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Evaluation of *ku* gene as a differential biomarker for rapid molecular detection of *Mycobacterium tuberculosis* complex using a real-time PCR assay

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ABSTRACT

Purpose: currently TB diagnosis is limited by some major limitations in low-income and less experienced hospitals. Recently, it has been proposed that the *ku* gene of mycobacterial strains has the potential to be a highly specific and sensitive candidate biomarker for molecular detection of *Mycobacterium tuberculosis* (Mtb). This study was aimed to evaluate the specificity and sensitivity of a real-time PCR assay for detection of *ku* gene in Mtb complex to determine its applicability for Mtb identification.
Materials and methods: The identification of Mtb was confirmed using GeneXpert assay. Specific primers for *ku* gene were designed and the cycle threshold (Ct) value from the real-time PCR was used as a proxy measure of the cut-off point. Receiver operating characteristic (ROC) curve analysis was conducted to determine the diagnostic performance of *ku* gene in detecting Mtb directly from clinical specimens.
Results: ku amplification was interpreted as positive and negative based on Ct values, in which a value <38 was considered positive and a value >40 was considered negative. Our findings revealed that the *ku* gene was found to be distributed in all Mtb-positive samples. Of note, none of the Mtb-negative exhibited a specific signal in a maximum of 40 cycles.
Conclusions: The *ku* gene amplification using real-time PCR indicated high sensitivity and specificity for the detection of Mtb complex in sputum samples.

Keywords: *Mycobacterium tuberculosis*, *ku* gene, biomarker, nucleic acid amplification tests (NAATs), GeneXpert MTB/RIF

1.Introduction

Tuberculosis (TB) is an important infectious disease that could affect the lungs and other parts of the body in almost all age groups. The disease is caused by *Mycobacterium tuberculosis* bacteria (Mtb), and with approximately 10 million infected cases and 1.4 million deaths in 2019, remains as a global public health concern [1]. Early diagnosis and treatment are critical to control and reduce the mortality rate of TB cases. Diagnosis of active TB infection is routinely performed based on clinical symptoms and radiological features, smear microscopy, culture and biochemical-based approaches, nucleic acid amplification tests (NAATs), and automated methods like GeneXpert MTB/RIF [2, 3]. Nevertheless, TB diagnosis is still accompanied by some major limitations, including low specificity of clinical signs, low sensitivity of sputum smear microscopy and culture, and inaccessibility to high-performance diagnostic techniques in most laboratories in developing countries [4]. Moreover, a major issue in TB prevention and control is the presence of a large reservoir of undiagnosed TB cases, which accounts for approximately half of all TB cases, signifying the need for more advanced diagnostic tools [5, 6].

In the past decades, several gene amplification assays have been developed for rapid detection of Mtb, which could identify mycobacterial isolates in culture or directly in clinical specimens [7, 8]. However, several of these approaches need high-performance technologies to provide an acceptable discriminatory power for accurate and rapid detection of Mtb; therefore, their application is limited because of their high costs, especially in developing countries. Moreover, genes targeted in these assays, such as *rpoB*, *IS6110*, and *23S rRNA*, may have some limitations for specific detection of Mtb. For instance, some of these genes may be absent in some Mtb strains, such as *IS6110*, or may be prone to mutation, such as *rpoB*, thereby causing false-negative detection [9, 10]. Hence, the rapid detection of Mtb in low-income countries with high infection rates is yet considered as a prominent problem. Accordingly, it seems necessary to develop novel methods based on single-gene biomarkers, which could be applied as an accurate, rapid, and culture-free molecular assays for Mtb detection.

Recently, it has been suggested that in prokaryotic error-prone non-homologous end-joining pathway (NHEJ), the *ku* gene could be considered as a highly specific and sensitive candidate biomarker and used in molecular detection of Mtb [11].The NHEJ system is coupled with CRISPR/Cas9 system and repairs double-strand breaks on DNA by inducing genetic modifications at the cleavage site through insertions and/or deletions of several nucleotides in order to repair the targeted genome [12]. In contrast to eukaryotes, the NHEJ DNA repair system is not ubiquitous among bacterial cells. Importantly, many of the bacterial strains that encode a *Ku* homolog spend much of their life cycle in stationary or dormant phases which are characterized as slow-growth, non-growth, or spore-formation periods [13, 14]. Prokaryotic NHEJ homologs have so far been detected in only a few bacterial genera, including *Bacillus*, *Mycobacterium*, and *Pseudomonas* [15, 16]. The scarcity of the Ku protein in most prokaryotes suggests that it could possibly be applied as a specific biomarker in Mycobacterium detection. Therefore, in the current study, the specificity and sensitivity of a real-time PCR method in detecting Mtb complex *ku* gene were investigated to evaluate its applicability in Mtb detection as a specific biomarker.

2. Materials and Methods

2.1. Sputum smear microscopy and culture

This study was performed on 300 respiratory sputum samples at the Tehran Regional Reference Laboratory for Tuberculosis from March 2019 to April 2021. The ethics committee of Tarbiat Modares University approved this study. The sputum decontamination process was performed for all sputum samples using N-acetyl-L-cysteine sodium hydroxide (NALC-NaOH %1). Ziehl-Neelsen staining was performed for all smears to determine the presence of acid-fast bacilli (AFB). Then 500 µL of the specimen obtained from the decontamination process was cultured in Lowenstein Jensen (LJ) culture medium and subjected to the incubation process at 37 °C. All cultures were inspected once a week for up to 8 weeks to determine the possible growth of Mtb strains.

2.2. GeneXpert MTB/RIF assay

The GeneXpert MTB/RIF assay was carried out for processed samples according to the recommendations of the manufacturer. Briefly, the decontaminated specimens were diluted with Xpert sample reagent (SR) at a ratio of 1:3 and incubated at room temperature for 10 min while shaking vigorously twice. The prepared mixture with the desired volume was transported into the Xpert test cartridge which was then placed into the Xpert machine. At last, the data obtained from the measured fluorescent signals were automatically interpreted by the GeneXpert DX system and represented within 90 min.

2.3. DNA extraction

DNA extraction was performed for sputum samples using a tissue genomic DNA extraction mini kit (FAVORGEN, Taiwan) according to the recommendations of the manufacturer. The yield, concentration, and purity of the obtained DNA were assessed by employing a Nanodrop (DeNovix Inc., USA). The extracted DNA was maintained at -20 °C until used for subsequent analysis. In the current study, DNA was used to detect Mtb by real-time PCR assay.

2.4. In Silico Analysis of the ku Gene

For the design of specific primers, the sequences of Mtb complex *ku* gene were acquired from NCBI GenBank (http://www.ncbi.nlm.nih.gov/) and aligned with those of other mycobacterial species using CLC Sequence Viewer 7. Subsequently, primers were designed via AlleleID6 and evaluated by the Primer-BLAST on NCBI (http://www. ncbi.nlm.nih.gov/).

2.5. Limit of Detection (LOD) of designed real-time PCR

The real-time PCR assay was initially evaluated by calculating the limit of detection (LOD). LOD was calculated for the ku gene in duplicate using DNA extracted from *M. tuberculosis H37Rv* strain. DNA was diluted in serial 10-fold dilutions covering the range of 100ng/µL to 10 fg/µL.

2.6. Evaluation of sputum by real‑time PCR

The real-time PCR was performed to amplify a 117 bp region by employing SYBR Green chemistry using specific primers (forward: 5'-CTGCTGGCTAAGACACTC -3'; reverse: 5'-CATCACCTCTCGCTTGC -3'). PCR amplification was performed in a total volume of 25 µL reaction mixture consisting of 1X SYBR green PCR master mix (AMPLIQON, Denmark), 0.5 µL of 10 pmol forward and reverse primers, and 100 ng of DNA template. The real-time PCR was carried out under the following thermal cycling conditions using a real-time PCR system (LightCycler 480, Roche): an initial denaturation step at 94 °C for 10 m, followed by 40 cycles of denaturation at 94°C for 20 s, 60 °C for 15 s, and 72 °C for 30 s. The accuracy and specificity of PCR amplification were confirmed by melting curve analysis through increasing the temperature by 0.5 °C from 40 to 95 °C. It is worth noting that DNA extraction and PCR assay were repeated for those samples that were positive in culture method but negative in PCR amplification of the ku gene. In addition to clinical mycobacterial isolates, the reference strain *M.tuberculosis H37Rv*(ATCC 27294), *Bacillus subtilis*(ATCC 10742), and *Escherichia coli*(ATCC 25922) were used in this study.

2.7. Statistical analysis

The cycle threshold (Ct) value in the real-time PCR was used as a proxy measure of the cut-off point. Samples with $Ct \leq 38$ were considered as positive for the ku gene. To evaluate the diagnostic potential of ku amplification in identifying Mtb compared to other methods (i.e., smear microscopy, microbiological culture, and GeneXpert), the sensitivity and specificity of the cut-off points calculated were recorded. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic potential of ku gene in detecting Mtb directly in clinical specimens. Accordingly, the areas under the ROC curve (AUC) were estimated for the real-time PCR assay of the ku gene and compared to the results of smear microscopy, culture, and GeneXpert MTB/RIF assays, separately.

3. Result

Of 300 sputum samples, 50 (16.7%) were identified as M. tuberculosis using GeneXpert MTB/RIF assay. Among 50 individuals from whom Mtb strains were approved, 27 (54%) cases were male, and 23 (46%) cases were female.

Real-time PCR optimization was conducted using standard curves prepared based on 10-fold serial dilutions of 100 ng/µL of *M. tuberculosis H37Rv* stock solution (Figure 1). The obtained results revealed that 1 fg/µL (~ 2.1 × 10 genomic equivalents) was the least DNA concentration (LOD) identified in real-time PCR analysis. The dynamic range of Ct values was from 16.5 (lower Ct value) to 38 (upper Ct value). The results of real-time PCR amplification of the ku gene were reported as positive and negative based on Ct values, in which a Ct value <38 was considered as positive, and a Ct value >40 was considered as negative. Ct values between 38 and 40 were reported as doubtful based on <100% reproducibility. Accordingly, the present study findings revealed that the *ku* gene was present in all Mtb-positive sputum samples.

In the ROC curve analysis, the AUC for ku amplification was 0.95 (95% CI: 0.91-0.99), 0.90 (95% CI: 0.80-0.99), and 0.97 (95% CI: 0.93-0.99) compared to AFB smear microscopy, culture, and GeneXpert MTB/RIF assays, respectively (Figure 2). In comparison with AFB smear microscopy, the best Ct as the cut-off value for ku amplification to diagnose Mtb was 38; this threshold value was associated with 93% sensitivity and 98% specificity. In comparison with the culture method, the best Ct value for ku amplification was 35 with 86% sensitivity and 91% specificity. Finally, compared to GeneXpert MTB/RIF assay, the best Ct value for ku amplification to diagnose Mtb was 38; this threshold value was also associated with 94% sensitivity and 100% specificity.

4.Discussion

Among the common methods used for detecting Mtb strains, AFB smear microscopy (e.g., Ziehl–Neelsen and Kinyoun staining) and culture methods remain as the most widely used laboratory diagnostic methods for detecting TB in developing countries. In Iran, sputum smear microscopy is frequently used as a simple, rapid, and inexpensive technique for diagnosing pulmonary TB. However, the sensitivity of AFB smear microscopy ranges from 20 to 78%, especially in those with human immunodeficiency virus (HIV) co-infection [5, 18]. Although considered as the conventional gold standard for tuberculosis diagnosis, culture method is labor-intensive and time-consuming and takes about 3 – 8 weeks to yield results, the results are often invariably delayed because of the slow growth rate of mycobacteria [19]. Hence, NAATs are more important in detecting and identifying Mtb complex in clinical samples of TB patients in areas with high burden of the disease. In this study, a real-time PCR assay was evaluated specifically to detect the *ku* gene in order for diagnosing Mtb complex in clinical samples.

Several NAATs have been developed based on species-specific insertion sequences, such as *IS6110* [20], *16S rRNA*, *rpoB*, and *hsp65* genes [21, 22], to specifically detect Mtb complex. However, the presence of some Mtb strains harboring no copies of *IS6110* elements have been reported in certain parts of the world, especially in Asia [23]. Moreover, identification approaches based on *16S rRNA*, *rpoB*, and *hsp65* genes commonly rely on restriction analysis or direct sequencing [24]. Although rpoB is used in GeneXpert assay that could be performed with little technical expertise on unprocessed sputum samples and represent data within 90 min, it requires special instruments and is still expensive for use in developing countries. Accordingly, it is necessary to identify a specific biomarker that could be used to detect Mtb complex without the need for sequencing.

The present study findings highlight the importance of the *ku* gene in Mtb detection as a diagnostic biomarker that is not ubiquitous among prokaryotes; thus, its rarity in bacteria, especially in other families of Corynebacteriales closely related to *Mycobacterium* (e.g., *Rhodococcus* and *Nocardia* spp.), suggests it as a potential mycobacterial-specific biomarker [11]. Herein, the Ct value of 38 obtained by real-time PCR assay for the *ku* gene could be comparable to AFB smear microscopy and culture method with acceptable sensitivity and specificity. In addition, the data obtained in this study confirmed that the Ct value of 35 could be considered as an indicative of the presence of Mtb strains with high sensitivity and specificity compared to GeneXpert MTB/RIF assay. It is well documented that real-time PCR-based methods show a good sensitivity in TB diagnosis. The main advantage of real-time PCR-based methods is that they enable sensitive, specific, fast, and safe detection of Mtb strains. Pinhata et al. (2015) previously reported that the sensitivity of an in-house real-time PCR method, performed by

targeting *mpt64* to identify Mtb complex, was 96.1 and 63.6% in smear-positive and smear-negative samples, respectively [25].

Given that the *ku* gene could be detected in almost every species of *Mycobacterium*. However, in this study, a specific primer set was designed based on Mtb complex-specific sequences that could differentiate Mtb complex from NTMs. Several validated real-time PCR assays have been developed to identify pathogenic mycobacteria. Richardson et al. (2009) previously designed a multiplex real-time PCR assay, resulting in the identification of 93% of the pathogenic mycobacteria in two parallel reactions [26].

There was no false-negative results in real-time PCR method employed in this study, indicating the high sensitivity of this approach. False-negative results in conventional PCR could occur due to the presence of inhibitors of enzymatic amplification and/or unequal distribution of bacilli in paucibacillary samples. On contrary, real-time PCR methods show more sensitivity and specificity in target detection than conventional PCR assays; in addition, the risk of environmental contamination during the process is negligible in these methods due to simultaneous amplification and detection of the nucleic acid in the same closed vessel [27].

5. Conclusion

The *ku* gene amplification using real-time PCR indicated high sensitivity and specificity for the detection of Mtb complex in sputum samples. This approach could be applied as an alternative approach for clinical laboratories with limited resources that do not have rapid cartridge-based nucleic acid amplification tests for the identification of Mtb, enabling the performance of TB diagnosis in up to five hours.

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Author Contributions

B Bakhshi, T Radaei, M Nikkhah, and GR Hamzehloo conceived and designed the study. T Radaei performed the experimental procedures and interpreted the data. T Radaei wrote a first draft of the paper, and B Bakhshi contributed to the revisions. M Nikkhah and GR Hamzehloo approved the final version of the manuscript and the authorship list.

Ethics approval code

The study was reviewed and approved by Medical Ethics Committee of Tarbiat Modares University (IR.MODARES.REC.1397.280).

Consent for publication

Not applicable.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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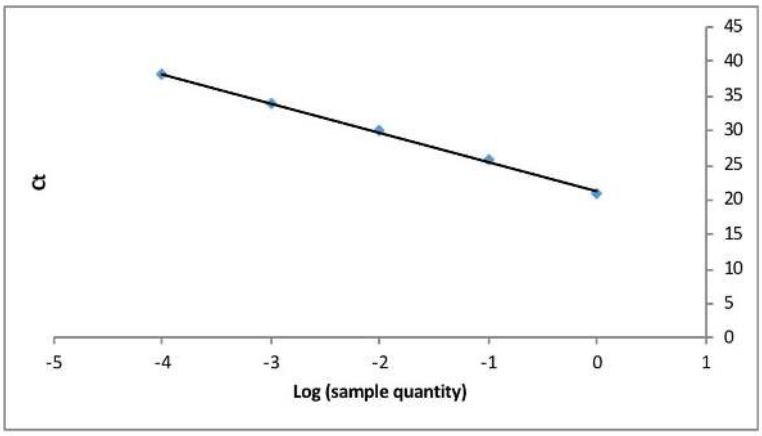
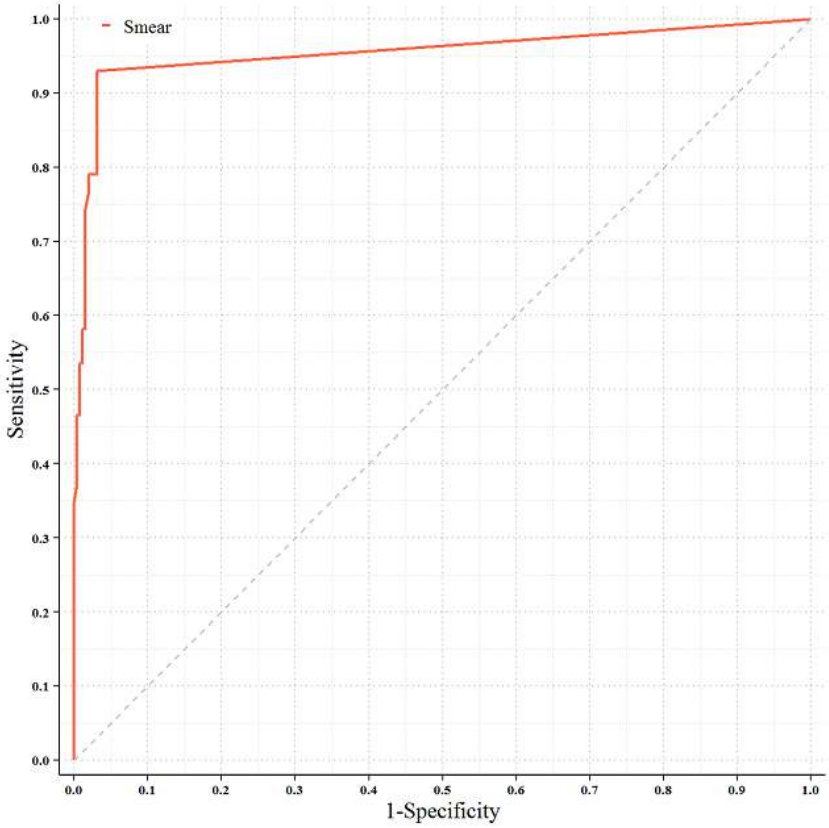


Figure 1: Standard curve used to evaluate the efficiency of real-time PCR for the *ku* gene detection



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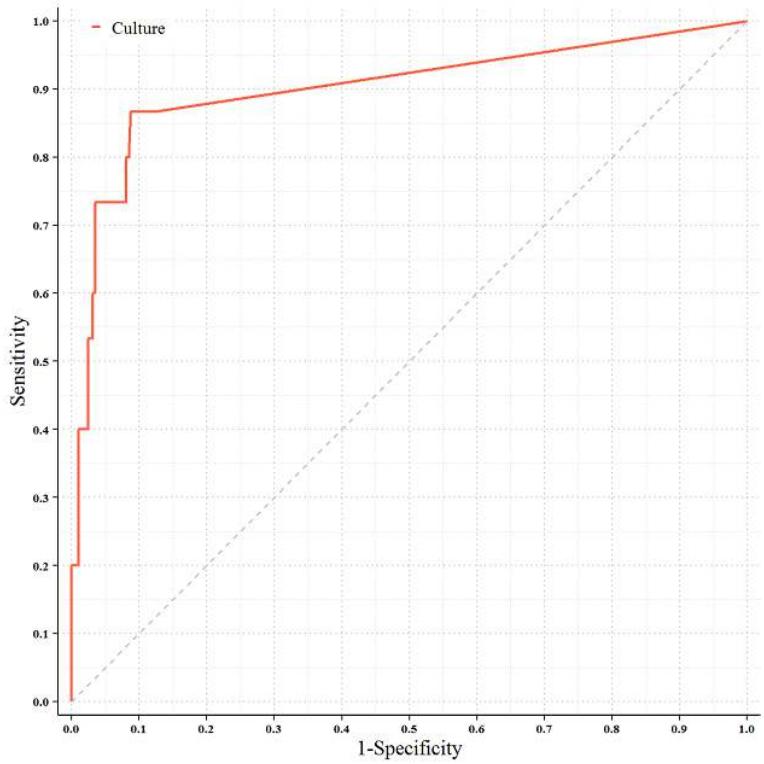

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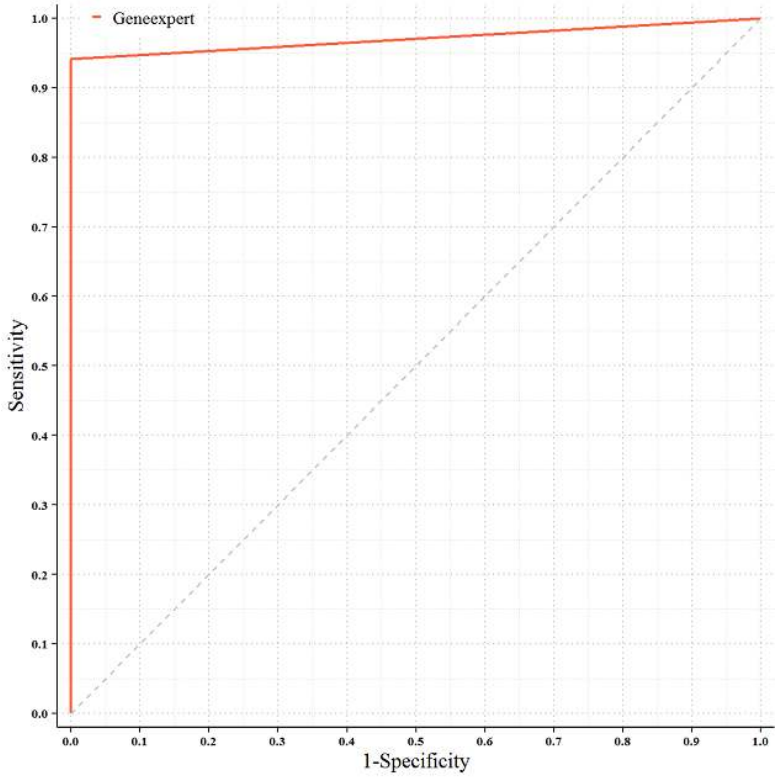

Figure 2: Receiver operating characteristic (ROC) curves for amplification of *ku* was reconstructed compared to A) smear microscopy, B) culture, and C) GeneXpert assay