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Conference report

The short-chain dehydrogenases/reductases (SDR) gene: A new specific target for rapid detection of *Mycobacterium tuberculosis* complex by modified comparative genomic analysis

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ABSTRACT

Background: Early detection of tuberculosis is one of the crucial steps for TB control. Although, the sensitivity of conventional methods like Lowenstein Jensen (LJ) culture and direct staining is quite low, molecular techniques like polymerase chain reaction (PCR) are more sensitive and be considered as useful tools for rapid detection of tuberculosis. Various genes like *IS6110 and mpb64* have been used as target for detection of *M. tuberculosis,* but more research is needed to find the most specific targets.

The short-chain dehydrogenases/reductases family (*SDR*) is one of a very large family of NAD- or NADP-dependent oxidoreductase enzymes which is present in all *M. tuberculosis* strains. The large part of *SDR* sequences in tuberculosis is completely conserved and different from non-tuberculosis mycobacterium. The aim of the study was to develop an in-house PCR assay using the *SDR* target for rapid detection of *M. tuberculosis* from clinical specimens.

Method: M. tuberculosis-specific sequences were found using modified genome comparison method and the primers were designed by the Primer Premier 5.0 software. A PCR assay was developed targeting the nucleotide sequences within the *SDR* gene. A total of 50 cultivated specimens and 120 clinical specimens were evaluated by PCR.

Results: The clinical evaluation of *SDR* PCR assay showed high specificity (100%) and high sensitivity (88.5%). The analytical sensitivity was 10 fg of template DNA which is theoretically equivalent to 2 copy of genomic DNA per microliter. The *SDR* is a new specific target of *M. tuberculosis* and no cross-reactivity was observed to non-tuberculosis mycobacterium and other pathogenic bacteria.

Conclusions: Based on our results, the *SDR* gene can be considered as a useful target for detection of *M. tuberculosis* complex from clinical specimens.

1. Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, remains as second most contagious disease around the world and about one-third of the world's population has been infected with *M. tuberculosis*. According to the WHO Global TB report 2016, there were 10.4 million new TB cases and estimated 1.7 million people died from tuberculosis, of which, 400,000 people were co-infected with HIV/TB. Although, about 2 billion people infected with tuberculosis never develop the active disease, the people with HIV who have latent TB infection, are at high risk for developing TB disease (Falzon et al., 2017). Due to the high prevalence of tuberculosis, the strong and cheap diagnostic strategies are needed for successful and effective treatment and TB management (Pauwels et al., 2001).

The traditional strategies like Acid fast bacilli (AFB) microscopy are rapid but have very low sensitivity and specificity leading to inefficient identification. Culture-based methods are more sensitive than AFB microscopy and able to detect 10–100 bacilli of mycobacterium per milliliter of samples (Palomino, 2006). So it continues to be remained as the reference standard procedure compared with all other diagnostic

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methods (Chegou et al., 2011). However, it is time consuming and takes 4–8 weeks to provide results. (Although both culture and microscopy remain essential for monitoring responses to treatment) (Greco et al., 2009).

Since *M. tuberculosis* grows so slowly, the rapid, accurate and sensitive identification of *M. tuberculosis* in clinical specimens is necessary in order to start the initial treatment and prevent the spread of the disease within the community.

The more current tests like Xpert® MTB/RIF assay, MGIT tubes, radiometric BACTEC test, serological tests and hybridization methods are expensive and require more instruments and technical experience which are not available in all laboratories (Creswell et al., 2014; Kim et al., 2015; Ravibalan et al., 2016).

Presentation of the nucleic acid amplification techniques (NAAT) particularly polymerase chain reaction (PCR) to recognize different infections in the clinical specimens have made a significant breakthrough in the rapid detection of diseases (Ieven and Goossens, 1997). PCR is a low-cost, fast, sensitive, specific and simple technology that uses a pair of primers to detect the specific DNA target in the specimens.

The choice of specific target genes and designing a pair of strong primers are two important steps for accurate detection of microorganism with a high sensitivity and specificity especially in clinical specimens. The efficacy of PCR detection method relies on the uniqueness of the sequence (no cross reaction to any other bacterial) and the specific binding of the primers to the targets.

Several studies have reported different regions/gene sequences of mycobacterial genome with various degrees of specificity and sensitivity which are used as targets e.g. *IS6110*, *MPB64*, *MPB70*, *rpoB*, 65 kDa, *devR* and 38 kDa antigen (*PhoS, CIE Ag78* or *Pab*) for detection of *M. tuberculosis* by PCR (Chakravorty et al., 2006; Kim et al., 2004; Negi et al., 2007; Therese et al., 2005).

The *IS6110* element is the most common target for detection by DNA amplification. Although, the multiple copies of the element exist in *M. tuberculosis* complex and causes the high sensitivity for detection of tuberculosis (Eisenach et al., 1990; van Soolingen et al., 1991), some of the strains particularly those identified in southeast Asia lack *IS6110* elements. Because these insertion sequences are not integrated in all of *M. tuberculosis* complex genome (Huyen et al., 2013). Some other targets yielded cross reactivity when they were tested against non-tuberculosis mycobacterium (Nimesh et al., 2013).

Due to these limitations of known targets, finding a new specific target is needed for rapid detection of *M. tuberculosis* in clinical specimens. Whereas most of the available target genes used for PCR detection of tuberculosis involving important genes or virulence factors, the modified comparative genomics method has led the way to increase the availability of whole genome sequences to customize the genome for specific DNA sequences and allows for the identification of unique sequences for the genus or species. Thus far, several studies have used comparative genomic methods to find new targets for detection of other pathogens and showing this to be a practical methodology for the recognition of specific sequences (Bolotin et al., 2004; Chen et al., 2003; Iguchi et al., 2009; Thomson et al., 2008). In this study, we used modified comparative genomic methods on *M. tuberculosis* whole genome for identification of specific and unique detection targets.

The short-chain dehydrogenases/reductases family (SDR) is a novel specific target and one of a very large family of enzymes in mycobacterium, which are known as NAD- or NADP-dependent oxidoreductase (Kallberg et al., 2002). These enzymes are present in all mycobacteria, but the large part of SDR sequence of *M. tuberculosis* complex is different from non-tuberculosis mycobacterium.

Until now, this enzyme has not been used as DNA target for detection of *M. tuberculosis* complex. The aim of study is introducing a new target gene which specific for *M. tuberculosis* complex and designing a strong primers with high sensitivity and specificity for rapid detection of *M. tuberculosis* in clinical specimens.

2. Materials and methods

2.1. Bacterial sample collection

A total of 50 cultivated specimens and 100 clinical sputum specimens including 50 smear- and culture- positive and 50 smear- and culture-negative were collected from tuberculosis laboratory of Qaem hospital in Mashhad. In order to confirm the identification of *M. tuberculosis* complex (MTBC) in solid culture, IS6110-PCR and mpb64-PCR were performed.

We also provide smear negative, culture-positive specimens to evaluate the sensitivity of the primers. We do this in such a way that processed negative specimen was added to the smear-positive culture-positive samples and 10-fold dilution was made. Then the dilutions that were negative in ziehl-neelsen staining, were cultured on Lowenstein Jensen medium (LJ) (Yamada et al., 2006). The lowest dilutions with positive culture were considered as smear-negative, culture-positive.

2.2. DNA extraction of specimens

Clinical samples were processed with 3% NaOH, and then centrifuged at 14,000 rpm. Then, 2 ml phosphate buffered saline (PBS) was added to their sediments.

In order to reduce processing time, contamination and costs in DNA extractions, we used an autoclave method for preparing mycobacterial DNA as PCR templates (Simmon et al., 2004). Samples were transferred to the microtubes and processed in a preheated autoclave set to 121 °C and a sterilization time of 3 min. The microorganism are lysed by rapid heating in an autoclave. Then, the debris, cleared by centrifuged at 14,000 rpm and the supernatant was used directly in the PCR reaction. The genomic DNA of *M. tuberculosis* H37Rv from colonies was extracted and purified by Dick Van Soolingen method (Huard et al., 2003) and was used to determine the analytical sensitivity.

2.3. Modified comparative genomic analysis and primer design

The workflow for selection of *M. tuberculosis* complex-specific target is illustrated in Fig. 1. Seven *M. tuberculosis* complex genome references sequences were obtained from nucleotide collection database (http://www.ncbi.nlm.nih.gov/nuccore) (Table 1). *M. tuberculosis* H37rv was used as the reference strain and the genome sequence was divided into 5000 bp fragments and checked by Basic Local Alignment Search Tool (BLAST) in silico (nucleotide collection database). The fragments that has ≥99% identity or query cover with seven *M. tuberculosis* complex were considered as *M. tuberculosis*-conserved sequences. Then, the conserved sequences cut into the shorter fragments and searched against the database of non-tuberculosis mycobacterium (NTM) and other bacterial genome sequences using blast nucleotide to determine the specificity of the sequences (DNA fragments with alignment scores < 40 for NTM and other bacteria were determined as specific sequences).

The fragment that show no cross reactions with other bacteria especially NTM, were considered as *M. tuberculosis* complex-specific DNA sequences. The longer specific target was picked and used to design primers by Primer Premier5.0 software (Premier Biosoft Intl., CA USA). Then, the primers were checked for secondary structure and predicted melting temperature using Oligo Analyzer 3.1 (https://eu.idtdna.com/calc/analyzer). Afterward, the search was limited to human sequences by selecting "*Homo sapiens*" to show not overlapping alignments, in order to confirm the primers specific to the genome of the *M. tuberculosis* complex in clinical specimens. The Primers were synthesized by Macrogen (Seoul, Korea).

2.4. Bioinformatics control

In this part of the study, two methods were used to evaluate the

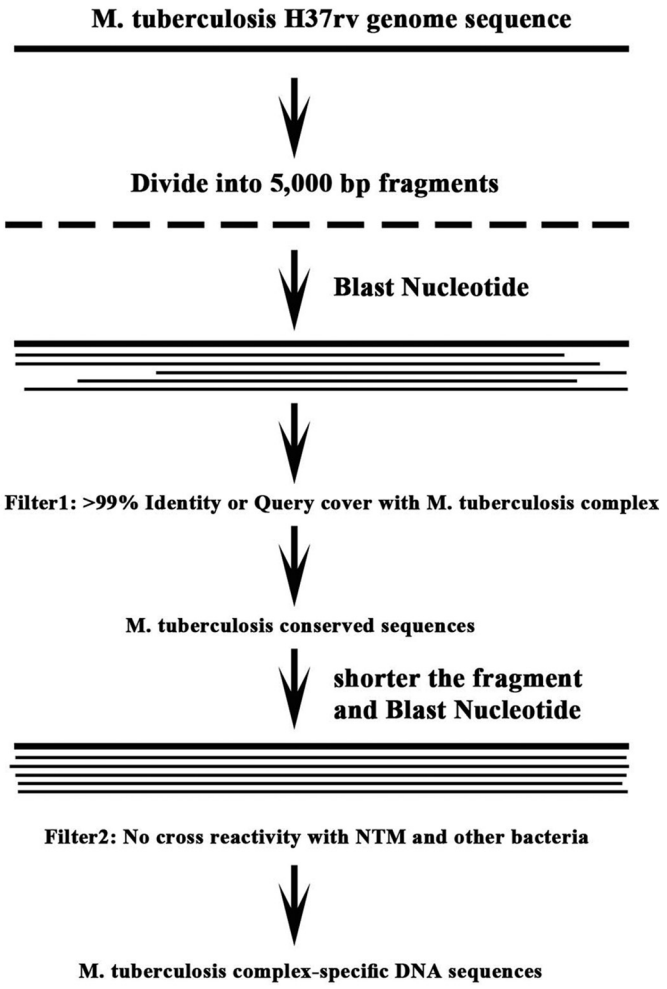

Fig. 1. Scheme for modified comparative genomics method.

Table 1
The accession number of MTBC members on nucleotide collection database.

Species	Accession number
Mycobacterium tuberculosis H37Rv	CP009480.1
Mycobacterium bovis strain ATCC BAA-935	CP009449.1
Mycobacterium bovis BCG Pasteur 1173P2	AM408590.1
Mycobacterium africanum GM041182	FR878060.1
Mycobacterium microti strain 12	CP010333.1
Mycobacterium caprae strain Allgaeu	CP016401.1
Mycobacterium canettii CIPT 140010059	FO203509.1

conservation of the *SDR* gene in the different strains of the *M. tuberculosis* complex:

1. Comparison with *rpoB* gene: At this stage, all strains identified by *rpoB* (as a conserved sequence) were compared with those strains identified by the *SDR* gene in nucleotide collection database using blastn search.
2. Determining the *SDR* conservation using the TB-ARC project data (https://olive.broadinstitute.org/projects/tb_arc/tree): At this stage, genomic sequences of 224 species which related to the TB Antibiotic Resistance Catalog project (Barletta et al., 2014) from around the world, were downloaded and the presence of the *SDR* gene among these strains was studied by blastn search. The geographical areas and the number of strains studied are shown in Table 2.

Table 2
The geographical areas and the number of strains, to prove the SDR conservation.

Location	Number of strains
India	46
Iran	16
Taiwan	30
Mali	24
USA	8
Sweden	30
Uganda and South Korea	24
Romania	8
Moldova	8
Africa	30
Total	224

2.5. Polymerase chain reaction

The reaction mixture consisting of 1× reaction buffer (50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2), 10 pmol each of primers, 200 mM each of dATP, dCTP, dTTP, and dGTP, 2 U of Taq DNA-polymerase (5 U/μl; Fermentas) were prepared in 25 μl volumes. 1 μl of extracted DNA from *M. tuberculosis*H37Rv was used as template for PCR amplification.

PCR was performed in a Mastercycler Gradient (ASTEC, Japan) using the following program: initial denaturation at 95 °C for 5 min and 40 cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 30 s (the annealing were tested at temperature 57–62 °C and the best result achieved at 59 °C), and extension at 72 °C for 30 s, and the final extension at 72 °C for 10 min. The 5 μl of PCR products were run on 2% agarose gel and stained with Green viewer™ and electrophoresis in 0.5 × TBE for 45 min at 110 mV. The predicted size (226 bp) of amplified targets were visualized by UV-transilluminator. For each PCR run, 1 ng of *M. tuberculosis* H37Rv genomic DNA and sterile water were used instead of template as positive and negative control, respectively.

2.6. Sensitivity and specificity of primers

First, the primers specificity was determined bioinformatically using the blastn software against all databases to check any cross-reactivity with other bacterial or human genome. The analytical specificity of the primers was evaluated against a total of 36 strain of *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*), 4 strains of NTM (*M. chelonae*, *M. smegmatis*, *M. simiae and M. kansasii*) and 10 other bacterial strains (Table 3). To check the specificity of the primers in clinical isolates, 50 smear- negative, culture- negative samples were also tested.

To determine the analytical sensitivity of the primers, serial dilutions of the *M. tuberculosis* H37rv genome (1 fg to 10 ng) were examined by PCR. The concentration of extracted DNA from culture of *M. tuberculosis* was calculated the absorbance at 260 nm by spectrophotometer (Thermo Scientific) and adjusted to 10 ng by adding sterile water. Then, 10-fold serial dilution series of 10 ng templates were

Table 3
The microorganism used to check the analytical specificity of the primers.

Mycobacterium species	Other bacteria
M. tuberculosis (34)	*Staphylococcus aureus*
M. bovis (2)	*Streptococcus pneumonia*
M. chelonae	*Enterococcus faecium*
M. kansasii	*Pseudomonas aeruginosa*
M. simiae	*Acinetobacter baumannii*
M. smegmatis	*Listeria monocytogenes*
	Corynebacterium diphtheriae
	Streptococcus pyogenes
	Shigella
	E-coli

prepared ranging from 1 fg to 1 ng. One microliter of each dilution was tested as sample for each PCR reaction. To check the clinical sensitivity of the primers, 50 smear and culture positive and 20 smear-negative, culture-positive sputum specimens were also evaluated.

2.7. Analysis of results

Data analysis was performed using Excel 2010. The specificity, sensitivity, negative predictive value (NPV) and positive predictive value (PPV) with "exact" Clopper-Pearson confidence intervals (CI) were calculated. Solid cultures of *M. tuberculosis* are considered as gold standard and the results of this study were compared with the results obtained with culture. The accuracy was also calculated, statistically (accuracy = (true positive + true negatives)/total samples).

2.8. The effect of inhibitors in clinical specimens

To investigate the inhibitory effect in clinical specimens, two groups of six dilutions were prepared by the pure genomic DNA. The first group included 10 pg, 1 pg, 100 fg, 10 fg, 5 fg, and 1 fg dilutions of the pure genomic DNA and the second group was prepared similar to the first group. Then, 1 µl of each dilution were used for the *SDR*-PCR test. Before performing the reactions, 1 µl pooled true negative processed sputum sample for the first group and 2 µl for the second group were added to the reactions. Each reaction was prepared in 25 µl volumes. So, the amount of clinical specimen was the only difference in the reactions and the concentration of DNA used in both groups was equal.

3. Results

3.1. The screening of M. tuberculosis-specific detection targets

A set of 4,396,119 sequences were checked by dividing the genome of *M. tuberculosis* H37rv into 5000 bp fragments. We looked only for sequences that exactly specific for *M. tuberculosis* complex. Then, the fragments were compared against other organisms within the database sequences using BLAST. The fragment that has ≥99% identity or query cover with *M. tuberculosis* complex and no cross reactivity (alignment scores < 40) with other bacterial genomes especially NTM were considered as *M. tuberculosis*-conserved sequences.

The best results were obtained for the short-chain dehydrogenases/reductases gene (SDR) (GenBank NC_000962.3, Region: 365234–366,142 (Rv0303)) which possess a high degree of sequence similarity to the *M. tuberculosis* complex genomes and did not represent high similarity with other species. So, the forward (SDR-F 5′-GACGTT CGGGGTAAACCACTTG-3′) and reverse (SDR-R 5′-CAGCTTGGACGTG GTGTATCG-3′) primers were designed with the lowest degree of secondary structure. The sequences of primers, amplicon and location were showed in Fig. 2. The expected size of the amplicon was 226 bp.

3.2. Bioinformatics control

All strains that were detected by *rpoB* were also identified by *SDR* gene (Additional file 1: Table A1). In addition, bioinformatics studies in 224 different strains throughout the world (TB-ARC project) showed that this sequence is completely conserved (100% Query cover and 99–100% identify) in the all strains (Additional file 1: Table A2).

3.3. Test specificity and sensitivity (Limit of detection)

The specificity of the primers was evaluated by template DNA from 50 strains including 36 strain of *M. tuberculosis* complex (*M. tuberculosis, M. bovis*), 4 strains of NTM (*M. chelonae, M. smegmatis, M. simiae and M. kansasii*) and 10 bacterial strains from other genera (Table 2). All DNA templates from *M. tuberculosis* and *M. bovis* strains showed a detectable amplicon, whereas no amplification was obtained with DNA from NTM and other genera (Fig. 3). Thus, the primers were specific for the detection of *M. tuberculosis* complex.

Analytical sensitivity test shows that the small amount of genomic DNA as template can be correctly detected by PCR assay. The detection limit of *SDR*-PCR was found to be 10 fg of extracted DNA of *M. tuberculosis* H37Rv (Fig. 4) which is theoretically equivalent to 2 copy of DNA in a reaction tube (Barletta et al., 2014). The *SDR*-PCR assay was not showed any detectable amplification in 5 fg and 1 fg of template DNA.

3.4. Detection of M. tuberculosis in clinical samples

After DNA extraction of the 120 samples, only 1 µl DNA was used as template to reduce the PCR inhibitors in clinical specimens. The PCR products showed the target 226 bp bands in gel electrophoresis and the same amplification was not observed with DNA from human cells. 47 out of 50 smear- and culture-positive and 15 out of 20 smear negative-culture positive samples indicated the amplicons. Five of them were randomly selected and confirmed by sequencing (accession numbers are MK369742, MK369743, MK369744, MK369745 and MK369746). All 50 smear- and culture-negative did not show any amplicons, too.

The specificity, sensitivity, NPV, PPV and accuracy were calculated. The sensitivity of the *SDR*-PCR assay for smear positive-culture positive and smear negative-culture positive samples were 94% and 75% respectively. Likewise, no amplification was found in smear- negative samples which yielded negative cultures. So, the specificity of the assay was 100%. The results obtained with clinical samples are summarized in Table 4.

The PPV and NPV of the *SDR*-PCR assay were 100% and 86.2%, respectively. The *SDR*-PCR showed high accuracy of 93.3%. So, in-house PCR assay confirmed to be extremely reliable with high specificity, sensitivity and accuracy. The *SDR*-PCR was also performed on colonies of each specimen (culture positive) and all of them were positive.

3.5. The effect of inhibitors in clinical specimens

The results indicated that the sensitivity of the first and second groups were 10 fg/µl and 20 fg/µl in *SDR*-PCR, respectively (Fig. 5). Therefore, the use of 2 µl DNA in comparison with 1 µl DNA in the PCR reaction increases the inhibitory effect of the clinical specimen and reduces the sensitivity.

4. Discussion

M. tuberculosis is still one of the most causes of mortality in the world. So, early and efficient detection of the agents followed by adequate treatment is important for at risk patients. In order to prevent continued transmission and misdiagnosis, better tests and more efficient diagnostic tools are essential in microbiological laboratory practice.

Although, conventional phenotypic methods are challenging in diagnosis of tuberculosis and not fast, the role of NAATs like PCR is clear in the rapid diagnosis of infectious disease. The usefulness of DNA amplification techniques is completely relying on the selection of the target sequence and specificity of the primer sets.

The sensitivity and specificity of the smear microscopy for detection of *M. tuberculosis* in clinical samples were lower than PCR methods (Haldar et al., 2011; Steingart et al., 2006). As reported in previous studies, the specificities of the PCR method ranged from 88.9% to 99.2% while sensitivities ranged from 63.7% to 95.9%which shows the efficiency of the PCR method for rapid detection of tuberculosis (Haldar et al., 2011; Nimesh et al., 2013). However, discovery the new targets helps us to improve the sensitivity and specificity of the PCR method especially for clinical samples and allows us to reduce the time for diagnosis.

Fig. 2. The sequences of primers and amplicon.

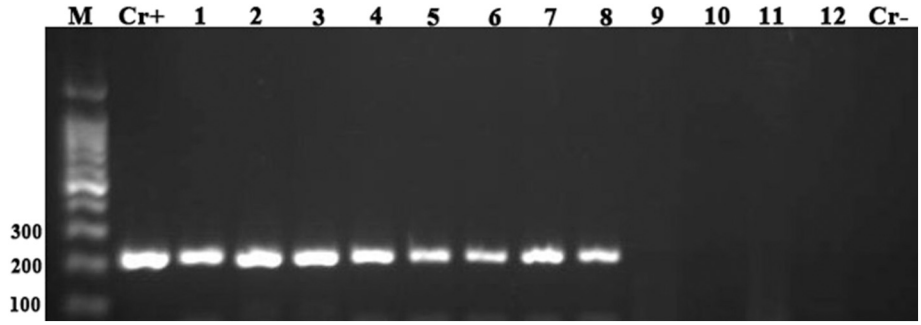

Fig. 3. Gel electrophoresis of PCR amplification of cultivated samples on 2% agarose gel. M: 100 bp Ladder, Cr+: DNA from *M. tuberculosis H37rv*, Cr-: negative control, 1–6*: M. tuberculosis*, 7–8: *M. bovis*, 9: *M. chelonae*, 10: *M. smegmatis*, 11: *M. simiae*, 12: *M. kansasii*.

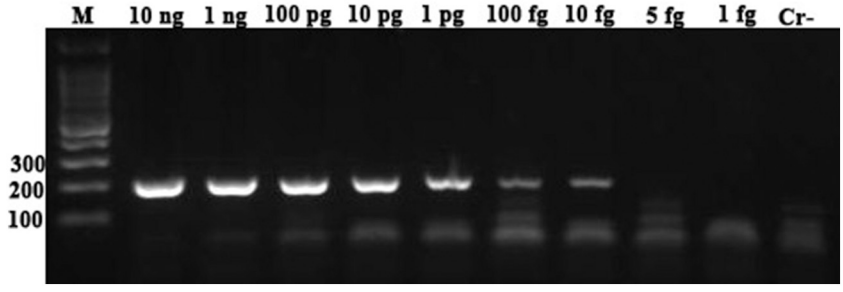

Fig. 4. Gel electrophoresis of PCR amplification of H37rv on 2% agarose gel. M: 100 bp Ladder, Cr-: negative control, lanes show amplicons using 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, 5 fg, 1 fg.

Here, we have employed a modified comparative genomics method to identify unique nucleotide sequences and used to develop PCR assay for direct detection of *M. tuberculosis* in clinical samples. Whereas most of the available target genes used for PCR detection of tuberculosis is limited to 4–5 targets, the modified comparative genomics method has led the way to increase the availability of whole genome sequences and find more specific and sensitive detection targets and allows for identification of unique genes and sequences for the genus or species. Bioinformatics studies on *SDR* gene showed a high specificity to the *M. tuberculosis* complex.

Table 4
The specificity and sensitivity of the primers.

Samples	Sensitivity			Specificity
	Cultivated isolate	Clinical isolate		Clinical isolate
	Culture- positive	Smear- positive Culture- positive	Smear- negative Culture- positive	Smear- negative Culture- negative
Positive NO./total no. (%)	36/36 (100%)	47/50 (94%)	15/20 (75%)	50/50 (100%)

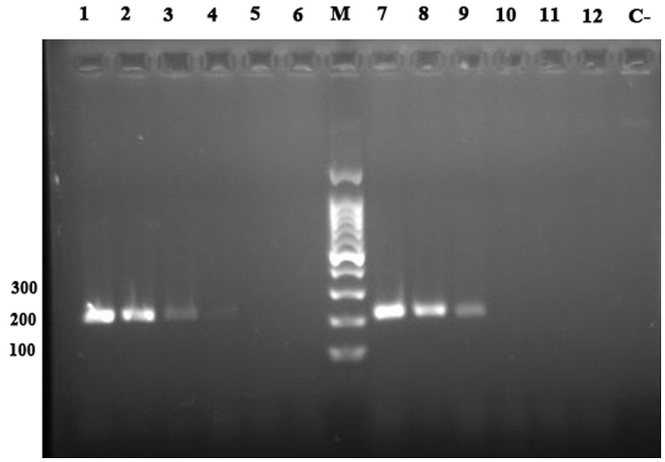

Fig. 5. The effect of inhibitors in clinical specimens by gel electrophoresis of *SDR*-PCR amplification of H37rv using 1 pg, 100 fg, 20 fg, 10 fg, 5 fg and 1 fg. M: 100 bp Ladder, Cr-: negative control, 1–6: first group with 1 µl of processed clinical specimen show amplicons until 10 fg 7–12: second group with 2 µl of processed clinical specimen show amplicons until 20 fg.

In the present study, specific primers were designed to amplify 226 bp region of *SDR* gene in *M. tuberculosis*. The *SDR*-PCR assay has been optimized to minimize time duration of PCR protocol and can detect even low template of genomic DNA.

The analytical specificity of the PCR assays was performed, initially with 50 strains of bacteria (36 strain of *M. tuberculosis* complex, 4 strains of NTM, 10 other bacterial strains). The results confirmed that the SDR gene is completely specific for *M. tuberculosis* complex.

The clinical evaluation of the primer set was further tested against 50 smear- and culture- negative, 20 smear- negative culture- positive and 50 smear- and culture- positive samples.

The values of sensitivity for smear- positive, smear- negative and specificity for clinical isolates are 94%, 75% and 100%, respectively. The high overall specificity (100%) and sensitivity (88.5%) of the target gene illustrates that *SDR* gene can be a good target for rapid detection of *M. tuberculosis* complex from sputum and possibly other clinical specimens.

The target used in this study is encoded chromosomally and all isolates have the target sequences. It belongs to a single-copy gene and the detection limit of *SDR*-PCR assays was 10 fg. So, this detection limit is sufficient to detect infections due to *M. tuberculosis* in clinical samples.

The most recent studies on clinical application of PCR for tuberculosis use multi-copy gene targets, such as *IS6110* sequences, and achieved detection limits up to 10 fg (Dalovisio et al., 1996; Nimesh et al., 2013). The sensitivity of the target is excellent but one limitation of this target is that not all isolates display multiple copies of these elements and some strains lack even a single copy. So, the *IS6110* PCR assay demonstrated low sensitivity when applied to clinical isolates (63%) that may be due to lack of the sequences in some strains (Nimesh et al., 2013).

The lack of specificity associated with other targets like *devR* and *rpoB* has recommended the use of another target sequences for detection of *M. tuberculosis* (Nimesh et al., 2013). The present study provides a novel target gene that can be used alone or in combination with other PCR targets to improve the accuracy of *M. tuberculosis* detection especially in clinical samples.

The PPV of the *SDR*-PCR assay was 100% which indicated that there was no false positive reaction and NPV was 86.2% which due to false negative of the PCR assay.

The rare failure of detection in these samples was caused by the errors in DNA extraction or PCR inhibitors that may have been derived from the human sputa. This is one of the most important drawbacks which cause the false-negative results and we confirmed the presence of these inhibitors in the clinical specimen. A study on sputum specimens showed that the presence of an inhibitor in the clinical specimen could lead to false negative results between 10% and 17% (Nolte et al., 1993; Neshani et al., 2018). In the present study, by increasing the amount of negative sputum in PCR reaction, the sensitivity decreased from 10 fg to 20 fg.

False-negative reactions are created when clinical components like mucus and blood interfere with the amplification of DNA. In order to decrease the effect of inhibitors, we only used 1 µl of the specimen in 25 µl of reaction, because the sensitivity of the primers is enough to detect the mycobacterial DNA even in the low concentration (10 fg). False positive results were not observed due to high specificity of the target which eliminates the false positive reactions.

In this study, we used autoclave method for DNA extraction. This method let us to reduce the processing time, cost and also preventing the transmission of *M. tuberculosis* during the extraction procedure. However, the probability of false negative results was increased because the washing procedure was not performed in this extraction technique and clinical components like mucus and blood can inhibit the PCR reaction.

Since the goal of this study was to diagnose TB rapidly in clinical specimens, the primers should be designed in such a way that they do not show any cross-reactivity with the human genome. Therefore, the primers were blast on the human genome and the results confirmed that there was no cross-reactivity with the human genome.

The successful detection of *M. tuberculosis* in clinical samples was achieved using the modified comparative genomics method. In silico analysis reveals that the *SDR* gene can detect the most whole genome of MTBC members including: 237 strains of *M. tuberculosis*, 5 strains of *M. bovis,*12 strains of *M. bovis BCG*, 3 strains of *M. africanum*, 1 strains of *M. canettii*, 1 strain of *M. caprae* and 1 strain of *M. microti*. The partial sequences were not found because the *SDR* target has not been used before. In addition, bioinformatics studies in 224 different strains around the world (TB-ARC project) indicated that the *SDR* gene is completely conserved (Additional file1: Table A2) and has 99–100% Query cover and identify with these strains.

In the present study, we have developed in-house PCR assay for the specific detection of *M. tuberculosis* complex using *SDR* gene specific primers which was found to be effective in this research. This gene encodes the enzymes of fundamental metabolic importance that often adopt a specific structure.

In conclusion, a modified comparative genomics approach for identifying specifies-specific nucleotide sequences was used to identify *M. tuberculosis*-specific target sequences. Several nucleotide sequences were investigated and one target sequence was used to develop a PCR

for the detection of *M. tuberculosis* in clinical samples.

The future studies are needed as follow: (1) comparison of the PCR test against *SDR*, *IS6110*, *sdaA*, *MPT64*, *rpoB* and *devR* in clinical samples. (2) Finding other specific targets for the diagnosis of tuberculosis through the modified comparative genomics procedure. (3) It could be used as new target for the modern TB diagnostic methods like LAMP, RFLP and etc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.01.012.

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