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Gene Xpert for Direct Detection of *Mycobacterium Tuberculosis* in Stool Specimens from Children with Presumptive Pulmonary Tuberculosis

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Abstract. ***Background.*** Gene Xpert(GX) is a novel real time polymerase chain reaction (RT- PCR) assay which was endorsed by the World Health Organization (WHO) in 2011 for tuberculosis (TB) diagnosis and susceptibility to refampicin(RIF). ***Objective.*** To evaluate GX for direct diagnosis of TB in stool samples from children with suspected pulmonary Tuberculosis (PTB). ***Methods.*** Children older than one year and younger than 16 years with presumptive PTB were enrolled and classified to five clinical categories based on clinical, radiological, and laboratory findings: confirmed TB, probable TB, possible TB, Unlikely TB, and not TB. Two stool samples were collected from each child and tested for the presence of *Mycobacterium tuberculosis* (MTB) by GX and the obtained results were compared to Lowenstien-Jensen (LJ) culture as a gold standard. ***Results.*** In total, 115 children were enrolled. 36 had been confirmed with TB, 61 probably TB, 10 possible TB, 5 unlikely TB, and 3 not TB. GX had a sensitivity of 83.33 and 80.56 % and specificity of 98.73 and 99.36 % by patients and samples respectively. GX was positive in 83.3% of confirmed TB as well as 1.6 and 0.8% of probable TB cases by patients and samples respectively. ***Conclusions.*** GX provided timely results with quit acceptable sensitivity and good specificity compared to LJ culture. In this study, sensitivity calculations take into account only children with confirmed TB. GX could not detect TB in children with probable TB, so it should not be used alone for TB diagnosis. Further studies for GX stool protocol optimization and assessment is required.

Key words: Gene Xpert, *Mycobacterium tuberculosis*, Pulmonary tuberculosis, Children.

Introduction

TB, caused by Mycobacterium tuberculosis (MTB), is one of the most devastating infectious diseases worldwide, with one-third of the world population being infected [1]. In 2013, globally 9.0 million people became infected and 1.5 million people died from this disease [2]. According WHO estimation, Egypt is ranked as a country with middle/ low level of TB incidence. It is estimated that 11 cases per 100 000 population develop active pulmonary smear positive TB annually, while 24 per 100 000 develop all types of TB annually [3].

The difficulty of establishing an accurate TB diagnosis is the greatest challenge to patient management and impedes the assessment of the true disease burden and the development of treatment and vaccination strategies appropriate for effective disease control in children [4). TB diagnosis in children usually follows discovery of a case in adults, tuberculin skin testing, chest radiograph, and clinical signs and symptoms, however, none of these are fully efficient to diagnose a case [5].

The standard diagnostic test for TB requires a sputum sample which is very difficult to obtain as young children are unable to expectorate sputum. Since good sputa are not accessible, MTB cultures and smears are not performed even though the disease is in progress. Procedures can be undertaken in order to obtain samples from a child's lung or stomach, but these can be traumatic for the child. It is, therefore, desirable to develop a non-sputum-based TB diagnostic test for young children that gives a fast and reliable result. In this context, children with high risk of dying from TB, can get facile treatment [6]. Given that children may swallow

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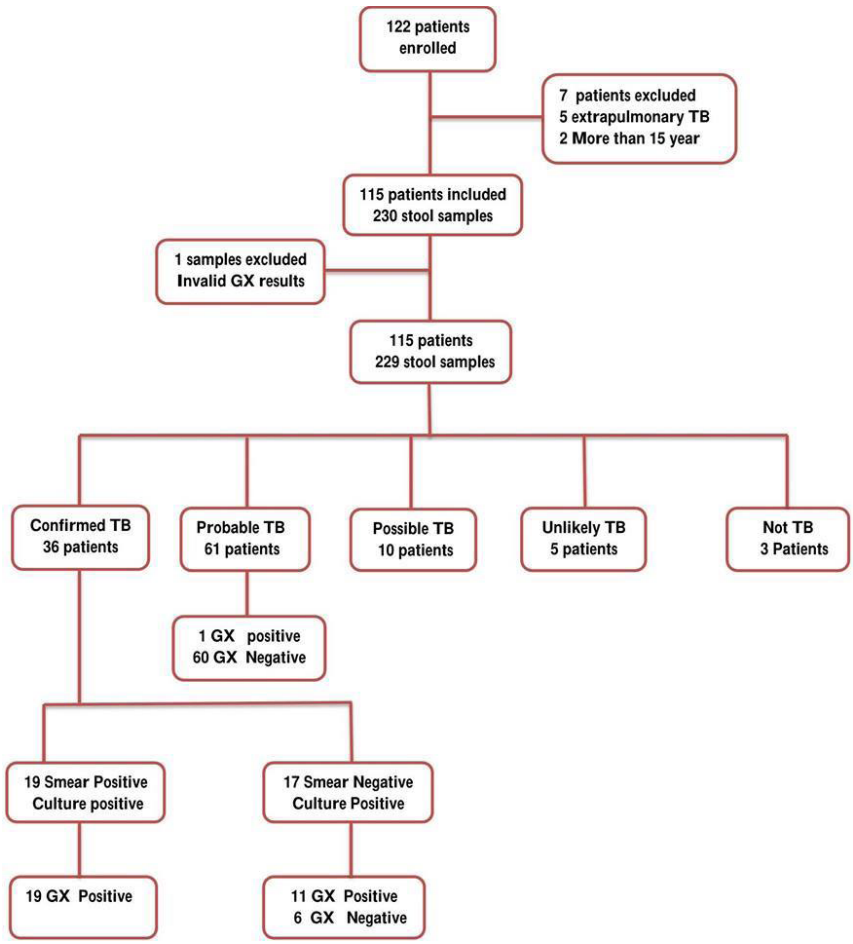

Figure 1. *Follow chart of study showing number and classification of participants and their lab results.*

sputum when they cough and TB DNA can survive intestinal transit [7], therefore, testing stool for TB DNA from swallowed sputum can be utilized to diagnose PTB.

WHO recommends using rapid molecular tests MTBDR*plus* and GX to diagnose TB in developing and high-burden countries [8,9]. In this sense, GX is a molecular test that uses RT-PCR to amplify a MTB specific sequence of the RNA polymerase gene (*rpoB*) [10]. This gene is probed with molecular beacons to detect rifampicin resistance mutations, which can be used as surrogate markers for multiple drug resistant TB (MDR-TB) [11]. The sensitivity of GX is directly related to the bacterial load in the specimen, and consequently correlate to sputum smear status [12].

In view of the aforementioned guidelines, we aimed, in this study, to evaluate the usage of GX for rapid diagnosis of TB and RIF resistance in children with suspected PTB.

Materials and Methods

This study was conducted in Abbassaia Chest Hospital, Cairo, Egypt between February 2013 to July 2015. Written informed consent was obtained from the parent or legal guardian. The study was approved by the local ethics committee of the Faculty of Women for Education, Arts and Sciences, at Ain Shams University.

Study participants. The inclusion criteria for this study were children older than one year and younger than 15 years old with clinical signs of PTB. Exclusion criteria were patients who received anti-TB treatment, clinical symptoms or physical signs suggestive of extrapulmonary TB or any disease other than PTB **Table 1**. Patients were classified into five categories based on their clinical, radiological, and laboratory results according to Gerham *et al* [13]. Confirmed TB has symptoms suggestive of TB and microbiological confirmation is obtained. Probable TB has symptoms suggestive of TB and chest radiograph consistent with TB and one of the following: a. positive response to anti-TB treatment, b. documented exposure to MTB, c. Immunological evidence of MTB infection. Possible TB has symptoms suggestive of tuberculosis and one of the following: a. positive response to anti-TB treatment b. Documented exposure to MTB c. Immunological evidence of MTB infection for TB. Unlikely TB is symptomatic but does not fit any of the previous definitions., Not TB alternative diagnosis proven [13]. All enrolled children in this study were HIV negative.

Clinical procedure. Patient were diagnosed and treated by National TB program following the recommendations of WHO. Anthropometric data were collected at each visit and chest radiograph and tuberculin test (TST) were performed at recruitment visit.

Specimens' collection and laboratory procedures.

Sputum specimens. Two sputum specimens were collected from each participant. They were asked to expectorate sputum and in case of expectoration is not

Table 1. *Demographic and clinical characteristics of enrolled children.*

Parameter	Total N=115	Confirmed TB N=36	Probable TB N=61	Possible TB N=10	Unlikely TB N=5	Not TB N=3
Gender						
Male	70	24	35	6	3	2
1-6 years	27	8	15	2	2	0
6-16 years	43	16	20	4	1	2
Female	45	12	26	4	2	1
1-6 years	14	2	10	1	1	0
6-15 years	31	10	16	3	1	1
TB contact						
Yes	29	11	9	4	3	2
Family member	15	3	6	3	2	1
No	68	21	45	2	0	0
Unknown	3	1	1	1	0	0
Fever (>38°C)						
<1week	27	9	12	3	2	1
≥1 week	76	22	48	5	1	0
No fever	12	5	1	2	2	2
Persistent cough						
Yes	99	32	52	10	3	2
No	16	4	9	0	2	1
Night sweats						
Yes	89	25	56	3	4	1
No	26	11	5	7	1	2
Weight loss						
Yes	45	12	26	4	1	2
No	70	24	35	6	4	1
Chest X ray						
Consistent with TB	96	36	60	0	0	0
Not Consistent with TB	15	0	0	8	5	2
Unclear	4	0	1	2	0	1
Tuberculin test						
<5mm	54	23	26	4	1	0
≥5mm	13	5	6	1	1	0
Not done	48	8	29	5	3	3

Table 2. *Stool GX and culture results by patients clinical classification.*

Parameter	Total	Confirmed TB N (%)	Highly probable TB N(%)	Probable TB N(%)	Unlikely TB N(%)	Not TB N(%)
By patient						
GX positive	31	30(83.3)	1(1.6)	0(0.0)	0(0.0)	0(0.0)
GX negative	84	6 (16.7)	60(98.4)	10(100)	5(100)	3(100)
Total	115	36	61	10	5	3
By sample						
GX positive	59	58(80.5)	1(0.8)	0(0.0)	0(0.0)	0(0.0)
GX negative	170	14 (19.4)	120(99.2)	20(100)	10(0.0)	6(0.0)
Total	229	72	121	20	10	6

Table 3. *Validity values of stool GX compared to LJ culture.*

Stool GX	Total	Both +ve	Both -ve	GX +ve LJ-ve	GX -ve LJ +ve	Sensitivity% (95% CI)	Specificity% (95% CI)	PPV% (95% CI)	NPV% (95% CI)
By patients	115	30	78	1	6	83.33 (67.19;93.63)	98.73 (93.15;99.97)	96.77 (83.30;99.92)	92.86 (85.10; 97.33)
By samples	229	58	156	1	14	80.56 (69.53;88.94)	99.36 (96.5;99.98)	98.31 (90.91;99.96)	91.76 (86.57; 95.42)

possible, induced sputum specimen (IS) was obtained by well-trained nurse as previously described by Zar *et al* [14]. The second IS specimen was obtained by the following day or when it was possible. Sputum Acid bacilli (AFB) smear, culture on Lowenstein-Jensen medium, and drug susceptibility testing of MTB isolates using the agar proportion on Middlebrook 7H11 medium conducted through hospital as routine procedures for TB diagnosis using standard procedures for mycobacterial laboratories [15,16]. MTB was confirmed by Genotype MTBDR*plus*® assay (Hain Lifescience, Nehren, Germany) for both acid fast smear positive and culture positive following manufacturer's instructions.

Stool specimen. Two distinct stool samples from different bowel movements were collected from each participant and analyzed immediately. About 2 cm^3 of stool specimen was suspended in 10 ml of distilled water and homogenized by vortex then left at room temperature until solid particles settle. Supernatant was taken and centrifuged at 4000 rpm for 20 minutes and the resulting sediment was decontaminated by 10 ml of 3% NALC-NaOH for 15 min at room temperature then mixed with 40 ml of phosphate buffer (PB) pH 6.8 and centrifuged for 20 min. The resulting sediment was suspended 1 ml of PB and mixed with 2 ml of sample reagent and left for 15 min at room temperature after that transferred to Gen Xpert/RIF cartridge and analyzed using GeneXpert® system (Cephid, USA) [17,18]. Aliquots were stored at 4°C for duplicate testing within one week if needed. The reading of all GX tests was blind to clinical information, culture, and radiological results.

Statistical analysis. Receiver operating characteristic (ROC curve) analysis was performed. Sensitivity, specificity, positive, and negative predicted values were calculated for TB diagnosis using GX, and the 95% confidence interval (CI) was also determined compared to gold standard (LJ culture) [19]. The data were analyzed with MedCalc software (MedCalc, Mariakerke, Belgium).

Results

A total of 122 HIV negative children with presumptive pulmonary tuberculosis were enrolled in this study; 7 were excluded from data analysis. **Figure 1** provides follow chart of study participants distributed on five clinical categories. Demographic and clinical characteristics of study participants were shown in **Table 1**.

We analyzed 230 stool samples by GX from 115 eligible children; one sample showed invalid result. GX detected MTB in 58 samples from 30 children with confirmed TB group. Detailed GX results distributed on patients clinical categories were shown in **Table 2**.
GX showed negative results in 10 samples from 5 children belonging to the confirmed TB group. One sample from a child belonging to the probable TB group was culture negative and showed positive results by GX. Validation results of GX compared to the gold standard were shown in **Table 3**.

GX detected MTB in all smear positive children and in 11 of 17 smear negative patients were illustrated in **Figure 1**.

Furthermore, four samples from two patients showed RIF resistance by both agar proportion method and GX.

Discussion

Diagnosis of tuberculosis still depends on time consuming culture-based method or AFB, which has a low detection rate [20]. These methods are based on sputum samples, which are very difficult to obtain

because young children are unable to expectorate sputum [21]. Stool could easily be obtained from clinics and the fields, which are appropriate samples for the diagnosis of PTB in children. Although there are many studies evaluated GX for PTB diagnosis [10,22-24], there is insufficient information about accuracy of GX for direct diagnosis of PTB using stool specimens.. In this study, we offer an evaluation of direct detection of MTB in stool samples from children with presumptive PTB using GX.

The performance analysis of GX for detection of PTB in children showed overall sensitivity of 83.33%, CI (67.19;93.63) and of 80.56%, CI (69.53;88.94) and specificity of 98.73%, CI (93.15;99.97) and of 99.36%, CI (96.5;99.98) by patients and samples respectively, compared to LJ culture results. Results obtained by Welday *et al.*: stool GX showed 100% sensitivity and 89.36% specificity for diagnosis of PTB in children + without missing any positive from AFB smear microscopy [6]. Stool heteroduplex PCR showed sensitivity 97% and specificity 100% for diagnosis of pulmonary TB [7]. Different results were obtained by Nicol *et al.*: stool GX testing from 115 children with suspected pulmonary tuberculosis (PTB) detected 8/17 (47%) culture-confirmed tuberculosis cases [18]. This low accuracy level may due to stool sample processing as they used small amount of specimen (about 0.15g) along with single stool specimen was collected from each participant. However, in this study, we used pair stool samples from each patient to avoid random absence of DNA. This slightly increased the sensitivity of stool GX as some cases showed only one GX positive specimen.

Stool GX results are closely related to sputum AFB smear results, as GX detected MTB in 11 children of 17 sputum AFB smear negative children. Results obtained by Kokuto *et al.*, revealed that the sensitivity of the fecal GX was 100% for detection of MTB in specimens from sputum AFB smear positive patients, 81.0% in specimens from sputum AFB smear scanty positive patients, and 50.0% in specimens from sputum AFB smear negative patients with 100% specificity.

The fact that stool GX is considerably rapid, non-invasive, and relatively bio-secure initial test for children with presumptive PTB [25], it can be considered as a good alternative to the invasive procedure for collecting respiratory samples or sputum from children. Pilot accuracy study performed in South Africa, indicated that stool Xpert is a promising test for diagnosis of PTB [18].

Moreover, in this study, Stool GX has enabled the detection of an extra one positive sample in the probable TB group, which has also showed 14 negative specimen in the confirmed TB group. These findings are considered major limitations of stool GX. Additionally, results of single sample stool GX could not be reliable for TB diagnosis due to probability of random absence of MTB DNA. Significantly, stool GX could not differentiate between active and dead MTB. This means that stool GX may give false positive results as MTB may be died but still exert DNA [7]. Stool GX detected only few patients among clinically diagnosed TB patients, which agrees with findings from previous studies [11,26]. Owing to these limitations, stool GX could not be used as a single care point in TB diagnosis and follow up clinical and radiological assessment still important.

In conclusion, the major outcome of this study highlighted that stool GX could be a powerful tool for PTB diagnosis when ease of sample collection and time were taken into consideration. In spite of that, stool GX could not be used alone for PTB diagnosis and clinical and radiological assessment remain important. Furthermore, future studies are required in order to evaluate stool GX as well as simplify and improve stool processing protocol to achieve more accurate stool GX results and eliminate invalid results. In this regard, we suggest that stool GX must be evaluated by analyze multiple stool specimens from each patient.

Study limitation. This study aimed to evaluate performance of GX for diagnoses PTB directly by detection of MTB in stool samples. In this study, 84% was confirmed TB and probable TB. This is due to selection bias and this does not reflect disease prevalence. Sensitivity and specificity are

affected by selection and sampling in this study; 19 smear positive and 17 smear negative patients were enrolled. In this study, sensitivity calculations take into account only children with confirmed TB. Results of stool GX are closely related to those of sputum AFB smear and implied that high positive rate will enhance stool GX sensitivity. However, in this study we did not examine the influence of complications such as gastrointestinal TB, which may affect the stool GX sensitivity. Importantly, only four samples from two patients showed resistance to RIF and because of the low number of RIF resistant specimens, we could not evaluate accuracy of GX in detection of RIF resistance.

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