Peer review file

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Comment 1: GeneXpert MTB/RIF is a nucleic acid amplification test recommended by the WHO to diagnose TB. Compare to GeneXpert MTB/RIF assay, what's the advantage of mNGS? And have these samples been tested with GeneXpert? Adding the diagnostic performance to GeneXpert MTB/RIF and mNGS will be better if available. And how is the cost?

Reply 1: Thanks very much for your comments. We summarized the main characteristics of 19 samples which were tested by mNGS, culture and GeneXpert MTB/RIF (Xpert) in Table S2, Table S3, and Table S4 (see Page 39-41). The tables presented the diagnostic performance of mNGS, culture, Xpert, mNGS/Xpert, and mNGS/Xpert/culture in TB, and the relationship and concordance between mNGS and Xpert. We found that mNGS performed better than Xpert in all clinical (76.9%, 95%CI 0.460-0.938 Vs. 61.5%, 95%CI 0.323-0.849), pulmonary (87.5%, 95%CI 0.467-0.993 Vs. 75.0%, 95%CI 0.356-0.955), and extrapulmonary samples (60.0%, 95%CI 0.170-0.927 Vs. 40.0%, 95%CI 0.073-0.830) (see Page 12, line 256-262). In terms of the cost, we highlighted that the testing time of mNGS has not surpassed Xpert, thus putting forward some suggestions in optimizing the turnaround time and the cost of mNGS (see Page 18-19, line 393-406).

Changes in the text: Title, the statements of "Comparison of metagenomic next-generation sequencing（mNGS）technology with culture method in the diagnosis of

tuberculosis "were corrected as "Comparison of metagenomic next-generation sequencing technology, culture and GeneXpert MTB/RIF assay in the diagnosis of tuberculosis".

Page 2, line 41-42, “Results of GeneXpert MTB/RIF assay (Xpert) were obtained from 19 patients.” were added.

Page 3, line 50-51, “and Xpert (76.9%, 95%CI 0.460-0.938 Vs. 61.5%, 95%CI 0.323-0.849)” were added.

Page 3, line 54, “outstanding (mNGS/culture 88.2%; mNGS/Xpert 100%)” were added.

Page 3, line 59; Page 5, line 100-102; Page 10, line 200; Page 13, line 270; Page 14, line 297; Page 16, line 330; Page 16, line 335; Page 17, line 359; Page 19, line 417 “or Xpert” were added.

Page 5, line 98, “and Xpert results from 19 patients.” were added.

Page 6, line 116; Page 10, line 198; Page 11, line 227; Page 14, line 292; Page 17, line 357; Page 19, line 415-416 “and Xpert” were added.

Page 6, line 126-128, “In all 19 samples which were further tested by Xpert, 8 were BALF samples, 7 were pleural fluid samples, 2 were sputum samples, and 2 were pus samples. ” were added.

Page 11, line 223-225, “. For 19 samples which were tested by mNGS, culture, and Xpert, the ratio of pulmonary (52.7%, 10/19) and extrapulmonary samples (47.4%, 9/19) was close (Table S2).” were added.

Page 11, line 230, “(Table 2 and S3).” were added.

Page 12, line 256-262, “In comparison with Xpert, mNGS also showed better sensitivity

in all clinical (76.9%, 95%CI 0.460-0.938 Vs. 61.5%, 95%CI 0.323-0.849), pulmonary (87.5%, 95%CI 0.467-0.993 Vs. 75.0%, 95%CI 0.356-0.955), and extrapulmonary samples (60.0%, 95%CI 0.170-0.927 Vs. 40.0%, 95%CI 0.073-0.830). The sensitivity and positive predictive value of mNGS, culture, and Xpert all reached 100% in patients who received all three tests. In terms of detecting Mtb by single method, the Youden index of mNGS ranked first, Xpert second, and culture third (Table S3).” were added.

Page 13-14, line 282-290, “The improvement of diagnostic performance of NGS in combination with Xpert in TB were also found. The sensitivity, specificity, positive predictive value, negative predictive value, and Youden index of mNGS plus Xpert in all clinical samples was 84.6% (95%CI 0.537-0.973), 100% (95%CI 0.517-1.00), 100% (95%CI 0.679-1.00), 75.0% (95%CI 0.356-0.955), 84.6%, respectively. The Youden index of mNGS/Xpert in pulmonary samples was outstanding (100%; Table 2). When combined mNGS with culture and Xpert, the diagnostic performance in TB was further enhanced in extrapulmonary samples (Youden index: mNGS/Xpert 66.7% Vs. mNGS/Xpert/culture, 80.0%; Table S3).” were added.

Page 14-15, line 306-311, “The significant correlation between mNGS and Xpert was observed only when all 19 samples were taken into account (p=0.020). The kappa value of mNGS and Xpert in all, pulmonary, and extrapulmonary samples was 0.582 (p=0.009), 0.348 (p=0.260), 0.727 (p=0.023), respectively. The kappa statistic illustrated satisfying concordance between mNGS and Xpert in all and extrapulmonary samples (Table S4).” were added.

Page 15, line 328, “among whom 19 patients also received Xpert test.” were added.

Page 16, line 332, “all mNGS, culture, and Xpert methods” were added.

Page 18-19, line 390-406, “When compared with Xpert which was recommended for Mtb detection by WHO, mNGS shown better performance in pulmonary and extrapulmonary samples. In addition, the combination of mNGS and Xpert could make up the defect of Xpert in detecting Mtb in extrapulmonary samples. By means of mNGS, the diagnostic speed can be nearly 5 times faster than it of traditional culture method in Mtb detection (3 days Vs. over 14 days). However, the testing time of mNGS has not surpass Xpert (3 days Vs. 2-3 hours). In consideration of the influences of samples types in the comparison of mNGS, culture and Xpert results, we applied the same batch of samples in these methods. Another limitation is that specimen preservation for few days may affect the accuracy of mNGS, culture and Xpert. Given these, we should appraise the mNGS report dialectically and make clinical diagnosis on the basis of various testing data. Moreover, in order to the better clinical application, the turnaround time and cost of mNGS can not be neglected as well. Automation, standardization process, and optimization of the connection between hospital departments are all good choices to advance the development of mNGS. In addition, the lower price of reagent and the parallel processing technique which refers to test different types of samples simultaneously,also do good to the turnaround time and the cost of mNGS.” were added.

Page 39-41, Table S2, Table S3, and Table S4 were added.

Table S2. Clinical characteristics of 19 patients received Xpert test (n=19)

	TB group(n=13)	Non-TB group(n=6)
Gender, n (%)		
Female	4(30.8%)	1(16.7%)
Male	9(69.2%)	5(83.3%)
Pulmonary samples, n		

BALF	6	2
Sputum	2	/
Extrapulmonary samples, n		
Pleural fluid	5	2
Pus	/	2

Abbreviation: BALF, bronchoalveolar lavage fluid; CSF, cerebrospinal fluid; TB, tuberculosis; Xpert, GeneXpert MTB/RIF assay.

Table S3. Diagnostic performance of mNGS, traditional culture, and Xpert in TB

	Sensitivity (95% CI)	Specificity (95% CI)	+PV (95% CI)	-PV (95% CI)	Youden index
All samples					
Traditional culture	46.2% (0.204-0.739)	100% (0.517-1.00)	100% (0.517-1.00)	46.2% (0.204-0.739)	46.2%
mNGS	76.9% (0.460-0.938)	100% (0.517-1.00)	100% (0.655-1.00)	66.7% (0.309-0.910)	76.9%
Xpert	61.5% (0.323-0.849)	100% (0.517-1.00)	100% (0.598-1.00)	54.5% (0.246-0.819)	61.5%
mNGS+Xpert	84.6% (0.537-0.973)	100% (0.517-1.00)	100% (0.679-1.00)	75.0% (0.356-0.955)	84.6%
mNGS+Xpert+Traditional culture	92.3% (0.621-0.996)	100% (0.517-1.00)	100% (0.699-1.00)	85.7% (0.420-0.992)	92.3%
Pulmonary samples					
Traditional culture	62.5% (0.259-0.898)	100% (0.198-1.00)	100% (0.463-1.00)	40.0% (0.073-0.830)	62.5%
mNGS	87.5% (0.467-0.993)	100% (0.198-1.00)	100% (0.560-1.00)	66.7% (0.125-0.982)	87.5%
Xpert	75.0% (0.356-0.955)	100% (0.198-1.00)	100% (0.517-1.00)	50.0% (0.092-0.908)	75.0%
mNGS+Xpert	100.0% (0.598-1.00)	100% (0.198-1.00)	100% (0.598-1.00)	100% (0.198-1.00)	100%
mNGS+Xpert+Traditional culture	100.0% (0.598-1.00)	100% (0.198-1.00)	100% (0.598-1.00)	100% (0.198-1.00)	100%
Extrapulmonary samples					
Traditional culture	20.0% (0.011-0.701)	100% (0.396-1.00)	100% (0.055-1.00)	50.0% (0.174-0.826)	20.0%
mNGS	60.0% (0.170-0.927)	100% (0.396-1.00)	100% (0.310-1.00)	66.7% (0.241-0.940)	60.0%
Xpert	40.0% (0.073-0.830)	100% (0.396-1.00)	100% (0.198-1.00)	57.1% (0.202-0.882)	40.0%
mNGS+Xpert	60.0% (0.170-0.927)	100% (0.396-1.00)	100% (0.310-1.00)	66.7% (0.241-0.940)	66.7%
mNGS+Xpert+Traditional culture	80.0% (0.396-1.00)	100% (0.396-1.00)	100% (0.396-1.00)	80.0% (0.299-0.989)	80.0%

Abbreviation: TB, tuberculosis; mNGS, metagenomic next-generation sequencing; Xpert, GeneXpert MTB/RIF assay; 95% CI, 95% confidence interval; +PV, positive predictive Value; -PV, negative predictive Value.

Table S4. Relationship between mNGS and Xpert

			Xpert			
			Negative	Positive	P_1	Kappa value (P_2)
All samples	mNGS	Negative	8(88.9%)	3(30.0%)	**<u>0.020</u>**	**<u>0.582(0.009)</u>**
		Positive	1(11.1%)	7(70.0%)		
Pulmonary samples	mNGS	Negative	2(66.7%)	2(28.6%)	0.500	0.348(0.260)
		Positive	1(33.3%)	2(71.4%)		
Extrapulmonary samples	mNGS	Negative	6(100%)	1(33.3%)	0.083	**<u>0.727(0.023)</u>**
		Positive	0(0.00%)	2(66.7%)		

Abbreviations: TB, tuberculosis; mNGS, metagenomic next-generation sequencing; Xpert, GeneXpert MTB/RIF assay; P_1, P value of McNemar's test; P_2, P value of Cohen's Kappa test.

Statistically significant data were marked with bold and underline.

Comment 2: 70 samples were tested in the experiment, but only 36 samples were diagnosed to be TB. For calculating the sensitivity of mNGS, more positive samples tested will be more convincing.

Reply 2: Thanks very much for your comments. We apologize for the rather small sample size of positive TB cases. This is one limitation of our study. This retrospective study incorporated a total of 70 patients at the Shanghai Pulmonary Hospital of Tongji University between 1 January and 30 September 2019. Among all enrolled patients, 36 patients (51.4%) were finally diagnosed with pulmonary or extrapulmonary TB. To confirm our present findings, we are planning to test the diagnostic performance of mNGS with larger sample size and would be published in our future paper.

Changes in the text: Page 19, line 411-412, "For further investigation and stronger credibility, a prospective and multi-centered study with larger sample size is necessary." were added. Page 19, line 420-421, "More researches need to be conducted to explore better clinical application of mNGS in the future." were added.

Comment 3: Are the same batch of samples used in culture method and mNGS? Will storage of samples for few days affect the accuracy of mNGS or culture?

Reply 3: Thanks very much for your comments. In consideration of the influences of samples types in the comparison of mNGS, culture and Xpert results, we applied the same batch of samples in these methods (see Page 18-19, line 396-398). Specimen preservation for few days could affect the accuracy of mNGS, culture and Xpert. We highlighted that automation, standardization process, and optimization of the

connection between hospital departments are worth expecting (see Page 18-19, line 402-406).

Changes in the text: Page 18-19, line 396-406, “In consideration of the influences of samples types in the comparison of mNGS, culture and Xpert results, we applied the same batch of samples in these methods. Another limitation is that specimen preservation for few days may affect the accuracy of mNGS, culture and Xpert. Given these, we should appraise the mNGS report dialectically and make clinical diagnosis on the basis of various testing data. Moreover, in order to the better clinical application, the turnaround time and cost of mNGS can not be neglected as well. Automation, standardization process, and optimization of the connection between hospital departments are all good choices to advance the development of mNGS. In addition, the lower price of reagent and the parallel processing technique which refers to test different types of samples simultaneously, also do good to the turnaround time and the cost of mNGS.” were added.

Comment 4: Please explain the phenomenon ‘the infection of Mtb was detected in patient nos.25 and nos.28 by culture, but not by mNGS’, and I can't find figure 3 in the pdf file. And there is no figure 2.

Reply 4: Thanks very much for your comments. We explained the opposite result between mNGS and culture in patient nos.25 and nos.28 as advised (see Page 18, line 386-390). In addition, we rechecked the part of figure 2 and figure 3 and removed the corresponding statement in the manuscript as advised.

Changes in the text: Page 18, line 386-390, “When compared with the positive culture result, the difficulty of releasing sufficient genomic DNA by Mtb may also lead to the false-negative result of mNGS. Besides, due to the insufficient number of Mtb in clinical samples which failed to meet the analytical concentration of mNGS, the opposite result between mNGS and culture in patient nos.25 and nos.28 was found (40).” were added.

Comment 5: In the conclusion part, “mNGS could recognize various kinds of pathogens and evaluate drug susceptibility” was not reflected in this paper.

Reply 5: Thanks very much for your comments. We checked the text again and removed the statement of “mNGS could recognize various kinds of pathogens and evaluate drug susceptibility” as advised (see Page 19, line 417).

Changes in the text: Page 19, line 417, “mNGS could recognize various kinds of pathogens and evaluate drug susceptibility” were deleted.

Comment 6: There are many spelling, grammar and format mistakes. Such as the names of bacteria should be in italic, “top ten killer in all diseases”, “which could also be used for drug susceptibility testing” and so on. The authors may find a professional scientist to polish or some companies to polish the manuscript.

Reply 6: Thanks very much for your comments. We asked two experts of Tongji University to help us revise the grammar and writing in the paper carefully. The spelling, grammatic, and format errors were corrected in the manuscript as advised.

Changes in the text:

Page 3, line 62-63, “Mycobacterium tuberculosis” were corrected as “*Mycobacterium tuberculosis*”.

Page 3, line 66, “top ten killer all diseases” were corrected as “top ten killers in all diseases”.

Page 4, line 73, “could also used for drug susceptibility identification” were corrected as “could also be used for drug susceptibility testing”.

Page 15, line 315, “Haemophilus influenzae, Candida albicans, Aspergillus” were corrected as “*Haemophilus influenzae*, *Candida albicans*, *Aspergillus*”.

Page 15, line 318-319, “non-tuberculous mycobacterium” were corrected as “*non-tuberculous mycobacterium*”.

Page 15, line 320, “Candida albicans” were corrected as “*Candida albicans*”.

Page15, line 323, “Epstein-Barr virus (EBV) and cytomegalovirus (CMV)” were corrected as “*Epstein-Barr virus* (EBV) and *cytomegalovirus* (CMV)”.

Page 16, line 346, “Varicella-zoster virus” were corrected as “*Varicella-zoster virus*”.

Page 19, line 418, “showed an outstanding advantage” were corrected as “showed outstanding advantages”.

Page 19, line 421, “of mNGS” were added.