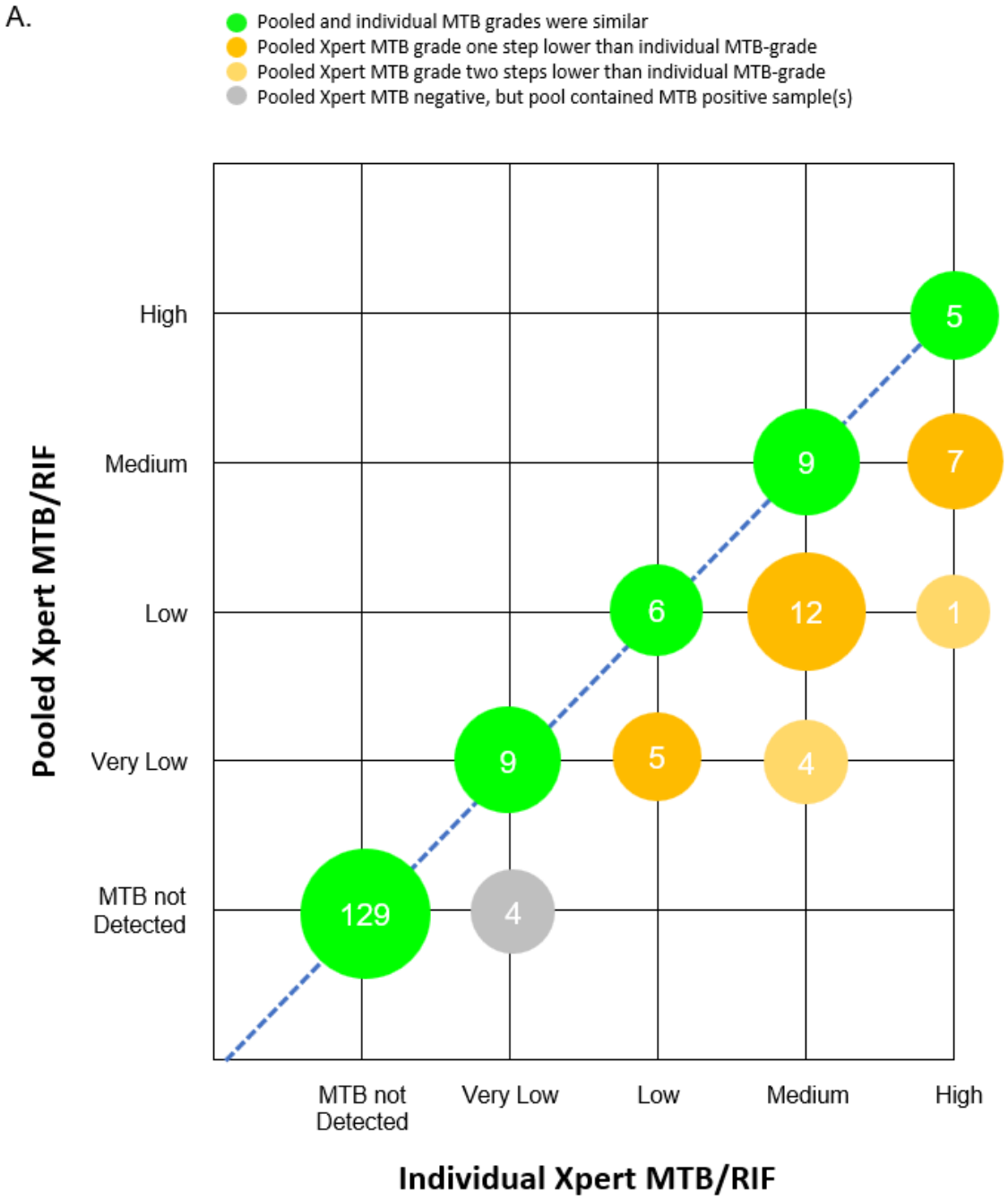
Individual samples were tested in 210 pools. Of these, 81 (38.6%) pools contained at least one Xpert MTB-positive sample and 129 (61.4%) had only Xpert MTB-negative samples. Sixty-two (75%) of the 81 pools with MTB-positive samples contained only one MTB-positive sample, 17 (21%) contained two MTB-positive and two (2.5%) pools contained three MTB-positive samples (Table 2.2). Seventy-seven (sensitivity 95.1%, 95%CI 87.8% - 98.6%) of the 81 pools with MTB-positive samples tested Xpert MTB-positive in the pooled assay and four (4.9%, 95%CI 1.4% - 12.2%) tested negative. None (0%) of the 129 pools containing only MTB-negative samples returned a pooled Xpert MTB-positive result, resulting in 100% (95%CI 97.2% - 100%) specificity. The agreement between the individual and pooled approaches was 98.1% (Kappa: 0.959).

Table 2.2. Distribution of positive individual samples among pooled results, by Xpert test

	Number of positive Xpert results included in a pool					
	All negative n (%)	**One positive n (%)**	**Two positive n (%)**	**Three positive n (%)**	**Four positive n (%)**	**All n (%)**
Pooled Xpert TB/RIF	**129**	**62**	**17**	**2**	**0**	**210**
Detected	0 (0%)	58 (93.5%)	17 (100%)	2 (100%)	0 (0%)	77 (36.7%)
Not detected	129 (100%)	4 (6.5%)	0 (0%)	0 (0%)	0 (0%)	133 (63.3%)
Pooled Xpert Ultra	**140**	**45**	**20**	**5**	**0**	**210**
Detected	0 (0%)	45 (100%)	20 (100%)	5 (100%)	0 (0%)	70 (33.3%)
Not detected	140 (100%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	140 (66.7%)

Thirteen individual samples tested with Xpert MTB/RIF had *very low*, 11 *low*, 25 *medium* and 13 *high* MTB-grades. The grades for the pools containing single MTB-positive samples are shown in Figure 2.2A. The MTB-grade was the same in 29/62 (46.8%) individual and pooled tests and discrepant in 33/62 (53.2%). The discrepancies were always in the same direction, with the pooled MTB-grade being one grade lower than the individual MTB-grade in 28/33 (85%) and two steps lower in five (15%) of the discrepant samples. The four pools testing MTB-negative by the pooled Xpert MTB/RIF but positive by the individual test had *very low* individual MTB-grades (Figure 2.2A).

Figure 2.2A. Correlation of individual and pooled Xpert MTB/RIF grades (positive pools only include those with one individual Xpert MTB-positive sample).



The median CT values of the Xpert MTB/RIF probes are shown in Table 2.3. Individual A-E probes had median CTs ranging from 20.4 to 21.9. Pooled assays had higher CT values with CT values ranging from 23.8 to 25.0, with difference between individual and pooled assays ranging from 2.8 to 3.6 CTs. Lastly, none of the 840 samples tested were Xpert RIF-positive or indeterminate. Consequently, all pools with

MTB-positive samples contained only RIF-negative samples and none of them reported pooled RIF-positive results.

Table 2.3. Median CT values of individual and pooled Xpert MTB/RIF and Xpert Ultra probe results.

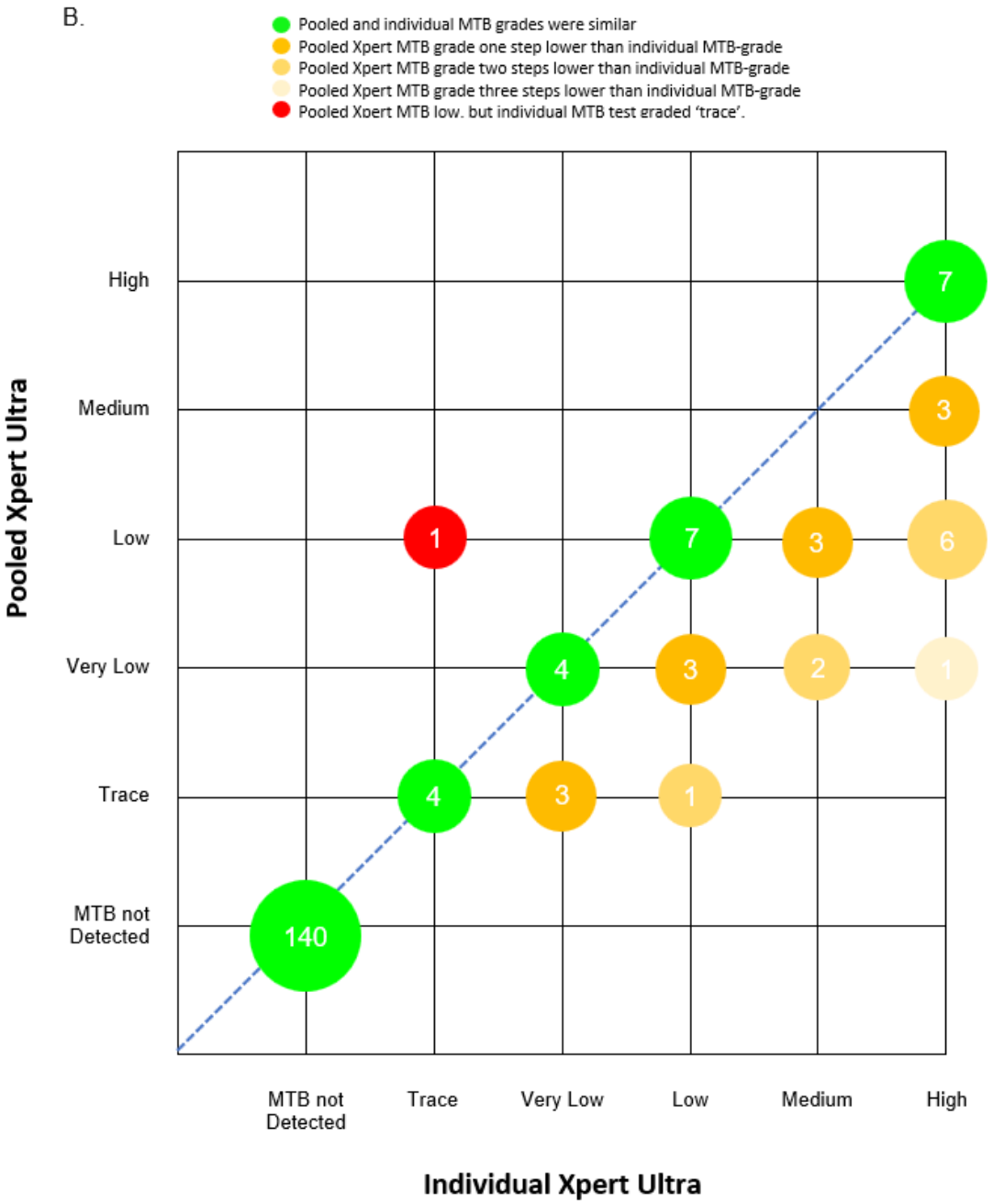
Xpert MTB RIF					
	Individual results n=102		**Pooled results n=77**		
Probe	**CT Median IQ range**	**Min-Max**	**CT Median IQ range**	**Min-Max**	**ΔCT**
Probe D	21.5 (18.1, 27.1)	11.9, 35.4	24.9 (20.6, 28.7)	13.2, 36.3	3.4
Probe C	20.7 (17.6, 26.3)	11.5, 34.1	24.3 (19.8, 28.0)	11.8, 33.5	3.6
Probe E	22.0 (18.6, 27.2)	12.8, 36.6	25.0 (21.0, 29.9)	13.6, 36.8	3.0
Probe B	21.7 (18.5, 26.5)	12.6, 33.8	24.5 (20.9, 27.8)	13.3, 34.3	2.8
Probe A	20.4 (16.9, 25.8)	11.2, 34.0	23.8 (19.9, 28.5)	11.7, 34.6	3.4
Xpert Ultra					
	Individual results n=100		**Pooled results n=70**		
Probe	**CT Median IQ range**	**Min-Max**	**CT Median IQ range**	**Min-Max**	**ΔCT**
Probe IS1081/ IS6110	16.8 (16.2, 21.3)	15.9, 31.0	18.1 (16.2, 22.2)	16.0, 30.0	1.3
Probe rpoB1	18.3 (17.4, 22.7)	0.0, 36.5	20.7 (17.9, 25.7)	0.0, 39.5	2.4
Probe rpoB2	18.2 (17.4, 22.5)	0.0, 36.0	20.6 (17.7, 25.6)	0.0, 35.7	2.4
Probe rpoB3	19.8 (18.6, 24.6)	0.0, 37.5	21.9 (19.0, 27.1)	0.0, 37.7	2.1
Probe rpoB4	21.4 (20.3, 26.4)	0.0, 37.8	24.1 (20.6, 28.8)	0.0, 39.4	2.7

Xpert Ultra survey

In the 2021 survey, 500/840 (59.5%) participants were male and 340/840 (40.5%) female and 100/840 (11.9%) were Xpert Ultra MTB-positive (Table 2.1). Males and females were equally likely to be Xpert Ultra MTB-positive (59/500 (11.9%) and 41/340 (12.1%), respectively, p-value > 0.1, Table 2.1). Individual samples were tested in 210 pools and of these, 70 contained at least one MTB-positive sample and 140 contained only MTB-negative samples. Among the 70 pools with MTB-positive samples, 45 contained one MTB-positive, 20 contained two MTB-positive and five contained three MTB-positive samples, as shown in Table 2.2. All 70/70 pools containing at least one MTB-positive sample tested Xpert MTB-positive in the pooled assay, resulting in 100% (95%CI 94.9% - 100%) sensitivity, and all 140/140 pools containing only MTB-negative samples tested Xpert MTB-negative (specificity 100%, 95%CI 97.4% - 100%) and 100% agreement (Kappa: 1). Seven individual samples tested with Xpert Ultra had *very low*, 11 *low*, 5 *medium* and 17 *high* MTB-grades, with 5 samples reporting *trace* results, as shown in Figure 2.2B. The MTB-grades coincided in 22/45 (48.9%) individual and pooled tests and was discrepant in 23/45 (51.1%). Similar to the Xpert MTB/RIF survey, in all but one of the discrepancies the pooled MTB-grade was lower than the MTB-grade of the individual test (Figure 2.2B). The CT values of the Xpert Ultra probes are shown in Table 2.3. Individual probes (IS1081/IS6110 and rpoB1-B4) had median CTs ranging from 16.7 to 21.4 and pooled CTs ranged from 18.1 to 24.1, with a CT difference between individual and pooled assays ranging from 1.3 to 2.7. Only one (1%) of the 100 MTB-positive samples was RIF-positive, 14/100 (14%) were RIF-indeterminate and 85/100 (85%) RIF-negative. Among the 70 pools containing MTB-positive samples, 1/70 (1.4%) had the single RIF positive sample, 12/70 (17.1%) contained a single RIF-

indeterminate and 1/70 (1.4%) two RIF-indeterminate samples. The RIF-positive sample tested RIF-positive in the pooled test. Four of the 12 pools containing single RIF-indeterminate samples tested pooled RIF-indeterminate and eight tested RIF-negative. The pool containing two RIF-indeterminate samples tested pooled RIF-indeterminate. Of the 56 pools containing solely RIF-negative samples, 49/56 (88%) tested pooled RIF-negative, 6/56 (11%) pooled RIF-indeterminate and 1/56 (2%) RIF-positive.

Figure 2.2B. Correlation of individual and pooled Ultra grades (positive pools only include those with one individual Xpert MTB-positive sample).



Xpert MTB/RIF and Xpert Ultra savings

The number of cartridges required to test the 840 individuals using the pooling method was estimated for both surveys. Testing 210 pools with Xpert MTB/RIF required 210 cartridges, and 77 were MTB-positive. The MTB-positive pools required re-testing the individual samples to identify the positive sample/s in the pool, and this required 308 (77x4) additional test and a total of 518 Xpert MTB/RIF cartridges. The pool method therefore resulted in a saving of 322/840 (38.3%) cartridges (840 - 518). Similarly, the pooling method required 210 cartridges to test in pools and 280 additional cartridges to test the individual samples of the 70 positive pools (70X4), resulting in a total of 490 (210 + 280) Xpert Ultra cartridges. The pooling method therefore would result in a saving of 350 (840 – 490, or 41.7%, n=350/840) cartridges. The results of the extrapolation to illustrate the cartridge savings achieved when screening 1,000 consecutive individuals and the number of individuals that could be tested with a fixed number of 1,000 cartridges are shown in Table 2.4. Cartridge costs for testing 1,000 individuals would amount USD 9,980, and the pooling method would cost USD 6,158 and USD 5,818 for Xpert MTB/RIF and Xpert Ultra, respectively, resulting in USD 3,822 and USD 4,161 savings, respectively. Alternatively, given its efficiency, using the pooling method with a fixed number of 1,000 Xpert MTB/RIF and Xpert Ultra cartridges would allow testing 1,620 and 1,715 patients, reducing the effective cartridge cost per individual screened to USD 6.16 and USD 5.80, respectively.

Table 2.4. Cost and savings to screen consecutive patients using the pooling method and number of patients that could be tested with Xpert MTB/RIF and Xpert Ultra cartridges.

	Individual Xpert		**Pooled Xpert**	
	MTB/RIF	**Ultra**	**MTB/RIF**	**Ultra**
Number of individuals tested	1000	1000	1000	1000
Sensitivity	reference	reference	95.1%*	100%*
Specificity	reference	reference	100%*	100%*
Proportion positive	12.1%*	11.1%*	36.7% pools	33.3% pools
Bacteriologically confirmed	121	111	115	111
Cartridges required	1000	1000	617	583
Cartridge costs (USD)	9,980	9,980	6,158	5,818
Cartridge savings (USD)	0	0	**3822 (38.3%)**	**4161 (41.7%)**
Number tested with 1000 cartridges				
Number tested	1000	1000	1620	1715
Cartridge cost per patient (USD)	9.98	9.98	**6.16**	**5.80**

* Assumes pools of 1:4; proportion positive taken from the surveys' findings

Discussion

This is the first report directly comparing testing pooling samples for TB using the Xpert MTB/RIF and Xpert Ultra in the same population setting and study methods. Samples for the surveys were collected and tested at the time the country had implemented quarantine measures to reduce the spread of SARS-CoV-2 infections and staff had been re-deployed in response to the COVID-19 pandemic and thus was conducted at a time when human resources were strained.

Our study adds to the emerging body of evidence that the pooling methods for testing with molecular assays can improve the efficiency of testing for TB, potentially enabling the screening and testing of larger numbers of people more cost-effectively. Our findings confirm that there is a good correlation between the results of the individual and pooled tests, with a low frequency of false-negative results and a high degree of specificity. Our findings support previous studies indicating that pooled Xpert MTB/RIF detects about 95% of MTB-positive samples and that pooled Xpert Ultra can yield full agreement between individual and pooled Xpert Ultra testing, as previously reported from Cambodia [32]. The higher agreement of Xpert Ultra is likely due to its higher sensitivity [29, 30], as its limit of detection (15.6 cfu/ml) [4] is lower than for Xpert MTB/RIF's (131 cfu/ml) [48], thus reducing the risk of the diluted TB DNA falling below the detection limit. All false Xpert MTB-negative results occurred among individual samples containing high CT values that were graded MTB-very low, which corresponded to the increasing CT values of the individual probes. Given the complete agreement between pooled and individual testing with Xpert Ultra, countries with limited testing resources could consider using the pooling method as a routine practice. Lao PDR's NTC will phase out Xpert MTB/RIF once stocks are depleted and will replace it with Xpert Ultra from 2022.

The program is considering the adoption of pooling for TB once endorsed by WHO guidance.

It is important to note that our study was conducted among adults with a low prevalence of HIV (0.17%) [49] and that very few participants had dual TB-HIV co-infections, as only 5% of new TB cases in Lao PDR occur among HIV-infected individuals [41]. Individuals with HIV often present with paucibacillary TB and a systematic review in HIV prevalent settings have reported that the sensitivity of pooled testing may be lower [50], with a higher sensitivity achieved when testing with Xpert Ultra (87.6%, 95%CI 75.4 - 94.1%) than with Xpert MTB/RIF (74.9%, 95%CI 58.7 - 86.2) [51]. Similarly, our findings may be different to those observed in studies conducted during active TB case finding interventions, where the proportion of individuals with positive tests is much lower (typically less than 5%) and paradoxically patients may be identified at very early or late stages of the disease [52, 53] and therefore further studies are needed among populations with high HIV prevalence and in locations where the proportion of individuals testing positive is low.

In terms of specificity, our results confirm the high specificity of pooled testing for both Xpert MTB/RIF and Xpert Ultra, with none of the MTB-negative samples becoming positive in the pooled tests. Some studies however have reported a slightly lower specificity [4, 54], which may be attributed to the increased manipulation of samples resulting in an increased risk of contamination and labelling errors and these varying results may reflect the competency and dedicated time available of laboratory staff for sample processing. Positive predictive value, the probability that subjects with a positive result truly have the disease, can be increased. This is because a truly negative sample will need to go through an additional round of testing in pooled testing strategy, and therefore the probability of a false-positive result will become

much smaller. In addition, a lower sensitivity and resultant negative predictive value (the probability that subjects with a negative result truly do not have the disease) showed that pooled testing is likely to miss the detection of paucibacillary samples. These results suggest potentially for missed case detection which could pose a risk of community transmission of TB from undetected sources.

Our results also confirm that testing for RIF resistance in the pooled assays is unreliable for Xpert Ultra, as four pools containing individual Xpert RIF-indeterminate samples tested RIF-negative when tested in a pool and pools containing only RIF-negative samples tested pooled RIF-indeterminate. The same issues have been reported for Xpert MTB/RIF, but all samples tested in our study were RIF-negative. False-positive rifampicin resistance is not unusual in paucibacillary samples [55, 56], and more than half of Xpert Ultra false-positive rifampicin resistance results were obtained from individuals with MTB trace results [54]. However, since the pooling method requires repeating individually all samples from pools testing MTB-positive, this issue would not have misclassified individuals in routine practice.

Interestingly, men were more likely to be Xpert MTB-positive (p-value < 0.1, Chi-Square test) but men and women were equally likely to be Xpert Ultra MTB-positive (p-value > 0.1, Chi-Square test). The higher sensitivity of Xpert Ultra may compensate for women not being comfortable when coughing and expectorate sputum, leading to sub-optimal specimen quality. The impact of Ultra on the diagnosis of TB in women is a finding that suggests the need for further studies, especially those which could use gender-disaggregated data to improve planning and resource prioritization for underserved populations as well as ensuring integration of gender and social inclusion dimensions.

The pooling method resulted in savings ranging from 38%-42% in cartridge costs, allowing testing more patients with a limited number of cartridges. Over the course of a year, potential savings from such an approach are large, even with a single machine, and many more people would be tested and diagnosed using the pooling method. In our setting, 620-715 (60-70%) additional TB patients could be tested with the same cost of resources, which would facilitate closing the country-wide testing gap. Cartridges and time savings however are directly related to the proportion of pools that are positive, and this proportion would change with TB prevalence and populations tested. Savings therefore may be larger in locations with low prevalence and during active case finding, when the proportion of pools testing positive may be lower. The savings presented here therefore may underestimate actual savings. Moreover, we did not estimate other savings, such as staff time, electricity, overhead costs, and costs to patients and their carers. The pooling method therefore can be particularly important at a time when procurement and importation of laboratory consumables is limited due to the pandemic, and when staff had been re-deployed to SAR-CoV-2 testing.

Conclusions

The pooling method has high sensitivity and specificity for both Xpert MTB/RIF and Xpert Ultra, with the latter resulting in full agreement between individual and pooled testing. Pooled testing resulted in significant cartridge savings and facilitated more efficient testing within the pandemic, when financial resources are stretched, and the health system is strained. These promising results call for more studies to assess the potential of the pooling method in populations with low TB prevalence, such as outreach active case finding campaigns, where the proportion of people with bacteriologically confirmed TB is usually lower, as it could result in significantly

higher savings. The pooling method would support the WHO End TB strategy, urging countries to expand access to rapid molecular tests for the detection of TB. In a context where countries may experience stock-outs or delays in laboratory commodities procurement due to the COVID-19 lockdown, pooling may be the optimal diagnostic option for individuals with presumptive TB.

Chapter III - Pooling sputum in Nigeria for Xpert MTB/RIF and Xpert Ultra testing for tuberculosis during the COVID-19 pandemic

As published in Public Health Action (Published: March 21, 2023)

Citations as follows: Bimba J[†], Adekeye OA[†], **Iem V[†]**, Eliya T, Osagie I, Kontogianni K, Edwards T, Dodd J, Squire SB, Creswell J, Cuevas LE. Pooling sputum samples for Xpert® MTB/RIF and Xpert® Ultra testing for TB diagnosis. *Public Health Action* 2023: 13(1): 12-16.

[†] **Equal contributions**

https://doi.org/10.5588/pha.22.0052

Abstract

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%, κ = 0.867). Five hundred additional participants 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%, κ = 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.

Introduction

Despite intensive efforts since 1993, when the WHO declared TB a global emergency [57], 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 [9].

The WHO recommends using molecular assays as the first test for examination of individuals with presumptive TB [33]. The assays most widely used are the Xpert® MTB/RIF (MTB/RIF; Cepheid, Sunnyvale, CA, USA) and Xpert® MTB/RIF Ultra (Ultra; Cepheid) assays [58]. 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 centralized 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 [17]. 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 [29, 32]. 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 [59]), has the second highest TB burden in Africa, with an estimated 467,000 people with TB in 2021 [9]. However, under-detection is a major problem, and only 204,700 (43.8%) people with TB were notified [9]. The country is thus one of the 10 countries accounting for 77% of the global gap in TB detection and notification [9]; 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.

Materials and 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. 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 3.1). 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 ($\chi 2$ 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 3.2).

Table 3.1. Baseline characteristics of participants with individual Xpert MTB/RIF and Ultra results

		Xpert MTB/RIF		Xpert Ultra	
		All n= (%)	**Positive n= (%)**	**All n= (%)**	**Positive n= (%)**
	Number of participants	**500**	**72**	**500**	**60**
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	**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	**Number of participants**	**500**	**72**	**495**	**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)

Table 3.2. Number of pools with 0, 1, 2, 3 and 4 positive results[1]

	Individual Xpert results included in a pool*					
	Four negatives n (%)	**One positive n (%)**	**Two positives n (%)**	**Three positives n (%)**	**Four positives n (%)**	**All n (%)**
Pooled results						
Xpert MTB RIF	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	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 shown in bold

[1] Non-valid results have not been recorded systematically. Tests with non-valid results reported on the initial test were repeated, and there were no non-valid results reported for individual MTB/RIF tests and only one error for Ultra after re-testing

Xpert MTB/RIF survey

Of 500 individuals who underwent MTB/RIF testing, 72 (14.4%) were MTB-positive and 428 MTB-negative (Table 3.1). 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 3.2). 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 3.3). The overall agreement was 93.6% (n = 117/125, κ = 0.867; Table 3.4).

Table 3.3. Results of pooled and individual Xpert testing

	Xpert MTB/RIF		**Xpert Ultra**	
	Individual	**Pooled**	**Individual**	**Pooled**
MTB Result	**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)

Table 3.4. Agreement of Xpert individual and pooled tests

	Pooled N = 125			
	Xpert MTB/RIF		Xpert Ultra	
Individual test	**Positive**	**Negative**	**Positive**	**Negative**
One or more positive	46	4	35	7
All negative	4	71	1	82
Agreement	117/125 (93.6%)		117/125 (93.6%)	
Kappa	0.867		0.851	
Sensitivity (95% CI)	0.920 (0.808 - 0.978)		0.8571 (0.715 - 0.946)	
Specificity (95% CI)	0.947 (0.869 - 0.985)		0.988 (0.935 - 0.998)	

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 3.1). The MTB grades of the individual and pooled samples were the same for 17 (47.2%) tests. The 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 3.2. 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.

Supplementary Table 3.1. Correlation of Individual and pooled Xpert MTB grades (only includes data for pools with one positive sample)

	Individual Xpert MTB grade included in pool					
	Trace n (%)	**Very low n (%)**	**Low n (%)**	**Medium n (%)**	**High n (%)**	**All n (%)**
Pooled Xpert MTB/RIF	**0**	**3**	**9**	**8**	**16**	**36**
Not detected	0 (0.0)	2 (66.7)	1 (11.1)	1 (12.5)	0 (0.0)	4 (11.1)
Very low	0 (0.0)	0 (0.0)	0 (0.0)	3 (75.0)	0 (0.0)	3 (8.3)
Low	0 (0.0)	1 (33.3)	4 (44.4)	1 (25.0)	0 (0.0)	6 (16.7)
Medium	0 (0.0)	0 (0.0)	2 (22.2)	2 (50.0)	5 (31.2)	9 (25.0)
High	0 (0.0)	0 (0.0)	2 (22.2)	1 (25.0)	11 (68.8)	14 (38.9)
Pooled Xpert Ultra	**5**	**2**	**6**	**6**	**13**	**27**
Not detected	5 (100)	1 (50.0)	5 (83.3)	0 (0.0)	0 (0.0)	6 (22.2.)
Trace	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Very low	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	1 (7.7)	2 (7.4)
Low	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	4 (30.8)	6 (22.2)
Medium	0 (0.0)	1 (50.0)	1 (16.7)	2 (33.3)	5 (38.5)	9 (33.3)
High	0 (0.0)	0 (0.0)	0 (0.0)	1 (16.7)	3 (23.1)	4 (14.8)

Supplementary Table 3.2. Probe results for Xpert MTB/RIF and Xpert Ultra in individual and pooled tests

	Xpert MTB/RIF				
	Individual tests n=72		Pooled tests n=50		ΔCT
Probe	**CT Median IQ range**	**Min-Max**	**CT Median IQ range**	**Min-Max**	
Probe D	18.75 (13.90 - 21.80)	0.00 - 34.40	18.00 (14.20 - 22.10)	0.00 - 33.40	-0.75
Probe C	18.35 (14.45 - 22.40)	9.60 - 32.70	18.75 (13.70 - 23.90)	11.60 - 38.30	0.4
Probe E	19.75 (15.10 - 23.65)	10.00 - 36.20	18.60 (14.60 - 23.50)	0.00 - 34.90	-1.15
Probe B	19.60 (15.00 - 23.45)	10.80 - 32.90	19.45 (14.80 - 23.90)	12.10 - 38.00	-0.15
Probe A	18.25 (14.60 - 22.10)	9.30 - 34.00	18.40 (13.60 - 23.50)	10.70 - 40.00	0.15
	Xpert Ultra				
	Individual tests n=60		Pooled tests n=36		ΔCT
Probe	**CT Median IQ range**	**Min-Max**	**CT Median IQ range**	**Min-Max**	
IS1081-IS6110	16.40 (16.10 - 23.10)	1.00 - 31.70	16.80 (16.20 - 23.20)	15.90 - 31.80	0.4
rpoB1	18.70 (17.60 - 21.30)	0.00 - 33.70	20.10 (0.00 - 25.60)	0.00 - 32.20	1.4
rpoB2	18.70 (17.40 - 21.60)	0.00 - 39.60	19.75 (17.30 - 24.90)	0.00 - 34.70	1.05
rpoB3	19.90 (18.30 - 23.80)	0.00 - 34.60	21.65 (0.00 - 26.80)	0.00 - 32.00	1.75
rpoB4	22.00 (20.30 - 25.70)	0.00 - 36.50	23.50 (0.00 - 28.30)	0.00 - 35.60	1.5

Xpert Ultra survey

Of 500 individuals tested using Ultra, 60 (12%) were MTB-positive and 440 (88%) MTB-negative (Table 3.1), 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 3.2). 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 3.2). 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, $\kappa = 0.851$; Table 3.4). 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 3.4).

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 3.1). 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 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 3.1). 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 3.2. 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 3.3). 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 x 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 770 and 929 individuals respectively.

Supplementary Table 3.3. Cartridge costs screening 500 consecutive patients using the pooling method and number of patients that could be tested with 500 Xpert MTB/RIF and Xpert Ultra cartridges

	Xpert MTB/RIF		**Xpert Ultra**	
	Individual N = 500	**Pooled** N = 125	**Individual** N = 500	**Pooled** N = 125
Sensitivity	reference	92.0%*	reference	83.3%*
specificity	reference	94.7%*	reference	98.8%*
Proportion positive	14.4%*	40% of pools	12.0%*	28.8 of pools
Bacteriologically confirmed	72	68	73	68
Cartridges required	500	325	500	269
Cartridge costs (USD)	4,990	3,243	4,990	2,685
Cartridge savings (USD)	Reference	1,747 (35%)	0	2,305 (46.2%)
Number of patients that could be tested with 500 cartridges	500	770	500	929
Cartridge cost per patient (USD)	9.98	6.48	9.98	5.37

** Assumes pools of 1:4; proportion positive taken from the surveys' findings*

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 [17]. Moreover, recent studies in Cambodia [32] and Lao PDR [60, 61], 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 [60, 61]. 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 [62]), 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, hypothesizing a 'carrier RNA' effect caused by increased total cellular RNA in the samples [63, 64]. 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.

Positive predictive value, the probability that subjects with a positive result truly have the disease, can be increased. This is because a truly negative sample will need to go

through an additional round of testing in pooled testing strategy, and therefore the probability of a false-positive result will become much smaller. In addition, a lower sensitivity and resultant negative predictive value (the probability that subjects with a negative result truly do not have the disease) showed that pooled testing is likely to miss the detection of paucibacillary samples. These results suggest potentially for missed case detection which could pose a risk of community transmission of TB from undetected sources.

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

Costs for repeating all Xpert tests with non-valid results (invalid, error, no results) were not included in the calculation. This is an important factor because the rate of non-valid Xpert results could make up ~10% of total results, impacting the cost-effectiveness of the pooling strategy [65].

Conclusions

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.

Chapter IV - Pooled testing of sputum with Xpert MTB/RIF and Xpert Ultra during tuberculosis active case finding campaigns in Lao People's Democratic Republic

As published in BMJ Global Health (Published: February 14, 2022)

Citations as follows: Iem V, Chittamany P, Suthepmany S, et al. Pooled testing of sputum with Xpert MTB/RIF and Xpert Ultra during tuberculosis active case finding campaigns in Lao People's Democratic Republic. BMJ Global Health 2022;7:e007592.

http://dx.doi.org/10.1136/bmjgh-2021-007592

Introductory section

Chapter II and III demonstrate that the pooling method has high sensitivity and specificity for both Xpert MTB/RIF and Xpert Ultra, with the latter resulting in full agreement between individual and pooled testing during routine passive case finding in Lao PDR. These promising results call for more studies to assess the potential of the pooling method in populations with low TB prevalence, which would be predicted to result in significantly higher savings.

Therefore, in Chapter IV, we assess the performance of pooled testing during outreach active case finding campaigns where the proportion of people with bacteriologically confirmed TB is usually lower.

The study in Chapter IV was conducted from March to April 2020 and from January to March 2021, in parallel with studies conducted in Chapters II and III.

It is also important to demonstrate that pooled testing can be generalized to other diseases and not just TB. In the next chapter V, we therefore assess the performance and the savings that could potentially be generated when pooling samples with Xpert Xpress SARS-CoV-2 during the COVID-19 pandemic.

Abstract

Introduction: Active case finding (ACF) of individuals with tuberculosis (TB) is a key intervention to find the 30% of people with TB who are missed every year. However, ACF requires screening large numbers of individuals who have a low probability of positive results, typically <5%, which makes using the recommended molecular tests expensive.

Methods: We conducted two ACF surveys (in 2020 and 2021) in high TB burden areas of Lao PDR. Participants were screened for TB symptoms and received a chest X-ray. Sputum samples of four consecutive individuals were pooled and tested with Xpert-MTB/RIF (2020) or Xpert-Ultra (2021). The agreement of the individual and pooled samples was compared and the reasons for discrepant results and potential cartridge savings were assessed.

Results: Each survey included 436 participants, which were tested in 109 pools. In the Xpert-MTB/RIF survey, 25 (sensitivity 89%, 95%CI 72.8%–96.3%) of 28 pools containing MTB-positive samples tested positive and 81 pools containing only MTB-negative samples tested negative (specificity 100%, 95%CI 95.5%–100%). In the Xpert-Ultra survey, all 32 (sensitivity 100%, 95%CI 89.3%–100%) pools containing MTB-positive samples tested positive and all 77 (specificity 100%, 95%CI 95.3%–100%) containing only MTB-negative samples tested negative. Pooling with Xpert-MTB/RIF and Xpert-Ultra saved 52% and 46% (227/436 and 199/436, respectively) of cartridge costs alone.

Conclusion: Testing single and pooled specimens had a high level of agreement, with complete concordance when using Xpert-Ultra. Pooling samples could generate significant cartridge savings during ACF campaigns.

Introduction

Despite being treatable and curable, tuberculosis (TB) remains one of the main infectious killers in the world, as ten million people fall ill and 1.4 million die from the disease each year [66]. Its diagnosis is usually reliant on passive case finding (PCF), in which health services wait for individuals with symptoms of TB to attend a health facility to initiate the diagnostic process. Although PCF identifies most people with TB in locations with adequate access to health services, it misses those unwilling or unable to attend the clinics, and is a major reason only seven of the ten million people with TB are diagnosed and notified [67]. Individuals missed by passive approaches often include vulnerable populations, such as internally displaced, migrant, or rural populations, women, the unemployed, and ethnic minorities [68, 69], who may face multiple societal and economic barriers to attend the service, including catastrophic costs [70, 71]. It is thus recognized that, to be inclusive and reduce the socioeconomic impact of TB [72], health services need to include active case finding (ACF) approaches that involve pro-active interventions to extend the reach of TB services for diagnosis [73] and treatment [74]. Although ACF interventions can be very effective [75, 76], they are less standardized than PCF, as they address the specific barriers of multiple target populations, and are more resource- and time-intensive than PCF [77].

The World Health Organization (WHO) recommends testing all individuals with presumptive TB with molecular assays, such as the Xpert MTB/RIF and Xpert Ultra (Cepheid Sunnyvale, CA, United States) [4], with the latter being preferred given its higher sensitivity [5]. Although the use of these assays is expanding, the assay cartridge unit costs of US$ 9.98 per test [47] remains one of the main hurdles for its wider implementation in low- and middle-income countries. Diagnostic test costs can

limit the expansion of ACF activities, as they require testing large numbers of individuals with relatively lower yields than PCF [78].

Since 2015, the Lao National Tuberculosis Control Center (NTC) has conducted ACF by implementing intensified case finding activities to increase the detection of individuals with TB in high burden districts of the country. These activities include the sensitization of the population, the local provision of chest X-rays for screening (independently of symptoms) and the identification of individuals with symptoms of TB who have not attended health facilities. Participants with abnormal chest X-rays or symptoms of TB are tested using Xpert MTB/RIF or Xpert Ultra [54]. The activities have increased case detection, although the cost of the Xpert cartridges is considered high and is the main limiting factor to implement the intervention on a larger scale.

One approach that could increase the affordability of Xpert testing is to test several samples together using the pooling method [29]. This procedure combines (or pools) the sputum of several individuals into one pot and tests them together with a single test. If the test is positive, the pool's samples are re-tested individually to identify the positive sample(s) while if the test is negative, all samples in the pool are considered negative, resulting in 30-40% savings in Xpert cartridge costs alone depending on the prevalence of TB in the population tested [17]. Therefore, pooling may hold great promise for ACF, but there are few reports of its performance under operational conditions [32].

Here, we report a prospective study to assess the sensitivity and specificity of the pooling method using Xpert MTB/RIF and Xpert Ultra during intensified case finding interventions, and its potential to increase the affordability of Xpert testing in Lao PDR.

Materials and methods

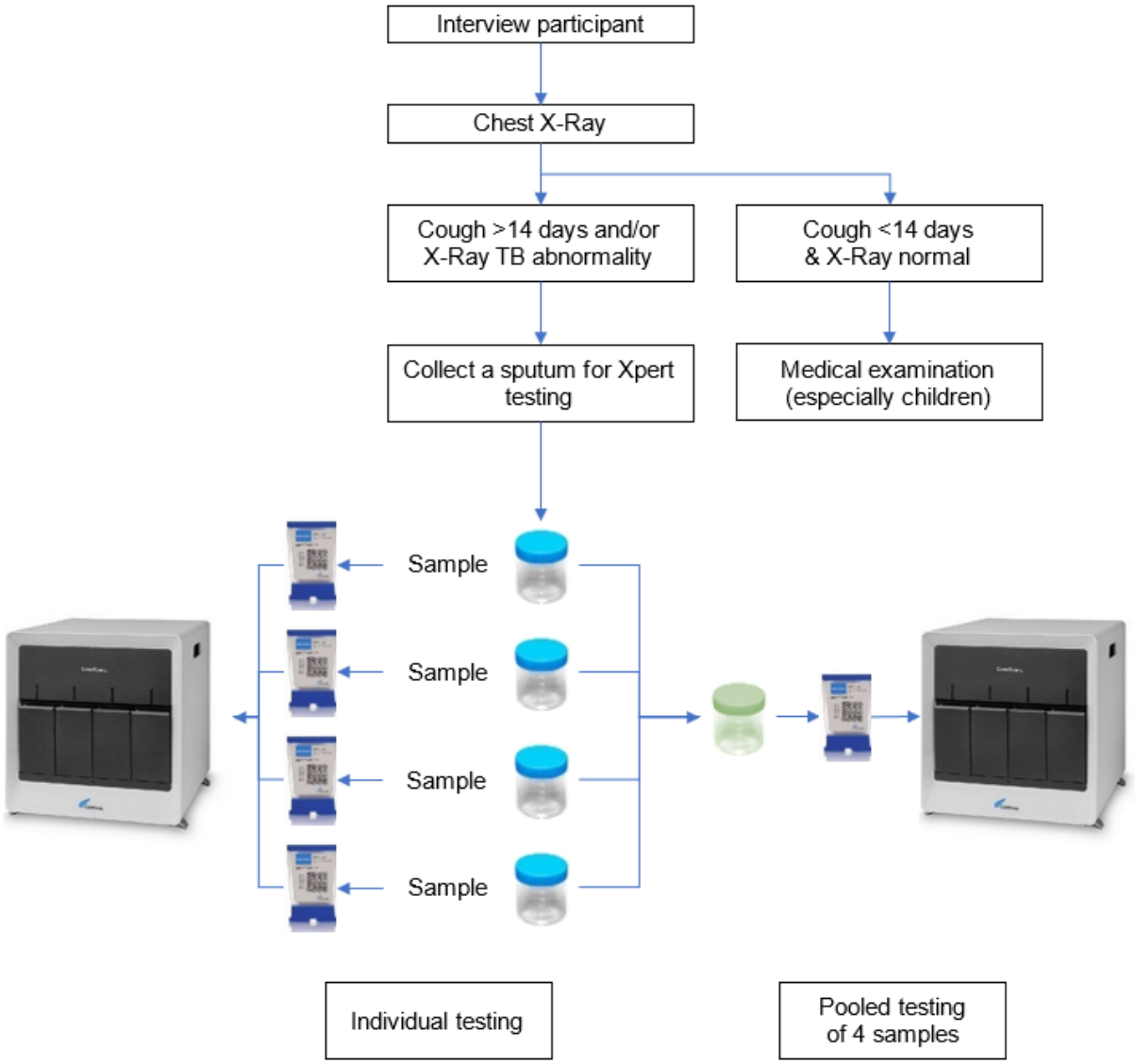
We conducted two independent prospective surveys embedded within the ACF activities of Lao's NTC, from March to April 2020 and from January to March 2021. Both surveys were cross-sectional and used the same recruitment and testing procedures. The 2020 survey aimed to assess the performance of the pooling method when testing samples with Xpert MTB/RIF, while the 2021 survey assessed the method when using Xpert Ultra, after its release for routine use by Lao's NTC.

Active case finding was conducted in Lao's high TB burden areas, which are programmatically defined as TB incidence ≥ 100 cases per 100,000 population. The 2020 survey was conducted in Vientiane Capital, Luang Prabang and Savannakhet provinces with estimated populations of 890,129, 468,375 and 1,051,675 inhabitants, respectively, and TB notification rate of 134, 88 and 102 cases per 100,000 population in 2020, respectively. The 2021 survey was conducted in Saravane and Oudomxay, with 430,428 and 333,934 population and TB notification rates of 127 and 110 cases per 100,000 population in 2020, respectively.

Both surveys were conducted in the same fashion. Before an ACF activity, the NTC team met the province and district health authorities and conducted preparation visits with the provincial TB coordinator, district TB manager and village authorities, distributed health education materials, obtained the addresses of individuals with TB and line listed household contacts. At an agreed date, the NTC team set up a digital chest X-ray machine and a 4-module GeneXpert platform in the village and invited all residents to complete a questionnaire on signs and symptoms, history and treatment of TB and offered chest X-rays for screening, independently of the presence of symptoms. Individuals with abnormal chest X-rays and those who indicated having

cough > 2 weeks duration were asked to provide sputum samples for Xpert testing and were managed according to the decision tree shown in Figure 3.1. Sputum samples were tested with Xpert following the manufacturer's instructions [3].

Figure 4.1. Flow diagram of the sputum processing.



Sputum samples tested individually with Xpert MTB/RIF or Xpert Ultra were processed in the village GeneXpert platform. Consecutive samples with remnant volumes ≥ 0.5 ml were included in the pooling studies and were transported to the National TB Reference Laboratory in Vientiane using a cold chain. Samples were transported after the sample reagent had been added. Turned around time to testing was < 48h after the sample reagents had been added and samples were maintained in a cold chain at all times. Sputum samples from four participants were pooled together, with a volume of 0.5 ml of sputum each added to a pot, to obtain an aggregated volume of 2 ml [3]. Samples for a pool were selected consecutively and staff were blind to the individual Xpert test results and the pooled specimen was tested using one new Xpert cartridge.

Statistical analysis

Categorical data were summarized using descriptive statistics and chi-squared tests were used to test for statistically significant differences. Individuals unable to produce sputum were excluded from the analysis. The pooled samples were compared with the four Xpert MTB/RIF and Xpert Ultra individual results and their agreement was tested using kappa statistics. The CT values and grades (trace, very low, low, medium, and high) of individual and pooled tests were compared to describe the effect of combining the samples. Cost differences were calculated on the basis of the number of cartridges required to test all specimens using pooled and individual testing.

Sample size for the surveys was not formally estimated as we were limited by the expected number of participants attending the campaigns before the COVID-19 lockdown, the capacity of staff to conduct additional testing to their routine activities and the number of spare cartridges available for research purposes. The study was

approved by the Lao NTC and the Liverpool School of Tropical Medicine Research Ethics Committee, UK (Ethical waiver 20-037) and informed consent waiver was obtained.

Patient and Public Involvement

It was not appropriate or possible to involve patients or the public in the design, or conduct, or reporting, or dissemination plans of our research.

Results

The 2020 survey included 436 participants, 334 (76.6%) males and 102 (23.4%) females, and 29 (6.7%, 95%CI 4.7-9.4%) were Xpert MTB/RIF MTB-positive. The 2021 survey also included 436 participants, 222 (50.9%) males and 214 (49.1%) females, and 37 (8.5%, 95%CI 6.5-11.5%) were Xpert Ultra MTB-positive (p-value >0.1, Table 4.1). Males were more likely to be MTB-positive than females in 2020 (26/334 (7.8%) males vs 3/102 (2.9%) females, respectively, $p = 0.014$); but females were more likely to be MTB-positive than males in 2021 (12/222 (5.4%) vs 12/214 (11.7%), respectively, $p < 0.008$). Each survey included 109 pools of four patients.

Table 4.1. Baseline characteristics of participants and Xpert MTB results

	Xpert MTB/RIF		Xpert Ultra	
	Individual n (%)	**Pool n (%)**	**Individual n (%)**	**Pool n (%)**
Sex	**436**	-	**436**	-
Male	334 (76.6)	-	222 (50.9)	-
Female	102 (23.4)	-	214 (49.1)	-
Age	**436**	-	**436**	-
Mean (sd) (range)	45 (16.1) (12-89)	-	54 (13.7) (10-90)	-
<35	131 (30.0)	-	41 (9.4)	-
35-54	184 (42.2)	-	159 (36.5)	-
>=55	121 (27.8)	-	236 (54.1)	-
Xpert MTB Result	**436**	**109**	**436**	**109**
Detected/≥1 MTB included	29 (6.7)	25/28 (22.9)	37 (8.5)	32/32 (29.4)
Not detected/≥1 MTB included	-	3 (2.7)	-	-
Not detected/Only MTB-negative	407 (93.3)	81 (74.3)	399 (91.5)	77 (70.6)
Xpert MTB Result by sex				
Male	26/334 (7.8)	-	12/222 (5.4)	-
Female	3/102 (2.9)	-	25/214 (11.7)	-
MTB Grade	**29**	**25**	**37**	**32**
Trace	NA	NA	6 (16.2)	21 (65.6)
Very low	6 (20.7)	15 (60.0)	8 (21.6)	11 (34.4)
Low	16 (55.2)	9 (37.5)	15 (40.5)	0 (0.0)
Medium	6 (20.7)	1 (4.2)	2 (5.4)	0 (0.0)
High	1 (3.4)	0 (0.0)	6 (16.2)	0 (0.0)
Rif Resistance	**29**	**25**	**37**	**32**
Detected	0	0	0	0
Not detected	27 (93.1)	24 (96.0)	30 (81.1)	10 (31.2)
Indeterminate	2 (6.9)	1 (4.0)	7 (18.9)	22 (68.8)

Xpert MTB/RIF survey

In 2020, 28 (25.7%) pools contained one or more Xpert MTB/RIF MTB-positive sample(s) (27 pools with one and one pool with two MTB-positive samples) and 81 (74.3%) pools contained solely MTB-negative samples (Table 4.2). The pool with two MTB-positive and 24 of 27 pools with one MTB-positive sample tested MTB-positive and three tested MTB-negative, resulting in a sensitivity of 89% (25/28, 95%CI 72.8%–96.3%). All 81 pools containing solely MTB-negative samples tested MTB-negative in the pooled assay (specificity 100%, 95%CI 95.5%-100%).

Table 4.2. Number of pools with 0,1,2,3,4 positive results.

	Individual Xpert results included in a pool					
	All negative n (%)	**One positive n (%)**	**Two positive n (%)**	**Three positive n (%)**	**Four positive n (%)**	**All**
Pooled Xpert MTB/RIF	**81**	**27**	**1**	**0**	**0**	**109**
Detected	0	24 (89%)	1 (100%)	0	0	25 (23%)
Not detected	81 (100%)	3 (11%)	0	0	0	84 (77%)
Pooled Xpert Ultra	**77**	**27**	**5**	**0**	**0**	**109**
Detected	0	27 (100%)	5 (100%)	0	0	32 (29%)
Not detected	77 (100%)	0	0	0	0	77 (71%)

Therefore, the accuracy performance of the 109 pools in correlation to the 436 individual results resulted in 97.3% agreement (kappa: 0.925). Among the 27 pools containing single MTB-positive samples, five contained very low, 15 low, 6 medium and one high MTB-grades. The pooled MTB-grade was similar to the individual test in four (14.8%), one grade lower in 21 (77.8%), two grades lower in one (3.7 %) and one grade higher in one (3.7%) of the pools. Of the five pools containing very low

individual MTB-grades, three tested MTB-not detected and two very low MTB-grade in the pooled assay (Table 4.3).

Table 4.3. Correlation of Individual and pooled Xpert MTB grades (positive pools only include those with only one positive Xpert).

	Individual Xpert grade included in pool					
	Not detected n (%)	**Trace n (%)**	**Very low n (%)**	**Low n (%)**	**Medium n (%)**	**High n (%)**
Pooled Xpert MTB/RIF	**81**	**NA**	**5**	**15**	**6**	**1**
Not detected	81 (100%)	NA	**3 (60%)**	0	0	0
Very low	0	NA	2 (40%)	**12 (80%)**	0	0
Low	0	NA	0	2 (13.3%)	**6 (100%)**	**1 (100%)**
Medium	0	NA	0	**1 (6.7%)**	0	0
High	0	NA	0	0	0	0
Pooled Xpert Ultra	**77**	**2**	**5**	**13**	**1**	**6**
Not detected	77 (100 %)	0	0	0	0	0
Trace	0	2 (100%)	**4 (80%)**	**7 (54%)**	**1 (100%)**	**2 (33%)**
Very low	0	0	1 (20%)	**6 (46%)**	0	**4 (67%)**
Low	0	0	0	0	0	0
Medium	0	0	0	0	0	0
High	0	0	0	0	0	0

The CT values for the Xpert MTB/RIF probes for both individual and pooled testing are shown in Table 4.4. The median CT values for probes A-E ranged from 23.4 to 24.8 for the individual tests and from 30.6 to 33.6 for the pooled tests, with an increase in CT values ranging from 5.4 to 7.1.

Table 4.4. Median CT values of individual and pooled Xpert MTB/RIF and Xpert Ultra probe results.

Xpert MTB RIF					
	Individual results n=29		Pooled results n=25		
Probe	**CT Median IQ range**	**Min-Max**	**CT Median IQ range**	**Min-Max**	**ΔCT**
Probe D	24.6 (22.7-27.3)	19.1-35.2	32.3 (28.5-34.2)	21.0-38.2	7.1
Probe C	23.7 (22.0-26.7)	18.9-34.7	30.3 (27.4-31.8)	20.0-35.7	6.5
Probe E	24.8 (23.0-28.1)	20.5-36.2	33.9 (29.3-34.9)	21.2-39.3	7.1
Probe B	24.6 (22.9-27.3)	20.1-33.8	30.1 (27.3-32.5)	20.9-34.7	5.4
Probe A	23.3 (22.0-26.0)	20.5-34.1	31.1 (27.1-32.9)	19.7-34.6	6.6
Xpert Ultra					
	Individual results n=37		Pooled results n=32		
Probe	**CT Median IQ range**	**Min-Max**	**CT Median IQ range**	**Min-Max**	**ΔCT**
Probe IS1081/ IS6110	19.6 (17.1-22.6)	16.0-32.0	24.9 (22.2-26.5)	19.9-29.3	4.6
Probe rpoB1	21.6 (17.9-24.9)	0-32.0	0 (0-30.2)	0-34.9	NA
Probe rpoB2	21.3 (17.9-25.8)	0-32.1	0 (0-29.8)	0-35.3	NA
Probe rpoB3	23.3 (19.2-27.0)	0-33.7	0 (0-32.9)	0-39.8	NA
Probe rpoB4	25.7 (21.2-29.7)	0-35.7	0 (0-33.5b)	0-37.7	NA

Two of the MTB-positive samples were RIF-indeterminate and 27 RIF-negative. Of the 28 pools with MTB-positive samples, 25 pools contained one MTB-positive RIF-negative sample, one had two MTB-positive RIF-negative samples and two had one MTB-positive RIF-indeterminate samples. Of the 25 MTB-positive RIF-negative pools, three tested MTB-negative and did not report RIF results and 22 tested RIF-negative. The pool containing two MTB-positive RIF-negative samples tested RIF-negative and the two pools containing RIF-indeterminate samples tested RIF-negative in one and RIF-indeterminate in the other.

Xpert Ultra survey

In 2021, 32 (29.4%) pools contained MTB-positive samples and 77 (70.6%) solely MTB-negative samples. Twenty-seven of the 32 MTB-positive pools contained one and five contained two MTB-positive samples and all tested positive in the pooled assay (sensitivity 100%, 95% CI 89.3-100%). All 77 pools containing only MTB-negative samples tested MTB-negative (specificity 100%, 95%CI 95.3%-100%), resulting in 100% agreement (Kappa: 1). Among the 27 pools with single MTB-positive samples, two contained trace, five very low, 13 low, one medium and six high MTB-grades. The pooled MTB-grades were the same as the individual grades in three (11%), one grade lower in ten (42%), two grades lower in seven (29%), three grades lower in five (21%) and four grades lower in two (8%) of the pooled assays (Table 4.3). The Xpert Ultra probes CT values are shown in Table 4.4. Probe IS1081/IS6110 had median CT of 19.6 for individual and 24.9 for pooled results, with a median increase of 4.6. Probes rpoB1-B4 median CT values ranging from 19.6 to 25.7 for the individual tests but CT values were not available for the pools. Among the 37 MTB-positive samples, 30 (81%) were RIF-negative and seven (18.9%) RIF-indeterminate and were distributed in 32 pools. Twenty-five of the 32 pools contained only RIF-negative and seven contained RIF-indeterminate samples. Fifteen of the 25 pools containing only RIF-negative samples tested RIF-indeterminate and 10 RIF-negative, while all seven pools containing RIF-indeterminate samples tested pooled RIF-indeterminate.

Xpert MTB/RIF and Xpert Ultra costs (Table 4.5)

The cartridges cost for testing individually the 436 participants with Xpert at USD 9.98 per test was USD 4351.28 for each survey. The pooling method in 2020 required 109 Xpert MTB/RIF cartridges to test 109 pools and 100 cartridges to test individual samples of 25 MTB-positive pools. The total of 209 (109 + 100) cartridges for pool testing would cost US$ 2,085.82, resulting in USD 2265.46 (52%) saving in cartridge costs. Similarly, testing 109 pools with Xpert Ultra in 2021 required 109 cartridges to test the pools and 128 cartridges to test individually the 32 positive pools. The total of 237 cartridges would cost US$ 2,365.26, resulting in USD 1986.02 (46%) savings in cartridge costs. If the number of cartridges is kept fixed, the pooling method could test more patients than testing samples individually, as 436 cartridges would allow testing 909 and 802 individuals with Xpert MTB/RIF and Xpert Ultra, respectively – an effective test per patient cost of USD 4.78.and 5.42 respectively.

Table 4.5. Costs and savings to screen consecutive patients using the pooling method and number of patients that could be tested with Xpert MTB/RIF and Xpert Ultra cartridges

	Individual Xpert		**Pooled Xpert**	
	MTB/RIF	**Ultra**	**MTB/RIF**	**Ultra**
Number of individuals tested	436	436	436	436
Sensitivity	reference	reference	89%*	100%*
Specificity	reference	reference	100%*	100%*
Proportion positive	6.7%	8.5%	22.9%	29.4%
Bacteriologically confirmed	29	37	26	37
Cartridges required	436	436	209	237
Cartridge costs (USD)	4,351.28	4,351.28	2,085.82	2,365.26
Cartridge savings (USD)	NA	NA	**2,265.46 (52%)**	**1,986.02 (46%)**
Number tested with 436 cartridges				
Number tested	436	436	909	802
Cartridge cost per patient (USD)	9.98	9.98	**4.78**	**5.42**

* Assumes pools of 1:4; proportion positive taken from the surveys' findings

Discussion

Our surveys compared pooling with single testing during ACF for TB in a low-income country. Our results confirm that testing individual and pooled samples with the GeneXpert platform can achieve a high level of concordance. Concordance was higher with Xpert Ultra than with Xpert MTB/RIF, which is in agreement to regional studies evaluating pooling with Xpert Ultra in Cambodia [32] and Vientiane, Lao PDR [61]. Discrepancies between individual and pooled Xpert MTB/RIF tests only occurred among pauci-bacillary samples with high Xpert CT values, suggesting that some samples with low DNA concentrations fall below the assay's limit of detection and that the better agreement of Xpert Ultra is due to its higher sensitivity. Consequently, some patients with paucibacillary disease could be missed by pooling, especially if testing is based on Xpert MTB/RIF.

The pooling strategy can lead to significant cost savings and facilitate testing of more individuals for a given number of cartridges. In our setting, pooling samples would double the number of people tested with the same number of cartridges. This is higher than in PCF studies, where pooling is reported to save up to 40% of cartridges [17]. Cartridge savings are a function of the proportion of people with MTB-positive results and their distribution within the pools. If the proportion positive is low, a low number of pools would need to be re-tested, resulting in higher cartridge savings. For example, in a survey in Lao's district clinics, 12% of individuals tested Xpert-positive, and pooling resulted in 38.3% and 41.7% cartridge saving costs with Xpert MTB/RIF and Xpert Ultra, respectively [61], while in our survey setting the proportion of positives was 8.5%, which led to higher savings. The proportion of participants with positive tests in ACF is often lower than reported from studies utilizing PCF, typically below 5% depending on the target population [78, 79], and

lower to 10-20% of individuals attending TB clinics in PCF [80, 81]. We have thus shown that pooling could be highly efficient when testing populations using ACF, and further studies amongst such populations are warranted. Since the pooling method is a laboratory change, it would not affect the screening algorithm and can be easily instituted without any major modifications.

Findings from the systematic review in Chapter I have highlighted that individual and pooled RIF results are often discordant, with pools containing RIF-negative samples often returning RIF-indeterminate pooled results [17] and our findings are in agreement with these observations. Although samples with pooled RIF results would be routinely confirmed at the time of re-testing the samples of a positive pool to identify the individual MTB-positive samples, it is important to highlight that pooled RIF results are unreliable and should not be used for clinical management.

Interestingly, men were more likely to be Xpert MTB-positive (p-value < 0.1, Chi-Square test) but women were more likely to be Xpert Ultra MTB-positive (p-value < 0.1, Chi-Square test). The higher sensitivity of Xpert Ultra may compensate for women not being comfortable when coughing and expectorate sputum, leading to sub-optimal specimen quality. The impact of Ultra on the diagnosis of TB in women is a finding that suggests the need for further studies, especially those which could use gender-disaggregated data to improve planning and resource prioritization for underserved populations as well as ensuring integration of gender and social inclusion dimensions.

Further studies could explore ways to further improve the efficiency of pooling when combined with other screening tools, such as C-Reactive Protein (CRP) [82] and digital chest X-rays with Computer Aided Diagnosis (CAD) [83, 84]. Both tools can

identify individuals with and without the traditional symptoms of TB, although their relatively lower specificity requires confirming the diagnosis with more specific molecular assays. Although using tests combinations could increase assay costs, individuals with a positive CRP or abnormal chest X-rays CAD could be confirmed using the pooling method, and its efficiency gains could increase the affordability of tests combinations.

Conclusions

In conclusion, we have shown pooling samples for TB diagnoses during ACF campaigns can replicate testing samples with individual tests. The approach can facilitate testing higher numbers of patients with lower cartridge costs, increasing the affordability of testing with molecular assays. The high level of agreement between individual and pooled samples obtained with Xpert Ultra demonstrates that pooling can be reliable and contribute to achieve the WHO End TB strategy targets in resource-limited settings.

Chapter V - Pooling samples to increase testing capacity with Xpert Xpress SARS-CoV-2 during the COVID-19 pandemic in Lao People's Democratic Republic

As published in PLOS ONE (Published: September 29, 2022)

Citations as follows: Iem V, Xangsayarath P, Chittamany P, et al. Pooling samples to increase testing capacity with Xpert Xpress SARS-CoV-2 during the Covid-19 pandemic in Lao People's Democratic Republic. PLOS ONE 2022: 17(9): e0275294.

https://doi.org/10.1371/journal.pone.0275294

Introductory section

Findings from all the studies we have conducted have demonstrated that the pooling method is reliable and can replicate the performance of individual testing with significant assay costs savings when it comes to TB diagnosis.

In this chapter V, we describe how pooled testing can be implemented beyond TB under programmatic conditions for SARS-CoV-2 screening given the emergency of the situation. We assess if the pooled testing was appropriate in situation of outbreaks where mass screening is needed with rapid turnaround time, in order to apply or lift quarantine and isolation policies.

The study in Chapter V was conducted from April 2021 to the May 2021, after lockdown and quarantine measures were put in place.

In all previous chapters, the savings described were limited to assay cost savings and were therefore not reflecting the actual cost-effectiveness of the pooled testing strategy. In the following chapter VI, we conduct a formal costs and cost-effectiveness analysis of the pooled testing compared to individual testing.

Abstract

The COVID-19 pandemic created the need for large-scale testing of populations. However, most laboratories do not have sufficient testing capacity for mass screening. We evaluated pooled testing of samples, as a strategy to increase testing capacity in Lao PDR. Samples of consecutive patients were tested in pools of four using the Xpert Xpress SARS CoV-2 assay. Positive pools were confirmed by individual testing, and we describe the performance of the test and savings achieved. We also diluted selected positive samples to describe its effect on the assays CT values. 1,568 patients were tested in 392 pools of four. 361 (92.1%) pools were negative and 31 (7.9%) positive. 29/31 (93.5% (95%CI 77–99%) positive pools were confirmed by individual testing of the samples but, in 2/31 (6.5%) the four individual samples were negative, suggesting contamination. Pools with only one positive sample had higher CT values (lower RNA concentrations) than the respective individual samples, indicating a dilution effect, which suggested an increased risk of false negative results with dilutions >1:10. However, this risk may be low if the prevalence of infection is high, when pools are more likely to contain more than one positive sample. Pooling saved 67% of cartridges and substantially increased testing capacity. Pooling samples increased SARS-CoV-2 testing capacity and resulted in considerable cartridge savings. Given the need for high-volume testing, countries may consider implementation of pooling for SARS-CoV-2 screening.

Introduction

The world is facing an unprecedented health crisis since the emergence of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) resulting in the coronavirus disease-19 (COVID-19) pandemic [85, 86]. Ministries of health have implemented unparalleled non-pharmacological (NPIs) and pharmacological interventions, with stay-at-home, curfews, masking, quarantine orders, and increasingly, new and repositioned treatments and immunizations. Since early in the pandemic, identifying infected individuals has been considered a key pillar to prevent onward transmission and to monitor the efficacy of NPIs. For this, testing is needed at a large scale. COVID-19 confirmation is based on the detection of SARS-CoV-2 RNA by nucleic acid amplification assays, such as real-time reverse-transcription polymerase chain reaction (RT-PCR) [87]. RT-PCR is highly sensitive and specific compared to rapid antigen testing [88], but the large number of tests required has generated test stockouts, delayed reporting and unmanageable workloads, outstripping the capacity of the laboratories [89-92].

COVID-19 was first reported in Lao People's Democratic Republic (PDR) in March 2020. Initial epidemic waves were controlled through NPIs but a large epidemic wave, which started in April 2021, resulted in the establishment of community transmission, leading to large numbers of test requests that exceeded the testing capacity of the country. In response, the National COVID-19 Task Force introduced pooled testing, to increase testing capacity and the efficiency of the diagnostic algorithm. Although ideally the method's performance should have been assessed before widespread implementation, the Task Force decided to implement the approach routinely, based on the urgent need to increase testing capacity.

In pooled testing, clinical samples of several patients are mixed (pooled) together and tested with a single test. If the test is negative, all the samples included in the pool are considered negative, while if the test is positive, all the samples are re-tested individually to identify the infected specimens [63]. Depending on the positivity rate of the pooled tests, pooling uses an overall lower number of tests than individual testing, increasing testing capacity, lowering costs, and saving time. This method has been used in diagnostic laboratories and blood banks to screen for infections, such as hepatitis B [14], and TB [17] and is increasingly reported for SARS-CoV-2 screening [93].

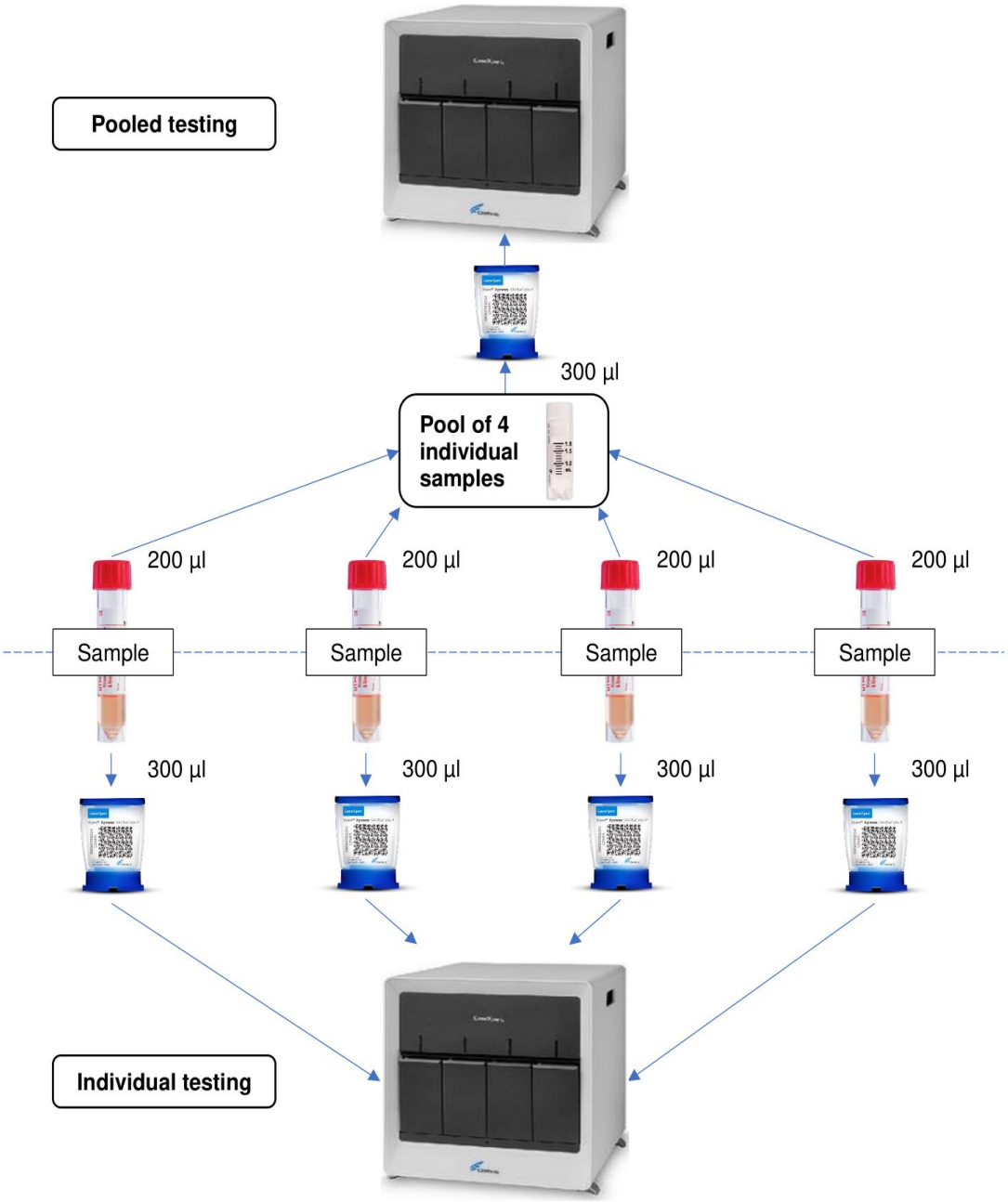
Here, we describe the agreement of pooled and individual testing in clinical specimens using the GeneXpert (Cepheid, US) with Xpert Xpress SARS-CoV-2 assays (Xpress) [7], changes in the assays cycle threshold (CT) values and the cost and processing time to detect SARS-CoV-2 within the context of the epidemic.

Materials and methods

We conducted a prospective cross-sectional study from the 26th of April 2021 to the 24th of May 2021 in Vientiane, Lao PDR. The study was conducted under the authority of the Ministry of Health National Center for Laboratory and Epidemiology, which was responsible at that time for the mass screening of the population in four large open-air sites in the capital. Participants were invited to participate if they had COVID-19 symptoms, close contact with individuals with confirmed COVID-19 less than 14 days prior to disease onset; a history of travel to/from other countries, or if they had a diagnosis of Severe Acute Respiratory Infections or confirmed COVID-19 before hospital discharge. Both nasopharyngeal and oropharyngeal swabs were collected from each participant and put together in a single viral transport media tube.

There were 1,568 consecutive samples included in the study, corresponding to the number of cartridges available at the National Tuberculosis Reference Laboratory during the study period. Samples were tested for SARS-CoV-2 using the pooling method with the Xpress cartridge, as shown in Fig 5.1. The Xpert Xpress is specifically designed to amplify sequences of the envelope (E) and the nucleocapsid (N2) of the virus to generate tests results. If one or more SARS-CoV-2 nucleic acid targets (E, N2) has a CT within the valid range, the test reports a positive result. Pools were created by pipetting equal amounts (200 μL) of the individual samples directly into a container with the virus transport medium (BD Universal Viral Transport System, catalogue number 220220), and mixed together into a single-use 2 mL cryovial tube. The pooled fluid was homogenized by soft pipetting-expelling to reduce risks of aerosols, loaded into an Xpress cartridge, and tested following the manufacturer's instructions [7]. If the pool tested positive, the four samples in the pool were then tested individually. If the pool tested negative, all samples were considered negative and were not re-tested. Individual Xpert Xpress results were notified for patient management by the Emergency Operation Centre.

Fig 5.1. Flow diagram of the sample processing



CT values of pooled and individual samples were available for positive pools, to describe changes in viral loads. In addition, we conducted a bench evaluation of five clinical samples with known CT values to describe the dilution effect of the samples on the overall CT values. Samples were purposely selected if they had CT values <20, 20–25, 25–30, 30–35 and >35 and were diluted 1:2, 1:4, 1:6, 1:8, 1:10, 1:15 and 1:20 before testing. For this bench evaluation, 200 µL from the individual positive samples with known CT values were diluted using multiples of 200 µL fresh virus transport medium to replicate the desired dilution of the pools. Each diluted sample was tested with Xpert Xpress cartridges using 300µL per sample.

Statistical analysis

All samples received were included in the analysis. Categorical data were summarized using descriptive statistics, with 95% confidence intervals (95% CI). We tested the agreement between the pools with positive Xpress results and the corresponding individual samples and estimated the cost and number of cartridges required to test all specimens using pooled and individual testing. Xpert Xpress costs were estimated at USD 19.80 per cartridge, as listed at wambo.org prices. Chi-squared tests were used to test for statistically significant differences between proportions. Changes in the CT values of the assays were described using correlations between the CT values of the non-diluted and diluted samples.

Sample size was not formally estimated as we were limited by the expected number of participants, the capacity of staff to conduct additional testing to their routine activities and the number of spare cartridges available for research purposes.

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable requests for guideline development and systematic reviews.

Ethics statement

Need for ethical approval and informed consent were waived by the National Center for Laboratory and Epidemiology, Lao PDR Ministry of Health. The Center is the delegated authority for COVID-19 testing. Permission was granted through the Emergency Operations Centre under Lao PDR Task force for COVID-19 Prevention, Control and Response.

Patient and Public Involvement

It was not appropriate or possible to involve patients or the public in the design, or conduct, or reporting, or dissemination plans of our research.

Results

The study included 898 (57.3%) males and 670 (42.7%) females. Young participants (under 35 years old) provided the majority of samples (1036, 66.1%), followed by 35–54-year-olds (446, 28.4%), with only a few samples belonging to participants ≥ 55 years (86, 5.5%), as shown in Table 5.1. The 1,568 samples were distributed into 392 pools, each pool containing four individual samples. Three hundred and sixty-one (92.1%) pools tested Xpress-negative and 31 (7.9%) Xpress-positive. The samples of the 31 Xpress-positive pools were tested individually. Twenty (64.5%) of them contained only one positive sample, six (19.3%) contained two, two (6.4%) contained three and one (3.2%) contained four positive samples, for a total of 42 Xpert SARS-CoV-2 positive samples. Two (6.4%) positive pools did not contain positive samples when tested individually and were further re-tested for a different gene target using a different RT-PCR assay (Novel Coronavirus nucleic acid diagnostic kit PCR fluorescence probing (Sansure, China), on the CFX platform (BioRad, US)). However, all eight samples were still negative by this further assay [94]. Therefore 29 of the 31 positive pools were confirmed by individual testing, with an agreement of 94% (95%CI 77 – 99%).

Table 5.1. Baseline characteristics of participants with single and pooled Xpert Xpress SARS-CoV-2 results.

		Xpert Xpress SARS-CoV-2	
		Individual n (%)	**Pools n (%)**
Sex		**1,568**	NA
Male		898 (57.3%)	NA
Female		670 (42.7%)	NA
Age		**1,568**	
Mean (sd) (range)		31.4 (12.6) (1-96)	NA
<35		1036 (66.1%)	NA
35-54		446 (28.4%)	NA
>=55		86 (5.5%)	NA
Individual Xpert Xpress SARS-CoV-2		**1,568**	**392**
Negative		1526 (97.3%)	361 (92.1%)
Positive		42 (2.7%)	31 (7.9%)
<35		32 (76.2%)	NA
35-54		10 (23.8%)	NA
>=55		0 (0.0%)	NA
SARS-CoV-2 positive		**42**	
Male		18 (42.9%)	NA
Female		24 (57.1%)	NA
Number of positive samples included in Xpress-positive pools			**31**
	0	-	2 (6.5%)
	1	-	20 (64.5%)
	2	-	6 (19.4%)
	3	-	2 (6.5%)
	4	-	1 (3.2%)

The proportion of positive tests was similar for males and females (18/898, 2%, 95%CI 1.2-3.2% versus 24/670, 3.6%, 95%CI 2.4-5.4%, respectively, p = 0.06) and among adults < 35 years and 34-54 years old (32/1036, 3%, 95%CI 2.2-4.4% versus 10/446, 2.2%, 95%CI 1.1-4.2%, respectively, p = 0.4). However, none of the adults ≥ 55 years old was positive.

The pooled median CT for probe E of the pools with single positive samples was 21.7 (range 17.7 - 39.4) and 19.5 (range 14.8 - 35.4) for the individual samples (Table 5.2). The pooled CT values for probe E were higher than the individual values by a median of 2.3 (range 0.9 – 6.1, p = 0.2), as shown in Supplementary Table 5.1, Supplementary Figure 5.1. The median pooled CT values for probe N2 were 23.4 (range 19.5 - 43.6) and 21.2 (range 17.1-37.4) for the individual samples, respectively. The pooled CT value for probe N2 was higher than the individual CT values by a median of 2.2 (range 0.7 – 8.7, p= 0.2), (Supplementary Table 5.1, Supplementary Figure 5.1). The CT values of the two pools that tested positive in the pool, but negative in the individual samples were 0 and 43.1 for probe E and N2 and 0 and 44.8 for the first and second pool, respectively, indicating they had high CT values and were late calls, corresponding to low SARS-CoV-2 RNA loads.

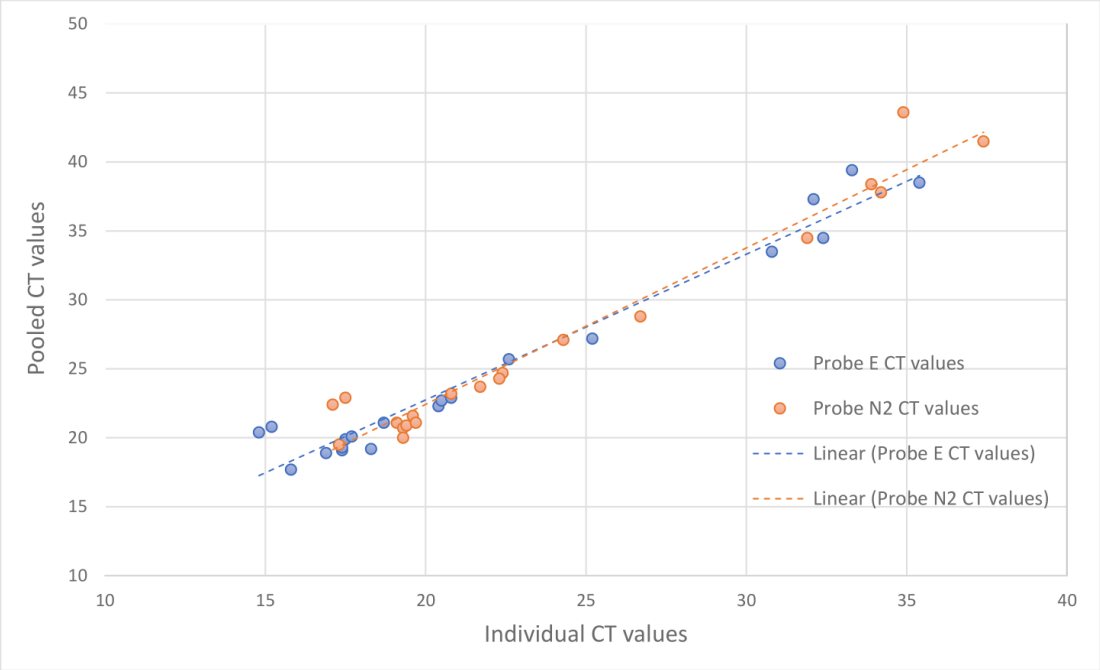
Table 5.2. Median CT values of individual and pooled Xpert Xpress SARS-CoV-2 probe results

Xpert Xpress SARS-CoV-2 CT values					
	Individual results n=42		Pooled results n=29		
Number of positive samples in the pool	**CT Median**	**Min-Max**	**CT Median**	**Min-Max**	**ΔCT Median**
1 positive (n=20)					
Probe E	19.5	14.8-35.4	21.7	17.7-39.4	2.3
Probe N2	21.2	17.1-37.4	23.4	19.5-43.6	2.2
2 positive (n=6)					
Probe E	22.7	15.1-39.9	19.7	17.2-32.9	-0.8
Probe N2	24.2	17.2-41.7	20.9	19.2-35.2	-0.8
3 positive (n=2)					
Probe E	33.3	30.1-37.1	32.3	31.7-33.0	-0.9
Probe N2	34.2	31.8-37.1	33.6	33.6-33.7	-0.5
4 positive (n=1)					
Probe E	37.0	31.9-38.2	NA	NA	NA
Probe N2	39.8	34.2-41.9	NA	NA	NA

Supplementary Table 5.1. CT values for the probes E and N2 for both individual and pooled results

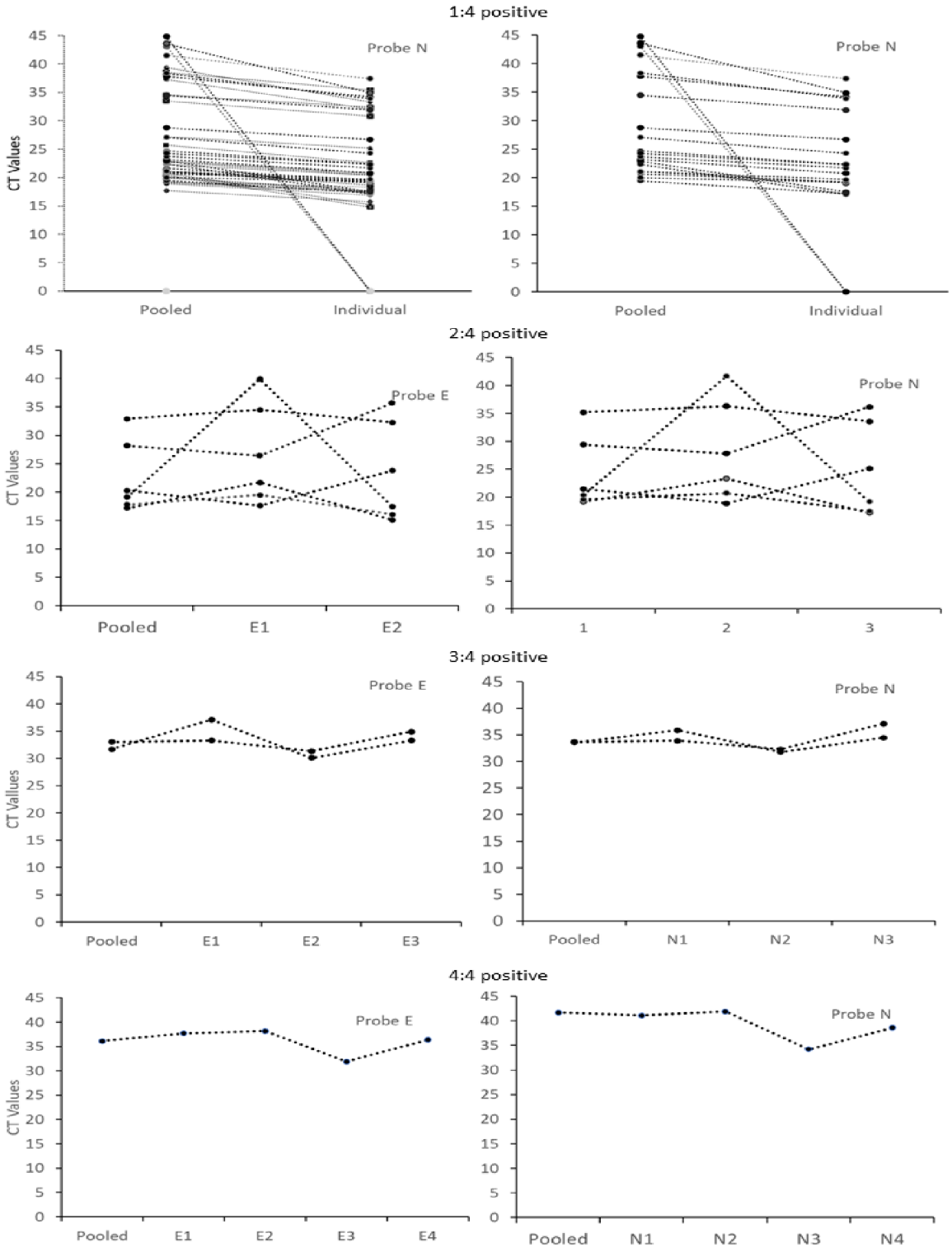
Number of positive samples included in Xpress-positive pools	Individual CT values		Pooled CT values	
	E	N2	E	N2
0	0	0	0	43.1
	0	0	0	44.8
1	15.8	17.3	17.7	19.5
	16.9	19.1	18.9	21.1
	17.4	19.3	19.1	20.7
	18.3	19.3	19.2	20
	17.4	19.4	19.3	20.9
	17.5	19.6	19.9	21.6
	17.7	19.7	20.1	21.1
	14.8	17.1	20.4	22.4
	15.2	17.5	20.8	22.9
	18.7	20.8	21.1	23.2
	20.4	21.7	22.3	23.7
	20.5	22.4	22.7	24.7
	20.8	22.3	22.9	24.3
	22.6	24.3	25.7	27.1
	25.2	26.7	27.2	28.8
	30.8	31.9	33.5	34.5
	32.4	34.2	34.5	37.8
	32.1	33.9	37.3	38.4
	35.4	37.4	38.5	41.5
	33.3	34.9	39.4	43.6
2	21.7	23.3	17.2	19.2
	15.1	17.2		
	19.5	20.7	17.8	19.6
	16.1	17.5		
	39.9	41.7	19.1	20.4
	17.5	19.2		
	17.6	18.9	20.3	21.5
	23.8	25.1		
	26.4	27.8	28.2	29.4
	35.7	36.2		
	34.5	36.3	32.9	35.2
	32.3	33.6		
3	37.1	35.9	31.7	33.6
	30.1	31.8		
	33.3	34.5		
	33.3	33.9	33	33.7
	31.3	32.3		
	34.9	37.1		
4	37.7	41.1	36.1	41.7
	38.2	41.9		
	31.9	34.2		
	36.4	38.6		

Supplementary Figure 5.1. Correlation of individual and pooled Xpert Xpress Sars-CoV2 (positive pools only include those with single individual Xpert Xpress SARS-CoV2-positive sample)



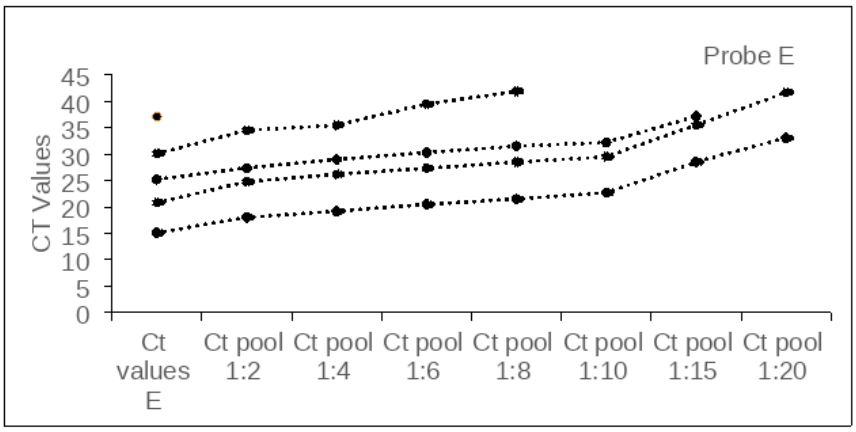
The CT values for the nine pools containing more than one positive sample are shown in Supplementary Table 5.1 and their paired combination are shown in Fig 5.2. Six of the pools that contained two positive samples had a median probe E CT of 19.7 (range 17.2 – 32.9) compared to 22.7 (range 15.1 – 39.9) for the 12 positive individual samples within the pools and a median CT difference of -0.8 (range -9.6 – 0, p = 0.5). Similarly, the median pooled probe N2 CT was 20.9 (range 19.2 – 35.2), compared to 24.2 (range 17.2 – 41.7) for the individual samples, and a median difference of -0.8 (range -10 – 0.5, p = 0.5). A similar pattern was observed for the pool containing three and four positive samples, with median CT being higher in the pools than the individual samples (Fig 5.2, Supplementary Table 5.1).

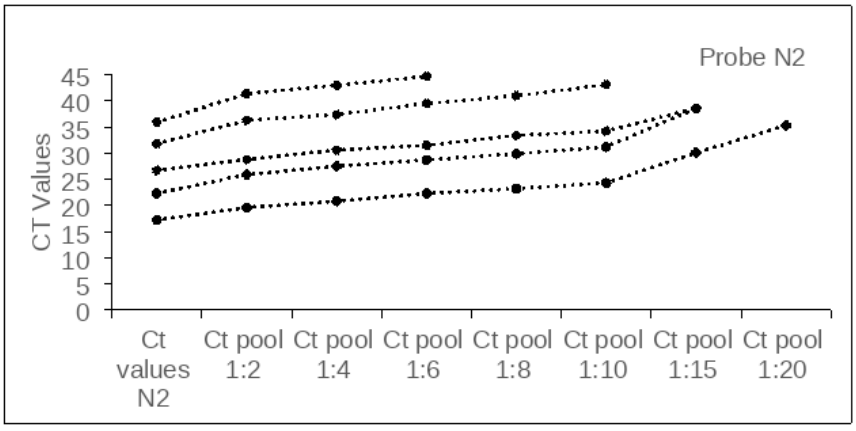
Fig 5.2. CT values of samples containing 1, 2, 3 and 4 positive samples in a pool



The changes in the CT values for Probes E and N of the five positive samples subjected to serial dilutions are shown in Supplementary Figure 5.2 and Supplementary Table 5.2. CT values followed an almost linear increase in CT values across all samples and an increasing number of samples becoming undetectable at 1:10 and 1:20 dilution.

Supplementary Figure 5.2. Effect of serial dilution on CT values of positive samples





Supplementary Table 5.2. Effect of dilution on CT values of positive samples

	Individual	**1:2**	**1:4**	**1:6**	**1:8**	**1:10**	**1:15**	**1:20**
Probe E				**CT value**				
Sample 1	15.1	18	19.2	20.5	21.5	22.7	28.5	33.1
Sample 2	20.8	24.8	26.2	27.3	28.5	29.5	35.5	41.7
Sample 3	25.2	27.4	29	30.3	31.5	32.2	37.2	NA
Sample 4	30.1	34.5	35.5	39.5	41.9	NA	NA	NA
Sample 5	37.1	NA	NA	NA	NA	NA	NA	NA
Probe N2				**CT value**				
Sample 1	17.2	19.6	20.8	22.3	23.2	24.3	30.1	35.3
Sample 2	22.3	25.9	27.5	28.7	29.9	31.2	38.6	NA
Sample 3	26.7	28.8	30.6	31.5	33.4	34.2	38.6	NA
Sample 4	31.8	36.3	37.4	39.5	41	43.1	NA	NA
Sample 5	35.9	41.4	43	44.7	NA	NA	NA	NA

Cost analysis (Table 5.3) indicated testing 1568 participants would have required 1568 cartridges at a cost of USD 31,046.40. The pooling method required 392 cartridges to test in pools of four and 124 cartridges to re-test individually the 31 positive pools, resulting in 516 cartridges at a cost of USD 10,217.00. This represents a savings of 1052 (67%) cartridges, equivalent to USD 20,829.60. Using these same estimates, testing 1000 consecutive patients would require 329 cartridges instead of 1000 at a cost of USD 6.51 per participant. In Lao PDR's context, where laboratories receive a fixed allocation of cartridges, 1,000 cartridges would allow testing 3,040 individuals, which significantly increased testing capacity.

Table 5.3. Cost and diagnostic savings to screen 1000 consecutive patients using the pooling method and number of patients that could be tested with 1000 Xpert Xpress SARS-CoV-2 cartridges

		Pool of 4
	Individual testing	**Pooled testing**
Number of individuals tested	1000	1000
Sensitivity	reference	NA
Specificity	reference	NA
Proportion positive	2.7%	7.9%
COVID-19 cases confirmed	27	27
Cartridges required	1000	329
Cartridge costs (USD)	19,800	6,514
Cartridge savings (USD)	0	**67.1%**
Number tested with 1000 cartridges		
Number tested	1000	3,040
Cartridge cost per patient (USD)	19.80	**6.51**

Discussion

SARS-CoV-2 testing is often limited by the number of tests available. Assay shortages are multifactorial, from the limited production capacity for new assays, delayed procurement, a global shortage of RNA extractions kits, insufficient number of RT-PCR platforms and limited staff for testing. It is thus unlikely that testing capacity will reach the number of tests required in the short-term.

This study demonstrates that pooling samples can significantly increase testing capacity, while simultaneously reducing the resources needed for mass screening of SARS-CoV-2. The savings documented in our study, close to two thirds of the number of cartridges required for individual testing, are significant and were documented at a time when the proportion of pools testing positive was close to 8%. With the same resources required for individual testing, pooling allowed triplicating the number of people tested. Pooling has been reported to generate significant resource and time savings when screening for other infections, such as TB [17, 29]. Savings are dependent upon the pool size and the proportion of pools that are positive. If the proportion of positive pools is low, e.g. at the nadir of an epidemic wave, most pools would be classified as negative and would not require further testing. However, if the proportion positive is high, many more pools would require individual testing [16]. Pooling therefore works well when there is a low prevalence of the pathogen, with more negative than positive results [95]. This is an important practical issue within the pandemic, as the proportion of positive pool varies rapidly during SARS-CoV-2 epidemic waves, with the introduction or removal of NPIs, the arrival of Variants of Concern, and mass gatherings [96].

All individual samples of two of the positive pools tested negative and remained negative when re-tested with a different assay. These pools had high CT values, suggesting they contained very low viral loads and were assumed to be false-positive, caused by accidental cross contamination during sample preparation [97]. Although we had planned to test all individual samples in parallel to the pools, with the aim of exploring whether negative pools contained missed positive samples, this was not possible at the time of the emergency, as supplies were limited, and the Task Force prioritized implementing the approach to increase testing capacity. Previous studies have shown that the RNA concentrations of the individual and pooled tests are correlated, with a dilution effect in the pooled sample due to the lower sample volume used from each patient. This dilution effect increases the possibility of false negative pools, when the RNA concentration is below the limit of detection [95], and our result confirm this trend, with pools with single positive samples having higher CT values than individual samples. PCR CT values, however, are predictable, in that with 100% efficiency, the fluorescence should double each cycle. Therefore, if half of the target RNA is present, the CT value would be one CT later, thus a 1:2 dilution would result in a CT value +1 the undiluted sample, and 1:4 dilution in a CT value of +2. Our data on serial dilutions (S5.2 Fig) seem to have increased CT values slightly more than expected, although these values are within the margin of error, and even though the increase of 1:10 to 1:20 is steeper, this would be expected at high dilution ratios. Interestingly, the dilution effect was not homogeneous, as pools with multiple positive samples often had the same or lower CT value than individual samples, thus indicating that the combination of multiple positive samples in a pool increases the total amount of genetic material and compensates for the dilution effect. Studies by others on pooling for SARS-CoV-2 testing from nasopharyngeal swabs have reported

reduced CT values for pooled samples containing a single positive, hypothesizing that the PCR efficiencies were increased by a "carrier-RNA" effect caused by increased total cellular RNA in the samples [63, 64]. Furthermore, pooling samples can lead to improved PCR efficiency and sensitivity in the case of a single positive sample containing PCR inhibitors, which are then diluted by pooling. However, no failed internal control results occurred on any of the Xpert Xpress runs of our study.

Our findings indicate that, in the Lao PDR context, the pooling method can increase the testing capacity by a factor of 3.04 compared to individual testing, with significant public health implications in situations of tests shortages and high demand for laboratory testing. In addition to resources savings, the rapid turnaround time with pooled testing can have a significant impact in terms of quarantine and isolation policies. By rapidly identifying positive clusters, the health authorities can trigger lockdowns in areas with confirmed outbreaks and ease the restrictions where there is no active transmission [98].

Other studies have reported that pooling with Xpert Xpress SARS-CoV-2 assay is reliable and that the dilution effect of multiple testing has limited effect on the sensitivity of the test [16, 99]. Here we assessed the impact of the dilution on the CT values by performing serial dilution on samples of known CT values. Our results indicate the likelihood of false negative samples increases with the increasing dilution ratios, and that diluting the sample >1:10 results in significant losses of sensitivity. With a 1:20 ratio dilution, there was an increase in CT values >10, and consequently individual samples with low viral load and high CT values were missed by falling under the limit of detection. However, if the proportion of positive samples is high, the risk of false negative results may be minimized by the increased likelihood of samples containing more than one positive specimen in the pool.

Study limitations

In other studies focusing on the assessment of the sensitivity and specificity of the pooling method, all samples are tested individually, and the pooling is conducted as an operational research to determine whether it could result in the same number of positive and negative patients as individual testing [100, 101]. This study took place at a time when there was a consortium allocation established by WHO for Xpress tests, with 1,000,000 tests available globally, and Lao PDR was entitled for only 10,000 cartridges. Consequently, laboratories in the country received a fixed allocation of cartridges based on the burden of the disease in their catchment area and testing capacity with the shared GeneXpert platform, to ensure essential services for TB and HIV were not diverted. Therefore, given the limited resources available for mass screening, pooling was used as the reference method, assuming its sensitivity and specificity was acceptable, and that pooling was warranted based on public health needs. Individual testing was thus only done for individual samples in positive pools and therefore, we did not assess the sensitivity and the specificity of the method. Consequently, we don't know if, among the negative pools, there were positive samples that were missed. Moreover, the country decided to apply pools of four samples without assessment of the optimal pool size. A prior epidemiology analysis to determine the proportion of positive samples by province and district for the different population groups would have allowed identifying whether larger pool sizes could have been more efficient. The pooling method is not a one-size-fits-all approach and statistical calculations using different combinations of pool sizes and positivity rate would have maximized the testing capacity and optimized the resources savings [99].

Furthermore, the cost analysis and the savings presented in this study did not include all savings that were generated around the pooling method, such as staff time,

electricity, consumables, laboratory maintenance, samples transportation, wastes management and life expectancy of the GeneXpert machines.

Conclusions

The pooling of samples for SARS-CoV-2 testing can be a useful strategy for testing when health systems are overwhelmed. This method can be rapidly implemented given the limited need for additional staff or sophisticated infrastructure. In a time where countries are facing shortage in laboratory supplies, with daily number of samples collected exceeding testing capacity, the pooling method can facilitate the expansion of testing in resource limited settings and accelerate the implementation or ease of NPIs based on the local incidence.

Chapter VI - Pooling sputum testing to diagnose tuberculosis using Xpert MTB/RIF and Xpert Ultra: a cost-effectiveness analysis

As published in BMC Infectious Diseases ((Published: May 22, 2023).

Citations as follows**:** Iem V, Bimba J, Santos V, Dominguez J, Creswell J, Somphavong S, Wingfield T, Khan JAM, Cuevas LE. Pooling sputum testing to diagnose tuberculosis using xpert MTB/RIF and xpert ultra: a cost-effectiveness analysis. *BMC Infectious Diseases* 2023: 23(1): 341

https://doi.org/10.1186/s12879-023-08330-9

Introductory section

This thesis has shown that pooled testing can result in significant savings, for both Xpert MTB/RIF and Xpert Ultra, and during both active and passive case finding. However, one of the limitations that was common to all these studies was that the estimations of the savings were limited to costs savings of the test assay cartridges only and therefore did not represent the actual cost effectiveness of the pooled testing.

In chapter VI, we aimed to investigate the magnitude of the savings of the pooled testing by conducting a formal costs and cost-minimization analysis.

The study in Chapter VI was conducted in 2022, after all results from the previous chapters were finalised.

Abstract

Background: The World Health Organization (WHO) recommends the diagnosis of tuberculosis (TB) using molecular tests, such as Xpert MTB/RIF (MTB/RIF) or Xpert Ultra (Ultra). These tests are expensive and resource-consuming, and cost-effective approaches are needed for greater coverage.

Methods: We evaluated the cost-effectiveness of pooling sputum samples for TB testing by using a fixed amount of 1,000 MTB/RIF or Ultra cartridges. We used the number of people with TB detected as the indicator for cost-effectiveness. Since the outcomes (identification of TB cases) of the intervention and control were not statistically significantly different, a cost-minimization analysis was conducted from the healthcare system perspective and included the costs to the healthcare system using pooled and individual testing.

Results: There was no significant difference in the overall performance of the pooled testing using MTB/RIF or Ultra (sensitivity, 93.9% vs 97.6%, specificity 98% vs 97%, p-value >0.1 for both). The mean unit cost across all studies to test one person was 34.10 international dollars for the individual testing and 21.95 international dollars for the pooled testing, resulting in a savings of 12.15 international dollars per test performed (35.6% decrease). The mean unit cost per bacteriologically confirmed TB case was 249.64 international dollars for the individual testing and 162.44 international dollars for the pooled testing (34.9% decrease). Cost-minimization analysis indicates savings are directly associated with the proportion of samples that are positive. If the TB prevalence is ≥30%, pooled testing is not cost-effective.

Conclusion: Pooled sputum testing can be a cost-effective strategy for diagnosis of TB, resulting in significant resource savings. This approach could increase testing

capacity and affordability in resource-limited settings and support increased testing towards achievement of WHO End TB strategy.

Introduction

Tuberculosis (TB) was the second leading cause of death by an infectious disease after Coronavirus disease 2019 (COVID-19) [1]. The World Health Organization (WHO) recommends to provide upfront molecular tests (mWRDs) for the diagnosis of TB and at least rifampicin resistance to all individuals with presumptive TB [2]. mWRDs include the Xpert MTB/RIF [3] (MTB/RIF) and Xpert MTB/RIF Ultra (Ultra) [4], which are semi-automated and simultaneously detect *Mycobacterium tuberculosis* complex and markers of rifampicin resistance using the GeneXpert platform. The Ultra assay is currently the recommended Xpert assay, based on its increased sensitivity, which improves the detection of paucibacillary TB [5]. Several high TB burden countries such as South Africa and Uganda have transitioned towards use of Xpert as the upfront test for TB diagnosis. However, despite efforts made by National TB Programmes, mWRDs are still not used globally as the upfront test for TB diagnosis for many people. This is because of the high cost ($US 9.98 per test at FIND negotiated price) and mWRDs being predominantly available only at higher levels of the TB laboratory network with better infrastructure and more qualified human resources [102]. Consequently, due to the high costs of the test, cartridges are often rationed, and many tests are only used as reflex tests once people have been diagnosed, and more centralized testing can lead to longer turnaround time.

To maintain sufficient TB testing capacity and cope with these challenges, one practice that has re-emerged during the COVID-19 pandemic is pooled testing. In this approach, several specimens collected from different presumptive TB cases are pooled (mixed) together and tested as a group in a single assay. If the pooled test is negative, it is then assumed all samples included in the pool are negative. If the pooled test is positive, it means at least one sample included in the pool is positive,

and individual re-testing of samples is needed to identify the positive sample(s) (Figure 6.1). A systematic review published in 2021 concluded this method was highly sensitive and specific and can substantially increase testing capacity with savings up to 27-31% in cartridges alone, depending on the prevalence of TB in the population tested [17]. However, data on cost-effectiveness are currently limited to assay savings on the basis of the number of cartridges that would have been required to test all specimens when using individual vs pooled testing as part of individual evaluations.

In this study, we conducted a cost-effectiveness analysis of the pooled testing strategy in comparison with Xpert MTB/RIF and Xpert Ultra individual testing, during passive case finding (PCF) routine activities. Between each method, we compared the costs to test 1,000 patients, the potential resources savings, the diagnostic accuracy, the cost to detect one person with bacteriologically confirmed TB, and the potential increase in testing capacity and TB case detection.

Materials and methods

In this cost-effectiveness analysis, a total of 3,076 individuals with presumptive TB were enrolled from two studies conducted in Lao PDR (840 individuals per study) [61], two studies in Nigeria (500 individuals per study) [103], and one study in Brazil (396 individuals) [104], which are described in more detail below.

WHO defines an individual with presumptive TB as anyone who shows symptoms or signs suggestive of TB. The most common symptom of pulmonary TB is persistent, productive cough, often accompanied by other non-specific respiratory symptoms (shortness of breath, chest and back pains, hemoptysis) and/or constitutional symptoms (loss of appetite, weight loss, fever, night sweats, and fatigue) however screening tests such as chest X-ray can also be used to identify people with presumptive TB despite lack of symptoms [105].

Pools were created by mixing four consecutive samples. Pooled samples were then tested with Xpert MTB/RIF or Ultra assays. Pools and their corresponding individual results were compared to determine the level of agreement, meaning if pools including only MTB-negative sample would return an MTB-negative pooled test result, and pools containing ≥ 1 MTB-positive sample would return an MTB-positive pooled test result.

Studies were cross-sectional surveys, conducted during PCF programmatic activities. In this approach, which is a patient-initiated pathway to TB diagnosis, individuals with symptoms suggestive of TB present spontaneously to the health facility for the health worker to initiate the investigation for TB using a diagnostic algorithm with sufficient sensitivity and specificity to diagnose TB [68].

In Lao PDR [61] and Nigeria [103], two independent studies were conducted in each country during PCF of people with presumptive TB, one using Xpert MTB/RIF, and the other Ultra. One study from Brazil assessed the performance of pooled testing with Xpert Ultra only [104].

Statistical analysis

Categorical data were summarized using descriptive statistics and chi-squared tests were used to test for statistically significant differences, where appropriate. Pooled test results (MTB-positive or MTB-negative) were compared with the four corresponding Xpert MTB/RIF or Xpert Ultra individual test results and their agreement was assessed by calculating the Kappa coefficient. The kappa values and their interpretations were as follows: <0, no agreement; 0–0.19, very weak agreement; 0.20–0.39, weak agreement; 0.40–0.59, moderate agreement; 0.60–0.79, substantial agreement; and 0.8–1.0, excellent agreement [106].

Cost-effectiveness analysis

We measured the cost-effectiveness of pooled testing vs individual testing by comparing the number of individuals that would be bacteriologically confirmed using each method. Since the outcomes (identification of TB cases) of the intervention and control were not statistically significantly different, the cost-effectiveness analysis turned to be cost-minimization analysis, where we estimated and compared the costs of individual and pooled TB detections.

Cost analysis is a technique that involves the systematic collection, categorization, and analysis of costs of any intervention [107]. Potential savings were calculated by comparing all resources required to test all specimens using pooled and individual testing by analyzing the costs of each TB detection method. We used an ingredient-

based, top-down approach, in which all categories of inputs were listed alongside all quantities needed to perform all tests annually, for both the individual and pooled testing approach (Table 6.1).

The GeneXpert instruments set, biosafety cabinet and autoclaves, and other small equipment (uninterruptible power supply, timer, vortex) were considered as “capital items”. The cost of equipment was determined by using the estimated lifetime of capital items in years to which we then applied an annuity factor to estimate the cost per year. The useful time of the capital items reported here was based on annual warranty cost with a 5-year expected lifetime [47].

We also listed and quantified all recurrent items needed to perform all the tests over one year, with the cost of all items needed annually. The base level cost of MTB/RIF and Ultra testing were the same. All the Xpert cartridges, laboratory supplies, disposable personal protective equipment, biosafety supplies, and human resources were considered as “recurrent items”. We then divided the total annual cost for capital and recurrent items by the number of tests performed annually to estimate the unit cost to perform one test. Values for each country were adjusted for international dollars by using DEC (World Bank's Development Economics department) alternative conversion factor (local currency units per US$) and purchasing power parity conversion factor, gross domestic product (local currency units per international $) from the World Bank (2021 data).

We then compared both approaches to calculate the difference in the money invested for testing 1,000 consecutive individuals, the number of people who could be tested for TB when using a fixed amount of 1,000 cartridges, and the costs per bacteriologically confirmed TB case detected. The cost of pooled testing also included

the cost of retesting all specimens from positive pools individually. Thus, our cost-minimization analysis was able to demonstrate a cost-saving outcome if pooled testing cost less than individual testing while detecting at least the same or higher numbers of TB cases.

Figure 6.1. Flow diagram of the sputum processing (same test was used for individual and pooled testing (either Xpert MTB/RIF or Xpert Ultra))

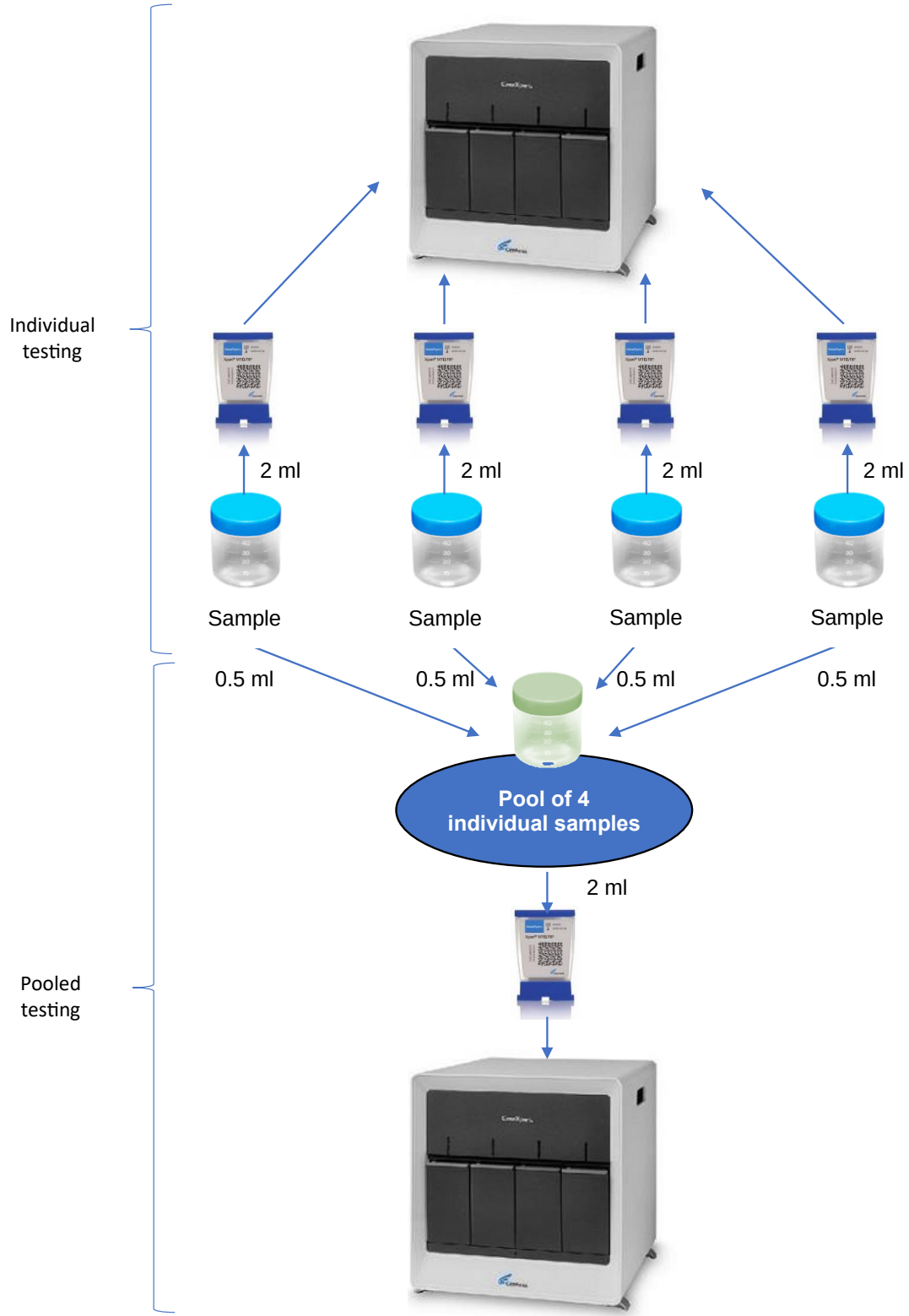


Table 6.1. Resources costs assumptions for TB diagnosis by Xpert MTB/RIF and Ultra (unit costs from the Global Drug Facility products catalogs "Ordering List of TB Medicines or Diagnostics, Medical Devices and other health products")

	Price (US$)	Cost per test (US$)	Cost per test (International $)		
			Lao PDR	Nigeria	Brazil
Supplies required for Xpert test: Provided by Cepheid					
GeneXpert instrument	15105	0.5815	1.977	1.522	1.242
Laptop HP or DELL brand	2395	0.1126	0.383	0.295	0.240
Biosafety equipment					
Laboratory coats	20	0.0018	0.006	0.005	0.004
Autoclave	22,000	0.5042	1.714	1.320	1.077
Equipment					
UPS 1500 VA	1,500	0.0577	0.196	0.151	0.123
Timer	30	0.0012	0.004	0.003	0.002
Vortex	220	0.0085	0.029	0.022	0.018
Stationery					
Indelible labelling marker	2	0.0033	0.011	0.009	0.007
Pens (red and blue or black)	1	0.0017	0.006	0.004	0.004
Supplies required for Xpert test					
MTB/RIF cartridges	499	9.9800	33.930	26.130	21.312
Laboratory supplies					
Sterile screw-capped specimen collection containers	83.5	0.0835	0.284	0.219	0.178
Paper towels	2	0.0067	0.023	0.017	0.014
Personal protective equipment					
Disposable gloves	20	0.0800	0.272	0.209	0.171
Surgical masks	21.5	0.0344	0.117	0.090	0.073
Biosafety supplies					
Disposable autoclave bags (LxW = 35"x25")	20	0.0320	0.109	0.084	0.068
Disposable autoclave bags (LxW = 19"x14")	20	0.0160	0.054	0.042	0.034
Tuberculocidal disinfectant solution 0.003 liters per test	45	0.0270	0.092	0.071	0.058
Human resources					
Laboratory technician (40 hours/week, 4 weeks/month)*	250	0.5000	1.700	1.309	1.068
Total number of test/years	**6000***	**11.7141**	**40.91**	**31.50**	**25.69**

* Lao PDR data

Results

Pooled testing diagnostic accuracy (Table 6.2)

In Lao PDR, in the Xpert MTB/RIF survey, 77/81 (sensitivity 95.1%, 95%CI 87.8%-98.6%) pools containing ≥1 positive sample tested MTB-positive and 4/81 (4.9%, 95%CI 1.4%-12.2%) tested MTB-negative. All 129/129 pools containing MTB-negative samples tested MTB-negative (specificity 100%, 95%CI 97.2%-100%), with 98.1% agreement (Kappa: 0.959). In the Xpert-Ultra survey, 70/70 (sensitivity 100%, 95%CI 94.9%-100%) pools containing ≥ 1 MTB-positive sample tested MTB-positive and 140/140 (specificity 100%, 95%CI 97.4%-100%) pools containing only MTB-negative samples tested MTB-negative, with 100% agreement (Kappa: 1).

In Nigeria, 46/50 (92%, 95%CI 80.8%-97.8%) positive pools tested Xpert MTB/RIF MTB-positive and 71/75 (94.7%, 95%CI 86.9%-98.5%) negative pools tested MTB-negative (agreement 93.6%, Kappa=0.867). In comparison, 36/42 (86%, 95%CI 71.5%-94.6%) positive pools tested Xpert-Ultra MTB-positive and 82/83 (98.8%, 95%CI 93.5%-99.8%) negative pools tested negative (agreement 94.4%, Kappa=0.871). There was no statistically significant difference in sensitivity (p-value=0.33) or specificity (p-value=0.14) for pooling with Xpert MTB/RIF or Xpert Ultra.

In Brazil, 99 pools were tested, of which 62 (62.6%) had MTB-detected and 37 (37.4%) MTB-not detected, including six (6.1%) with MTB-trace. The agreement of individual and pooled testing was 96.0% (Kappa of 0.913). Pooling had sensitivity of 95.3% (95%CI 86.9%–99%) and specificity of 97.1% (95%CI 85.1%–99.9%).

There was no significant difference in the overall agreement across all studies with individual testing when pooling either Xpert MTB/RIF (96.4% agreement

(n=323/335, CI 95% 93.7%-98.1%) or Ultra (97.2% agreement (n=422/434, CI 95% 95.1%-98.5%), p-value=0.529.

There was also no significant difference in the overall performance across all studies when pooling with either Xpert MTB/RIF or Ultra (sensitivity 93.9% (n=123/131, CI 95% 87.9%-97.1%) vs 97.6% (n=166/170, CI 95% 93.7%-99.2%), p-value=0.105, and specificity 98% (n=200/204, CI 95% 94.7%-99.4%) vs 97% (n=256/264, CI 95% 93.9%-98.6%, p-value=0.467, respectively).

Table 6.2. Agreement of individual and pooled tests

	MTB/RIF					ULTRA						
	Lao PDR Pooled n=210		Nigeria Pooled n=125		Overall	Lao PDR Pooled n=210		Nigeria Pooled n=125		Brazil Pooled n=99		Overall
Individual	**Neg**	**Pos**	**Neg**	**Pos**		**Neg**	**Pos**	**Neg**	**Pos**	**Neg**	**Pos**	
All four negative	129	0	71	4	-	140	0	82	7	34	1	-
At least one positive	4	77	4	46	-	0	70	1	35	3	61	-
Agreement	206/210 (98.1%)		117/125 (93.6%)		323/335 (96.4%)	210/210 (100%)		117/125 (93.6%)		95/99 (96.0%)		422/434 (97.2%)
Kappa	0.959		0.867		-	1.000		0.851		0.913		-
Sensitivity (95% CI)	95.1% (87.8-98.6%)		92.0% (80.8-97.8%)		93.9% (87.9-97.1%)	100% (94.9%-100%)		85.7% (71.5-94.6%)		95.3% (86.9%-99.0%)		97.6% (93.7%-99.2%)
Specificity (95% CI)	100% (97.2-100%)		94.7% (86.9-98.5%)		98% (94.7-99.4%)	100% (97.4-100%)		98.8% (93.5%-99.8%)		97.1% (85.1%-99.9%)		97% (93.9%-98.6%)

Testing capacity and number of bacteriologically confirmed TB cases (Table 6.3)

In Lao PDR, pooled testing using a fixed number of 1,000 Xpert MTB/RIF cartridges would miss 5.1% (n=10/197) of the TB cases. However, pooled testing would generate an increase of 62% in the number of people screened (1,000 vs 1,620) leading to an increase of 54% in the absolute number of the TB cases identified despite the 10 missing TB cases (121 vs 187 (197-10)). Pooled testing using a fixed number of 1,000 Ultra cartridges would generate an increase of 71.5% in the number of people tested (1,000 vs 1,715) and 71.5% in the absolute number of TB cases identified (111 vs 191), with no missing TB cases.

In Nigeria, pooled testing using a fixed number of 1,000 Xpert MTB/RIF cartridges would miss 5.8% (n=13/223) of the TB cases. However, pooled testing would generate an increase of 44% in the number of people screened (1,000 vs 1,440) leading to an increase of 45.3% in the absolute number of TB cases identified despite the 13 missing TB cases (144 vs 210 (223-13)). Pooled testing using a fixed number of 1,000 Ultra cartridges would miss 9.6% (n=27/280) of the TB cases. However, pooled testing would generate an increase of 85.8% in the number of people screened (1,000 vs 1,858) leading to an increase of 110.7% in the absolute number of TB cases identified despite the 27 missing TB cases (120 vs 253 (280-27)).

In Brazil, pooled testing using a fixed number of 1,000 Ultra cartridges would miss 3.3% (n=9/275) of the TB cases. However, pooled testing would generate an increase of 14.2% in the number of people screened (1,000 vs 1,142) and 10.4% in the number of TB cases identified despite the 9 missing TB cases (240 vs 265).

Table 6.3. Cost analysis of each strategy (individual vs pooled) by country, by assay (MTB/RIF vs Ultra) using a fixed amount of 1,000 cartridges

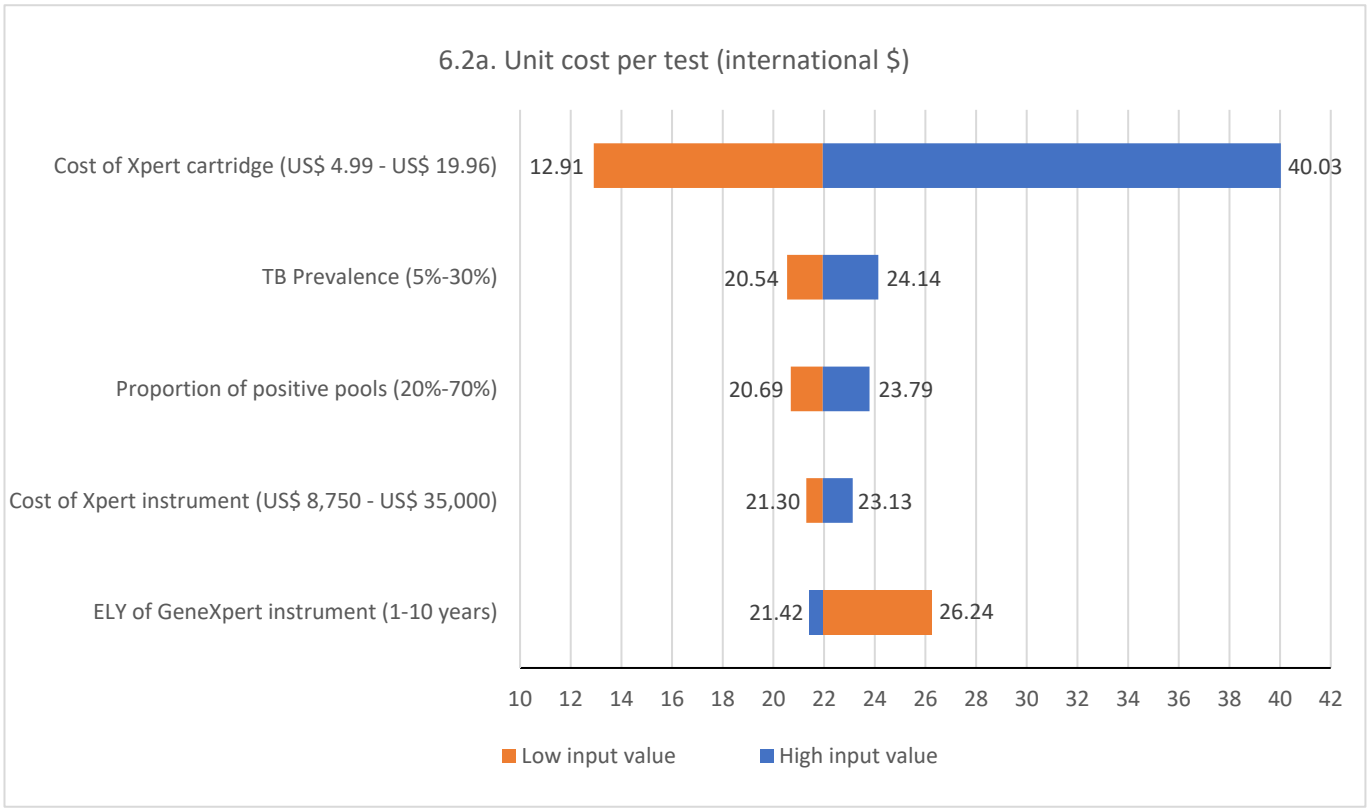
Assay	Country	Testing Strategy	Number of individuals tested	Potential missed among TB cases	Bacteriologically confirmed cases
Xpert MTB/RIF	Lao PDR	Individual	N=1,000	Reference	121
		Pooling	**N=1,620 (62% increase)**	**5.1%, n=10/197**	**187 (54% increase)**
	Nigeria	Individual	N=1,000	Reference	144
		Pooling	**N=1,440 (44% increase)**	**5.8%, n=13/223**	**210 (45.3% increase)**
Xpert ULTRA	Lao PDR	Individual	N=1000	Reference	111
		Pooling	**N=1,715 (71.5% increase)**	**-**	**191 (71.5% increase)**
	Nigeria	Individual	N=1,000	Reference	120
		Pooling	**N=1,858 (85.8% increase)**	**9.6%, n=27/280**	**253 (110.7% increase)**
	Brazil	Individual	N=1,000	Reference	240
		Pooling	**N=1,142 (14.2% increase)**	**3.3%, n=9/275**	**265 (10.4% increase)**

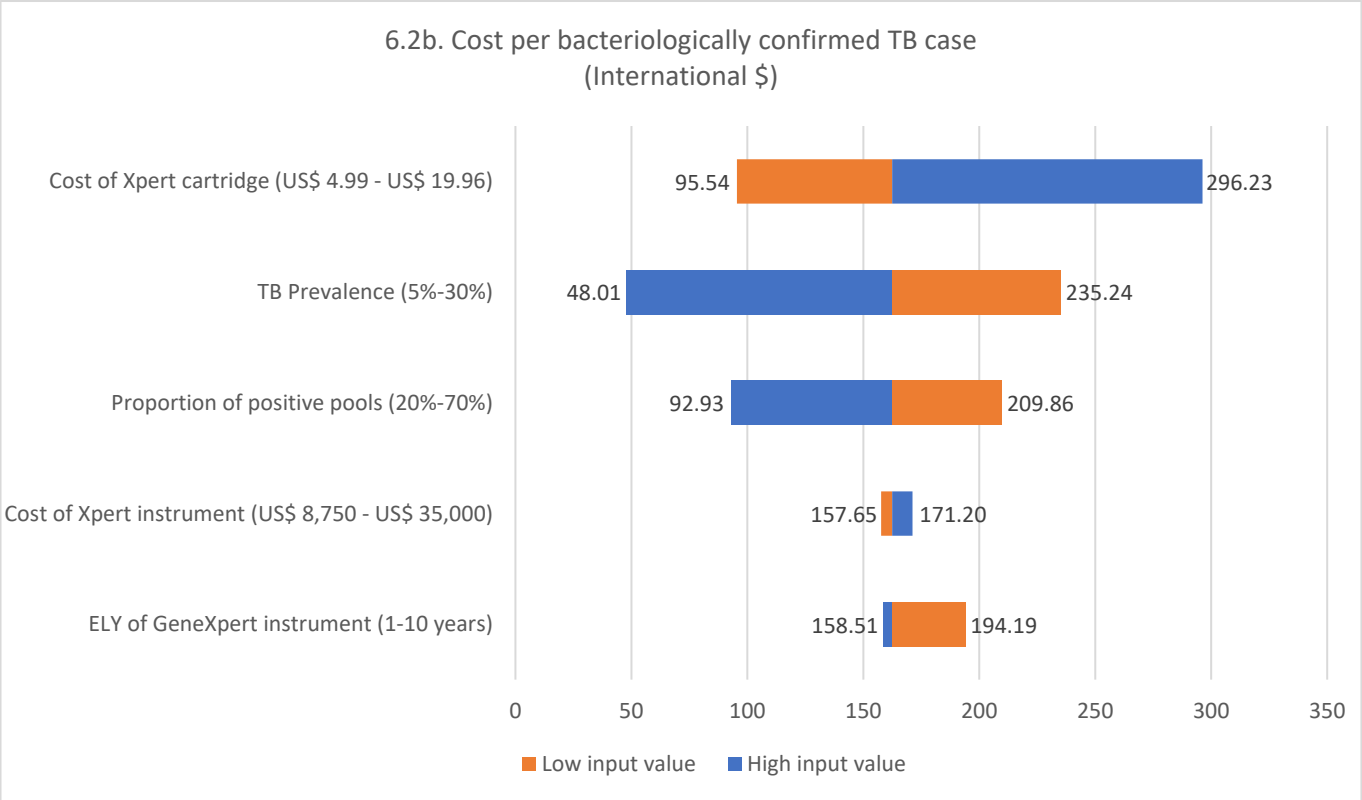
Costs of detection methods (Table 6.4)

Cost-minimization analysis

Since the detection of TB cases by individual and pooled testing, with both Xpert MTB/RIF and Ultra was not significantly different, we compare only the costs of tests and accept the least costly one as the cost-effective method by utilizing the cost-minimization analysis technique [107]. The univariate sensitivity analysis (Figure 6.2a and Figure 6.2b) on other parameters that could affect the cost effectiveness and that would vary among different settings shows costs of the cartridge assay was the major determinant in the unit cost per test variation, accounting for 85.2% of the cost to test one person with presumptive TB.

Figure 6.2. Parameters affecting the pooled testing cost-effectiveness





The overall unit cost across all studies (1,000 individual sample size population) to test one person was 34.10 international dollars for the individual testing and 21.95 international dollars for the pooled testing, resulting in a savings of 12.15 international dollars per test performed (35.6% decrease). The overall unit cost per bacteriologically confirmed TB case was 249.64 international dollars for the individual testing and 162.44 international dollars for the pooled testing (34.9% decrease).

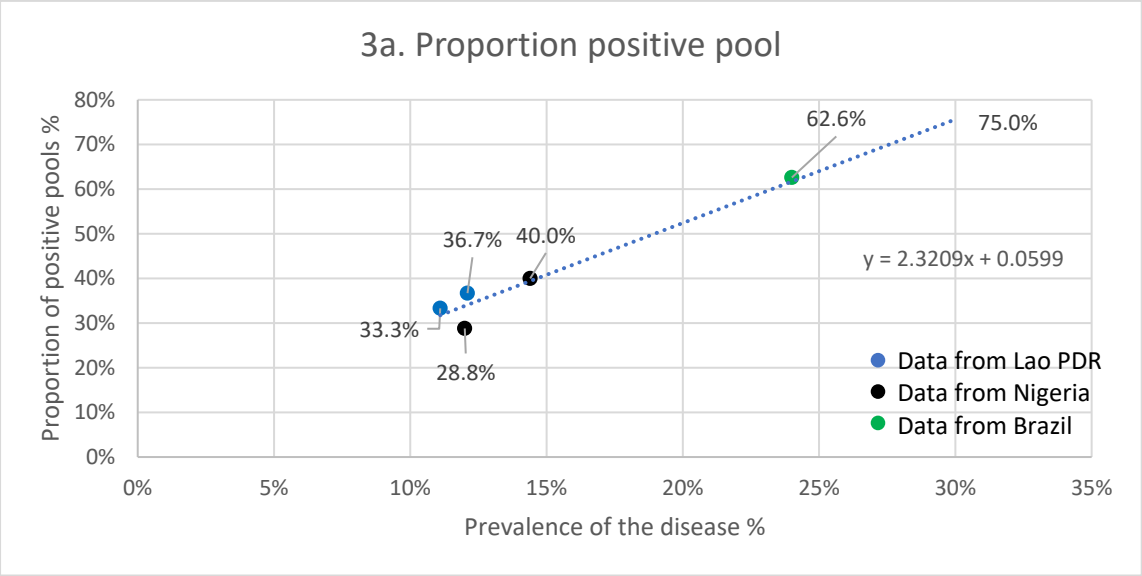
Table 6.4. Cost-analysis of each strategy (individual vs pooled) by country, by assay (MTB/RIF vs Ultra) to test 1,000 presumptive TB patients

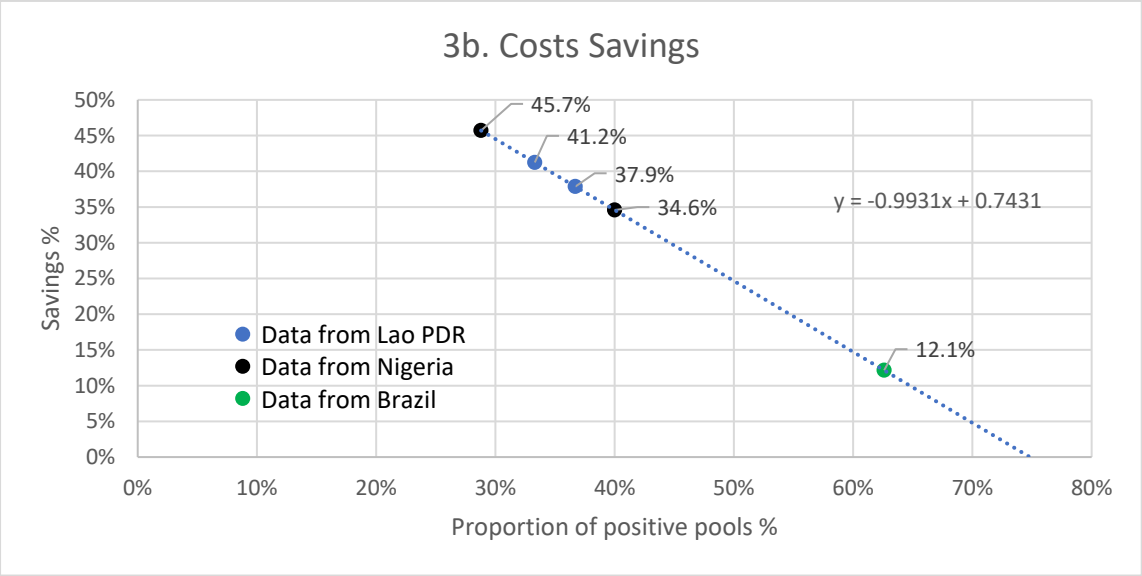
Assay	Country	Testing Strategy	Proportion positive	Savings (%)	Cost per test (International $)	Nb of cartridges	Bacteriologically confirmed cases	Cost per bacteriologically confirmed TB case (International $)
Xpert MTB/RIF	Lao PDR	Individual	12.1%	Reference	40.91	1,000	121	338.07
		Pooling	36.7%	**37.9%**	**25.42**	**617**	**115**	**221.04**
	Nigeria	Individual	14.4%	Reference	31.50	1,000	144	218.77
		Pooling	40%	**34.6%**	**20.61**	**650**	**136**	**151.53**
Xpert ULTRA	Lao PDR	Individual	11.1%	Reference	40.91	1,000	111	368.53
		Pooling	33.3%	**41.2%**	**24.04**	**583**	**111**	**216.56**
	Nigeria	Individual	12.0%	Reference	31.50	1,000	146	215.77
		Pooling	28.8%	**45.7%**	**17.10**	**538**	**136**	**125.77**
	Brazil	Individual	24%	Reference	25.69	1,000	240	107.06
		Pooling	62.6%	**12.1%**	**22.57**	**876**	**232**	**97.30**
OVERALL		Individual			**34.10**			**249.64**
		Pooling			**21.95**			**162.44**

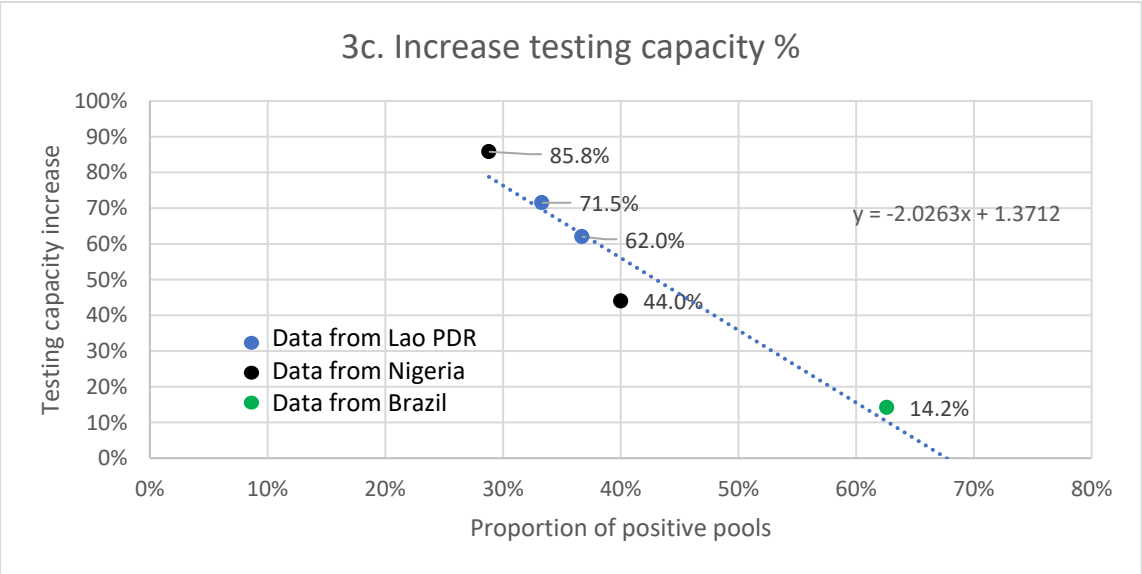
Figure 6.3a shows there is a linear correlation between the prevalence of the disease in the population tested and the proportion of positive pools. This has a direct impact on the savings: the lower the proportion of positive pools, the higher the savings in assay costs (Figure 6.3b), since fewer pools require individual testing. Consequently, the lower the proportion of positive pools, the higher the increase of testing capacity (Figure 6.3c).

Based on these findings, by applying a forecast forward from the trendline of the graph in Figure 6.3b, we can observe savings disappear when the proportion of positive pools is ≥ 75%. Inductively, when applying a forecast forward on graph 6.2a, a 75% proportion of positive pools corresponds to a 30% prevalence of TB. Therefore, when the prevalence of TB is ≥ 30%, pooled testing is unlikely to still be cost-effective.

Figure 6.3. Effect of the prevalence of the disease on the amount of savings by pooling method







Discussion

Depending on the local TB prevalence, pooled testing could potentially enable the screening and testing of larger numbers of people more cost-effectively. Varying the number of samples per pool may also help improve cartridge savings [108]. Pooled testing demonstrated high sensitivity and specificity with both Xpert MTB/RIF and Xpert Ultra. At a time when international donors are increasingly requesting countries to commit to co-financing mechanisms for the procurement of tests from government-funded schemes, the pooling method is relevant to help National TB Programs cope with these funding gaps.

Discrepancies between individual and pooled tests only occurred among pauci-bacillary samples with high Xpert CT values. This suggests that some samples with low DNA concentrations fall below the assay's limit of detection once mixed in the pool. Consequently, some patients with paucibacillary disease could be missed by pooling, especially if testing is based on Xpert MTB/RIF. However, if we look at the resources needed to screen this fixed number of patients, the savings will allow a higher number of patients to be tested using the same amount of resources. Therefore, under the pooling approach, a higher number of individuals could be tested leading to a higher absolute number of bacteriologically confirmed cases within a fixed time period with a fixed amount of resources, despite the number of missed TB cases. Pooled testing will allow a faster catch-up and more cost-effective strategy to find the people with TB compared to individual testing. Moreover, Cepheid will discontinue the production of the Xpert MTB/RIF assay in 2023, and the Global Laboratory Initiative from the Stop TB Partnership, has issued practical guidance to plan and implement a smooth transition from use of Xpert MTB/RIF to Xpert MTB/RIF Ultra cartridges, ensuring uninterrupted service and avoiding cartridge wastage [109]. If

countries choose to implement pooled testing going forward, only Xpert Ultra will be available, which has better sensitivity and agreement compared to Xpert MTB/RIF.

A small number of individual samples included in MTB-positive pooled test results would return an MTB-negative result when re-tested individually. These are unexpected results since the Xpert MTB/RIF and the Ultra are highly specific and are not expected to yield false-positive results [5]. However, in other studies assessing the performance of the pooled testing for SARS-Cov-2 [62], these false-positive pooled test results happened on rare occasions with pools displaying borderline high CT values suggesting very small quantity of genetic material, and the authors have attributed it to cross-contamination during samples handling and processing. In general, for all diagnostic tests, false-positive results occur more frequently in low prevalence settings [110], and this is why for instance the WHO recommends repeat Xpert test with a fresh sample whenever rifampicin resistance is detected for an individual from groups with low risk of RR/MDR-TB, despite the high specificity of the assay [2]. It is therefore important to properly organize the workflow of samples with adequate laboratory commodities, clear standard operating procedures to avoid any clerical errors or risks of contamination. The evaluation of trace results should also be interpreted cautiously. If a pool returns a trace result, all samples included in that pool should be retested individually in order to determine if the pooled trace result is due to a very low load of bacilli that became trace due to the dilution effect, or if a trace sample was indeed included in the pool. If individual testing of samples from the trace pooled test shows there was a sample with a very low result, or one or more trace results samples, those patients need to be managed according to their national diagnostic algorithm considering pulmonary or extrapulmonary TB, HIV status, age, and prior TB treatment. However, since all samples included in MTB-

positive pools are re-tested individually, the false-positive pooled test results would have no impact since the individual test result is used to guide the clinical management of the patients.

Our results demonstrate that pooling samples can significantly increase testing capacity, while simultaneously reducing the resources needed for TB mass testing. The unit cost for testing each person with presumptive TB and the savings were a function of the estimated underlying prevalence of the disease (proportion of people with MTB-positive results) in the setting where the pooled testing was implemented and their distribution within the pools. When the proportion of individuals with positive tests is lower, there are more MTB-negative pooled tests which do not require further testing, leading to higher savings. In the study from Brazil [104], the proportion of individual samples MTB-detected was much higher (24%), and many more MTB-detected pools required further individual testing (62.6%), resulting in reduced cost savings (12%). Pooling therefore works well when there is a low TB prevalence, with more negative than positive results [16]. This is an important practical factor to consider before implementation of pooled testing, as the proportion of positive pools varies significantly according to the population to be tested. Extrapolations from results reported here confirm the findings from previous study showing that in population where the disease prevalence is above 30%, the proportion of pools returning an MTB-positive results would be high (75%), leading to no savings due to the high number of deconvolution [46]. Adjusting the number of samples per pool may increase the efficiency of pooling based on the expected prevalence [108]. Pooling is not a universal solution and National TB Programmes need to be cautious as to where and when to apply it. Laboratories should determine the TB prevalence based on a rolling average of the positivity rate of their own testing

and for different populations/groups. Indeed, the clinical history of the patients to be tested by the pooling method must be considered, especially in settings where HIV is prevalent. PLHIV have low sputum bacillary loads, and mixing those samples into a pool with MTB-negative samples will increase the risk of getting a false negative pooled test result due to the dilution factor. However, if the proportion of TB-HIV coinfected is high, the risk of false-negative results may be minimized by the increased likelihood of samples containing more than one positive specimen in the pool. Other studies have shown the dilution effect was not homogeneous, as pools with multiple positive samples often had the same or lower CT value than individual samples [62], thus indicating that the combination of multiple positive samples in a pool increases the total amount of genetic material and compensates for the dilution effect. Laboratories can then determine when the positivity rate is low enough to justify the implementation of a pooling strategy [108]. Moreover, the use of the pooling method should be a dynamic strategy following the evolution of the TB prevalence in the selected area and the positivity rate of laboratory results.

Study limitations

Results reported here are focused on the cost-minimization of the pooled testing for TB diagnosis, but parameters included for the analysis did not encompass all actual costs. For example, the costs for maintenance of the instruments were not included. These costs comprise the price of the spare parts such as the module (900$ per refurbished module, 3000$ for new module) or the annual calibration (Xpert Check calibration kit at 450$ per kit per machine), shipment, purchasing and supply management (PSM) costs and the manpower to carry out the calibration or replace faulty elements. After purchasing new modules and annual calibration kits, National TB Programmes need to plan budget for engineers to go on-site and replace the faulty module. This would incur labour cost, travel costs, accommodations, and daily subsistence allowance. Maintenance and servicing were recognized as major bottlenecks for the scale up of the GeneXpert instrument to a lower level in the laboratory network [102]. The absence of local authorized service providers from Cepheid and limited capacity of end-users for maintenance have led to high rates of module failures in different settings [111]. As part of their after sales service, Cepheid proposes a warranty extension agreement for the GeneXpert instrument, accompanying accessories, software, and the computer used in connection therewith. This service comes at a price of USD2,898 per year for one 4-module instrument, which can be a burden for LMICs given the resources constraints. Including maintenance costs in the analysis would therefore significantly increase the actual unit cost of the test.

Secondly, we have not included the costs for repeating all Xpert tests with non-valid results (invalid, error, no results) in the calculation. This is an important factor because the rate of non-valid Xpert results can significantly vary from one setting to

another, impacting the costs and cost-effectiveness of the pooled testing. Some studies have reported abnormally high rates of non-valid results, with 10.6% (range 5.9–16.3%) in nine countries implementing Xpert MTB/RIF [65], 7.2% (range 4–17%) in India [102], and 11% for Nigeria [112]. These high rates of non-valid results were attributed to either the environment with high temperature and/or dust, or due to poor adherence to standard operating procedures. In addition, to guarantee reliability of tests results, every laboratory is required to be part of a quality programme. Quality Assurance is important to ensure accurate results, identify problems early, improve patient care and enhance public health. Setting up and maintaining a performant quality assurance programme requires a comprehensive action plan for all the training, shipments, corrective actions, and monitoring plan, which can sometimes be compromised due to the lack of financial support.

Thirdly, this study focuses on the cost minimization for the diagnosis of TB using pooled testing compared to individual testing. We therefore did not assess the impacts of earlier TB diagnosis and TB treatment initiation, nor did we incorporate into the analysis the cost-effectiveness of preventing additional disease transmission. Cost-effectiveness analyses are more robust when the number of people correctly diagnosed and started on treatment is included along with costs and outcomes related to treatment, survival and disability, using cost per disability-adjusted life year [113]. Our cost analysis study provides a partial economic analysis because it does not consider the consequences of interventions. We considered only program perspective in cost calculation as we compared with two methods of detecting TB and intend to find the cost-effective one for implementation in future in the health system. However, future research would benefit from including a patient cost analysis on direct medical, direct non-medical and indirect costs (such as time, productivity, and

income loss), as well as coping mechanisms. This would provide insights into the socioeconomic impact and cost effectiveness of the pooled testing strategy from a societal rather than program or health system perspective. Many model-based economic evaluations predicted that Xpert would be cost-effective through a reduction in tuberculosis-related mortality and/or reduction in the overtreatment of tuberculosis [114, 115]. Given that more cases are detected with pooling, more patients will be initiated on treatment potentially leading to less transmission, so likely that the pooling strategy would be more cost-effective if these parameters are incorporated into the model.

Conclusions

Our results demonstrate the repeatability, reliability, consistency, and accuracy of the pooling method in a variety of settings with both Xpert MTB/RIF and Xpert Ultra, in PCF approach. The low frequency of false-negative results and the high degree of specificity makes this approach a cost-effective strategy for large scale TB testing at reduced costs. This can allow resource limited countries to catch up with the WHO End TB strategy targets despite the reversal of progress due to the COVID-19 pandemic.

Final discussion and Conclusions

Our findings show that there is a good correlation between the results of the individual and pooled tests, with pooled Xpert MTB/RIF detecting about 95% of MTB-positive samples and pooled Xpert Ultra able to yield full agreement between both methods. Therefore, pooling samples could increase the resilience of TB diagnostic services when health systems are being challenged by the COVID-19 pandemic or indeed future epidemics and pandemics. The systematic review we conducted synthesized the available literature on the performance of the pooling method using sputum for GeneXpert testing for detecting pulmonary TB. Our studies conducted afterwards added to the emerging body of evidence that the pooling methods for testing with molecular assays can improve the efficiency of testing for TB, potentially enabling the screening and testing of larger numbers of people more cost-effectively.

The studies included in the systematic review reported high sensitivity and specificity for 1:2 and 1:4 pooling ratios, replicating single test results, but pooling >4 specimens decreased sensitivity. This trend was further confirmed by the studies we conducted in both passive and active case finding settings, in which we applied pools of four samples.

Cartridges and time savings are directly related to the proportion of pools that are positive, a proportion which is dynamic depending on the local TB prevalence, testing strategy, and specific populations being tested. Savings were higher in low prevalence settings and during active case finding, when the proportion of pools testing positive were lower. The savings presented in our studies only described money saved in cartridges cost alone from 38%-52% when pooling Xpert MTB/RIF, from 42%-46%

when pooling with Ultra, and up to 67% when pooling Xpert Xpress SARS Cov-2. These cost savings are higher than those found in our systematic review (27%-31%), which were already considered to be substantial. Positive predictive value (PPV) and negative predictive value (NPV) are useful indicators that help to interpret test results within a particular context. PPV and NPV are prevalence-dependent parameters that take into consideration other factors such as the pool size, test sensitivity, test specificity, and testing procedures. Thus, high PPVs and NPVs indicate reliable positive and negative results, respectively and are useful in designing approaches for difference prevalence settings. Pooling can increase the PPV of testing compared to individual testing because pooling strategies result in repeat testing of positive specimens, and thus the diagnostic specificity by way of additional confirmatory testing.

Controversies

Our studies and others reported a slight increase in CT values and corresponding lower MTB/RIF semiquantitative results for pooled samples. This is expected, as testing samples together necessarily dilutes individual samples. Findings also showed that this dilution effect can lead to discrepancies between individual and pooled Xpert MTB/RIF tests, as false-negative pooled results could occur among pauci-bacillary samples with high Xpert CT values. This suggests that some individual samples with low DNA concentrations may fall below the assay's limit of detection once diluted in the pool. Consequently, some patients with paucibacillary disease – including, for example, vulnerable groups including children or people living with HIV, could be missed by pooling, especially if testing is based on Xpert MTB/RIF.

Pooled testing allows to conserve testing reagents but other consumables such as sputum containers, and other consumables used for TB testing are not necessarily conserved. In addition, since all specimens need to be manipulated when creating the pool, pooling does not provide significant savings in time or efficiency for laboratory personnel. The additional manual pipetting required during the testing procedure may result in an increase in the hands-on needed per specimen. Pooling can reduce turn-around time for pooled samples returning negative results but because positive pools must go through a multistage testing process, pooling can actually lengthen the time it takes to process results when they are positive. Unfortunately, this somewhat negates the benefit of the GeneXpert being a 2-hour test. However, while the cartridge is being read by the module, the laboratory technician can do other activities and so time is not lost as compared to smear preparation time and reading which requires their involvement until requisite fields have been read. The pooling strategy cost-effectiveness may vary if the background TB prevalence fluctuates over time. More

positive pools will be found as TB prevalence increases, necessitating more samples to be re-tested individually and eventually longer reporting turnaround times.

Despite this limitation, the use of pooled testing can still be rationalized for National TB Programs due to the significant savings generated. With the same amount of resources available, TB laboratories are able to significantly increase their testing capacity, reduce the turnaround time for release of results and increase the number of same-day diagnosed TB patients. Individual testing with Xpert MTB/RIF may miss a few cases but to reduce transmission and end TB, solutions to ensure diagnosis as early as possible are required in order to initiate prompt treatment and isolation.

In our studies, discrepancies occurred mainly when pooling with Xpert MTB/RIF and the better agreement of Xpert Ultra was attributed to its higher sensitivity. This is why the WHO recommends countries to transition to Xpert Ultra. In addition, Cepheid will discontinue the production of the Xpert MTB/RIF and produce the Xpert Ultra exclusively. Therefore, it is expected using only Xpert Ultra will increase the efficiency of the pooling method and reduce the risk of false-negative pools.

Another limitation of the pooled testing is its unreliability for the detection of Rifampicin resistance. Our results showed pooled testing can lead to RIF Indeterminate status, especially when the bacillary load is too low for the assay to give a definitive answer as to the RIF resistance status. However, this limitation has no impact on the sensitivity or specificity of the pooled testing, given all individual samples included in RIF Indeterminate pooled results are re-tested individually. Consequently, patients will not be misclassified as RIF Sensitive if they are RIF resistant.

There is also the possibility of false-positive pools on rare occasions. This is more likely related to human errors with possible cross-contamination during sample handling and processing, which could also happen when testing individually and therefore is not a limitation related to the pooled testing per say. From the laboratory perspective, it is important to set up a technical working group to assess the pros and cons of using the pooled testing, update guidelines/testing algorithm, set up clear SOPs and organize the workflow in an efficient manner. Clerical errors such as mislabeling, and cross-contamination may hamper the perception from the general population on the reliability of the pooled testing. However, since samples included in positive pools are always retested individually to identify the positive sample(s), there are no patients that could be misclassified due to false positive pools since individual testing would reveal all samples are actually MTB-negative.

Implications for the field of TB

The pooling method for the diagnosis of TB has never been tested during a real health crisis, such as the COVID-19 pandemic. The study took place at a time laboratory resources were being diverted and healthcare workers were repurposed for SARS-CoV2-testing. Our results show pooled testing is fully suitable under routine conditions for TB passive and active case finding strategies, and easily adaptable since it only requires creating a pool by mixing four samples together.

Our results showed pooled testing can increase the testing capacity with an insignificant loss of sensitivity, which can virtually reduce the unit cost per test and increase the affordability and access to molecular rapid diagnostics which are recommended by the WHO. These findings are significant because internationals donors are increasingly requesting countries to commit to co-financing mechanisms for the procurement of tests from government-funded schemes despite individual tests being perceived to be expensive by national TB programs. These findings therefore contribute to recognize gaps in funding sources for the procurement of sufficient cartridges for testing all individuals with presumptive TB, which jeopardizes access to high sensitivity WHO-recommended rapid molecular diagnostic tests, such as the GeneXpert Xpert MTB/RIF and Xpert Ultra.

Countries are also reluctant to implement the pooling method until the approach is endorsed by WHO guidelines. Our study is part of an initiative funded by the Stop TB Partnership in Geneva, to generate further evidence that the pooling approach is reliable and capable of increasing the affordability of the tests.

If the WHO chose to endorse the pooled testing in future consolidated guidelines, that means our studies could play a major role in the field of TB and our findings could

serve as evidence-based for the endorsement of the pooling method in national TB testing algorithm globally. At the time of writing this thesis, colleagues from STOP TB Partnership in Geneva are leading sensitisation sessions with policymakers at WHO about sputum pooling, which include many of the results from this thesis. Small progress has been made as the recently released WHO diagnostic standards mention pooling as to be feasible and accurate compared to individual testing and associated with reductions in both time and costs [116]. The Liverpool School of Tropical Medicine is currently conducting a project (START4ALL) that is expected to add a lot more evidence on the value of the pooled testing, with hopefully start having real conversations with the WHO TB Programme about it in the coming year.

Key populations and communities are at the heart of the fight against infectious diseases, to ensure a people-centred integrated system for health to deliver on impact, resilience, and sustainability and to maximize health equity, gender equality and human rights. Interestingly, men were more likely to be Xpert MTB-positive, but women were either equally likely (Chapter II) or more likely (Chapter IV) to be Xpert Ultra MTB-positive. The higher sensitivity of Xpert Ultra may compensate for women not being comfortable when coughing and expectorate sputum, leading to sub-optimal specimen quality. This can contribute to addressing some of the persistent bottlenecks such as quality of care, including the limited package of services offered, inadequate efforts to address gender and cultural norms of ethnic groups. The impact of Ultra on the diagnosis of TB in women is a finding that suggests the need for further studies, especially those which could use gender-disaggregated data to improve planning and resource prioritization for underserved populations as well as ensuring integration of gender and social inclusion dimensions.

Implications for other fields of study

The pooling method has been described since World War II when the economist Robert Dorfman first developed the theory and practice of pooled testing to detect syphilis among US soldiers [13]. Pooling strategies have also been used for the detection of pathogens including hepatitis B and C viruses [14], HIV [21], and Neisseria gonorrhoeae [25].

Findings from our study are relevant to any infectious diseases program with regards to maintaining stocks and sustaining essential services, increasing testing capacity and, more importantly, saving time and resources in settings where budgets are already stretched to maximum capacity.

Our promising results call for more studies to evaluate the pooled testing for other notable global infectious diseases that cause significant morbidity and mortality, such as lower respiratory tract infections, diarrheal diseases, HIV/AIDS, and malaria. In the light of the findings from our study, Ministries of Health globally and other national disease programs may be interested in evaluating the method for other diseases screening and diagnosis.

SARS-CoV-2 testing is often limited by the number of tests available and our study demonstrates that pooling samples can significantly increase testing capacity, while simultaneously reducing the resources needed for mass screening of SARS-CoV-2. The savings documented in our study, close to two thirds of the number of cartridges required for individual testing, are significant and were documented at a time when the proportion of pools testing positive was close to 8%. With the same resources required for individual testing, pooling allowed triplicating the number of people tested.

The higher efficiency of the pooling method can mitigate the challenges of testing during a pandemic by substantially increasing testing capacity, lowering the cost per test, and conserving reagents during times of sudden and heavy inflow of test requests and large-scale population testing. If all TB and COVID-19 samples were processed by pooled testing, laboratories would be able to optimize the use of the GeneXpert shared platform and technicians will have more opportunity to run other individual tests on the GeneXpert such as HIV VL, HBV VL, HCV VL. By reducing the turnaround time for TB and COVID-19, this will automatically impact and reduce the turnaround time for other tests that need to run on the same platform, which is likely to be of benefit to both the health system and the populations which it serves.

Pooled testing can also have a positive impact on the mental health and well-being of healthcare workers. A systematic review has shown poor wellbeing and moderate to high levels of burnout are associated with poor patient safety outcomes such as medical errors [117]. By saving time, healthcare workers are less overwhelmed, feel less pressure in their daily tasks which can make them more careful and more competent, reducing clerical errors and risk of laboratory acquired infection due to rush. Workplaces with high levels of mental wellbeing are more productive since health care workers also have more time to take care of other tasks (stock management, equipment maintenance, administrative tasks, …) which may improve the overall quality management system of the entire laboratory [118].

Limitations of our study

A first limitation of our study that could have impacted on the interpretation of the findings was the samples inclusion process. Xpert MTB/RIF and Xpert Ultra were not available at the same time in Lao PDR. We had to conduct the first survey in 2020 with Xpert MTB/RIF, then the second survey in 2021 with Xpert Ultra. Therefore, the samples were not the same between the surveys, with different characteristics of the participants, different semi-quantitative results, different constitutions of the pools. However, there were no statistically significant differences in the proportion of males and females recruited between 2020 and 2021, the proportion of males with MTB detected, nor the proportion of females with MTB detected. In general, TB is more frequent among males. Annual data reported by the country indicates incidence/prevalence is higher among males. If we had used the same population samples, we would be able to make a head-to-head comparison of the sensitivity and specificity of pooling Xpert MTB/RIF vs Xpert Ultra, using the individual test as the reference.

The second limitation of the study is the small sample size. We were limited by the expected number of participants attending the TB campaigns, the capacity of staff to conduct the study in addition to their routine activities and the number of spare cartridges we could use for research purposes. We included all samples from the moment we had the approval to conduct the study until COVID-19 related lockdown measures were introduced. Although there was no formal sample size calculation, we targeted a minimal sample size of 400 participants per survey. Assuming a type 1 error of 0.05, an expected proportion of the population to test positive for TB of 10% and an absolute level of precision of 0.9, the sample size required would be 426 participants. Thus, the sample size would have been adequate.

In the study “Pooling samples to increase testing capacity with Xpert Xpress SARS-CoV-2 during the COVID-19 pandemic in Lao People’s Democratic Republic” we were also limited by the number of cartridges available. This study took place at a time of a consortium allocation established by WHO for Xpert Xpress SARS-CoV-2 tests, with 1,000,000 tests available globally, and Lao PDR was entitled to only 10,000 cartridges. Therefore, given the limited resources available for mass screening, pooling was used as the reference method, assuming its sensitivity and specificity was acceptable. Individual testing was thus only done for individual samples in positive pools and therefore, we did not assess the sensitivity and the specificity of the method. Consequently, we don’t know if, among the negative pools, there were positive samples that were missed.

Another limitation is that we chose to apply pools of four samples without assessment or validation of the optimal pool size in this setting, time, and population. This decision was made based on previous evidence from other studies that helped guide us, such as in Cambodia with similar settings [119]. However, a prior epidemiological analysis to determine the proportion of positive samples by province and district for the different population groups would have allowed identifying whether larger pool sizes could have been more efficient. The pooling method is not a one-size-fits-all strategy and statistical calculations using different combinations of pool sizes and positivity rate would have maximized the testing capacity and optimized the resources savings.

Finally, the cost analysis and the savings presented in this work did not include all savings that were generated around the pooling method. Those savings therefore underestimate actual savings since less time spent on the bench necessarily lead to other savings, such as staff time, electricity, overhead costs, laboratory maintenance,

laboratories commodities, wastes management and life expectancy of the GeneXpert machines, and direct and indirect costs to patients' and their cares. Adding a cost-utility analysis, where the results are evaluated in quality-adjusted life years (QALY) and/or disability-adjusted life years (DALY) would have given more insights on the cost-effectiveness of the pooled testing. This analytical tool can be important when it is necessary to choose one of several alternatives due to financial constraints. The cost analysis would be more robust by including patient costs related to TB care and evaluate whether pooled testing could reduce the proportion of TB-affected households that faced catastrophic costs.

Recommendations

As the world continues to struggle with COVID-19 pandemic outbreak, it is crucial to ensure that essential services to protect the lives of people infected with TB are maintained. In the World Health Organization's 2022 Global TB report, TB deaths were reported to have risen for the first time in more than a decade due to the COVID-19 pandemic. The first challenge described by WHO is disruption in access to TB services and a reduction in resources allocated to TB services, with human, financial and other resources reallocated from tackling TB to the COVID-19 response, limiting the availability of essential services. Results presented here demonstrate pooling samples could facilitate maintenance of stocks and sustain essential services during the pandemic and provide cost and time savings during and post-pandemic.

Despite all the advantages, pooled testing is not a one-size-fit-all solution and countries need to conduct their own assessment prior to introducing this method. Pooled testing is not appropriate for every setting because the TB burden and the resources available vary from one country to another. Even within one country, the TB burden can vary from one region and/or subpopulation to another. In settings with very low TB incidence, governments may also have different public health priorities regarding TB elimination compared to higher TB incidence countries. We would recommend countries that have a low TB incidence and enough available resources to not use the pooling method. In such countries, testing capacity will be sufficient to test all samples individually and reduce the risk of getting a false-negative pooled test result. However, in high TB burden countries where resources are usually limited and number of samples to be processed are above testing capacity, pooled testing could be rationalized. In such settings, there is a significant gap between the estimated incidence and the notification rate, meaning that a significant number of TB cases are

missed. For those settings, the benefit of identifying and notifying more TB patients per day can outweigh the potential reduced sensitivity of the pooled testing compared to individual testing. Pooling method despite the few missing cases could help those countries reduce the gap between incidence and notification rate faster than if they were using individual testing.

Since the pooling method is a laboratory change, it would not affect the screening algorithm and can be easily instituted without any major modifications. Laboratory personnel already familiar with individual Xpert testing will require minimal training as the only change is the creation of a pool before testing. It is important to have clear SOPs and bench aids to record correctly all samples included in different pools for results reporting and individual testing in case of a pool returning a positive result. It should be ensured that such SOPs are followed correctly, and all samples are tested, recorded, and reported accordingly.

It is also recommended to implement a strong quality assurance and quality control programme. The National Reference Laboratory or other competent authority need to have regular on-site supervisions for monitoring, evaluation, and data quality assessment.

We also recommend countries to monitor the evolution of disease epidemiology over time if they decide to apply the pooling method. If the notification rate is similar to the estimated incidence of TB, a reversion to individual testing may be appropriate to ensure no missed cases due to false negative pooled results. In order to optimize the pooling method, it is recommended to establish a real-time electronic surveillance system in the country for all diseases expected to be tested by the pooling method. This database should be updated in a real-time manner and include data such as

notification rate, incidence, prevalence, number of people screened, number of positive tests, for a specific disease for all catchment area in the country, starting from the lowest peripheral level up to the central level, by village, district, provinces, …. Geospatial technologies and modelling should be used to guide countries on the use of the pooled method in certain areas centres in a targeted manner while using individual testing in other areas depending on the incidence. The amount of savings being directly dependent upon on the proportion of positive tests, country can decide when pooling is appropriate (low prevalence of the disease) or not recommended (high prevalence of the disease) in order to optimize the use of the pooling method.

The real-time surveillance system should link with or have in-built within it some modelling outputs that can give an estimate of the current costs and cost-effectiveness of the pooled method in their particular setting at that particular time. This could help to make policy fluid/flexible over time and with seasons/outbreaks potentially. Each resource saved can be reallocated afterwards, which will help the country implement more effective action plans to target TB interventions. A country can plan more effective local interventions with TB active case campaigns by conducting mass screening campaigns using the pooling method where prevalence is low. The end result is pooling approach can significantly reduce the burden of TB and its transmission in communities.

Future directions

To avoid further reversal of progress towards eradicating TB, WHO is urging for new knowledge and lessons from successful programmatic innovations to improve TB diagnosis, prevention, and care in the context of the pandemic and beyond. The WHO Global TB Programme has established a compendium of resources on TB and COVID-19 which comprises research projects on TB and COVID-19 in various countries. This report is meant to be submitted to the WHO along with results from other studies to support evidence-based adaptation of TB services to the contexts created by the pandemic.

Promising results from this study call for more pooling studies in other settings with GeneXpert for TB testing. It is important to provide evidence that the pooling method can be applied in any environment where individual testing is usually applied. It is therefore useful to enrich the body of literature on pooling especially when mass screening is expected, such as in prisons or among migrants.

It is also useful to gather more information on pooling methods using other platforms for TB diagnosis. The pooled testing for TB diagnosis is mainly described on the GeneXpert platform. In the latest WHO consolidated guidelines on TB (Module 3: diagnosis - rapid diagnostics for tuberculosis detection, 2021 update) [2] other platforms have been endorsed such as the Truenat MTB, MTB Plus (Molbio Diagnostics, Goa, India) in adults and children with signs and symptoms of pulmonary TB. Given the similarity of the operational characteristics with the Xpert MTB/RIF and Xpert Ultra, the WHO has approved the use of Truenat MTB, MTB Plus at the same health system level as Xpert MTB/RIF and Xpert Ultra. It can therefore be useful for countries who have implemented the Truenat MTB, MTB Plus

to conduct some operational research studies on pooled testing with this newly endorsed platform, so that evidence on the performance and reliability of the pooled testing can be extended to a wider range of platform and not limited to GeneXpert for TB diagnosis.

More recently, pooled testing has been advocated to address testing resource constraints during the ongoing COVID-19 pandemic for SARS CoV-2 [16]. A more recent recommendation from the US-CDC to use SARS-CoV-2 Ag-RDTs for self-testing is believed to help alleviate the burden on laboratory, leading to an expected reduced number of samples to be tested by RT-PCR and reduced turnaround time for patients' management [110]. It could therefore be interesting to combine pooled testing and use of the SARS-CoV-2 Ag-RDTs in the same testing algorithm, in order to optimize the use of those tools to facilitate linkage to clinical care and therapeutics. For mass screening purposes, individuals who test negative by pooled testing with RT-PCR or SARS-CoV-2 Ag-RDTs could be authorized to take part in group activities, indoor gatherings. In some settings, samples included in a pool that tests SARS-CoV-2-positive could be re-tested by Ag-RDTs to identify the positive sample(s) more rapidly. As the pandemic evolves rapidly, SARS-CoV-2 testing strategies need to be adjusted accordingly and countries need find the best combination between pooled testing, individual testing and SARS-CoV-2 Ag-RDTs for a timely, reliable and accurate diagnostic testing for SARS-CoV-2.

In parallel, there is a need for more studies on pooled testing for SARS-CoV-2 on all RT-PCR approved platforms with all test kits. All countries do not have the same RT-PCR platform nor the same test kits for SARS-CoV-2 testing and even within the same country, it is not uncommon that several platform and tests kits be implemented. In order to make the pooled testing a generic and systematic approach, it is important

that all nucleic acid detection tests referenced in the WHO Emergency Use Listing for In vitro diagnostics (IVDs) Detecting SARS-CoV-2 be validated for pooled testing. This will facilitate the update of countries testing algorithms without the need for purchasing new tests kits.

Another variant of the pooled testing would be the targeted multiplex pooled testing of infectious respiratory pathogens in certain settings/populations and potentially at certain times/seasons. Existing data showed that TB status might play a role in the development of severe acute respiratory syndrome in SARS-CoV-2 co-infection and that TB is associated with a 2.1-fold increased risk of severe COVID-19 disease [120]. TB prevalence among COVID-19 patients has been found to be 0.37 – 4.47% in several studies [121]. Early identification of respiratory infections (COVID-19, TB, ILI) can reduce morbidity and mortality. It would therefore be appropriate to explore opportunities to provide integrated respiratory health screening to people with COVID-19, TB and ILI/SARI symptoms using Antigen Rapid Diagnostic Tests (AgRDTs) and pooled molecular diagnosis to improve standard of care and increase testing capacity. Cepheid has recently released a new Xpert Xpress SARS-CoV-2/Flu/RSV (Xpert 4-in-1) assay for the simultaneous qualitative detection and differentiation of SARS-CoV-2, flu A, flu B, and RSV in one cartridge with in vitro diagnostic emergency use authorization (EUA) by the U.S. Food and Drug Administration (FDA) in September 2020. The use of such multiplex diagnostic tools can increase testing coverage for respiratory diseases and minimize disruptions to TB testing through integrated testing systems that leverage TB and COVID-19 diagnostics infrastructure.

To optimize the impact of the pooled testing strategies, it is also important to demonstrate that this approach is suitable at all health system level especially at the

most decentralized locations where human resource capacities are usually the most limited. The lower the level in the health system, the lower the number of healthcare workers available and the more healthcare workers are multitasked. The adaptation of the testing algorithm to integrate the pooled testing needs to be operated smoothly at any level of the health system, to support the transition for countries interested in adopting the pooled testing. There is therefore the need for more evidence on the feasibility and acceptability of the pooled testing method at these different health system levels.

Universal access and implementation of the pooled testing needs to be generalized to all infectious diseases diagnosis that requires mass screening and rapid turnaround time for optimal patient management, disease surveillance and epidemiology purposes. The theory and practice of pooled testing has been described since World War II when there was a need for mass screening of syphilis among US soldiers. The approach was then abandoned and has only re-emerged recently when it was advocated to address limited testing capacities during the COVID-19 pandemic. Despite all the burden and chaos created by the pandemic, this crisis was an opportunity to strategically strengthen/scale-up or initiate interventions that can address gaps and bottlenecks in existing pandemic such as HIV and TB, and also prepare countries for future waves of COVID-19 and/or other disease outbreak of similar proportions.

Conclusions

The pooling method replicates individual testing and has high sensitivity and specificity for Xpert MTB/RIF and Xpert Ultra, with the latter able to result in full agreement between individual and pooled testing. Pooled testing resulted in significant cartridge savings and more efficient testing within the pandemic. In a context where countries experience stock-outs or procurement delays in laboratory commodities during times of crisis such as during the COVID-19 pandemic, the pooling method may be considered as an interim option to strengthen testing capacity and to achieve the WHO End TB strategy targets in resource-limited settings. Hopefully the world will not wait for a new global crisis before re-considering pooled testing as an effective approach to reduce turnaround time and not only save resources but lives.

References

1. World Health Organization. Global tuberculosis report 2022. 2022.
2. World Health Organization. WHO consolidated guidelines on tuberculosis. Module 3: diagnosis - rapid diagnostics for tuberculosis detection, 2021 update. 2021.
3. Lawn SD, Nicol MP. Xpert MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. 2011(1746-0921 (Electronic)).
4. Chakravorty S, Simmons AM, Rowneki M, et al. The New Xpert MTB/RIF Ultra: Improving Detection of Mycobacterium tuberculosis and Resistance to Rifampin in an Assay Suitable for Point-of-Care Testing. *mBio* 2017: 8(4).
5. World Health Organization. WHO meeting report of a technical expert consultation: non-inferiority analysis of Xpert MTF/RIF Ultra compared to Xpert MTB/RIF. 2017.
6. World Health Organization. Programmatic innovations to address challenges in tuberculosis prevention and care during the COVID-19 pandemic. 2021.
7. Loeffelholz MJ, Alland D, Butler-Wu SM, et al. Multicenter Evaluation of the Cepheid Xpert Xpress SARS-CoV-2 Test. LID - 10.1128/JCM.00926-20 [doi] LID - e00926-20. 2020(1098-660X (Electronic)).
8. Venkatesan P. COVID-19 diagnostics-not at the expense of other diseases. *The Lancet Microbe* 2020: 1(2): e64.
9. World Health Organization. Global tuberculosis report 2021. 2021.
10. World Health Organization. Lao People's Democratic Republic: Tuberculosis Country profile 2021.
11. Uplekar M, Weil D, Lonnroth K, et al. WHO's new end TB strategy. *Lancet* 2015: 385(9979): 1799-1801.
12. Singh S, Dey B, Sachdeva KS, et al. Challenges in tuberculosis diagnosis and management: recommendations of the expert panel. *J Lab Physicians* 2015: 7(1): 1-3.
13. Dorfman R. The Detection of Defective Members of Large Populations. *The Annals of Mathematical Statistics* 1943: 14(4): 436-440.
14. Cardoso MS, Koerner K Fau - Kubanek B, Kubanek B. Mini-pool screening by nucleic acid testing for hepatitis B virus, hepatitis C virus, and HIV: preliminary results. 1998(0041-1132 (Print)).
15. Raboud JM, Sherlock C, Schechter MT, et al. Combining pooling and alternative algorithms in seroprevalence studies. *J Clin Microbiol* 1993: 31(9): 2298-2302.
16. Centers for Disease Control and Prevention. Interim Guidance for Use of Pooling Procedures in SARS-CoV-2 Diagnostic and Screening Testing. 2021.
17. Cuevas LE, Santos V, Lima SVMA, et al. Systematic Review of Pooling Sputum as an Efficient Method for Xpert MTB/RIF Tuberculosis Testing during the COVID-19 Pandemic. *Emerging Infectious Disease journal* 2021: 27(3): 719.
18. World Health Organization. WHO monitoring of Xpert MTB/RIF roll-out. 2019.
19. Van Deun A, Tahseen S, Affolabi D, et al. Sputum smear microscopy in the Xpert(®) MTB/RIF era. *Int J Tuberc Lung Dis* 2019: 23(1): 12-18.
20. World Health Organization. WHO: Global TB progress at risk. 2020.

21. Emmanuel JC, Bassett MT, Smith HJ, et al. Pooling of sera for human immunodeficiency virus (HIV) testing: an economical method for use in developing countries. *J Clin Pathol* 1988: 41(5): 582-585.
22. Morandi PA, Schockmel GA, Yerly S, et al. Detection of human immunodeficiency virus type 1 (HIV-1) RNA in pools of sera negative for antibodies to HIV-1 and HIV-2. *J Clin Microbiol* 1998: 36(6): 1534-1538.
23. Peeling RW, Toye B, Jessamine P, et al. Pooling of urine specimens for PCR testing: a cost saving strategy for Chlamydia trachomatis control programmes. *Sex Transm Infect* 1998: 74(1): 66-70.
24. Mine H, Emura H, Miyamoto M, et al. High throughput screening of 16 million serologically negative blood donors for hepatitis B virus, hepatitis C virus and human immunodeficiency virus type-1 by nucleic acid amplification testing with specific and sensitive multiplex reagent in Japan. *J Virol Methods* 2003: 112(1-2): 145-151.
25. Lindan C, Mathur M, Kumta S, et al. Utility of pooled urine specimens for detection of Chlamydia trachomatis and Neisseria gonorrhoeae in men attending public sexually transmitted infection clinics in Mumbai, India, by PCR. *J Clin Microbiol* 2005: 43(4): 1674-1677.
26. Westreich DJ, Hudgens MG, Fiscus SA, et al. Optimizing screening for acute human immunodeficiency virus infection with pooled nucleic acid amplification tests. *J Clin Microbiol* 2008: 46(5): 1785-1792.
27. Santos P SA, Verma R, Oliveira R, Camioli C, Lemos E, et al. The utility of pooling sputum samples for mass screening for tuberculosis in prisons using Xpert MTB/RIF Ultra. *The International Journal of Tuberculosis and Lung Disease* 2019.
28. Zishiri V, Chihota V, McCarthy K, et al. Pooling sputum from multiple individuals for Xpert® MTB/RIF testing: a strategy for screening high-risk populations. *Int J Tuberc Lung Dis* 2015: 19(1): 87-90.
29. Abdurrahman ST, Mbanaso O, Lawson L, et al. Testing Pooled Sputum with Xpert MTB/RIF for Diagnosis of Pulmonary Tuberculosis To Increase Affordability in Low-Income Countries. *Journal of clinical microbiology* 2015: 53(8): 2502-2508.
30. Ho J, Jelfs P, Nguyen PT, et al. Pooling sputum samples to improve the feasibility of Xpert MTB/RIF in systematic screening for tuberculosis. *Int J Tuberc Lung Dis* 2017: 21(5): 503-508.
31. Phuong NTB, Anh NT, Van Son N, et al. Effect of two alternative methods of pooling sputum prior to testing for tuberculosis with genexpert MTB/RIF. *BMC Infect Dis* 2019: 19(1): 347.
32. Chry M, Smelyanskaya M, Ky M, et al. Can the High Sensitivity of Xpert MTB/RIF Ultra Be Harnessed to Save Cartridge Costs? Results from a Pooled Sputum Evaluation in Cambodia. *Trop Med Infect Dis* 2020: 5(1).
33. World Health Organization. Automated real-time nucleic acid amplification technology for rapid and simultaneous detection of tuberculosis and rifampicin resistance: Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children. Policy update. 2013.
34. World Health Organization. Global tuberculosis report 2019. 2019.
35. Dowdy DW, Basu S, Andrews JR. Is passive diagnosis enough? The impact of subclinical disease on diagnostic strategies for tuberculosis. *Am J Respir Crit Care Med* 2013: 187(5): 543-551.
36. Sismanidis C, Glaziou P, Bloss E, et al. Understanding and addressing the tuberculosis case detection gap : national inventory studies to improve estimates of incidence and strengthen surveillance. In; 2016; 2016.

37. World Health Organization. Xpert MTB/RIF Implementation Manual: Technical and Operational 'How-To'; Practical Considerations. Geneva, 2014.
38. Cuevas LE, Al-Sonboli N, Lawson L, et al. LED fluorescence microscopy for the diagnosis of pulmonary tuberculosis: a multi-country cross-sectional evaluation. *PLoS Med* 2011: 8(7): e1001057.
39. Bloom BR, Atun R, Cohen T, et al. Tuberculosis. *In:* rd, Holmes KK, Bertozzi S, Bloom BR, Jha P, eds. Major Infectious Diseases, Washington (DC), 2017.
40. Iem V, Dean A, Zignol M, et al. Low prevalence of MDR-TB in Lao PDR: results from the first national anti-tuberculosis drug resistance survey. *Trop Med Int Health* 2019: 24(4): 421-431.
41. World Health Organization. Lao People's Democratic Republic: Tuberculosis Country profile 2019.
42. Comella-Del-Barrio P, De Souza-Galvão ML, Prat-Aymerich C, et al. Impact of COVID-19 on Tuberculosis Control. *Arch Bronconeumol* 2021: 57 Suppl 2: 5-6.
43. Sohrabi C, Alsafi Z, O'Neill N, et al. World Health Organization declares global emergency: A review of the 2019 novel coronavirus (COVID-19). *Int J Surg* 2020: 76: 71-76.
44. Rakotosamimanana N, Randrianirina F, Randremanana R, et al. GeneXpert for the diagnosis of COVID-19 in LMICs. *Lancet Glob Health* 2020: 8(12): e1457-e1458.
45. Glaziou P. Predicted impact of the COVID-19 pandemic on global tuberculosis deaths in 2020. *medRxiv* 2020: 2020.2004.2028.20079582.
46. Williams BG. Optimal pooling strategies for laboratory testing. 2010; p. arXiv:1007.4903.
47. Foundation for Innovative New Diagnostics. GeneXpert negotiated prices. 2021.
48. Helb D, Jones M, Story E, et al. Rapid detection of Mycobacterium tuberculosis and rifampin resistance by use of on-demand, near-patient technology. *J Clin Microbiol* 2010: 48(1): 229-237.
49. World Health Organization. Lao People's Democratic Republic: HIV Country Profile. 2019.
50. Steingart KR, Schiller I Fau - Horne DJ, Horne Dj Fau - Pai M, et al. Xpert MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. 2014(1469-493X (Electronic)).
51. Zifodya JS, Kreniske JS, Schiller I, et al. Xpert Ultra versus Xpert MTB/RIF for pulmonary tuberculosis and rifampicin resistance in adults with presumptive pulmonary tuberculosis. *Cochrane Database of Systematic Reviews* 2021(2).
52. Ward HA, Marciniuk Dd Fau - Pahwa P, Pahwa P Fau - Hoeppner VH, et al. Extent of pulmonary tuberculosis in patients diagnosed by active compared to passive case finding. 2004(1027-3719 (Print)).
53. Abdurrahman ST, Lawson L, Blakiston M, et al. Are patients with pulmonary tuberculosis who are identified through active case finding in the community different than those identified in healthcare facilities? 2016(2052-2975 (Print)).
54. Dorman SE, Schumacher SG, Alland D, et al. Xpert MTB/RIF Ultra for detection of Mycobacterium tuberculosis and rifampicin resistance: a prospective multicentre diagnostic accuracy study. *The Lancet Infectious diseases* 2018: 18(1): 76-84.
55. Ngabonziza JCS, Decroo T, Migambi P, et al. Prevalence and drivers of false-positive rifampicin-resistant Xpert MTB/RIF results: a prospective observational study in Rwanda. *The Lancet Microbe* 2020: 1(2): e74-e83.

56. Sharna M, Rahman A, Uddin K, et al. Discordance in Xpert MTB/RIF assay results among low bacterial load clinical specimens in Bangladesh. *The International Journal of Tuberculosis and Lung Disease* 2018: 22.
57. World Health Organization. Tuberculosis: a global emergency. *World Health Forum* 1993: 14(4): 438.
58. Cepheid. Critical Infectious Disease. 2022.
59. The World Bank. Population, total - Nigeria. 2020.
60. Iem V, Chittamany P, Suthepmany S, et al. Pooled testing of sputum with Xpert MTB/RIF and Xpert Ultra during tuberculosis active case finding campaigns in Lao People's Democratic Republic. *BMJ Global Health* 2022: 7(2): e007592.
61. Iem V, Chittamany P, Suthepmany S, et al. Pooling sputum for Xpert MTB/RIF and Xpert Ultra testing during the Covid-19 pandemic in Lao People's Democratic Republic. *PLOS Global Public Health* 2022: 2(4): e0000116.
62. Iem V, Xangsayarath P, Chittamany P, et al. Pooling samples to increase testing capacity with Xpert Xpress SARS-CoV-2 during the Covid-19 pandemic in Lao People's Democratic Republic. *PLOS ONE* 2022: 17(9): e0275294.
63. Lohse S, Pfuhl T, Berkó-Göttel B, et al. Pooling of samples for testing for SARS-CoV-2 in asymptomatic people. *The Lancet Infectious diseases* 2020: 20(11): 1231-1232.
64. Lohse S, Pfuhl T, Berkó-Göttel B, et al. Challenges and issues of SARS-CoV-2 pool testing - Authors' reply. 2020(1474-4457 (Electronic)).
65. Creswell J, Codlin AJ, Andre E, et al. Results from early programmatic implementation of Xpert MTB/RIF testing in nine countries. *BMC Infectious Diseases* 2014: 14(1): 2.
66. World Health Organization. Global tuberculosis report 2020. 2020.
67. Shewade HD, Gupta V, Satyanarayana S, et al. Patient characteristics, health seeking and delays among new sputum smear positive TB patients identified through active case finding when compared to passive case finding in India. *PLOS ONE* 2019: 14(3): e0213345.
68. World Health Organization. WHO consolidated guidelines on tuberculosis. Module 2: screening – systematic screening for tuberculosis disease. 2021.
69. de Vries SG, Cremers AL, Heuvelings CC, et al. Barriers and facilitators to the uptake of tuberculosis diagnostic and treatment services by hard-to-reach populations in countries of low and medium tuberculosis incidence: a systematic review of qualitative literature. 2017(1474-4457 (Electronic)).
70. Wingfield T, Boccia D, Tovar M, et al. Defining Catastrophic Costs and Comparing Their Importance for Adverse Tuberculosis Outcome with Multi-Drug Resistance: A Prospective Cohort Study, Peru. *PLOS Medicine* 2014: 11(7): e1001675.
71. Chittamany P, Yamanaka T, Suthepmany S, et al. First national tuberculosis patient cost survey in Lao People's Democratic Republic: Assessment of the financial burden faced by TB-affected households and the comparisons by drug-resistance and HIV status. *PLOS ONE* 2020: 15(11): e0241862.
72. Gurung SC, Dixit K, Rai B, et al. The role of active case finding in reducing patient incurred catastrophic costs for tuberculosis in Nepal. *Infectious Diseases of Poverty* 2019: 8(1): 99.
73. Ho J, Fox GJ, Marais BJ. Passive case finding for tuberculosis is not enough. *Int J Mycobacteriol* 2016: 5(4): 374-378.

74. Chan G, Triasih R, Nababan B, et al. Adapting active case-finding for TB during the COVID-19 pandemic in Yogyakarta, Indonesia. *Public Health Action* 2021: 11(2): 41-49.
75. Mhimbira FA, Cuevas LE, Dacombe R, et al. Interventions to increase tuberculosis case detection at primary healthcare or community-level services. *Cochrane Database of Systematic Reviews* 2017(11).
76. Arshad A, Salam RA, Lassi ZS, et al. Community based interventions for the prevention and control of tuberculosis. *Infectious Diseases of Poverty* 2014: 3(1): 27.
77. Datiko DG, Yassin MA, Theobald SJ, et al. Health extension workers improve tuberculosis case finding and treatment outcome in Ethiopia: a large-scale implementation study. 2017(2059-7908 (Print)).
78. Gurung SC, Dixit K, Rai B, et al. Comparative Yield of Tuberculosis during Active Case Finding Using GeneXpert or Smear Microscopy for Diagnostic Testing in Nepal: A Cross-Sectional Study. *Trop Med Infect Dis* 2021: 6(2).
79. Yassin MA, Datiko Dg Fau - Tulloch O, Tulloch O Fau - Markos P, et al. Innovative community-based approaches doubled tuberculosis case notification and improve treatment outcome in Southern Ethiopia. 2013(1932-6203 (Electronic)).
80. National Tuberculosis Control Center of Lao PDR. Lao PDR District Health Information Software 2 (DHIS2). 2020.
81. National Tuberculosis and Leprosy Control Programme of Nigeria. 2019 Annual TB Report. 2019.
82. Yoon C, Chaisson LH, Patel SM, et al. Diagnostic accuracy of C-reactive protein for active pulmonary tuberculosis: a meta-analysis. *Int J Tuberc Lung Dis* 2017: 21(9): 1013-1019.
83. Qin ZZ, Sander MS, Rai B, et al. Using artificial intelligence to read chest radiographs for tuberculosis detection: A multi-site evaluation of the diagnostic accuracy of three deep learning systems. *Scientific Reports* 2019: 9(1): 15000.
84. MacPherson P, Webb EL, Kamchedzera W, et al. Computer-aided X-ray screening for tuberculosis and HIV testing among adults with cough in Malawi (the PROSPECT study): A randomised trial and cost-effectiveness analysis. *PLOS Medicine* 2021: 18(9): e1003752.
85. Huang C, Wang Y, Li X, et al. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. *Lancet* 2020: 395(10223): 497-506.
86. Coronaviridae Study Group of the International Committee on Taxonomy of V. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat Microbiol* 2020: 5(4): 536-544.
87. Wang W, Xu Y, Gao R, et al. Detection of SARS-CoV-2 in Different Types of Clinical Specimens. *JAMA* 2020: 323(18): 1843-1844.
88. Brümmer LE, Katzenschlager S, Gaeddert M, et al. Accuracy of novel antigen rapid diagnostics for SARS-CoV-2: A living systematic review and meta-analysis. *PLOS Medicine* 2021: 18(8): e1003735.
89. Younes N, Al-Sadeq DW, AL-Jighefee H, et al. Challenges in Laboratory Diagnosis of the Novel Coronavirus SARS-CoV-2. *Viruses* 2020: 12(6): 582.
90. McMahon DE, Peters GA, Ivers LC, et al. Global resource shortages during COVID-19: Bad news for low-income countries. *PLOS Neglected Tropical Diseases* 2020: 14(7): e0008412.
91. Pullano G, Di Domenico L, Sabbatini CE, et al. Underdetection of cases of COVID-19 in France threatens epidemic control. *Nature* 2021: 590(7844): 134-139.
92. Fredriksson M, Hallberg A. COVID-19 Testing in Sweden During 2020-Split Responsibilities and Multi-Level Challenges. *Front Public Health* 2021: 9: 754861.

93. Majid F, Omer SB, Khwaja AI. Optimising SARS-CoV-2 pooled testing for low-resource settings. *Lancet Microbe* 2020: 1(3): e101-e102.
94. Banko AA-OX, Petrovic G, Miljanovic DA-O, et al. Comparison and Sensitivity Evaluation of Three Different Commercial Real-Time Quantitative PCR Kits for SARS-CoV-2 Detection. LID - 10.3390/v13071321 [doi] LID - 1321. 2021(1999-4915 (Electronic)).
95. US Food and Drug Administration. Pooled Sample Testing and Screening Testing for COVID-19. 2020.
96. Haug N, Geyrhofer L, Londei A, et al. Ranking the effectiveness of worldwide COVID-19 government interventions. *Nature Human Behaviour* 2020: 4(12): 1303-1312.
97. Carl Mayers KB. Impact of false-positives and false-negatives in the UK's COVID-19 RT-PCR testing programme. 2020.
98. Reilly M, Chohan B. Pooled testing for SARS-CoV-2, options for efficiency at scale. 2021(1564-0604 (Electronic)).
99. Becker MG, Taylor T, Kiazyk S, et al. Recommendations for sample pooling on the Cepheid GeneXpert® system using the Cepheid Xpert® Xpress SARS-CoV-2 assay. *bioRxiv* 2020: 2020.2005.2014.097287.
100. Mahmoud SA, Ibrahim E, Thakre B, et al. Evaluation of pooling of samples for testing SARS-CoV- 2 for mass screening of COVID-19. *BMC Infectious Diseases* 2021: 21(1): 360.
101. Mulu A, Alemayehu DH, Alemu F, et al. Evaluation of sample pooling for screening of SARS CoV-2. 2021(1932-6203 (Electronic)).
102. Raizada N, Sachdeva KS, Sreenivas A, et al. Feasibility of decentralised deployment of Xpert MTB/RIF test at lower level of health system in India. *PLoS One* 2014: 9(2): e89301.
103. Bimba JS, Adekeye OA, Iem V, et al. Pooling sputum samples for Xpert® MTB/RIF and Xpert® Ultra testing for TB diagnosis. *Public Health Action* 2023: 13(1): 12-16.
104. Santos VS, Allgayer MF, Kontogianni K, et al. Pooling of sputum samples to increase tuberculosis diagnostic capacity in Brazil during the COVID-19 pandemic. *Int J Infect Dis* 2023.
105. World Health Organization. Implementing the WHO Stop TB Strategy: A Handbook for National Tuberculosis Control Programmes. 2008: 1, Case detection.
106. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977: 33(1): 159-174.
107. Drummond MF, Sculpher MJ, Claxton K, et al. Methods for the Economic Evaluation of Health Care Programmes. Oxford University Press, 2015.
108. Abdalhamid B, Bilder CR, McCutchen EL, et al. Assessment of Specimen Pooling to Conserve SARS CoV-2 Testing Resources. *Am J Clin Pathol* 2020: 153(6): 715-718.
109. Global Laboratory Initiative. Planning for country transition to Xpert MTB/RIF Ultra cartridges. 2017.
110. Centers for Disease Control and Prevention. Guidance for Antigen Testing for SARS-CoV-2 for Healthcare Providers Testing Individuals in the Community. 2022.
111. Albert H, Nathavitharana RR, Isaacs C, et al. Development, roll-out and impact of Xpert MTB/RIF for tuberculosis: what lessons have we learnt and how can we do better? *European Respiratory Journal* 2016: 48(2): 516.
112. Gidado M, Nwokoye N, Nwadike P, et al. Unsuccessful Xpert MTB/RIF results: the Nigerian experience. *Public Health Action* 2018: 8(1): 2-6.

113. Sohn H, Sweeney S, Mudzengi D, et al. Determining the value of TB active case-finding: current evidence and methodological considerations. *Int J Tuberc Lung Dis* 2021: 25(3): 171-181.
114. Vassall A, van Kampen S, Sohn H, et al. Rapid diagnosis of tuberculosis with the Xpert MTB/RIF assay in high burden countries: a cost-effectiveness analysis. *PLoS Med* 2011: 8(11): e1001120.
115. Menzies NA, Cohen T, Lin HH, et al. Population health impact and cost-effectiveness of tuberculosis diagnosis with Xpert MTB/RIF: a dynamic simulation and economic evaluation. *PLoS Med* 2012: 9(11): e1001347.
116. Ruvandhi Nathavitharana, Abarna Pearl, Matthew O'Bryan, et al. Web Annex A. Uniting innovation and implementation: a mixed methods systematic review of implementation solutions to increase the uptake and impact of molecular WHO-recommended rapid diagnostic tests. 2023(WHO standard: universal access to rapid tuberculosis diagnostics.).
117. Hall LH, Johnson J, Watt I, et al. Healthcare Staff Wellbeing, Burnout, and Patient Safety: A Systematic Review. 2016(1932-6203 (Electronic)).
118. Søvold LE, Naslund JA, Kousoulis AA, et al. Prioritizing the Mental Health and Well-Being of Healthcare Workers: An Urgent Global Public Health Priority. *Frontiers in Public Health* 2021: 9.
119. Chry M, Smelyanskaya M, Ky M, et al. Can the High Sensitivity of Xpert MTB/RIF Ultra Be Harnessed to Save Cartridge Costs? Results from a Pooled Sputum Evaluation in Cambodia. *Tropical Medicine and Infectious Disease* 2020: 5(1): 27.
120. Crisan-Dabija R, Grigorescu C, Pavel CA, et al. Tuberculosis and COVID-19: Lessons from the Past Viral Outbreaks and Possible Future Outcomes. *Can Respir J* 2020: 2020: 1401053.
121. Gao Y, Liu M, Chen Y, et al. Association between tuberculosis and COVID-19 severity and mortality: A rapid systematic review and meta-analysis. *J Med Virol* 2021: 93(1): 194-196.

Appendix 1. LSTM Ethical waiver (Chapter II, III, IV)

Professor Luis Cuevas
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool
L3 5QA

LSTM
LIVERPOOL SCHOOL OF TROPICAL MEDICINE
Pembroke Place,
Liverpool, L3 5QA, UK
Tel: +44(0)151 705 3100
Fax: +44(0)151 705 3370
www.lstmed.ac.uk

Wednesday, 25 March 2020

Dear Professor Cuevas,

Re. Ethical waiver for 20-037 'Testing samples using the pooling method to increase resilience of TB laboratory diagnostic services at the time of the COVID-19 epidemic'

Thank you for your letter dated 2 March 2020, which outlines the detail of the above-named study.

On behalf of LSTM Research Ethics Committee, it is acknowledged that LSTM REC approval is not required for this study. If there is any change to the proposed study that affects the status of this waiver, you must provide LSTM REC with the relevant details.

Should you wish to contact LSTM REC in relation to this study in the future, please contact Lindsay Troughton, Secretary, Research Ethics Committee at lstmrec@lstmed.ac.uk.

Yours sincerely,

Professor Graham Devereux
Chair
Research Ethics Committee

Appendix 2. Lao PDR Ethical waiver (Chapter II, IV)

LAO PEOPLE'S DEMOCRATIC REPUBLIC
"PEACE, INDEPENDENCE, DEMOCRACY, UNITY AND PROSPERITY"

Ministry of Health of Lao PDR
Department of Communicable Disease Control
National Tuberculosis Center of Lao PDR
Tel office: +85621 41 42 59, Fax +85621 45 28 55

Vientiane Capital, Date: 10-03-2020

RE: ETHICS WAIVER

To whom it may concern,

- Within the frame of a collaboration between the National Tuberculosis Center (NTC) of Lao PDR, the National Tuberculosis Reference Laboratory (NRL) of Lao PDR, the Liverpool School of Tropical Medicine (LSTM), UK,

The NTC/NRL is willing to provide access to the cartridges as well as to the left-over samples collected by the NRL as part of its routine diagnostics activities without the approval notice of the National Ethics Committee for Health Research beforehand with the purpose of conducting an assessment on whether the method of pooling sputum samples is adequate for TB diagnosis.

The objectives of this study are:

- To determine whether and to what extent testing specimens of four patients in a pool (combined) cup will result in the same number of patients confirmed to have TB as testing patients individually
- To describe changes in the Ct values of single and pooled specimens and,
- To estimate the theoretical cost and processing time savings of a pooled testing strategy to detect TB.

I confirm all samples will be anonymized for the pool procedure and that the result of the Xpert test of the pooled specimen will not be used for diagnostic purposes.

Director of the National Tuberculosis Center

Appendix 3. Nigeria Ethical approval (Chapter III)

FEDERAL CAPITAL TERRITORY

Health Research Ethics Committee

Research Unit, Room 10 Block A Annex, HHSS, FCTA Secretariat,
No. 1 Kapital Street 11, Garki, Abuja-Abuja.

Notice of Expedited Approval of Research
Approval Number: FHREC/2020/01/29/10-04-20

Full Study Title: **Pooling Method to Increase Resilience of TB Laboratory Diagnostic Services at the Time of COVID-19 Epidemic.**

Principal Investigator: **Dr. John S. Bimba**

Address of Principal Investigator: Zankli Research Centre, Bingham University, Karu, Nasarawa State, Nigeria,

Date of receipt of valid application: 26/03/2020

The Federal Capital Territory (FCT, Nigeria) Health Research Ethics Committee (FCT HREC) has given expedited approval to the research described in the above stated protocol. The FCT HREC has determined that this research qualifies for expedited review pursuant to the National Code of Health Research Ethics.

This approval is valid from **10/04/2020** to **09/04/2021**.

Note that no activity related to this research may be conducted outside of these dates. Only the FCT HREC approved informed consent forms may be used when written informed consent is required. They must carry FCT HREC assigned protocol approval number and duration of approval of the study. The FCT HREC reserves the right to conduct compliance visit to your research site without prior notification.

The National Code of Health Research Ethics requires the investigator to comply with all guidelines, rules and regulations regarding the conduct of health research, and with the tenets of the code.

Modifications: Subsequent changes are not permitted in this research without prior approval by the FCT HREC.

Problems: All adverse events or unexpected side effects arising from this project must be reported promptly to FCT HREC.

Renewal: This approval is valid until the expiration date. If this project is to proceed beyond the expiration date, an annual report should be submitted to FCT HREC early in order to request for a renewal of this approval.

Closure of Study: At the end of the project, a copy of the final report of the research should be forwarded to FCT HREC for record purposes, and to enable us close the project.

For queries and further information contact FCT HREC office. I wish you best of luck with your research.

Desmond Emereonyeokwe
Secretary, FCT HREC
April 10, 2020.

ABUJA
The Heart of Nigeria

Appendix 4. Informed consent form for the study in Nigeria (Chapter III)

Informed Consent Form

A copy of this information should be kept by all individuals invited to participate

This Consent Form is for adults who have cough and presented at a Health facilities to see a health care giver.

The study is called **Pooling Method to increase resilience of TB Laboratory Diagnostic Services at the time of COVID-19 Epidemic**

The investigators are Dr. John S Bimba[1], Dr. Sadiq Abdulrahman[1], Prof. Luis Cuevas[2]
, [1]Zankli Research Centre, Bingham university karu Nigeria, [2]Liverpool School of Tropical Medicine, UK

This Informed Consent Form has two parts:

- Information Sheet (to share information about the study with you)
- Certificate of Consent (for signatures if you agree to participate)

You will be given a copy of the Full Informed Consent Form.

PART I: Information Sheet

I am [*Name*], and work for [*Research Institute*]. We are doing a study to improve the diagnostic services for the patients attending the centre who may have tuberculosis, or TB.

TB is an illness that is very common. I would like to give you information about this illness and the study and then invite you to take part. You do not have to decide today whether to participate or not and you can talk to anyone you feel comfortable with to help you make a decision.

If you decide to participate today, we will ask you some questions about your illness and collect some sputum for examination. If there are words in these pages or in the interview that you do not understand, please ask me, as we are happy to take time to explain what they mean. If you have questions later on, you can ask me. You are free not to answer questions which you find embarrassing or to stop the interview at any time if you change your mind.

Purpose: TB is disease caused by a germ that infects the lungs. It causes cough, tiredness and night sweats. We can find out if you have the disease by examining your sputum (phlegm). If the germ is there, then we can start treatment, but if we cannot see it, then we need to do other tests. The doctor asked you to provide phlegm for smear microscopy. This is a test that is done by a person in the laboratory and takes time. We want to ask your permission to use some of the phlegm to evaluate whether a similar test that is done by another method call Sample Pooling works the same as the conventional method. If you agree to participate, we will then do the normal tests AND the new tests and compare how they work.

Type of Research: This research will aim to understand if the diagnosis of TB could be improved and increased resiliently through Sample Pooling.

Participant Selection: All persons older than 18 years with symptoms of TB.

Voluntary Participation: Your participation in this research is entirely voluntary. It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive will continue and will be the same. You may also change your mind later and stop participating even if you had already agreed.

Procedures and Protocol: As some patients face difficulties during treatment, we will invite some patients that have experienced problems to return here to discuss their experiences and problems in more detail. The decisions about which treatment and examinations are needed will be made by the clinic and not by me. The treatment offered to participating patients is the same as for patients that do not participate.

Risks or discomforts: There are no risks or discomforts associated with participation in this study.

Benefits: If you participate, you may have more access to information and more opportunity to ask questions about the disease than others. However, we are unable to pay you.

Incentives: There are no extra payments for your participation in the study. Participants will still have to pay for routine tests if the hospital charges for these

Confidentiality: This research is being done in your community and the hospitals. It is possible that if others are aware that you are participating, they may ask you questions. You are free to answer any of these questions, but we will not share the identity of those participating in the research, nor respond questions about your disease from other persons.

The information that we collect from you will be confidential. Only the research sponsors, the institutional officials and ethics committee members may have access to the data. Any information about you will be kept locked and in a safe place. It will not be shared with or given to anyone except other investigators that need to examine the databases in more detail in the future. The information will not have any names or addresses attached to it.

Right to Refuse or Withdraw: You do not have to take part in this research if you do not wish to do so and refusing to participate will not affect your treatment at this clinic in any way. You will still have all the benefits that you would otherwise have at this clinic. You may stop participating in the research at any time without losing any of your rights as a patient here. Your treatment at this clinic will not be affected in any way.

How the community will be informed of the study results: At the end of the study, and once all the information is analysed, the Ministry of Health will be informed of the result of the study, and how this information could be used to improve the community-based treatment of TB.

Who to Contact: If you have any questions you may ask them now or later, at any time during the study. If you wish to ask questions later, you may contact 08065400855 0r bimbajs@yahoo.com

PART II: Certificate of Consent

I have been invited to participate in research trying to understand the issues faced by patients undergoing TB diagnosis and treatment. I am aware that there may be no benefit to me personally and that I will not be compensated. I have been provided with the name of a researcher who can be easily contacted using the number and address I was given for that person.

I have read the preceding information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to participate in this research and understand that I have the right to withdraw from the research at any time without in any way affecting my medical care. I am also happy to "gift" the sputum samples to the research study to allow storage for use in the current and future research projects on TB.

Print Name of Participant _____

Signature of Participant _____

Date _____ **(Day/month/year)**

If illiterate

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of witness _____ **Name and thumb print of participant**

Signature of witness _____

Date _____ **(Day/month/year)**

Person taking consent to complete this section

I have read or witnessed the accurate reading of the consent form to the potential participant. The individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Name of person taking consent _____ **Signature** _____

Date _____ **(Day/month/year)**

A copy of this Informed Consent Form has been provided to participant ____ (initialled by the researcher/assistant).